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Chikere CB, Omoni VT and Chikere BO (2008). Distribution of potential nosocomial pathogens in a hospital environment. Afr. J. Biotechnol. 7: 3535-3539.

Moran GJ, Amii RN, Abrahamian FM, Talan DA (2005). Methicillinresistant Staphylococcus aureus in community-acquired skin infections. Emerg. Infect. Dis. 11: 928-930.

Pitout JDD, Church DL, Gregson DB, Chow BL, McCracken M, Mulvey M, Laupland KB (2007). Molecular epidemiology of CTXM-producing Escherichia coli in the Calgary Health Region: emergence of CTX-M-15-producing isolates. Antimicrob. Agents Chemother. 51: 1281-1286.

Pelczar JR, Harley JP, Klein DA (1993). Microbiology: Concepts and Applications. McGraw-Hill Inc., New York, pp. 591-603.

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African Journal of Microbiology Research

Table of Content: Volume 9 Number 10, 11 March, 2015

## ARTICLES

**Principal criteria for selection of lactic acid bacteria for potential use as probiotics in foods**
Washington Luiz Gonçalves de Almeida Júnior, Íris da Silva Ferrari, Jane Viana de Souza, Aynoanne Leandro Barbosa, Mateus Matiuzzi da Costa, Daniel Ribeiro Menezes and Francesca Silva Dias

**Microbial diversity analysis of subclinical mastitis in dairy cattle in Northeast China**
Yufeng Yang, Qingyu Jiang, Kerui Xin, Xiudong Bian, Mengchao Li, Bei Zhang, Chunqiu Li, Zhenyu Yao, Jianhua Hu and Dongbo Sun

**Growth potential of common foodborne bacterial pathogens on some selected vegetables sold in Jimma Town, Southwestern Ethiopia**
Alemayehu Dugassa, Ketema Bacha and Tsige Ketema

**Effects of plant growth promoting bacterial isolates from Kavango on the vegetative growth of *Sorghum bicolor***
Daniel Hosea Haiyambo, Barbara Reinhold-Hurek and Percy Maruwa Chimwamurombe

**Screening of microbial isolates for extracellular fructosyltransferase production**
Seema A. Belorkar, A. K. Gupta and Vibhuti Rai

**Biochemical characteristics and microbial association of Isabgol (*Plantago ovata* Forks.) growing soils in Western Arid region of India**
B. K. Mishra, Balraj Singh, P. N. Dubey, Arunabh Joshi, Krishna Kant and S. R. Maloo

**Screening of Amazon fungi for the production of hydrolytic enzymes**
Marta Maria Guedes da Silva Mendes, Salony Aquino Pereira, Rafael Lopes e Oliveira, Leonor Alves de Oliveira da Silva, Sergio Duvoisin Jr. and Patrícia Melchionna Albuquerque

**Bacterial pathogens associated with hand-dug wells in Ibadan city, Nigeria**
Odeleye, F. O. and Idowu, A. O.

African Journal of Microbiology Research

## Table of Content: Volume 9 Number 10, 11 March, 2015

**Prevalence of *Salmonella*, *Escherichia coli* and coliforms on bell peppers from the field to the packing house process**
Marcela Soto-Beltran, Nohelia Castro-del Campo, Juan Campos-Sauceda, Roberto Avena-Bustillos and Cristobal Chaidez

**Effect of *Eichhornia crassipes* on coliforms load in small water bodies within Lake Victoria basin, Kenya**
Veronica Ombwa, Patrick Orwa, Alice Mutie, Reuben Omondi, Kenneth Werimo and John Ouko

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African Journal of Microbiology Research

*Review*

# Principal criteria for selection of lactic acid bacteria for potential use as probiotics in foods

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**The use of lactic acid bacteria (LAB) in different food products has been driving the consumer market due to the potential probiotic action of these bacteria, which raises the foods to the status of functional foods. A selection of potential probiotic strains can be obtained from different food matrices and environments. However, these microorganisms must be confirmed as safe while retaining characteristics that make them technologically, functionally and physiologically capable of benefitting the food and health of the host. Characterisation of a potential probiotic strain should include knowledge of the source, pathogenicity, infectivity, virulence factors, viability during processing, storage stability, phage resistance, contribution to sensory properties, tolerance within the gastrointestinal tract, cell adhesion, antimutagenic, anticarcinogenic and antagonistic activity against gastrointestinal pathogens and immunomodulation. Desirable physiological effects of probiotic strains include aiding in lactose intolerance and prevention and reduction of diarrhoea, inflammatory bowel disease and allergies. In this context, this paper reviews the current literature referring to principal criteria for the selection of LAB for potential use as probiotics in foods.**

**Key words:** Lactic acid bacteria, screening, safety, functional foods.

## INTRODUCTION

Probiotics are defined by FAO/WHO as live microorganisms which, when administered in adequate amounts, confer a health benefit on the host by improving the intestinal microbial balance (FAO/WHO, 2002). The value of consumption of lactic acid bacteria (LAB) appeared in the early 20th century when Metchnikoff suggested that the ingestion of these living microorganisms, present in yoghurt, increased the longevity of the consumer. Metchnikoff attributed the positive effects observed on the host health to a reduction in the population of spoilage bacteria and/or of bacteria producing toxins in the digestive tract (Burgain et al.,

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2014).

Interest is now growing in the development of novel foods containing probiotic microorganisms (Sidira et al., 2015). The most frequently studied LAB for probiotic usage belong to the genera *Lactobacillus* and *Enterococcus* (Coman et al., 2012; Jensen et al., 2012; Gregoret et al., 2013; Babot et al., 2014). The majority of the commercialized and most studied probiotics have been isolated from dairy products and from the human gastrointestinal tract (GIT). In fact, LAB strains are already used in many probiotic dairy products (García-Ruiz et al., 2014). However, commercial use of genres as probiotics in food requires screening of microorganisms and selected strains must meet safety, technological, functional and physiological requirements (Morelli, 2007; Vasiljevic and Shah, 2008).

The safety criteria of a probiotic include a documented origin and a lack of characteristics such as pathogenicity and infectivity and, virulence factors including resistance to antibiotics and metabolic activity (Saarela et al., 2000; Muñoz-Atienza et al., 2014; Rubio et al., 2014). From the technological aspect, probiotic cultures must remain viable during processing and show storage stability and phage resistance, while contributing to good sensory properties of the products, such as the aromatic profile (Vasiljevic and Shah, 2008; Champagne et al., 2011). Fermentation by the culture should preserve and enhance the quality while beneficially altering the flavour of the food (Rivera-Espinoza and Gallardo-Navarro, 2010), thereby generating a higher added-value product.

As a functional criterion, probiotic strains must survive within the gastrointestinal tract; they must be able to tolerate the acidic conditions of the stomach, resist digestive enzymes and bile acid at the start of the small intestine, adhere to the intestinal mucosal surface and ensure clinically validated benefits to consumer health (Erkkilä and Petäjä, 2000; Cotter and Hill, 2003; Jensen et al., 2012). Desirable physiological requirements for probiotic cultures are antimutagenicity, anticarcinogenicity, antagonism against gastrointestinal pathogens, promotion of immunomodulation and action on the metabolism of lactose and cholesterol (Zago et al., 2011; Burns et al., 2012; Ren et al., 2012). In this context, this paper reviews the current literature, referring to these principal criteria for the selection of LAB for potential use as probiotics in foods.

## SAFETY CRITERIA: ORIGIN, PATHOGENICITY AND VIRULENCE FACTORS

### Origin

To act as a safe probiotic microorganism, a strain should be a species and genera normally present in the human GIT (Kolozyn-Krajewska and Dolatowski, 2012). The selection of an appropriate strain for each food matrix is essential. The first step in the selection of a probiotic is the determination of its taxonomic classification, which may give an indication of the origin, habitat and physiology of the strain. All these characteristics have important consequences on the selection of novel strains (Morelli, 2007).

The LAB, especially *Lactobacillus* and *Enterococcus,* are commonly used as probiotics (Barbosa et al., 2010; Tulumoğlu et al., 2014). They are classified as prokaryotic, Gram-positive, non-sporulating cells that have the common characteristic of producing lactic acid as a main or sole product of fermentation during the homofermentative or heterofermentative metabolism (Balciunas et al., 2013). A host of current scientific papers report selections of LAB from different sources (for example, vegetables, milk and meat) for potential probiotic use (Table 1). Although LAB are generally regarded as safe (GRAS), their capability of producing toxic compounds must also be taken into account. Furthermore, the use of enterococci, part of the LAB community, is still controversial due to the presence of virulence genes in some species. For these reasons, toxicity and pathogenic determinants must be assessed (Landeta et al., 2013).

### Pathogenicity

The selection of microorganisms for use as probiotics must consider that some species of LAB can be pathogenic and infective. Some species of LAB can cause human diseases such as tooth decay, rheumatic complications, infectious endocarditis, septicaemia and vascular disease.

The identification of potential pathogenic characteristics can facilitate the use of organisms for probiotic purposes (Harty et al., 1994). Most lactobacilli are non-pathogenic, so identification of inherent strain properties related to health risks can be difficult. Vesterlund et al. (2007) suggested studying the properties in probiotic strains that might indicate a potential health risk in populations and that are known virulence factors in ‘true’ pathogens; namely, the ability to adhere to immobilized human collagen type IV, fibrinogen or intestinal mucus, α- or β-haemolysis, low induction of the respiratory burst in peripheral blood mononucleocytes and resistance to serum-mediated killing.

### Virulence factors

Bacterial virulence factors may include secreted proteins (for example protein toxins and enzymes), and cell-surface structures (for example capsular polysaccharides, lipopolysaccharides and outer membrane proteins) and further hydrolytic enzymes that may contribute

**Table 1.** Selection of LAB in different food matrix for potential probiotic use.

| Source | Microorganism | Product elaborated | Reference |
|---|---|---|---|
| Cheese | *L. delbrueckii, Lactococcus lactis, Leuconostoc mesenteroides* and *Streptococcus thermophiles* | Cheese | Gaglio et al. 2014 |
| Artisan cheese | *L. lactis* and *E. faecalis* | Fresh cheese | Coelho et al. (2014) |
| Traditional fermented yak milk | *E. durans*, *L. fermentum* and *L. paracasei* | Fermented milk | Ao et al. (2012) |
| Siahmazgi cheese | *L. plantarum* | Dry-fermented sausage | Kargozari et al. (2014) |
| Indonesian local beef | *L. plantarum* | Fermented lamb sausages | Arief et al. (2014) |
| Salami | *L. curvatus* | Salami | De Souza Barbosa et al. (2015) |
| Pork sausage | *L. plantarum* | Sausage | Dias et al. (2013) |
| Artisanal dry sausages manufactured | *L. curvatus* | Cooked pork meat | Rivas et al. (2014) |
| Chinese dry-cured hams | *L. salivarius* | Fresh pork | Luo et al. (2013) |
| Industrially fermented olives | *L. pentosus* and *L. plantarum* | Green olives | Blana et al. (2014) |
| kimchi | *L. brevis*, *L. curvatus*, *L. plantarum* and *L. sakei* | Fermented sausage | Paik and Lee (2014) |
| Fermented caper berries | *L. plantarum* | Fermented caper | Palomino et al. (2014) |
| Infant faeces | *L. casei, L. paracasei* and *L. rhamnosus* | Fermented sausages | Rubio et al. (2014) |

to the pathogenicity of the microorganism. Many genes encode virulence traits (e.g. secretion machineries, siderophores, catalases, regulators, etc.) that are also indirectly involved in pathogenesis and are equally important for bacteria to establish infection (Brogden et al., 2000; Chen et al., 2005). The identification of virulence genes in a potential candidate probiotic is especially important, since the possibility of horizontal transfer to other bacteria has become a concern in the food industry (Perin et al., 2014).

Haemolysis is a common virulence factor among pathogens, facilitating iron availability to the microbe and causing anaemia and oedema in the host (Vesterlund et al., 2007). The haemolytic reaction is observed on blood agar supplemented with 5% defibrinated whole horse blood. A clear zone of hydrolysis around the colonies is indicative of β-haemolysis, a partial hydrolysis indicates α-haemolysis and no reaction indicates γ-haemolysis. The determination of haemolytic activity is required in recognition of the importance of assuring safety, even among a group of bacteria with GRAS status (FAO/WHO, 2002). The absence of haemolytic activity should be a selection criterion for starter strains for use in dairy products (Giraffa, 1995).

Regarding the safety of probiotic strains, antibiotic resistance is one aspect requiring analysis because of the serious concerns about the growing level of resistance to antibiotics in regular use in human medicine (Monteagudo-Mera et al., 2012). The antibiotic resistance genes can be passed by some LAB through conjugative plasmids, bacteriophages or transposons to other micro-organisms by horizontal transfer. This type of transfer commonly occurs between bacteria in the gastrointestinal tract, with the potential to occur between innocuous species and harmful pathogens. If beneficial *Lactobacillus* species have antibiotic resistance genes, then those genes could possibly be transferred to other microbiota, including pathogens, living in the same niche (the GIT) (Shao et al., 2015).

Selection criteria for probiotic microorganisms must include characteristics of intrinsic or natural resistance to antimicrobials because this is chromosomally encoded and, therefore, non-transmissible (Argyri et al., 2013). The intrinsic resistance of LAB to several antibiotics may be partially due to genes that encode multidrug resistance efflux pumps (MDR), which expel different types of antibiotics and other chemicals (dyes, organic solvents, detergents, biocides and metabolic products) (Muñoz et al., 2014). Four mechanisms are recognised that confer intrinsic resistance in bacteria to a given antibiotic: enzymatic inactivation or modification of the antibiotic, antibiotic removal by efflux pumps and outer membrane (OM) permeability changes, alteration of bacterial target sites and intracellular metabolic rearrangement. Most of these mechanisms have been observed and studied in various bacteria; however, no specific studies have dealt with these mechanisms in probiotics (LAB or *Bifidobacterium*) (Sharma et al., 2014).

The probiotic bacteria should not produce harmful substances during metabolic activities. One test is to determine whether the bacteria convert food components or biological secretions into secondary substances that are potentially harmful to the host. For example, some intestinal bacteria act on proteins and their digested

products to produce ammonia, indoles, phenols and amines (Ishibashi and Yamazaki, 2001). Toxic products such as biogenic amines can be derived from the decarboxylation of free amino acids arising from the catabolism of lactic acid; for example, by the decarboxylases enzymes of *Lactobacillus* spp. (Pereira et al., 2001). Excessive intake of these biogenic amines can have toxicological effects on human health, such as hypotension or hypertension, headache, nausea, allergic reactions, cardiac palpitation and even intracerebral haemorrhage and death in very severe cases (Shalaby, 1996). However, selected LAB strains can act as amine removers; for example, Zhang et al. (2013) used *Lactobacillus plantarum* to decrease the accumulation of biogenic amines in silver carp sausage and achieved a significant reduction. Thus, the production of biogenic amines should be considered in the selection of microorganism for potential probiotic use due to the potential for triggering disorders in the host health (Pereira et al., 2001).

The ability of the microorganism to produce D-lactic acid is reported as a selection criterion for probiotic strains; however, the strains should not produce quantities exceeding 2 g/l. The D isomer of this acid is not hydrolysed by human lactate dehydrogenase and can be damaging to health by triggering responses such as metabolic acidosis. Only strains that produce L-lactic acid isomer should be selected (Ammor et al., 2007; Ruiz-Moyano et al., 2009).

LAB play a role in reducing fat absorption by the body by lowering serum cholesterol through the activity of bile salt hydrolase (BSH), the enzyme responsible for bile salt deconjugation in the enterohepatic circulation (Anandharaj and Sivasankari, 2014). However, excessive metabolism of bile salts in the human small bowel can be detrimental, as secondary (dehydroxylated) bile salts are cytotoxic and co-carcinogenic, and can induce cellular lesions in the small intestine (Marteau et al., 1995). Begley et al. (2006) reported that microbial BSH activity may have various impacts on the host in the form of altered digestive functions, lowered cholesterol, cancer/activation of carcinogens and formation of gallstones. Thus, the desirability of BSH activity as a trait in a probiotic bacterium is not completely clear. Considering this aspect, the absence or limited extent of bile salt transformation capacity of bacteria added to food is suggested as a pre-requisite for labelling a product as GRAS (Marteau et al., 1995).

## TECHNOLOGICAL CRITERIA

### Viability during processing and storage

In Brazil, fermented milks must show viability of LAB and *Bifidobacterium* at the end of the product shelf life of at least $10^7$ and $10^6$ CFU/g or mL, respectively (Brazil, 2007). In the preparation of fermented foods, probiotics show optimum growth at temperatures within 40-42°C; temperatures above 45-50°C during processing are detrimental to probiotic survival (Tripathi and Giri, 2014). The viability during operations, storage and processing, the survival during intestinal transit and the health benefits for consumers are the main criteria for the selection of appropriate strains of probiotic bacteria (Talwalkar et al., 2004). The physiological state of the probiotic cultures added to a product can also be a major factor affecting the overall culture viability. In this respect, the induction of stress responses in probiotic strains can have a dramatic effect on the ability of the cultures to survive processing (such as freeze drying and spray drying) and gastric transit (Ross et al., 2005).

Acid stress may significantly affect the viability of a culture (Shah, 2000). Consequently, dairy products are preferred for use as carriers of probiotic strains for enhancement of microbial survival in gastric juice, likely due to a buffering or protective effect (Ross et al., 2005). In general, *Bifidobacterium* cultures are less acid tolerant than *Lactobacillus* cultures and this is reflected by their reduced tolerance to human gastric juice. Tolerance to an acidic environment may also be a useful predictor of technological performance in fermented foods (Ross et al., 2005; Mättö et al., 2006).

Many other factors influence the viability of probiotic microorganisms in food products during production, processing and storage. Identified factors include food parameters (molecular oxygen, water activity, presence of salt, sugar and chemicals like hydrogen peroxide, bacteriocins, artificial flavouring and colouring agents), processing parameters (heat treatment, incubation temperature, cooling rate of the product, packaging materials and storage methods, and scale of production) and microbiological parameters (strains of probiotics, rate and proportion of inoculation) (Tripathi and Giri, 2014).

Oxygen content and redox potential are also important factors for the viability of probiotic species (microaerophilic and anaerobic) during storage (Shah, 2000). In the presence of oxygen, lactic acid bacteria are able to generate more hydrogen peroxide via the action of flavoprotein-containing oxidases, nicotinamide adenine dinucleotide oxidases (NADH) and superoxide dismutase (SOD) than they do under anaerobic conditions (Miller and Britigan, 1997; Kulisaar et al., 2002). Overall, oxygen affects probiotics in three ways: (i) it is directly toxic to some cells, (ii) certain cultures produce toxic peroxides in the presence of oxygen, and (iii) free radicals produced from the oxidation of components (e.g., fats) are toxic to probiotic cells (Korbekandi et al., 2011; Tripathi and Giri, 2014). One solution is the addition of ascorbic acid (vitamin C), which can act as an oxygen scavenger (Shah, 2000).

Another factor that may affect the viability of probiotics

is freezing. The freezing step in food processing is especially critical as it negatively affects both viability and physiological state of the bacteria. Lowering the temperature affects the structure and the properties of the cellular membrane. During freezing, the liquid phase moves to a liquid-crystalline phase, thereby reducing membrane fluidity. The formation of ice crystals induces mechanical damage that leads to cellular death. In addition, the crystallization of water leads to a cryo-concentration of the solutes, which induces some osmotic damage (McGann, 1978; Foschino et al., 1996; Beal et al., 2001). However, the ability of lactic acid bacteria to survive freezing and storage differs with strain (Fonseca et al., 2000). Fonseca et al. (2000) demonstrated that small spherical cells of enterococci are more stable during freezing and freeze-drying as compared to the large rods of lactobacilli. Senz et al. (2015) reported that short rods were significantly more stable than elongated rods during freeze-drying because the cell membrane damage increased with the enhanced cell surface area due to extracellular ice crystal formation during freezing. The addition of various protective compounds to probiotic cultures can improve their viability during manufacture: examples include glucose to energize cells upon exposure to acid (Corcoran et al., 2005) and cryoprotectants such as glycerol or inulin to improve survivability during freeze-drying (Fonseca et al., 2000; Carvalho et al., 2004).

## Phage resistance

Bacteriophages are a consistent and major threat to food fermentations, and particularly dairy fermentations, where infection of starter cultures may result in slow vats, low quality, inconsistent products and even complete fermentation failure (Whitehead and Cox, 1935; Mahony and Sinderen, 2014). Phages infecting LAB provide some of the most advanced model systems for (tailed) phage-host interactions (Mahony and Sinderen, 2014). In recent years, a number of strategies based on strain diversity, bacteriophage-insensitive mutants, and plasmids bearing phage-resistance mechanisms (Barrangou et al., 2007) were designed and implemented to minimize both the presence of phages in the dairy industry and their economic impact on fermentation processes (Briggiler-Marcó et al., 2014).

Bacteria have developed a variety of natural defence mechanisms that target diverse steps of the phage life cycle, notably blocking adsorption, preventing DNA injection, restricting the incoming DNA and abortive infection systems (Barrangou et al., 2007). Briggiler-Marcó et al. (2011) used two methodologies to isolate phage-resistant mutants: the agar plate (AP) and the secondary culture (SC) methods. Characterization of the phage resistance phenotype, genetic analysis and characterization of phage-resistant mutants identified a mutant MC4 that can be used in the manufacture of fermented milk.

Clustered regularly interspaced short palindromic repeats (CRISPR) are hypervariable loci widely distributed in bacteria and archaea that provide acquired immunity against foreign genetic elements. CRISPRs represent a family of DNA repeats which typically consist of short and highly conserved repeats, interspaced by variable sequences called spacers, and are often times adjacent to *cas* (CRISPR-associated) genes (Sorek et al., 2008; Horvath et al., 2009). The CRISPR-*cas* system may accordingly be exploited as a virus defence mechanism and also potentially used to reduce the dissemination of mobile genetic elements and the acquisition of undesirable traits such as antibiotic resistance genes and virulence markers. From a phage evolution perspective, the integrated phage sequences within CRISPR loci may also provide additional anchor points to facilitate recombination during subsequent phage infections, thereby increasing the gene pool to which phages have access (Barrangou et al., 2007). CRISPR loci appear to be a key distinguishing feature in LAB, from a phylogenetic and genome evolutionary standpoint. The ability of the CRISPR system to provide immunity against foreign genetic elements is essential and actively involved in the microbe's propensity to survive phage predation and to adapt to its environment (Horvath et al., 2009).

## Contribution of sensory properties to foods

LAB are not considered as strongly proteolytic bacteria; however, a part of their proteolytic system has been extensively explored for the last few decades because it is essential for the optimal growth of LAB in milk (Lopez-Kleine and Monnet, 2011). Intra- and inter-specific variability in acid production, autolysis and proteolysis are commonly reported for strains from natural sources (Franciosi et al., 2009).

Proteolysis is considered one of the most important biochemical processes involved in the manufacture of many fermented products. In cheese manufacture, the proteolysis of casein is thought to play a pivotal role because amino acids resulting from proteolysis are the major precursors of specific flavour compounds, such as various alcohols, aldehydes, acids, esters and sulphur compounds. Bitterness, which results from the accumulation of hydrophobic peptides (peptides rich in proline), is a serious quality concern facing the manufacture of Gouda and Cheddar cheeses (Smukowski et al., 2003; Smit et al., 2005; Savijoki et al., 2006). The correlation between the starter culture and the flavour of fermented sausages has been well established (Sidira et al., 2015). A number of potential precursors are responsible for the

flavour and odour, which may be produced by lipid hydrolysis and autoxidation, proteolysis and transformation of amino acids to aromatic compounds and carbohydrate metabolism (Ammor et al., 2007). Sah et al. (2014) showed that probiotics with proteolytic activity increased the generation of peptides with antioxidant potential and antimutagenic properties. Screening for proteinases, peptidases and aminopeptidases activities is therefore recommended.

Exopolysaccharides (EPS) are biopolymers with wide distribution in nature. Their occurrence is well documented in all organisms (viz. animals, plants, fungi and bacteria) and they are involved in various biological functions such as storage of energy (starch), cell wall architecture (cellulose) and cellular communication (glycosaminoglycans) (Badel et al., 2011). Microbial EPS produced by LAB are receiving increasing attention because of their GRAS status (Ahmed et al., 2013; Li et al., 2014). The EPS can be used as food additives to improve texture, which aids the development of new food products with improved appearance, stability and rheological properties (De Vuyst et al., 2001).

*Lactobacillus* produces EPS molecules in relatively large amounts (>100 mg/l), predominantly in media containing glucose. The viscosity and biological activity of an EPS depends on its molecular weight, sugar composition and primary structure (Kodali et al., 2009). The EPS can perform functions that go beyond technological characteristics; for example, Liu et al. (2010) and Nikolic et al. (2012) observed that EPS secreted by probiotic bacteria such as *Bacillus licheniformis* and *Lactobacillus paraplantarum* have immunomodulatory capacity, immunosuppressive and anti-inflammatory actions.

Antitumor effects, reduced blood cholesterol and enhanced colonization of probiotic bacteria in the gastrointestinal tract have also been observed (Welman and Maddox, 2003). EPS may function as part of a defence mechanism against bacteriophages by preventing their adsorption (Lamothe et al., 2002).

LAB have evolved mechanisms to support various concentrations of NaCl that generally involve the absorption or synthesis of a limited number of solutes (Bremer and Kraemer, 2000). Ge et al. (2011) reported that osmotic stress may become the major inhibiting factor for bacterial growth and fermentation. Reale et al. (2015) added information on intra-species variability in osmotolerance in *Lactobacillus casei*, *Lactobacillus paracasei* and *Lactobacillus rhamnosus* and highlighted that osmotolerance is an important criterion for the selection of strains for technological applications. In particular, several strains isolated from cheese and human faeces appeared to be very tolerant of high NaCl concentrations and are worth investigating for their performance in fermented foods. Ammor et al. (2007) also affirmed that the ability of a starter culture to compete with the natural microbiota of a raw material (sausage) and to undertake the expected metabolic activities is conditioned by its growth rate and survival in the conditions prevailing in the sausage, including rather high salt concentrations ranging from 2 to 15% in the final product.

Gas production by LAB may be required in some products, such as kefir and some cheeses like Gouda, Edam and Danbo (Leite et al., 2013; Pedersen et al., 2013). Gas production can increase the number and/or size of holes in semi-hard cheeses. Eye formation in cheese is a complex process that is influenced by a number of factors such as gas formation, gas diffusion, the presence of eye-forming nuclei, pH and elasticity of the cheese body, as well as technological parameters (Fröhlich-Wyder et al., 2013). Tammam et al. (2001) reported that the anaerobic production of $CO_2$ by lactobacilli prevails in cheese. In contrast, heterofermentative LAB are not suitable for sausage production because the formation of large amounts of carbon dioxide which leads to holes of different sizes in the product. In addition, these LAB produce concentrations of acetic acid that causes a pungent off-flavour (Buckenhüskes, 1993; Ammor et al., 2007).

Aroma compounds play a major role in the perception of flavour, which is an important property for consumer choice of food products (Yee et al., 2014). Diacetyl (2,3-butanedione) is a volatile product of citrate metabolism produced by lactic acid bacteria, and when taken in conjunction with the lactic acid and textural effects, enhances the sensorial profile of fermented foods (Rincon-Delgadillo et al., 2012; Xião and Lu, 2014). It can also inhibit pathogenic microorganisms by penetrating the targeted bacterial membranes and interfering with essential metabolic functions (Hor and Liong, 2014). High diacetyl levels are associated with a flavour imbalance, bitter taste and harsh aroma (Clark and Potter, 2007), but it is used as an ingredients in the formulation of many food products such as cottage cheese, margarine, vegetable oil spreads, processed cheese and sour cream to increase the levels of the naturally occurring buttery aroma associated with fermentation (Rincon-Delgadillo et al., 2012).

## FUNCTIONAL CRITERIA

### GIT tolerance

Some LAB possess probiotic properties beneficial to human health. However, both gastric acid and bile salt have adverse influences on the survival and viability of ingested probiotics. Most microbes have a low rate of survival and viability in acidic conditions, such as those occurring in the human gastric environment, where pH values range from 1.5 to 3.5 (Huang et al., 2014).

Probiotic strains must be able to survive high concentrations of lysozyme in human saliva, the acid stress conditions and digestive enzymes (pepsin) of the stomach, and the bile in the upper intestine (Corzo and Gilliand, 1999; Marco et al., 2006; Liu et al., 2013). Acid resistance and bile tolerance are considered the basic criteria for screening potential probiotic strains (Liu et al., 2013; Ren et al., 2014), and these features are the focus of several papers on the screening of potential probiotic strains. For example, *Lactobacillus* strains were found resistant to acidic conditions for 3 h, pH 2.5, and strains isolated from food samples were able to grow at a physiological concentration of bile for incubation periods of up to 4 h (Federici et al., 2014). *L. plantarum* N8, N9, ZL5 and *L. casei* ZL4 were resistant to simulated intestinal juice (Wang et al., 2014). *L. acidophilus* La-5 and *Bifidobacterium animalis* subsp. *lactis* BB-12 in goat milk showed higher tolerance to simulated gastric fluid when tested in combination, suggesting a synergistic effect between strains (Ranadheera et al., 2014). *L. fermentum*, *L. casei*, *L. paracasei* and *L. rhamnosus* were able to grow in intestinal juice, and possibly showed a better resistance to bile acid at the beginning of the small intestine (Rubio et al., 2014).

The capacity of lactobacilli to survive at low pH remains controversial (Ren et al., 2014). Mechanisms that may contribute to an adaptive acid tolerance response include the induction of a new pH homeostasis system and the synthesis of a new set of proteins known as stress proteins. Stress proteins can be transiently or constitutively produced and contribute to the adaptive acid tolerance response. Proteins induced after heat shock, including the chaperonin class proteins DnaK and GroEL, have shown homology with proteins produced during acid shock. These proteins are capable of refolding heat-denatured proteins into their native states and can protect proteins from denaturation during cellular stress (Foster, 1993; Shah, 2000). Further, acid tolerance of microorganisms involves multiple genes and mechanisms of protection and regulation (Patnaik et al., 2002; Ye et al., 2013).

Ye et al. (2013) reported that an error-prone whole genome amplification technique was successful in improving the acid tolerance of *L. pentosus*. The possible mechanisms for the increased acid tolerance of the generated mutants included decreased membrane conductivity to protons, increased proton extrusion or an increased buffering capacity of the cytoplasm.

Resistance to bile salts is also an important property for probiotic strains (FAO/WHO, 2002). Ren et al. (2014) affirmed that the most bile-tolerant strains are strongly conducive for relieving the symptoms of lactose intolerance. This statement is in agreement with the study of Noh and Gilliland (1993), who asserted that the permeability of cells of *L. acidophilus* increases in presence of bile, thereby permitting more substrate to enter the cells and increasing the β-galactosidase activity of whole cells.

The bile tolerance by LAB shows a generally greater sensitivity to the deleterious effects of bile in Gram-positive than in Gram-negative bacteria. However, conditions encountered in the external environment or in the host prior to entry into the small intestine will determine the effects of bile on a strain. Exposure to various pH levels, temperatures and growth atmospheres may either ‘‘harden’’ bacteria to the effects of bile or alternatively increase their susceptibility. The presence of food in the intestine may also affect survival, as bacteria may not be exposed to bile in certain microenvironments created by the food matrix or food constituents may even bind bile acids and prevent them from exerting toxicity. Pre-exposure of bacteria to low bile acid levels may increase their tolerance to high levels. Pre-exposure to one stress may also confer protection against other stresses, a phenomenon termed cross-adaptation. Many stresses have similar effects on cellular physiology and may well cause induction of the same set of stress proteins. Many stress management response systems may possibly overlap and be interconnected (Begley et al., 2005). Therefore, the acid and bile tolerance in probiotic strains could likely involve similar mechanisms.

## Adherence to the intestinal epithelial cells

The probiotic adhesion process involves various biophysical and biochemical properties of both probiotics and epithelial cell layers. These include electrostatic interactions, passive and steric forces, hydrophobicity, autoaggregation capacity and specific cellular structures such as external appendages. These properties vary among the probiotic strains and are reported as species-specific characteristics (Schillinger et al., 2005; Collado et al., 2007; Ranadheera et al., 2014). Apart from surviving the passage into the gastrointestinal tract, the probiotic microorganisms must also be able to adhere and to colonize the intestinal epithelium in order to compete with pathogenic organisms (Monteagudo-Mera et al., 2012; Ranadheera et al., 2014). This competition can result in exclusion (Tuomola, 1999), thereby reducing the risk of diseases arising from pathogenic organisms (Chapman et al., 2014) and making this an important selection criterion.

Strains of *L. reuteri* showed a high adherence capacity that could possibly enhance the epithelial barrier *in vitro* within 24 h (Jensen et al., 2012). *L. pentosus* E108, *L. plantarum* B282 and *L. paracasei* subsp. *paracasei* E94 showed good adhesion to Caco-2 cells (Argyri et al., 2013), while *L. casei* 12668-1 and *E. faecalis* 18156-3 showed strong adhesion ability to intestinal cells (Federici et al., 2014). *P. pentosaceus* CIAL-86 showed excellent adhesion and good anti-adhesion activity against

*Escherichia coli* CIAL-153 (García-Ruiz et al., 2014).

Strains of *L. plantarum* N8, N9, ZL5 ZL4 and *L. casei* were able to exert a significant *in vitro* inhibitory activity against *Campylobacter jejuni* and effectively inhibited the adhesion and invasion of HT-29 cells by *C. jejuni* (Wang et al., 2014). The adhesion ability of *Lactobacillus* probiotics could promote good residence time in the intestine, exclusion of pathogens and interaction with host cells to protect epithelial cells and induce immune modulation (Ouwehand et al., 1999).

The difficulties of studying bacterial adhesion *in vivo* led to the development of *in vitro* models for preliminary studies of adherent strains (Vesterlund et al., 2005). Tests such as hydrophobicity and autoaggregation are performed because these cell surface traits are considered necessary for adhesion. These traits facilitate temporary colonization as well as protection of the host system because of the biofilm formation over the host tissue. Some studies have shown that hydrophobicity and autoaggregation are important for promoting the colonization of probiotics in ecological niches such as the intestinal tract or the urogenital tract (Pelletier et al., 1997; Del Re et al., 2000; Giaouris et al., 2009).

Cell surface hydrophobicity is one of the physico-chemical properties that facilitate the first contact between the microorganism and the host cells (Schillinger et al., 2005). The initial and reversible contact stage is mediated by a complex of physicochemical interactions, including hydrophobicity and charges, which are ubiquitously thought to be nonspecific but important properties (Pelletier et al., 1997). Isolates that exhibit high hydrophobicity values are associated with high capacity for autoaggregation and adherence to Caco-2 cells (Kotzamanidis et al., 2010). *Lactobacillus* that showed an affinity for an apolar solvent above 40% generally presented more elevated hydrophobic characteristics (Giaouris et al., 2009). Dias et al. (2013) and Ren et al. (2014) reported *Lactobacillus* strains with high hydrophobicity of 59% and 54.75%, respectively.

The autoaggregation and coaggregation of probiotics are necessary processes for the occurrence of adhesion to the intestinal epithelium and the formation of barriers that prevent colonization by pathogenic microorganisms (Del Re et al., 2000). Aggregation is a phenotype related to cell adherence properties (Pelletier et al., 1997; Kos et al., 2003). According to Del Re et al. (2000), strains with values lower than 10% are designated as non-autoaggregating. Strains of *L. salivarius* and *L. plantarum* showed high capacity for autoaggregation (Ren et al., 2012). *L. salivarius* subsp. *salicinius, L. acidophilus* and *L. plantarum* showed relatively high values for autoaggregation (46, 45 and 34%, respectively) as compared to the reference *L. rhamnosus* strain (33%) (Ren et al., 2014). Four isolates of *Lactobacillus* showed autoaggregation values higher than 90% (Bautista-Gallego et al., 2013).

The coaggregation abilities of the *Lactobacillus* species with potential pathogens might prevent the colonization of the gut by pathogenic bacteria (Bao et al., 2010). Thus, probiotic strains should show the ability to coaggregate with the pathogenic strains tested, but the percentage of coaggregation is strain-specific (Collado et al., 2007). In general, lactobacilli have a higher coaggregation with *Listeria monocytogenes* (Dias et al., 2013). This property may be related to the formation of mixed species biofilms, as reported by Veen and Abee (2011) for *L. monocytogenes* and *Lactobacillus.*

## PHYSIOLOGICAL DESIRABLE CRITERIA

Probiotics can improve the function of the immune system and general health of the host. This review examines the main effects of physiologically desired criteria for a probiotic strain. Some of the health effects are listed in Table 2.

### Lactose intolerance

Primary lactose malabsorption, which is accompanied by a continuous drop in lactase activity during childhood and adolescence and hypolactasia in adulthood, is a normal physiological process in humans. It can result in gastrointestinal discomfort after consuming lactose-containing products, a condition called lactose intolerance (LI).

Symptoms can be unpleasant and include abdominal pain, bloating, flatulence and diarrhoea. Some dairy products, like cheese, contain little or no lactose, whereas yoghurt is often better tolerated than milk (De Vrese, 2014; Kies, 2014). The European Food Safety Authority (EFSA) gave a positive opinion on lactose-reduced foods, the use of lactase-enzyme as a dietary supplement and yoghurt containing viable yoghurt-bacteria (Kies, 2014). The ability of microorganisms to ferment the lactose in milk is an important techno-logical property (Zárate and Chaia, 2012). Accordingly, many studies have focused on the metabolism of lactose to potentially probiotic microorganisms (Iqbal et al., 2010; Rhimi et al., 2010; Zárate and Chaia, 2012).

The lactose-hydrolysing enzyme β-galactosidase (β-D-galactoside galactohydrolase, EC 3.2.1.23) is an enzyme that catalyses the hydrolysis of lactose (a disaccharide found abundantly in milk) to glucose and galactose (Ustok et al., 2010). Possible sources of the enzyme are plants, animal organs, bacteria, yeasts, fungi and moulds. Amongst these, the microbial sources are clearly preferable and are mostly used for both reactions because of their ease of fermentative production, high activities and generally good stability. β-Galactosidase has long been used as an important biocatalyst in the dairy

**Table 2.** Some of the beneficial effects on the health of the host and mechanisms of probiotic strains reported in scientific studies.

| Health effect | Mechanism | Authors |
|---|---|---|
| Relief lactose intolerance | Action of the enzyme β-galactosidase | De Vrese et al. (2014) |
| Treatment and prevention of allergy | Pre-hydrolyzing β-lactoglobulin | Pescuma et al. (2015) |
| Decline in risk associated with tumor development | Decreased levels of enzymes: β- glucuronidase, azoreductase, nitroreductase and activation of pro-carcinogens | Vasiljevic and Shah (2008) |
| Hypocholesterolemic effect | BSH activity | Peres et al. (2014) |
| Inhibition of *Helicobacter pylori* and intestinal pathogens | Secretion protein components or organic acid, decreases the ability of adhesion of H. pylori to gastric epithelial cells, reduces inflammation of the mucosa, and stabilizes the gastric barrier. | Kabir et al. (1997); Zheng et al. (2014) |
| Intestinal immunomodulating effect | β-Supra-regulation of IL-6 in the cecum | Lähteinen et al. (2014) |
| Decreased HIV infection | Exposure of the CD4 receptor by lactobacilli cells by inhibition of HIV-1 | Su et al. (2013) |

industry as well as in synthetic glycochemistry (Iqbal et al., 2010). β-Galactosidases produced by pure and mixed cultures of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus* 77 were characterized as stable at the pH range of 7-9 and the temperature range of 20-37°C, retaining 80-90% of their initial activities. As a result, these enzymes could be considered as potential candidates for the hydrolysis of lactose in milk and milk products (Ustok et al., 2010).

Rhimi et al. (2010) conducted a study where the gene encoding β-galactosidase from the dairy *S. thermophilus* strain LMD9 was cloned, sequenced and expressed in *E. coli*. The purified recombinant enzyme was a powerful biocatalyst for the lactose from whey extract, which is a cheap source of D-galactose and D-glucose. The relevant importance is seen when one considers that these monosaccharides are used in the production of low-calorie or hypocaloric sweeteners. Moreover, this protein efficiently cleaves lactose in milk, and may thus aid in overcoming the lactose intolerance problem generated by milk products. The LI disease affects nearly 70% of the world population, so the consumption of treated products can be a good way to incorporate dairy foods and nutrients into the diets of individuals with lactose intolerance (Vasiljevic and Shah, 2008; Kailasapathy, 2013).

## Prevention and reduction of diarrhoea

Disruption of intestinal microbiota by antibiotics is a major risk factor for diarrhoea and colonization by *Clostridium difficile* (Lawley et al., 2012). This is a common complication for most types of antibiotics, especially broad spectrum antibiotics such as clindamycin, beta lactams and 3rd generation cephalosporins. Rates of *C. difficile* infection have increased worldwide over the years (McFarland, 2009).

Probiotics have several beneficial health applications, such as reduction of colonization of pathogens (Vandenplas et al., 2014). Diarrhoea can be prevented by interrupting one of the potential contributing mechanisms: competition to inhibit the related receptor for growth of pathogens (nutrients, adhesion site), production of defensin or bacteriocin, reduction of the pH of the gut and stimulation of the host immune system (Ng et al., 2009). The effectiveness of probiotics is specific to the species and strain of disease, so the chosen probiotic strain should be linked to the disease (McFarland, 2009).

Studies with strains of *L. plantarum, S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus* in BALB/c mice have shown their importance in strengthening the colonization resistance of the faeces bacteria, suppressing proliferation of opportunistic pathogens (including enteric bacterium and *Enterococcus*) and restoration of the microbiota balance (Tian et al., 2014). A mixture of *Lactobacillus plantarum, Lactobacillus kefir, Lc. lactis, Kluyveromyces marxianus* and *Saccharomyces cerevisiae* was tested in an animal model of infection by *C. difficile* and demonstrated the ability to prevent diarrhoea and enterocolitis triggered by *C. difficile* (Bolla et al., 2013).

## Prevention of inflammatory bowel disease

Crohn's disease (CD) and ulcerative colitis (UC) are the two major forms of inflammatory bowel disease (IBD) and both diseases lead to high morbidity and health care costs (Reiff and Kelly, 2010). Evidence suggests that an abnormality in the response of the innate and adaptive immunity plays an important role in intestinal inflammation (Liu, 2009). Probiotic cultures may reduce the relapse of

the disease after treatment with steroids and/or surgery. Thus, the presence of a diverse microbial population in the gastrointestinal tract and changes that occur during IBD provide adequate support for the use of probiotics in the treatment of this disease (Fedorak and Demeria, 2012).

Huang et al. (2013) reported *in vitro* and *in vivo* anti-inflammatory and immunomodulatory effects of *L. plantarum* K68 isolated from *fu-tsai*, a traditional fermented food in Taiwan. *L. plantarum* K68 ameliorated dextran sulphate sodium induced UC in BALB/c mice through its anti-inflammatory and immunomodulatory activities and may be effective in treating other inflamematory diseases. Juarez et al. (2013) also reported a beneficial effect of *L. reuteri* CRL1101 in decreasing tumor necrosis factor alfa (TNF-α) production in lipopolysaccharide-treated macrophages, indicating it to be a candidate probiotic for the prevention and control of systemic inflammatory diseases.

## Prevention of allergies

The use of probiotics in treatment and prevention of allergies, allowing an adequate stimulation of the immune system, is once again an attractive proposition. Several authors have reported promising results that clearly support the efficacy of probiotics in the prevention of some allergic diseases, thereby justifying experimental trials with a focus on selection of strains to be used and the time of administration of these probiotics (Pelucchi et al., 2012).

Prescott and Bjorkstén (2007) affirmed that allergic disease results from a fundamental failure of underlying immune regulation. Microbial exposure arguably provides the strongest environmental signal for normal postnatal maturation of the immune system and also induces the maturation of antigen presenting cells and T-regulatory cells, which are essential for programming and regulating the T-cell response.

*Lactobacillus rhamnosus* GG reduced the prevalence of atopic eczema in children (Rautava et al., 2002). Specific probiotics may modulate early microbial colonization, which represents the first intervention target in allergic disease, together with their ability to reverse the increased intestinal permeability characteristics in children with atopic eczema and food allergy. Probiotics also enhance gut-specific IgA responses, which are frequently defective in children with food allergy. In addition, probiotics have the potential to alleviate allergic inflammation both locally and systemically.

However, unfortunately, contradictory results among studies do not currently allow a recommendation for probiotic use in the prevention of allergy. These contradictions arise from study variations such as the type of population studied in terms of age, type of allergic disease, disease stage, number of patients included, genetic background, environment (Butel, 2014). Therefore, a cautionary note should be made amidst the continuing public enthusiasm for probiotics until the results are more conclusive (Prescott and Bjorkstén, 2007).

## Probiotics and *Helicobacter pylori*

*H. pylori* is a pathogenic intestinal agent that causes infection over time, which can trigger chronic gastritis and peptic ulcer, and increase the risk of gastric malignancies. Probiotics do not seem to eliminate the population of *H. pylori*, but they can reduce the inflammation associated with these bacteria in both animals and humans (Vasiljevic and Shah, 2008; Kailasapathy, 2013).

A recombinant *L. lactis* expressing Urease B could serve as an antigen-delivering vehicle for the development of an edible vaccine to control *H. pylori* Infection. Urease is an important virulence factor that is required for colonisation of the gastric mucosa by *H. pylori* as it catalyses the conversion of urea into ammonium and $CO_2$, thereby raising pH close to neutrality. Urease B is an effective and common antigen of *H. pylori* and it has been widely investigated as a potential antigen for the development of prophylactic and therapeutic vaccines against *H. pylori* infection (Del Giudice et al., 2001; Zhang et al., 2014).

LAB increases the eradication rate of *H. pylori* infection. They inhibit *H. pylori* growth through secretion of protein components or organic acid, decrease the adherence capacity of *H. pylori* to gastric epithelial cells, reduce mucosal inflammation and stabilize the gastric barrier (Kabir et al., 1997; Zheng et al., 2014). Zheng et al. (2014) reported that *L. pentosus* LPS16 had a broad spectrum action against *H. pylori*.

## Modulation of the immune system

Probiotic cultures may influence the body's immune function. Evidence from *in vitro* systems and animal and humans models suggests that these microorganisms can increase both specific non-specific immune responses, possibly by the activation of macrophages, increased levels of cytokines, increased activity of natural killer cells and/or increased levels of immunoglobulins (Singh et al., 2011; Kailasapathy, 2013).

Lee et al. (2011) evaluated the activity of selected probiotic lactobacilli on immune function by measuring lymphocyte proliferation and IFN-γ. Strains of *L. gasseri* and *L. plantarum* induced higher release of interferon-γ cytokines. *L. fermentum* LA12 and *L. plantarum* (CJMA1, CJLP56, CJLP133, CJLP243, BJ53 and CJNR26) were

more effective at inducing lymphocyte proliferation than the positive control. These strains were characterized as beneficial in terms of immune modulation.

A popular Brazilian fresh cheese (Minas Frescal cheese) containing *L. acidophilus* LA14 and *Bifidobacterium longum* BL05 was fed for 2 weeks to adult Wistar rats. The authors concluded that this probiotic cheese may be a viable alternative to for enhancing the immune system and could be used to prevent infections, particularly those related to the physical overexertion of athletes (Lollo et al., 2012).

Kotzamanidis et al. (2010) affirmed that *L. reuteri* DC421, *L. rhamnosus* DC429 and *L. plantarum* 2035 strains exhibited regulatory activity of early immune responses in both air pouches and intestines that was characterized by stimulation of polymorphonuclear chemotaxis, phagocytic activity, combination of TLR2/TLR4/TLR9 signalling and secretion of a certain cytokine profile. The activation of these specific immune responses by these non-pathogenic microorganisms aids in achievement of homeostasis and provides the healthy host with a higher capacity to resist any inflammatory response.

### Reducing the risk associated with mutagenicity and carcinogenicity

Antigenotoxicity, antimutagenicity and anticarcinogenicity are potential functional properties of probiotics that have received much attention recently. Substantial evidence has been obtained from *in vitro* and animal model studies that support significant anticancer effects of probiotics (Kailasapathy, 2013; Serban, 2014). Genotoxic substances or environmental or autobiotic agents generated within the body can induce genetic changes or damage, leading to mutations and carcinogenesis (Wogan et al., 2004). The best candidates as antimutagens could be natural dietary components, including LAB (Ambalam et al., 2011).

Butyrate is generated from lactate formed by LAB and *Bifidobacterium* (Duncan and Flint, 2013). Butyrate exerts potent effects on a variety of colonic mucosal functions such as inhibition of inflammation and carcinogenesis, reinforcement of various components of the colonic defence barrier and decreasing oxidative stress. In addition, butyrate may promote satiety (Hamer et al., 2008). *L. plantarum* and *Bifidobacterium Bb12* have intrinsic antigenotoxic potential and may have protective effects against the early stages of colon cancer (Burns and Rowland, 2004).

A *L. rhamnosus* (Lr 231) strain isolated from human faeces possesses the ability to bind with acridine orange (AO), N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) and 2-amino-3,8-dimethylimidazo-[4,5-f]-quinoxaline (MeIQx) but not 4-nitro-o-phenylenediamine (NPD), indicating a variation in binding and antimutagenic activity. Instantaneous binding of AO and MNNG by Lr 231 can assist in the rapid removal of mutagens and may thereby prevent their absorption in the intestine. The binding of MNNG and MeIQx by Lr 231 results in a modification of the spectrum of mutagenic agents, implying residual dysmutagenesis associated biotransformation and subsequent detoxification of mutagenic agents. Thus, a potential probiotic human strain of Lr 231 possesses the ability to bind, biotransform and detoxify mutagens, and this property can be useful in formulating fermented foods for removal of potent mutagens (Ambalam et al., 2011).

Sah et al. (2014) reported that probiotic organisms had a statistically significant effect on proteolytic activity that enhanced the generation of peptides with potential antioxidant and antimutagenic properties, with good correlation between proteolytic and antioxidant or antimutagenic activities. The peptides generated by milk fermentation with *Lactobacillus* strains may contribute a variety of bioactive compounds that can have a positive effect on human health and could be applied commercially in new products or in the production of new anticancer peptides.

## CONCLUSION

Probiotic strains can be selected from different environments and food matrices for incorporation into various food products, thereby increasing food functionality and promoting beneficial effects on consumer health. However, these effects require rigorous selection criteria for the establishment of probiotic strains that are safe and functional and that have desirable technological and physiological characteristics. Some screening methods were discussed in this review. Although knowledge has been gained from the existing trials and scientific publications, human studies are still needed to elucidate the mechanisms of interaction between strains and host, since probiotic effects may be strain specific.

**Abbreviations: LAB,** Lactic acid bacteria; **GIT,** gastrointestinal tract; **GRAS,** generally regarded as safe; **CFU,** colony forming units; **MDR,** multidrug resistance; **OM,** outer membrane; **CRISPR,** clustered regularly interspaced short palindromic repeats; **BSH,** bile salt hydrolase; **NADH,** nicotinamide adenine dinucleotide oxidases; **SOD**, superoxide dismutase; **AP,** agar plate; **SC,** secondary culture; **EPS,** exopolysaccharides; **EFSA,** European Food Safety Authority; **CD,** Crohn's disease; **UC,** ulcerative colitis; **IBD,** inflammatory bowel disease; **TNF-α,** Tumor necrosis factor alfa; **NPD,** 4-nitro-o-phenylenediamine, **MNNG,** N-methyl-N'-nitro-N-nitrosoguanidine; **MeIQx,** 2- amino-3,8-dimethylimidazo-

[4,5-f]-quinoxaline; **AO,** acridine orange.

## Conflict of interests

The authors have not declared any conflict of interest.

### REFERENCES


Ahmed Z, Wang Y, Anjum N, Ahmad A, Khan ST (2013). Characterization of exopolysaccharide produced by *Lactobacillus kefiranofaciens* ZW3 isolated from Tibet kefir - Part II. Food Hydrol. 30(1):343-350.

Ambalam P, Dave JM, Nair BM, Vyas BRM (2011). *In vitro* mutagen binding and antimutagenic activity of human *Lactobacillus rhamnosus* 231. Anaerobe 17(5):217-222.

Ammor MS, Flórez AB, Mayo B (2007). Antibiotic resistance in non-enterococcal lactic acid bacteria and bifidobacteria. Food Microbiol. 24(6):559-570.

Anandharaj M, Silvasankari B (2014). Isolation of potential probiotic *Lactobacillus oris* HMI68 from mother's milk with cholesterol-reducing property. J. Biosci. Bioeng. 118(2):153-159.

Ao X, Zhang X, Zhang X, Shi L, Zhao K, Yu J, Dong L, Cao Y, Cai Y (2012). Identification of lactic acid bacteria in traditional fermented yak milk and evaluation of their application in fermented milk products. J. Dairy Sci. 95(3):1073-1084.

Argyri AA, Zoumpopoulou G, Karatzas KAG, Tsakalidou E, Nychas GJE, Panagou EZ, Tassou CC (2013). Selection of potential probiotic lactic acid bacteria from fermented olives by *in vitro* tests. Food Microbiol. 33(2):282-291.

Arief II, Wulandari Z, Aditia EL, Baihaqi M (2014). Physicochemical and microbiological properties of fermented lamb sausages using probiotic *Lactobacillus plantarum* IIA-2C12 as starter culture. Procedia Environ. Sci. 20(1):352-356.

Babot JD, Argañaraz-Martínez E, Saavedra L, Apella MC, Perez Chaia A (2014). Selection of indigenous lactic acid bacteria to reinforce the intestinal microbiota of newly hatched chicken - relevance of *in vitro* and *ex vivo* methods for strains characterization. Res. Vet. Sci. 97(1):8-17.

Badel S, Bernardi T, Michaud P (2011). New perspectives for Lactobacilli exopolysaccharides. Biotech. Adv. 29(1): 54-66.

Balciunas EM, Martinez FAC, Todorov SD, Franco BDGM, Converti A, Oliveira RPS (2013). Novel biotechnological applications of bacteriocins: A review. Food Control 32(1):134-142.

Bao Y, Zhang Y, Zhang Y, Yong L, Shuiquan W, Ximei D, Yanyan W, Zhang H (2010). Screening of potential probiotic properties of *Lactobacillus fermentum* isolated from traditional dairy Food Control products. Food Control 21(5):695-701.

Barbosa J, Gibbs PA, Teixeira P (2010). Virulence factors among enterococci isolated from traditional fermented meat products produced in the North of Portugal. Food Control 21(5):651-656.

Barrangou R, Fremaux C, Deveau H, Richards M, Boyaval P, Moineau S, Romero DA, Horvath P (2007). CRISPR provides acquired resistance against viruses in prokaryotes. Sci. 315(5819): 1709-1712.

Bautista-Gallego J, Arroyo-López FN, Rantsiou K, Jiménez-Díaz R, Garrido-Fernández A, Cocolin L (2013). Screening of lactic acid bacteria isolated from fermented table olives with probiotic potential. Food Res. Int. 50(1):135-142.

Beal C, Fonseca F, Corrieu G (2001). Resistance to freezing and frozen storage of *Streptococcus thermophiles* is related to membrane fatty acid composition. J. Dairy Sci. 84(11): 2347-2356.

Begley M, Gahan CGM, Hill C (2005). The interaction between bacteria and bile. FEMS Microbiol. Rev. 29(4):625-651.

Begley M, Hill C, Gahan CCM (2006). Bile Salt Hydrolase Activity in Probiotics. Appl. Environ. Microbiol. 72(3):1729-1738.

Blana VA, Grounta A, Tassou CC, Nychas GJE, Panagou EZ (2014). Inoculated fermentation of green olives with potential probiotic *Lactobacillus pentosus* and *Lactobacillus plantarum* starter cultures isolated from industrially fermented olives. Food Microbiol. 38(1):208-218.

Bolla PA, Carasi P, Bolla MDLA, De Antoni GL, Serradell MDLA (2013). Protective effect of a mixture of kefir-isolated lactic acid bacteria and yeasts in a hamster model of *Clostridium difficile* infection. Anaerobe 21(1):28-33.

Brazil (2007). Ministério da Agricultura, Pecuária e Abastecimento, Instrução Normativa nº46, from November 24th. Aprova o Regulamento técnico de identidade e qualidade de Leites Fermentados. Diário Oficial da União, Nov 24, 2007. Available at http://sistemasweb.agricultura.gov.br/sislegis /action/detalhaAto.do?method=gravarAtoPDF&tipo=INM&numeroAto =00000046&seqAto=000&valorAno=2007&orgao=MAPA&codTipo=& desItem=&desItemFim= Accessed September 10, 2014. (Online).

Bremer E, Kramer R (2000). Coping with osmotic challenges: osmoregulation through accumulation and release of compatible solutes in bacteria. In Storz G, Hengge-Areonis R (Eds.), Bacterial stress response. ASM Press, Washington, D.C.

Briggiler-Marcó M, Mercanti D, Reinheimer JA, Quiberoni A (2011). Performance of spontaneous phage-resistant derivatives of *Lactobacillus plantarum* in fermented milk manufacture. Int. Dairy J. 21(11):857-862.

Briggiler-Marcó M, Zacarías MF, Vinderola G, Reinheimer JA, Quiberoni A (2014). Biological and probiotic characterisation of spontaneous phage-resistant mutants of *Lactobacillus plantarum*. Int. Dairy J. 39(1):64-70.

Brogden KA, Roth JA, Stanton TB, Bolin CA, Minion FC, Wannemuehler MJ (2000). Virulence Mechanisms of Bacterial Pathogens, 3rd edn. ASM Press, Washington DC.

Buckenhüskes HJ (1993). Selection criteria for lactic acid bacteria to be used as starter cultures for various food commodities. FEMS Microbiol. Rev. 12(1-3):253-271.

Burgain J, Scher J, Francius G, Borges F, Corgneau M, Revol-Junelles AM, Cailliez-Grimal C, Gaiani C (2014). Lactic acid bacteria in dairy food: Surface characterization and interactions with food matrix components. Adv. Colloid Interface Sci. 213(1):21-35.

Burns AJ, Rowland IR (2004). Antigenotoxicity of probiotics and prebiotics on faecal water-induced DNA damage in human colon adenocarcinoma cells. Mutat. Res. 551(1-2):233-243.

Burns P, Cuffia F, Milesi M, Vinderola G, Meinardi C, Sabbag N, Hynes E (2012). Technological and probiotic role of adjunct cultures of non-starter lactobacilli in soft cheeses. Food Microbiol. 30(1):45-50.

Butel MJ (2014). Probiotics, gut microbiota and health. Med. Maladies Infect. 44(1):1-8.

Carvalho AS, Silva J, Ho P, Teixeira P, Malcata FX, Gibbs P (2004). Effects of various sugars added to growth and drying media upon thermotolerance and survival throughout storage of freezedried *Lactobacillus delbrueckii* ssp. *bulgaricus*. Biotechnol Prog. 20(1): 248-254.

Champagne CP, Ross RP, Saarela M, Hansen KF, Charalampopoulos D (2011). Recommendations for the viability assessment of probiotics as concentrated cultures and in food matrices. Int. J. Food Microbiol. 149(3):185-193.

Chapman CMC, Gibson GR, Rowland I (2014). Effects of single- and multi-strain probiotics on biofilm formation and *in vitro* adhesion to bladder cells by urinary tract pathogens. Anaerobe 27(1):71-76.

Chen L, Yang J, Yu J, Yao Z, Sun L, Shen Y, Jin Q (2005). VFDB: a reference database for bacterial virulence factors. Nucleic Acids Res. 33(1):325-332.

Clark S, Potter ED (2007). Cottage Cheese. In: Handbook of food products manufacturing: health, meat, milk, poultry, seafood and vegetables. hoboken. pp. 618-633.

Coelho MC, Silva CCG, Ribeiro SC, Dapkevicius MLNE, Rosa HJD (2014). Control of *Listeria monocytogenes* in fresh cheese using protective lactic acid bacteria. Int. J. Food Microbiol. 191(1):53-59.

Collado MC, Meriluoto JS, Salminen S (2007). Measurement of aggregation properties between probiotics and pathogens: *In vitro* evaluation of different method. J. Microbiol. Methods. 71(1):71-74.

Coman MM, Cecchini C, Verdenelli MC, Silvi S, Orpianesi C, Cresci A (2012). Functional foods as carriers for SYNBIO®, a probiotic bacteria combination. Int. J. Food Microbiol. 157(3):346-352.

Corcoran BM, Stanton C, Fitzgerald GF, Ross RP (2005). Survival of probiotic lactobacilli in acidic environments is enhanced in the presence of metabolizable sugars. Appl. Environ. Microbiol. 71(6):3060-3067.
Corzo G, Gilliland SE (1999). Bile salt hydrolase activity of three strains of *Lactobacillus acidophilus*. J. Dairy Sci. 82(3):472-480.
Cotter PD, Hill C (2003). Surviving the acid test: responses of Gram-positive bacteria to low pH. Microbiol. Mol. Biol. R. 67(3):429-453.
De Souza Barbosa MDS, Todorov SD, Ivanova I, Chobert JM, Haertlé T, de Melo Franco BDG (2015). Improving safety of salami by application of bacteriocins produced by an autochthonous *Lactobacillus curvatus* isolate. Food Microbiol. 46(1): 254-262.
De Vrese M, Laue C, Offick B, Soeth E, Repenning F, Thoß A, Schrezenmeir J (2014). A combination of acid lactase from *Aspergillus oryzae* and yogurt bacteria improves lactose digestion in lactose maldigesters synergistically: A randomized, controlled, double-blind cross-over trial. Clin. Nutr. 14(1):1-6.
De Vuyst L, Vin FD, Degeest FVB (2001). Recent developments in the biosynthesis and applications of heteropolysaccharides from lactic acid bacteria. Int. Dairy J. 11(9):687-707.
Del Giudice G, Covacci A, Telford JL, Montecucco C, Rappuoli R (2001). The design of vaccines against *Helicobacter pylori* and their development. Annu. Rev. Immunol. 19(1): 523-563.
Del Re B, Sgorbati B, Miglioli M, Palenzona D (2000). Adhesion, autoaggregation and hydrophobicity of 13 strains of *Bifidobacterium longum*. Lett. Appl. Microbiol. 31(6):438-442.
Dias FS, Duarte WF, Santos MRRM, Ramos EM, Schwan RF (2013). Screening of Lactobacillus isolated from pork sausages for potential probiotic use and evaluation of the microbiological safety of fermented products. J. Food Protect. 76(6):991-998.
Duncan SH, Flint HJ (2013). Probiotics and prebiotics and health in ageing populations. Maturitas 75(1):44-50.
Erkkilä S, Petäjä E (2000). Screening of commercial meat starter cultures at low pH and in the presence of bile salts for potential probiotic use. Meat Sci. 55(3): 297-300.
FAO / WHO (2002). Orientações para a avaliação de probióticos na alimentação. London, Ontario: Organização para Alimentação e Agricultura do relatório das Nações Unidas e do Grupo de Trabalho da Organização Mundial da Saúde (2002). pp 1-11.
Federici S, Ciarrocchi F, Campana R, Ciandrini E, Blasi G, Baffone W (2014). Identification and functional traits of lactic acid bacteria isolated from Ciauscolo salami produced in Central Italy. Meat Sci. 98(4):575-584.
Fedorak R, Demeria D (2012). Probiotic bacteria in the prevention and the treatment of inflammatory bowel disease. Gastroenterol Clin. North A. 41(4):821-842.
Fonseca F, Beal C, Corrieu G (2000). Method of quantifying the loss of acidification activity of lactic acid starters during freezing and frozen storage. J. Dairy Res. 67(1):83-90.
Foschino R, Fiori E, Galli A (1996). Survival and residual activity of *Lactobacillus acidophilus* frozen cultures under different conditions. J. Dairy Res. 63(2):295-303.
Foster JW (1993). The acid tolerance response of *Salmonella typhimurium* involves transient synthesis of key acid shock proteins. J. Bacteriol. 175(7):1981-1987.
Franciosi E, Settanni L, Cavazza A, Poznanski E (2009). Biodiversity and technological potential of wild lactic acid bacteria from raw cows' milk. Int. Dairy J. 19(1):3-11.
Fröhlich-Wyder MT, Guggisberg D, Badertscher R, Wechsler D, Wittwer A, Irmler S (2013). The effect of *Lactobacillus buchneri* and *Lactobacillus parabuchneri* on the eye formation of semi-hard cheese. Int. Dairy J. 33(2): 120-128.
Gaglio R, Scatassa ML, Cruciata M, Miraglia V, Corona O, Di Gerlando R, Portolano B, Moschetti G, Settanni L (2014). *In vivo* application and dynamics of lactic acid bacteria for the four-season production of Vastedda-like cheese. Int. J. Food Microbiol. 177(1): 37-48.
García-Ruiz A, González de Llano D, Esteban-Fernández A, Requena T, Bartolomé B, Moreno-Arribas MV (2014). Assessment of probiotic properties in lactic acid bacteria isolated from wine. Food Microbiol. 44(1):220-225.
Ge XY, Yuan J, Qin H, Zhang WG (2011). Improvement of L-lactic acid production by osmotic-tolerant mutant of *Lactobacillus casei* at high temperature. Appl. Microbiol. Biotechnol. 89(1):73-78.
Giaouris E, Chapot-Chartier MP, Briandet R (2009). Surface physicochemical analysis of natural *Lactococcus lactis* strains reveals the existence of hydrophobic and low charged strains with altered adhesive properties. Int. J. Food Microbiol. 131(1):2-9.
Giraffa G (1995). Enterococcal bacteriocins: their potential as anti-Listeria factors in dairy technology. Food Microbiol. 12(1): 291-299.
Gregoret V, Perezlindo MJ, Vinderola G, Reinheimer J, Binetti A (2013). A comprehensive approach to determine the probiotic potential of human-derived *Lactobacillus* for industrial use. Food Microbiol. 34(1):19-28.
Hamer HM, Jonkers D, Venema K, Vanhoutvin S, Troost FJ, Brummer RJ (2008). Review article: the role of butyrate on colonic function. Aliment Pharm. Ther. 27(2): 104-119.
Harty DWS, Oakey HJ, Patrikakis M, Hume EBH, Knox KW (1994). Pathogenic potential of lactobacilli. Food Microbiol. 24(1-2):179-189.
Hor YY, Liong MT (2014). Use of extracellular extracts of lactic acid bacteria and bifidobacteria for the inhibition of dermatological pathogen *Staphylococcus aureus*. Dermatol. Sin. 32(3):141-147.
Horvath P, Coûté-Monvoisin AC, Romero DA, Boyaval P, Fremaux C, Barrangou R (2009). Comparative analysis of CRISPR loci in lactic acid bacteria genomes. Int. J. Food Microbiol. 131(1): 62-70.
Huang HY, Hsieh HY, King VAE, Chi LL, Tsen JH (2014). To pre-challenge lactic acid bacteria with simulated gastrointestinal conditions is a suitable approach to studying potential probiotic properties. J. Microbiol. Methods. 107(1):138-146.
Huang HY, Korivi M, Tsai CH, Yang JH, Tsai YC (2013). Supplementation of *Lactobacillus plantarum* K68 and fruit-vegetable ferment along with high fat-fructose diet attenuates metabolic syndrome in rats with insulin resistance. Evid. Based Complement. Alternat. Med. 2013(1):1-12.
Iqbal S, Nguyen TH, Nguyen TT, Maischberger T, Haltrich D (2010). beta-Galactosidase from *Lactobacillus plantarum* WCFS1: biochemical characterization and formation of prebiotic galacto-oligosaccharides. Carbohydr. Res. 345(10):1408-1416.
Ishibashi N, Yamazaki S (2001). Probiotics and safety. Am. J. Clin. Nutr. 73(2):465-470.
Jensen H, Grimmer S, Naterstad K, Axelsson L (2012). *In vitro* testing of commercial and potential probiotic lactic acid bacteria. Int. J. Food Microbiol. 153(1-2):216-222.
Juarez GE, Villena J, Salva S, de Valdez GF, Rodriguez AV (2013). *Lactobacillus reuteri* CRL1101 beneficially modulate lipopolysaccharide-mediated inflammatory response in a mouse model of endotoxic shock. J. Funct. Foods 5(4):1761-1773.
Kabir AM, Aiba Y, Takagi A, Kamiya S, Miwa T, Koga Y (1997). Prevention of *Helicobacter pylori* infection by lactobacilli in a gnotobiotic murine model. Gut 41(1):49-55.
Kailasapathy K (2013). Commercial sources of probiotic strains and their validated and potential health benefits-a review. Int. J. Fermented Foods 2(1):1-17.
Kargozari M, Moini S, Akhondzadeh Basti A, Emam-Djomeh Z, Gandomi H, Revilla Martin I, Ghasemlou M, Carbonell-Barrachina AA (2014). Effect of autochthonous starter cultures isolated from Siahmazgi cheese on physicochemical, microbiological and volatile compound profiles and sensorial attributes of sucuk, a Turkish dry-fermented sausage. Meat Sci. 97(1):104-114.
Kies AK (2014). Authorised EU health claims related to the management of lactose intolerance: reduced lactose content, dietary lactase supplements and live yoghurt cultures. Foods, Nutrients and Food Ingredients with Authorised Eu Health Claims. pp. 177-211.
Kodali VP, Das S, Sen R (2009). An exopolysaccharide from a probiotic: Biosynthesis dynamics, composition and emulsifying activity. Food Res. Int. 42(5-6):695-699.
Kołozyn-Krajewska D, Dolatowskib ZJ (2012). Probiotic meat products and human nutrition. Process Biochem. 47(12): 1761-1772.
Korbekandi H, Mortazavian AM, Iravani S (2011). Technology and stability of probiotic in fermented milks. In Shah N, Cruz AG, Faria JAF (Eds.), Probiotic and prebiotic foods: Technology, stability and

benefits to the human health. Nova Science Publishers. NewYork. pp. 131-169.
Kos B, kovic JS, Vukovic S, Simpraga M, Frece J, Matosic S (2003). Adhesion and aggregation ability of probiotic strain *Lactobacillus acidophilus* M92. J. Appl. Microbiol. 94(6):981-987.
Kotzamanidis C, Kourelis A, Litopoulou-Tzanetaki E, Tzanetakis N, Yiangou M (2010). Evaluation of adhesion capacity, cell surface traits and immunomodulatory activity of presumptive probiotic *Lactobacillus* strains. Int. J. Food Microbiol. 140(2-3):154-163.
Kulisaar T, Zilmer M, Mikelsaar M, Vihalemm T, Annuk H, Kairane C, Kilk A (2002). Two antioxidative strains as promising probiotics. Int. J. Food Microbiol. 72(3):215-224.
Lähteinen T, Lindholma A, Rinttilä T, Junnikkalaa S, Kanta R, Pietilä TA, Levonenc K, Ossowskia I, Solano-Aguilarb G, Jakava-Viljanenc M, Palva A (2014). Effect of *Lactobacillus brevis* ATCC 8287 as a feeding supplement on the performance and immune function of piglets. Vet. Immunol. Immunop. 158(1-2):14-25.
Lamothe GT, Jolly L, Mollet B, Stingele F (2002). Genetic and biochemical characterization of exopolysaccharide biosynthesis by *Lactobacillus delbrueckii subsp. bulgaricus*. Arch. Microbiol.178(3):218-228.
Landeta G, Curiel JA, Carrascosa AV, Muñoz R, De Las Rivas V (2013). Technological and safety properties of lactic acid bacteria isolated from Spanish dry-cured sausages. Meat Sci. 95(2):272-280.
Lawley TD, Clare S, Walker AW, Stares MD, Connor TR, Raisen C, Gouldin D, Rad R, Schreiber F, Brandt C, Deakin LJ, Pickard DJ, Duncan SH, Flint HJ, Clark TG, Parkhill J, Dougan G (2012). Targeted restoration of the intestinal microbiota with a simple, defined bacteriotherapy resolves relapsing *Clostridium difficile* disease in mice. PLoS Pathog. 8(10):995-1002.
Lee J, Yun HS, Cho KW, Oh S, Kim SH, Chun T, Kim B, Whang KY (2011). Evaluation of probiotic characteristics of newly isolated *Lactobacillus spp*.: immune modulation and longevity. Int. J. Food Microbiol. 148(2):80-86.
Leite MO, Leite DC, Del Aguila EM, Alvares TS, Peixoto RS, Miguel ML, Silva JT, Paschoalin VMF (2013). Microbiological and chemical characteristics of Brazilian kefir during fermentation and storage processes. J. Dairy Sci. 96(7):4149-4159.
Li W, Ji J, Rui X, Yu J, Tang W, Chen X, Jiang M, Dong M (2014). Production of exopolysaccharides by *Lactobacillus helveticus* MB2-1 and its functional characteristics *in vitro*. LWT-Food Sci. Technol. 59(2): 732-739.
Liu C, Lu J, Lu L, Liu Y, Wang F, Xiao M (2010). Isolation, structural characterization and immunological activity of an exopolysaccharide produced by *Bacillus licheniformis* 8-37-0-1. Bioresour. Technol. 101(14):5528-5533.
Liu X, Liu W, Zhang Q, Tian F, Wang G, Zhang H, Chen W (2013). Screening of lactobacilli with antagonistic activity against enteroinvasive *Escherichia coli*. Food Control 30(2):563-568.
Liu ZJ (2009). Potential role of Th17 cells in the pathogenesis of inflammatory bowel disease. World J. Gastroenterol. 15(46):5784-5788.
Lollo PCB, Cruz AG, Morato PN, Moura CS, Carvalho-Silva LB, Oliveira CAF, Faria F, Amaya-Farfan J (2012). Probiotic cheese attenuates exercise-induced immune suppression in Wistar rats. J. Dairy Sci. 95(7):3549-3558.
Lopez-Kleine L, Monnet V (2011). Lactic Acid Bacteria Proteolytic System. Encyclopedia of Dairy Sciences (Second Edition), Four-Volume Set. Academic Press. pp. 49-55.
Luo Z, Gasasira V, Huang Y, Liu D, Yang X, Jiang S, Hu W (2013). Effect of *Lactobacillus salivarius* H strain isolated from Chinese dry-cured ham on the color stability of fresh pork. Food Sci. Hum. Wellness 2(3-4):139-145.
Mahony J, van Sinderen D (2014). Novel strategies to prevent or exploit phages in fermentations, insights from phage-host interactions. Curr. Opin. Biotechnol. 32(1):8-13.
Marco ML, Pavan S, Kleerebezem M (2006). Towards understanding molecular modes of probiotic action. Curr. Opin. Biotechnol. 17(2):204-210.
Marteau P, Gerhardts MF, Myaras A, Bouvieri E, Trivins F, Rambaud JC (1995). Metabolism of Bile Salts by Alimentary Bacteria during Transit in the Human Small Intestine. Microb. Ecol. Health D. 8(4):151-157.
Mättö J, Alakomi HL, Vaari A, Virkajärvi I, Saarela M (2006). Influence of processing conditions on *Bifidobacterium animalis subsp. Lactis* functionality with a special focus on acid tolerance and factors affecting it. Int. Dairy J. 16(9):1029-1037.
McFarland LV (2009). Evidence-based review of probiotics for antibiotic-associated diarrhea and *Clostridium difficile* infections. Anaerobe 15(6):274-280.
McGann LE (1978). Differing actions of penetrating and non-penetrating cryoprotective agents. Cryobiology 15(4):382-390.
Miller RA, Britigan BE (1997). Role of oxidants in microbial pathophysiology. Clin. Microbiol. 10(1):1-18.
Monteagudo-Mera A, Rodríguez-Aparicio L, Rúa J, Martínez-Blanco H, Navasa N, García-Armesto MR, Ferrero MÁ (2012). *In vitro* evaluation of physiological probiotic properties of different lactic acid bacteria strains of dairy and human origin. J. Funct. Foods 4(2):531-541.
Morelli L (2007). *In vitro* assessment of probiotic bacteria: From survival to functionality. Int. Dairy J. 17(11):1278-1283.
Muñoz-Atienza E, Araújo C, Magadán S, Hernández PE, Herranz C, Santos Y, Cintas LM (2014). *In vitro* and *in vivo* evaluation of Lactic Acid Bacteria of aquatic origin as probiotics for turbot (*Scophthalmus maximus* L.) farming. Fish Shellfish Immunol. 41(2):570-580.
Muñoz MCC, Benomar N, Lerma LL, Gálvez A, Abriouel H (2014). Antibiotic resistance of *Lactobacillus pentosus* and *Leuconostoc pseudomesenteroides* isolated from naturally-fermented Aloreña table olives throughout fermentation process. Int. J. Food Microbiol. 172(1):110-118.
Ng SC, Hart AL, Kamm MA, Stagg AJ, Knight SC (2009). Mechanisms of action of probiotics: recent advances. Inflamm. Bowel Dis. 15(2):300-310.
Nikolic M, López P, Strahinic I, Suárez A, Kojic M, Fernández-García M, Topisirovic L, Golic N, Ruas-Madiedo P (2012). Characterisation of the exopolysaccharide (EPS)-producing *Lactobacillus paraplantarum* BGCG11 and its non-EPS producing derivative strains as potential probiotics. Int. J. Food Microbiol. 158(2):155-162.
Noh DO, Gilliland S (1993). Influence of Bile on Cellular Integrity and Galactosidase Activity of *Lactobacillus acidophilus*. J. Dairy Sci. 76(5):1253-1259.
Ouwehand AC, Kirjavainen PV, Grönlund MM, Isolauri E, Salminen SJ (1999). Adhesion of probiotic micro-organisms to intestinal mucus. Int. Dairy J. 9(9):623-630.
Paik HD, Lee JY (2014). Investigation of reduction and tolerance capability of lactic acid bacteria isolated from kimchi against nitrate and nitrite in fermented sausage condition. Meat Sci. 97(4):609-614.
Palomino JM, Toledo del Árbol J, Benomar N, Abriouel H, Martínez Cañamero M, Gálvez A, Pulido R ( 2014). Application of *Lactobacillus plantarum* Lb9 as starter culture in caper berry fermentation. LWT - Food Sci. Technol. 60(2):788-794.
Patnaik R, Louie S, Gavrilovic V, Perry K, Stemmer WPC, Ryan CM, del Cardayré S (2002). Genome shuffling of *Lactobacillus* for improved acid tolerance. Nat. Biotechnol. 20(7): 707-712.
Pedersen TB, Ristagn D, McSweeney PLH, Vogense FKY, Ard Y (2013). Potential impact on cheese flavour of heterofermentative bacteriafrom starter cultures. Int. Dairy J. 33(2):112-119.
Pelletier C, Bouley CC, Bouttier S, Bourlioux P, Bellon-Fontaine MN (1997). Cell surface characteristics of *Lactobacillus casei subsp. casei, Lactobacillus paracasei subsp. paracasei*, and *Lactobacillus rhamnosus* strains. Appl. Environ. Microbiol. 63(5):1725-1731.
Pelucchi C, Chatenoud L, Turati F, Galeone C, Moja L, Bach JF, La Vecchia C (2012). Probiotics supplementation during pregnancy or infancy for the prevention of atopic dermatitis: a meta-analysis. Epidemiology 23(3):402-414.
Pereira CI, Crespo MTB, Romao MVS (2001). Evidence for proteolytic activity and biogenic amines production in *Lactobacillus curvatus* and *L. homohiochii.* Int. J. Food Microbiol. 68(3):211-216.
Peres CM, Alves M, Mendoza AH, Moreira L, Silva S, Bronze MR, Vilas-Boas L, Peres C, Malcata X (2014). Novel isolates of lactobacilli

from fermented Portuguese olive as potential probiotics. LWT - Food Sci. Technol. 59(1):234-246.
Perin LM, Miranda RO, Todorov SD, Franco BDGM, Nero LA (2014). Virulence, antibiotic resistance and biogenic amines of bacteriocinogenic lactococci and enterococci isolated from goat milk. Int. J. Food Microbiol. 185(1):121-126.
Pescuma M, Elvira MH, Haertlé T, Chobert JM, Mozzi F, Valdez GF (2015). *Lactobacillus delbrueckii subsp. bulgaricus* CRL 454 cleaves allergenic peptides of b-lactoglobulin. Food Chem. 170(1):407-414.
Prescott S, Björkstén B (2007). Probiotics for the prevention or treatment of allergic diseases. J. Allergy Clin. Immunol. 120(2):255-262.
Ranadheera CS, Evans CA, Adams MC, Baines SK (2014). Effect of dairy probiotic combinations on *in vitro* gastrointestinal tolerance, intestinal epithelial cell adhesion and cytokine secretion. J. Funct. Foods 8(1):18-25.
Rautava S, Kalliomäki M, Isolauri E (2002). Probiotics during pregnancy and breast-feeding might confer immunomodulatory protection against atopic disease in the infant. J. Allergy Clin. Immunol. 109(1):119-121.
Reale A, Di Renzo T, Rossi F, Zotta T, Lacumin L, Preziuso M, Parente E, Sorrentino E, Coppola R (2015). Tolerance of *Lactobacillus casei*, *L. paracasei* and *L. rhamnosus* strains to stress factors encountered in food processing and in the gastro-intestinal tract. LWT-Food Sci. Technol. 60(1):721-728.
Reiff C, Kelly D (2010). Inflammatory bowel disease, gut bacteria and probiotic therapy. Int. J. Med. Microbiol. 300(1):25-33.
Ren D, Li C, Qin Y, Yin R, Du S, Ye F, Liu C, Liu H, Wang M, Li Y, Sun Y, Li X, Tian M, Jin N (2014). *In vitro* evaluation of the probiotic and functional potential of *Lactobacillus* strains isolated from fermented food and human intestine. Anaerobe 30(1):1-10.
Ren D, Li C, Qin Y, Yin R, Li X, Tian M, Dub S, Guob H, Liu C, Zhub N, Sunb D, Li Y, Jin N (2012). Inhibition of *Staphylococcus aureus* adherence to Caco-2 cells by lactobacilli and cell surface properties that influence attachment. Anaerobe 18(5):508-515.
Rhimi M, Boisson A, Dejob M, Boudebouze S, Maguin E, Haser R, Aghajari N (2010). Efficient bioconversion of lactose in milk and whey: immobilization and biochemical characterization of a β-galactosidase from the dairy *Streptococcus thermophilus* LMD9 strain. Res. Microbiol. 161(7):515-525.
Rincon-Delgadillo MI, Lopez-Hernandez A, Wijaya I, Rankin SA (2012). Diacetyl levels and volatile profiles of commercial starter distillates and selected dairy foods. J. Dairy Sci. 95(3):1128-1139.
Rivas FP, Castro MP, Vallejo M, Marguet E, Campos CA (2014). Sakacin Q produced by *Lactobacillus curvatus* ACU-1: functionality characterization and antilisterial activity on cooked meat surface. Meat Sci. 97(4):475-479.
Rivera-Espinoza Y, Gallardo-Navarro Y (2010). Non-dairy probiotic products. Food Microbiol. 27(1):1-11.
Ross RP, Desmond C, Fitzgerald G, Stanton C (2005). Overcoming the technological hurdles in the development of probiotic foods. J. Appl. Microbiol. 98(6):1410-1417.
Rubio R, Jofré A, Martín B, Aymerich T, Garriga M (2014). Characterization of lactic acid bacteria isolated from infant faeces as potential probiotic starter cultures for fermented sausages. Food Microbiol. 38(1):303-311.
Ruiz-Moyano S, Martín A, Benito MJ, Casquete R, Serradilla MJ, Córdoba MG (2009). Safety and functional aspects of pre-selected lactobacilli for probiotic use in Iberian dry fermented sausages. Meat Sci. 83(3):460-467.
Saarela M, Mogensen G, Fondén R, Mättö J, Mattila-Sandholm T (2000). Probiotic bacteria: safety, functional and technological properties. J. Biotechnol. 84(3):197-215.
Sah BNP, Vasiljevic T, McKechnie S, Donkor ON (2014). Effect of probiotics on antioxidant and antimutagenic activities of crude peptide extract from yogurt. Food Chem. 156(1):264-70.
Savijoki K, Ingmer H, Varmanen P (2006). Proteolytic systems of lactic acid bacteria. Appl. Microbiol. Biotechnol. 71(4):394-406.
Schillinger U, Guigas C, Holzapfel HW (2005). *In vitro* adherence and other properties of lactobacilli used in probiotic yoghurt-like products. Int. Dairy J. 15(12): 1289-1297.
Senz M, Lengerich BV, Bader J, Stahl U (2015). Control of cell morphology of probiotic *Lactobacillus acidophilus* forenhanced cell stability during industrial processing. Int. J. Food Microbiol. 192(1):34-42.
Serban DE (2014). Gastrointestinal cancers: Influence of gut microbiota, probiotics and prebiotics. Cancer Lett. 345(2):258-270.
Shah NP (2000). Probiotic Bacteria: Selective Enumeration and Survival in Dair. Foods J. Dairy Sci. 83(4):894-907.
Shalaby AR (1996). Significance of biogenic amines to food safety and human health. Food Res. Int. 29(7):675-690.
Shao Y, Zhang W, Guo H, Pan L, Zhang H, Sun T (2015). Comparative studies on antibiotic resistance in *Lactobacillus casei* and *Lactobacillus plantarum*. Food Control 50(1):250-258.
Sharma P, Tomar SK, Goswami P, Sangwan V, Singh R (2014). Antibiotic resistance among commercially available probiotics. Food Res. Int. 57(1):176-195.
Sidira M, Kandylis P, Kanellaki M, Kourkoutas Y (2015). Effect of immobilized *Lactobacillus casei* on the evolution of flavor compounds in probiotic dry-fermented sausages during ripening. Meat Sci. 100(1):41-51.
Singh K, Kallali B, Kumar A, Thaker V (2011). Probiotics: A review. Asian. Pac. J. Trop. Biomed. 1(2):287-290.
Smit G, Smit BA, Engels WJM (2005). Flavour formation by lactic acid bacteria and biochemical flavour profiling of cheese products. FEMS Microbiol. Rev. 29(3):591-610.
Smukowski M, Wendorff WL, Ping Y, Rao RD (2003). Impact of cheese defects on U.S. graded cheeses. J. Dairy Sci. 86(suppl. 1): 364.
Sorek R, Kunin V, Hugenholtz P (2008). CRISPR—a widespread system that provides acquired resistance against phages in bacteria and archaea. Nat. Rev. Microbiol. 6(3):181-186.
Su Y, Zhanga B, Su L (2013). CD4 detected from *Lactobacillus* helps understand the interaction between *Lactobacillus* and HIV. Microbiol. Res. 168(5):273-277.
Talwalkar A, Miller CW, Kailasapathy K, Nguyen MH (2004). Effect of packaging materials and dissolved oxygen on the survival of probiotic bacteria in yoghurt. Int. J. Food Sci. Technol. 39(6):605-611.
Tammam JD, Williams AG, Banks J, Cowie G, Lloyd D (2001). Membrane inlet mass spectrometric measurement of O2 and CO2 gradients in cultures of *Lactobacillus paracasei* and a developing Cheddar cheese ecosystem. Int. J. Food Microbiol. 65(1-2):11-22.
Tian P, Xu B, Sun H, Li X, Li Z, Wei P (2014). Isolation and gut microbiota modulation of antibiotic-resistant probiotics from human feces. Diagn. Micr. Infect. Dis. 79(4):405-412.
Tripathi MK, Giri SK (2014). Probiotic functional foods: Survival of probiotics during processing and storage. J. Funct. Foods 9(1):225-241.
Tulumoğlu S, Kaya HI, Şimşek Ö (2014). Probiotic characteristics of *Lactobacillus fermentum* strains isolated from tulum cheese. Anaerobe 30(1):120-125.
Tuomola E (1999). The effect of probiotic bacteria on the adhesion of pathogens to human intestinal mucus. Fems. Immunol. Med. Mic. 26(2):137-142.
Ustok FI, Tari C, Harsa S (2010). Biochemical and thermal properties of b-galactosidase enzymes produced by artisanal yoghurt cultures. Food Chem. 119(3):1114-1120.
Vandenplas Y, Huys G, Daube G (2014). Probiotics: an update. J. Pediatr. doi:10.1016/j.jped.2014.08.005
Vasiljevic T, Shah NP (2008). Probiotics—from Metchnikoff to bioactives. Int. Dairy J. 18(7):714-728.
Veen SVD, Abee T (2011). Mixed species biofilms of *Listeria monocytogenes* and *Lactobacillus plantarum* show enhanced resistance to benzalkonium chloride and peracetic acid. Int. J. Food Microbiol. 144(3):421-431.
Vesterlund S, Paltta J, Karp M, Ouwehand AC (2005). Measurement of bacterial adhesion-*in vitro* evaluation of different methods. J. Microbiol. Method. 60(2): 225-233.
Vesterlund S, Vankerckhoven V, Saxelin M, Goossens H, Salminen S, Ouwehand AC (2007). Safety assessment of *Lactobacillus* strains: presence of putative risk factors in faecal, blood and probiotic isolates.

Int. J. Food Microbiol. 116(3):325-331.
Wang G, Zhao Y, Tian F, Jin X, Chen H, Liu X, Zhanga Q, Zhaoa J, Chena Y, Zhang H, Chen W (2014). Screening of adhesive lactobacilli with antagonistic activity against *Campylobacter jejuni*. Food Control 44(1):49-57.
Welman AD, Maddox IS (2003). Exopolysaccharides from lactic acid bacteria perspectives and challenges. Trends Biotechnol. 21(6):269-274.
Whitehead HR, Cox GA (1935). The occurrence of bacteriophage in cultures of lactic Streptococci: A preliminary note. NZJ. Dairy Sci. Technol. 16(1):319-320.
Wogan GN, Hecht SS, Felton JS, Conney AH, Loeb LA (2004). Environmental and chemical carcinogenesis. Semin. Cancer Biol. 14(6):473-486.
Xião Z, Lu RJ (2014). Strategies for enhancing fermentative production of acetoin: A review. Biotechnol. Adv. 32(2):492-503.
Ye L, Zhao H, Li Z, Wu JC (2013). Improved acid tolerance of *Lactobacillus pentosus* by error-prone whole genome amplification. Bioresource Technol. 135(1):459-463.
Yee AL, Maillard M, Roland N, Chuat V, Leclerc A, Pogačić T, Valence F, Thierry A (2014). Great interspecies and intraspecies diversity of dairy propionibacteria in the production of cheese aroma compounds. Int. J. F. Microbiol. 191(1):60-68.
Zago M, Fornasari ME, Carminati D, Burns P, Suàrez V, Vinderola G, Reinheimer J, Giraffa G (2011). Characterization and probiotic potential of *Lactobacillus plantarum* strains isolated from cheeses. Food Microbiol. 28(5):1033-1040.
Zárate G, Pérez Chaia A (2012). Feeding with dairy *Propionibacterium acidipropionici* CRL 1198 reduces the incidence of Concanavalin-A induced alterations in mouse small intestinal epithelium. Food Res. Int. 47(1):13-22.
Zhang H, Qiu Y, Zhao Y, Liu X, Liu M, Yu A (2014). Immunogenicity of oral vaccination with *Lactococcus lactis* derived vaccine candidate antigen (UreB) of *Helicobacter pylori* fused with the human interleukin 2 as adjuvant. Mol. Cell. Probes 28(1):25-30.
Zhang Q, Lin S, Nie X (2013). Reduction of biogenic amine accumulation in silver carp sausage by an amine-negative *Lactobacillus plantarum*. Food Control 32(2):496-500.
Zheng PX, Fang HY, Yang HB, Tien NY, Wang MC, Wu JJ (2014). *Lactobacillus pentosus* strain LPS16 produces lactic acid, inhibiting multidrug-resistant *Helicobacter pylori*. J. Microbiol. Immunol. Infect. 1-7. doi: http://dx.doi.org/10.1016/j.jmii.2014.04.014

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# Microbial diversity analysis of subclinical mastitis in dairy cattle in Northeast China

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**The microbial diversity of a pooled milk sample (H) from healthy dairy cows in the Daqing region of Heilongjing Province in northeastern China was compared to that of four pooled milk samples (M1-M4) from dairy cows from the same area previously diagnosed with subclinical mastitis, based on the California mastitis test, using pyrosequencing of the metagenomic *16S* ribosomal RNA genes. A total of 22 759 valid sequences were obtained from the five pooled milk samples, and the average length of a valid sequence was 484 bp. The composition of the bacterial communities of the M1, M2, M3, and M4 samples was significantly different from that of the H sample. The bacterial diversity of the M1 sample was highly similar to that of M2 sample; the bacterial diversity of the M3 sample was highly similar to that of M4 sample. The *Streptococcus* and *Staphylococcus* spp. were highly prevalent in the M1, M2, and M4 samples, and *Pseudomonas* spp. were highly prevalent in the M1, M2, and M3 samples. The levels of *Acinetobacter*, *Lactococcus*, and *Flavobacteriaceae* spp. in the M1, M2, M3, and M4 samples were lower than those of the H sample, which represents potential risk factors for subclinical mastitis. Our findings provide valuable information relevant to the prevention and treatment of subclinical mastitis in dairy cows in northeastern China.**

**Key words:** subclinical mastitis, microbial diversity, pyrosequencing.

## INTRODUCTION

Mastitis is the inflammation of the mammary gland and udder tissue, and is a major endemic disease of dairy cattle in cows-producing countries. Dairy cow mastitis causes economic losses due to reduced milk production, discarded milk, premature culling, and increased antibiotic usage (Halasa et al., 2007). The incidence rate of subclinical mastitis can reach 36.4%~85.7% on some diary farms in China (Ding et al., 2011). Compared with clinical mastitis, subclinical mastitis causes greater economic losses because of delays in treatment caused

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by the apparent lack of symptoms (Annapoorani et al., 2007).

Environmental microbial infections are considered to be the primary risk factors for mastitis in dairy cattle (Smith et al., 2005; Ericsson et al., 2009; Braem et al., 2012; Hogan and Smith, 2012). In previous decades, conventional bacterial culture, polymerase chain reaction (PCR), and PCR-denaturing gradient gel electrophoresis were widely used to identify and analyze the pathogenic bacteria associated with mastitis in dairy cattle (Kuang et al., 2009;Koskinen et al., 2010; Braem et al., 2011; Gurjar et al., 2012). In a recent study, Bhatt and others (2012) analyzed the microbiome signatures of milk samples from cows with subclinical mastitis using a shotgun pyrosequencing method, and Oikonomou et al. (2012) analyzed the microbial diversity of bovine mastitis-associated bacteria using pyrosequencing of the metagenomic 16s ribosomal RNA genes (16SrDNA). Metagenomic sequencing strategies obtain substantial information regarding the microbial composition of milk samples from dairy cows with mastitis.

The incidence of subclinical mastitis in dairy cows is closely related with environmental conditions. In different regions, the pathogenic bacteria associated with mastitis in dairy cattle may exhibit significant differences. In our current study, we used pyrosequencing of metagenomic 16S rDNA to analyze the microbial diversity of milk samples from dairy cows with subclinical mastitis in the Daqing area of Heilongjiang Province in northeastern China. Our findings provide valuable information for the prevention and treatment of subclinical mastitis in dairy cattle.

## MATERIALS AND METHODS

### Milk sampling

Twenty (20) milk samples (approximately 5 mL each) were collected aseptically in sterile centrifuge tube from Holstein cows with subclinical mastitis that were diagnosed using the California mastitis test (CMT) in 2013. The udder was washed thoroughly with potassium permanganate solution (1: 1000), and the teats were wiped with 70% ethyl alcohol prior to sampling. Based on their CMT rating, the CMT±, CMT+, CMT++, and CMT+++ cases were assigned to the M1, M2, M3, and M4 group, respectively, with five cows in each group. Milk samples from each cow were combined to form a pooled sample for the respective group. Milk samples were also collected from five healthy, unaffected cows from the affected dairy herd, and combined to form one pooled sample (H), which served as a control for the comparisons of microbial diversity. The H, M1, M2, M3, and M4 pooled samples were stored at -80°C.

### DNA extraction, PCR amplification, and pyrosequencing

Genomic DNA was extracted from 1 ml of the H, M1, M2, M3, and M4 milk samples using the EZNA bacterial DNA isolation kit (Omega Biotek, Guangzhou, China), according to the manufacturer's instructions. The extracted bacterial genomic DNA was analyzed by electrophoresis on 1% agarose gel. The V1-V3 region of the 16S rDNA in each sample, which contains a unique 10-bp barcode sequence used for multiplexed pyrosequencing, was amplified using the 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 533R (5'-TTACCGCGGCTGCTGGCAC-3') oligonucleotide primers (Oikonomou et al., 2012). The PCR was performed in triplicate using 5 µl of 5× buffer, 0.5 µl of extracted genomic DNA, 0.4 µM each primer, 2.5 U TransStart Fastpfu DNA Polymerase (TransGen Biotech, Beijing, China), 0.25 mM deoxynucleotide triphophates, and deionized water in a total volume of 25 µl. The protocol was optimized to reduce the number of cycles to ensure acceptable levels of accuracy and reliability in the subsequent data analysis. Thermal cycling was performed as follows: 95°C for 5 min; 25 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s; and 72°C for 5 min. The PCR triplicates were pooled, and 2 µl of the pooled PCR products was analyzed on a 2% agarose gel. The PCR products were purified using a DNA gel extraction kit (Axygen, Hangzhou, China), and quantified using a QuantiFluor-ST Fluorometer (Promega, Madison, WI, USA). Pyrosequencing was performed from the A-end by a commercial service provider (Majorbio Bio-Pharm Technology, Shanghai, China) using a Roche 454A sequencing primer kit on a Roche GS-FLX Titanium platform genome sequencer (Roche Diagnostics, Indianapolis, IN, USA).

### Bioinformatics analysis

The trimmed sequences were clustered to operational taxonomic units (OTUs) at phylotype similarity level of 97% by using the furthest neighbor method in the Mothur software package (www.mothur.org). Rarefaction analysis was performed using Mothur, and the rarefaction curve was generated using the R software (R Foundation for Statistical Computing, Vienna, Austria). The Shannon diversities and the Chao1 richness estimations were calculated using Mothur. The microbial-community barplot was generated using the R software, based on the data in the tax-level files. A heatmap figure and Venn diagrams were constructed using the R software. The principal component analysis (PCA) was performed to demonstrate clustering using the VEGAN computational tool in the R software. The similarity data were analyzed using the Jest method (Jaccard coefficient using richness estimators) and the Mothur software, and a similarity tree was generated using the R software.

## RESULTS

### Pyrosequencing of bacteria in milk from cows with subclinical mastitis

The 16S rDNA in the H, M1, M2, M3 and M4 samples were analyzed by metagenomic pyrosequencing of the V1-V3 region. A total of 22 759 valid sequences were obtained from the five pooled PCR samples, and the average length of a valid sequence was 484 bp (Table 1). After trimming and quality control, 20 470 sequences were obtained, and the average trimmed sequence length was 498 bp, accounting for 89.95% of the valid sequences. The number of the trimmed sequences in the H, M1, M2, M3, and M4 samples ranged from 3747 to 4464. A total of 1195 OTUs were obtained, with 97% sequence identity (0.03 cutoff distance) based on the Chao 1 and Shannon indices in Table 2. The OTUs ranged

**Table 1.** Valid sequences and trimmed sequences of bacterial 16S rRNA genes identified from milk samples with subclinical mastitis by pyrosequencing.

| Sample | Valid sequences | | Trimmed sequences | | Percent (trimmed/valid) |
|---|---|---|---|---|---|
| | Sequence no. | Average length (bp) | Sequence no. | Average length (bp) | |
| H | 5003 | 466 | 4339 | 496 | 86.72 |
| M1 | 4393 | 491 | 3995 | 501 | 90.94 |
| M2 | 4257 | 476 | 3747 | 498 | 88.02 |
| M3 | 4820 | 492 | 4464 | 498 | 92.59 |
| M4 | 4286 | 493 | 3925 | 498 | 91.58 |
| Total | 22759 | 484 | 20470 | 498 | 89.95 |

**Table 2.** Chao1 and Shannon indices for different cutoff distances (0.01, 0.03 and 0.05) and for different samples.

| Sample name | Distance cutoff | Number of samples/sequences analyzed | Clusters (OTUs) | Chao1 | Shanon index |
|---|---|---|---|---|---|
| H | 0.01 | 1/4228 | 446 | 1075 | 2.71 |
| | 0.03 | | 236 | 404 | 2.27 |
| | 0.05 | | 173 | 257 | 1.97 |
| M1 | 0.01 | 1/3963 | 510 | 1168 | 4.00 |
| | 0.03 | | 301 | 583 | 3.38 |
| | 0.05 | | 233 | 383 | 3.2 |
| M2 | 0.01 | 1/3689 | 452 | 1246 | 3.52 |
| | 0.03 | | 260 | 550 | 2.93 |
| | 0.05 | | 203 | 350 | 2.75 |
| M3 | 0.01 | 1/4449 | 399 | 1168 | 2.37 |
| | 0.03 | | 201 | 407 | 2.02 |
| | 0.05 | | 145 | 291 | 1.83 |
| M4 | 0.01 | 1/3901 | 382 | 1248 | 2.98 |
| | 0.03 | | 197 | 448 | 2.58 |
| | 0.05 | | 141 | 254 | 2.39 |

from 197 to 301. The rarefaction curves showed a trend toward a saturation plateau, demonstrating the validity of the data with regard to a greater number of sequences producing fewer OTUs (Figure 1).

## Classification and analysis of bacteria in milk from cows with subclinical mastitis

The bacterial community barplot of the H, M1, M2, M3, and M4 samples at the genera level are shown in Figure 2. The bacterial genera in the H, M1, M2, M3, and M4 samples included *Acinetobacter*, *Lactococcus*, *Pseudomonas*, *Sphingobacterium*, *Streptococcus*, *Staphylococcus*, *Empedobacter*, *Chryseobacterium*, *Flavobacterium*, and *Brochothrix*. The low-abundance bacterial genera in each sample were grouped as “others.” The relative abundance of bacterial genera among the five samples was significantly different, especially with regard to the levels of *Streptococcus* and *Staphylococcus* spp., which are commonly reported pathogens associated with mastitis in dairy cattle.

The hierarchical heatmap was generated based on the identified bacterial community in the samples at the genera level (Figure 3). The heatmap of the five samples indicated the existence of two different groups, one of

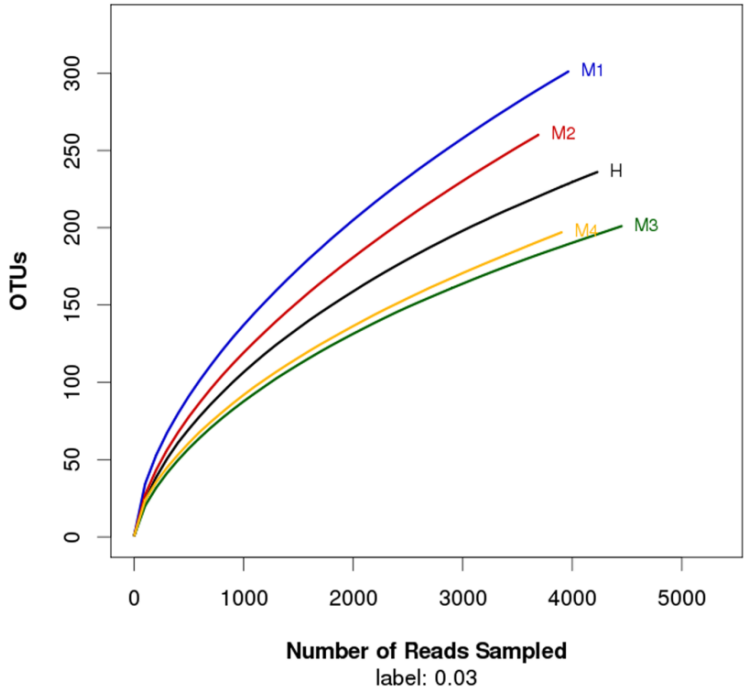


**Figure 1.** Rarefaction analysis of the H, M1, M2, M3, and M4 milk samples. Rarefaction curves of the OTUs clustered at a phylotype similarity level of 97%.

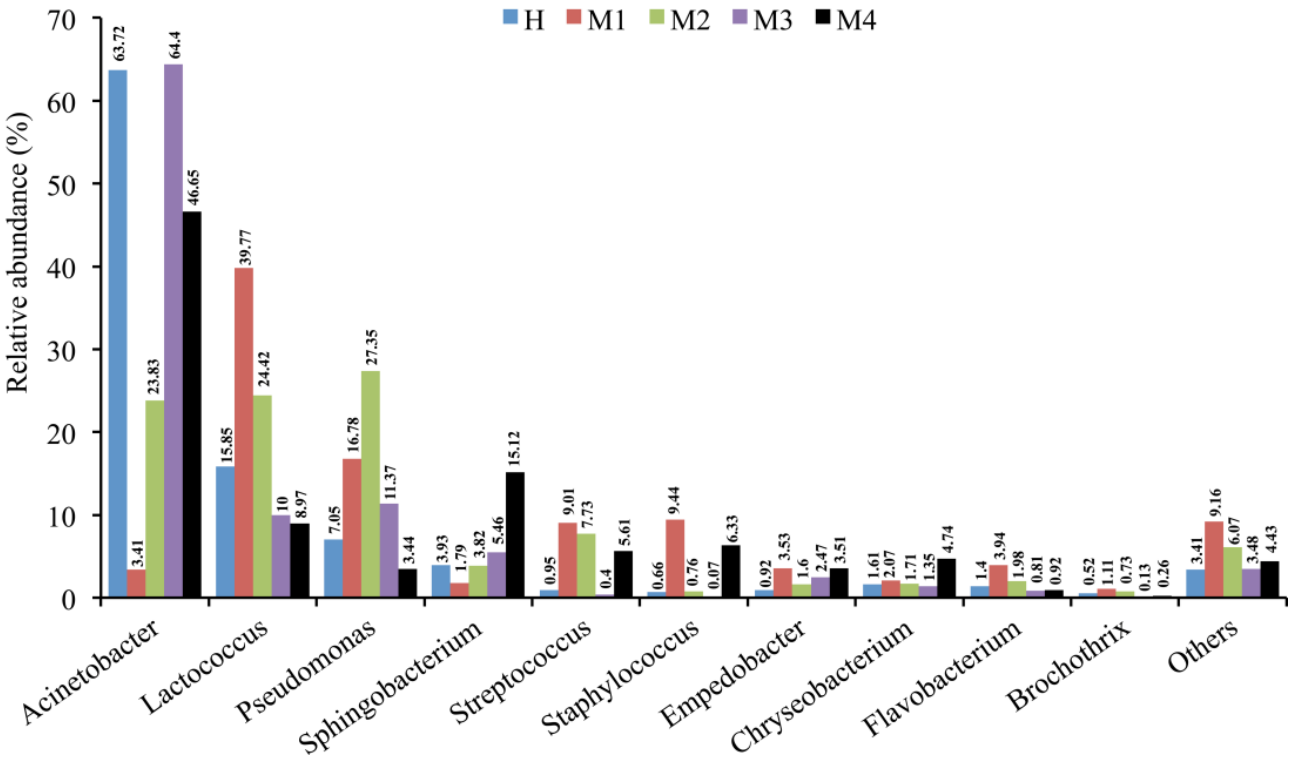


**Figure 2.** Bacterial community barplot of the H, M1, M2, M3, and M4 milk samples at the genera level. The relative abundance (%) was calculated based on the number of sequences of each bacterial genera in each sample.

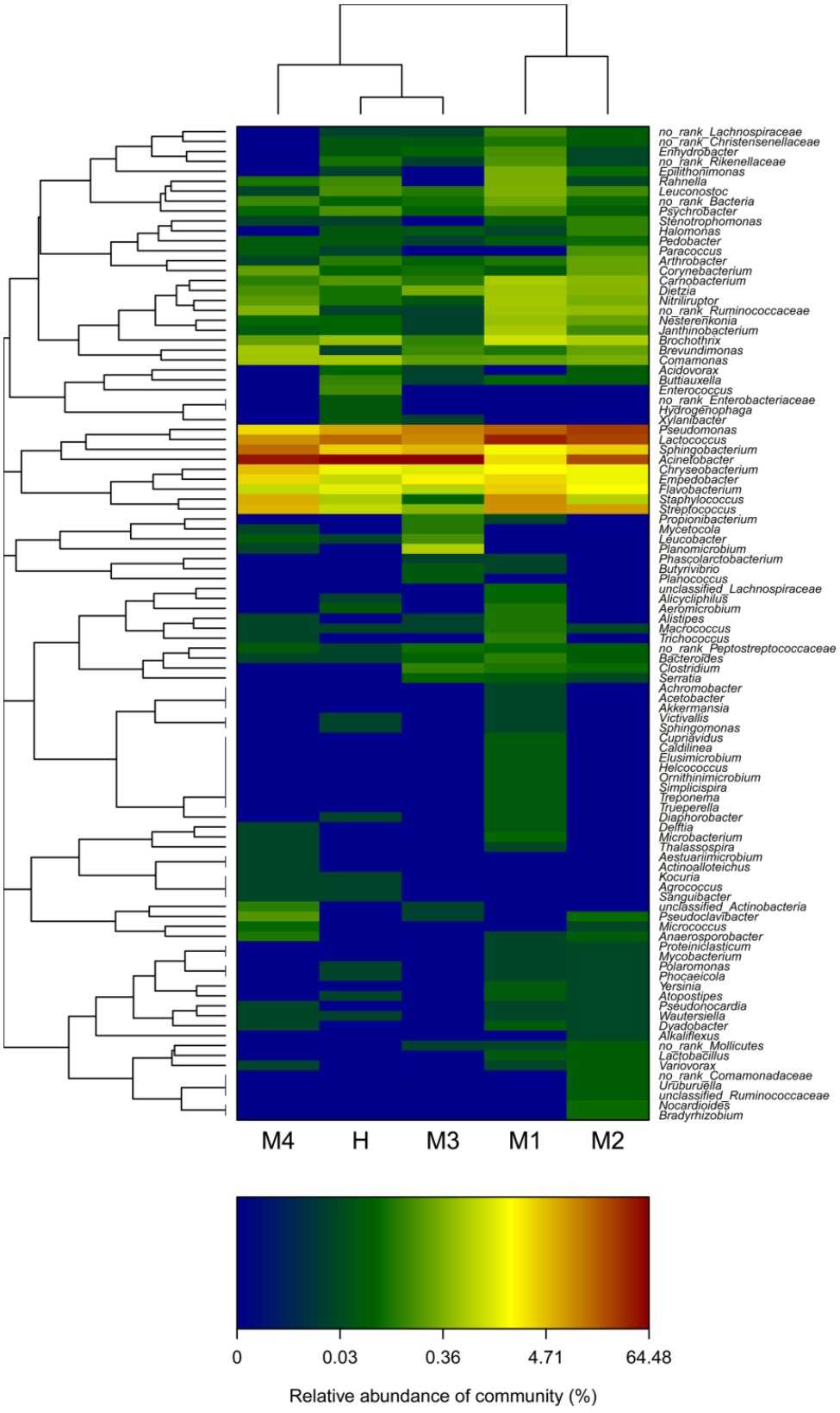


**Figure 3.** Heatmap figure of the bacterial-community composition of the H, M1, M2, M3, and M4 milk samples.

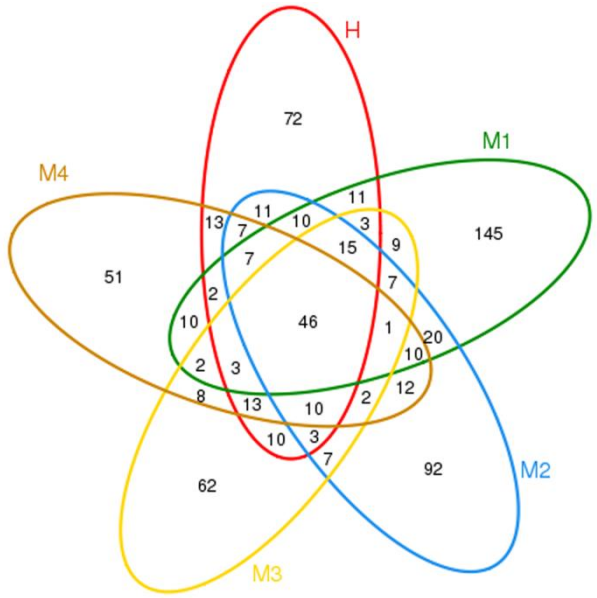


**Figure 4.** Venn diagram of the OTUs of the H, M1, M2, M3, and M4 milk samples at a distance level of 3%.

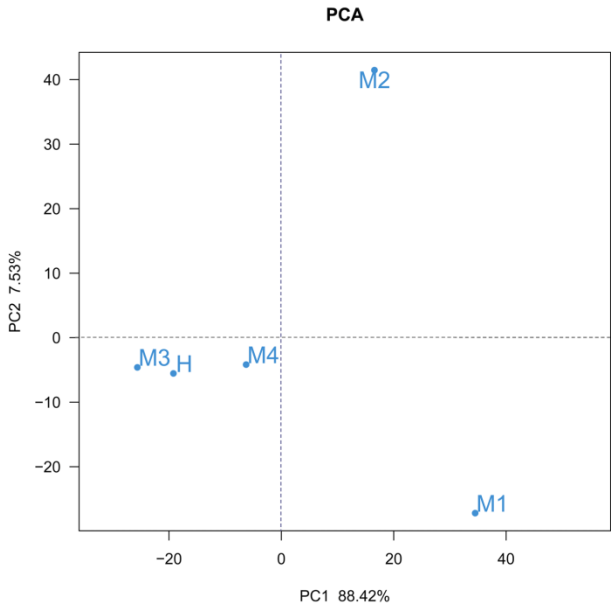


**Figure 5.** Scatter plot of the principal components analysis of the H, M1, M2, M3, and M4 milk samples. The PC1 and PC2 exhibited 88.42 and 7.53% of the variance, respectively.

which included the H, M3, and M4 samples, and the other included the M1 and M2 samples. The bacterial community of the H sample from the healthy dairy cows did not occupy a separate branch in the heatmap, and


**Figure 6.** Similarity tree of the H, M1, M2, M3, and M4 milk samples.

showed a high level of similarity with the M3 sample. The M1 sample had a high level of similarity with the M2 sample; the M3 sample had a high level of similarity with the M4 sample.

The shared communities among the five samples are depicted in the Venn diagrams in Figure 4. The shared OTUs of the M1, M2, M3, and M4 subclinical mastitis samples accounted for 4.9% of the 959 OTUs obtained for the four samples, of which the M1 and M2 samples shared the greatest number, at 116. The H, M1, M2, M3, and M4 samples shared 46 OTUs which accounted for 3.85% of the 1195 OTUs obtained for the five samples, and the shared OTUs of the H and M2 samples is 109.

The bacterial communities of the H, M3, and M4 samples were grouped in the bottom left of the PCA graph along the PC1, whereas the bacterial communities of the M1 and M2 samples were scattered throughout different areas of the PCA plot (Figure 5). Compared with the M1 and M2 samples, the bacterial communities of the H, M3, and M4 samples were more similar, which was consistent with the results of the heatmap analysis. In addition, the structure of similarity tree of the H, M1, M2, M3, and M4 samples was consistent with the PCA plot and heatmap (Figure 6).

## DISCUSSION

Among the genera comprising the bacterial communities in the H, M1, M2, M3, and M4 samples, *Acinetobacter*, *Lactococcus*, *Empedobacter*, *Pseudomonas*, and *Chryseodobacterium* spp. have been reported as bacterial isolates from raw cow's milk (Hagi et al., 2013), and pathogenic *Streptococcus*, *Staphylococcus*, and *Flavobacterium* spp. have been reported in dairy cows (Rampon and Barbesier, 1960; Sela et al., 2007; Rato et

al., 2013; Wang et al., 2013). In addition, *Lactococcus* is an increasingly reported cause of bovine mastitis (Plumed-Ferrer et al., 2013). However, *Sphingobacterium* and *Brochothrix* spp., which were also identified in the five samples, have not been previously reported in dairy cows with mastitis.

Compared with the milk in the H sample from healthy cows, the proportions of *Lactococcus* (39.77%), *Pseudomonas* (16.78%), *Streptococcus* (9.01%), *Staphylococcus* (9.44%), *Empedobacter* (3.53%), and *Flavobacterium* (3.94%) were higher in the M1 sample, and the proportions of *Lactococcus* (24.42%), *Pseudomonas* (27.35%), and *Streptococcus* (7.73%) were higher in the M2 sample. In addition, the proportions of *Acinetobacter* (64.4%), *Pseudomonas* (11.37%), *Empedobacter* (2.47%), and *Sphingobacterium* (5.46%) in the M3 sample were higher than those in the H sample, and the proportions of *Sphingobacterium* (15.12%), *Streptococcus* (5.61%), *Staphylococcus* (6.33%), *Empedobacter* (3.51%), and *Chryseobacterium* (4.74%) in the M4 sample were higher than those in the H sample.

*Streptococcus* and/or *Staphylococcus* spp. were abundant in the M1, M2, and M4 samples, which is consistent with previous reports of *Staphylococcus* or *Streptococcus* associated with mastitis in dairy cattle (Saei et al., 2009; Kozytska et al., 2010; Piccinini et al., 2010; Demon et al., 2012). *Pseudomonas* spp., which was highly abundant in the M1, M2, and M3 samples, were reported to be associated with an outbreak of mastitis. In addition, the relative abundance of *Acinetobacter* was significantly lower in the M1, M2, and M4 samples, compared with that of the H sample. The relative abundance of *Lactococcus* in the M3 and M4 samples was lower than that of the H sample. Thus, the differences in the abundance of *Acinetobacter* and *Lactococcus* spp. may be related to the incidence of subclinical mastitis.

The *Flavobacterium*, *Empedobacter*, and *Chryseobacterium* genera belong to the family *Flavobacteriaceae*, which primarily include environmental bacteria. The presence of *Flavobacterium*, *Empedobacter*, and *Chryseobacterium* in the milk of cows with subclinical mastitis might provide novel information for the pathogenic bacteria associated with mastitis in dairy cattle. The Gram-positive firmicutes are generally considered as contagious mastitis pathogens, and the Gram-positive bacteria *Streptococcus* and *Staphylococcus* are the most common cause of bovine mastitis in China (Yang et al., 2013; Alkasir et al., 2013). In our current study, *Streptococcus* and *Staphylococcus* had the higher relative abundance in the milk of cows with subclinical mastitis. However, these genera comprised only 0.07% to 9.44% of the total bacteria in each sample. The relative abundance of *Streptococcus* and *Staphylococcus* in milk samples may provide novel understanding between these bacteria and both clinical and subclinical mastitis in dairy cattle.

Oikonomou et al. (2012) reported that high numbers of anaerobic bacterial sequences were obtained from all of the mastitis cases in their study using pyrosequencing of metagenomic 16s rDNA, suggesting a possible role of anaerobic pathogens, such as *Fusobacterium*, in bovine mastitis. In our current study, *Fusobacterium* spp. was not found in any of the milk samples. Such differences in the prevalence of anaerobic pathogens in mastitis cases might be associated with geographical differences. *Pseudomonas* spp. were highly abundant in three of the milk samples from cows with subclinical mastitis, suggesting that *Pseudomonas* spp. are a common cause of subclinical mastitis, which is consistent with a previous study of the microbiome signatures of milk samples from cows with subclinical mastitis (Bhatt et al., 2012).

The California mastitis test (CMT) is the most commonly used indirect method of evaluating somatic cell count. The ±, +, ++, and +++ scores of the CMT provide a qualitative assessment of subclinical mastitis in dairy cattle. In our current study, the M1, M2, M3, and M4 milk samples, which represented the ±, +, ++, and +++ CMT scores, respectively, exhibited different bacterial-community compositions. The M3 and M4 samples had similar bacterial communities, and the bacterial communities of the M1 and M2 samples were also similar. The bacterial-community composition of the H, M3, and M4 samples was clustered together in the PCA and the similarity tree. This finding suggests that subclinical mastitis may be caused by a variety of different pathogenic bacteria. Thus, other factors, such as viruses, may contribute to the development of subclinical mastitis.

In conclusion, our pyrosequencing analysis of 16SrDNA sequences revealed a high level of microbial diversity in the milk from cows with subclinical mastitis in northeastern China. Although the interpretation of our findings might be limited by the small number of milk samples tested and the small geographic area from which the milk was sampled, our results provide important basic information regarding microbial diversity that increase our understanding of the etiology of subclinical mastitis in dairy cattle.

## Conflict of interests

The authors did not declare any conflict of interest.

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**REFERENCES**

Alkasir R, Liu X, Zahra M, Ferreri M, Su J, Han B (2013). Characteristics of *Staphylococcus aureus* small colony variant and its parent strain isolated from chronic mastitis at a dairy farm in Beijing, China. Microb. Drug Resist. 19(2):138-145.

Annapoorani C, Dante S, Douglas D (2007). Antimicrobial activity of bovine bactericidal permeability-increasing protein-derived peptides against gram-negative bacteria isolated from the milk of cows with clinical mastitis. Am. J. Vet. Res. 68(11):1151-1159.

Bhatt VD, Ahir VB, Koringa PG, Jakhesara SJ, Rank DN, Nauriyal DS, Kunjadia AP, Joshi CG (2012). Milk microbiome signatures of subclinical mastitis-affected cattle analysed by shotgun sequencing. J. Appl. Microbiol. 112(4):639-650.

Braem G, DE Vliegher S, Supré K, Haesebrouck F, Leroy F, DE Vuyst L (2011). (GTG) 5-PCR fingerprinting for the classification and identification of coagulase-negative Staphylococcus species from bovine milk and teat apices: a comparison of type strains and field isolates. Vet. Microbiol. 147(1-2):67-74.

Braem G, DE Vliegher S, Verbist B, Heyndrickx M, Leroy F, DE Vuyst L (2012). Culture-independent exploration of the teat apex microbiota of dairy cows reveals a wide bacterial species diversity. Vet. Microbiol. 157(3-4):383-390.

Demon D, Ludwig C, Breyne K, Guédé D, Dörner JC, Froyman R, Meyer E (2012). The intramammary efficacy of first generation cephalosporins against Staphylococcus aureus mastitis in mice. Vet. Microbiol. 160(1-2):141-150.

Ding BL, Feng JZ, Zhang GW (2011). Mastitis in dairy cattle. China Agriculture Press, pp. 40.

Ericsson Unnerstad H, Lindberg A, Persson Waller K, Ekman T, Artursson K, Nilsson-Ost M, Bengtsson B (2009) Microbial aetiology of acute clinical mastitis and agent-specific risk factors. Vet. Microbiol. 137(1-2):90-97.

Gurjar A, Gioia G, Schukken Y, Welcome F, Zadoks R, Moroni P (2012). Molecular diagnostics applied to mastitis problems on dairy farms. Vet. Clin. North Am. Food Anim. Pract. 28(3):565-576.

Hagi T, Sasaki K, Aso H, Nomura M (2013). Adhesive properties of predominant bacteriain raw cow's milk to bovine mammary gland epithelial cells. Folia Microbiol. (Praha) 58(6):515-522.

Halasa T, Huijps K, Osteras O, Hogeveen H (2007). Economic effects of bovine mastitis and mastitis management: A review. Vet. Q. 29(1):18-31.

Hogan J, Smith KL (2012). Managing environmental mastitis. Vet. Clin. North. Am. Food Anim. Pract. 28(2):217-224.

Koskinen MT, Wellenberg GJ, Sampimon OC, Holopainen J, Rothkamp A, Salmikivi L, van Haeringen WA, Lam TJ, Pyörälä S (2010). Field comparison of real-time polymerase chain reaction and bacterial culture for identification of bovine mastitis bacteria. J. Dairy Sci. 93(12):5707-5715.

Kozytska S, Stauss D, Pawlik MC, Hensen S, Eckart M, Ziebuhr W, Witte W, Ohlsen K (2010). Identification of specific genes in Staphylococcus aureus strains associated with bovine mastitis. Vet. Microbiol. 145(3-4):360-365.

Kuang Y, Tani K, Synnott AJ, Ohshima K, Higuchi H, Nagahata H, Tanji Y (2009). Characterization of bacterial population of raw milk from bovine mastitis by culture-independent PCR–DGGE method. Biochem. Eng. J. 45(1):76-81.

Oikonomou G, Machado VS, Santisteban C, Schukken YH, Bicalho RC (2012). Microbial diversity of bovine mastitic milk as described by pyrosequencing of metagenomic 16s rDNA. PLoS One 7(10):e47671.

Piccinini R, Borromeo V, Zecconi A (2010). Relationship between *S. aureus* gene pattern and dairy herd mastitis prevalence. Vet. Microbiol. 145(1-2): 100-105.

Plumed-Ferrer C, Uusikylä K, Korhonen J, von Wright A (2013). Characterization of *Lactococcus lactis* isolates from bovine mastitis. Vet. Microbiol. 167(3-4):592-599.

Rampon R, Barbesier J (1960). Contribution to the bacteriologic study of milk of cows affected with mastitis in Algeria (1955-1959). Arch Inst. Pasteur Alger 38:216-221.

Rato MG, Bexiga R, Florindo C, Cavaco LM, Vilela CL, Santos-Sanches I (2013). Antimicrobial resistance and molecular epidemiology of *Streptococci* from bovine mastitis. Vet. Microbiol. 161(3-4): 286-294.

Saei HD, Ahmadi M, Mardani K, Batavani RA (2009). Molecular typing of Staphylococcus aureus isolated from bovine mastitis based on polymorphism of the coagulase gene in the north west of Iran. Vet. Microbiol. 137(1-2):202-206.

Sela S, Hammer-Muntz O, Krifucks O, Pinto R, Weisblit L, Leitner G (2007). Phenotypic and genotypic characterization of *Pseudomonas aeruginosa* strains isolated from mastitis outbreaks in dairy herds. J. Dairy Res. 74(4):425-429.

Smith EM, Green LE, Medley GF, Bird HE, Dowson CG (2005). Multilocus sequence typing of Staphylococcus aureus isolated from high-somatic-cell-count cows and the environment of an organic dairy farm in the United Kingdom. J. Clin. Microbiol. 43(9):4731-4736.

Wang S, Wu C, Shen J, Wu Y, Wang Y (2013). Hypermutable *Staphylococcus aureus* strains present at high frequency in subclinical bovine mastitis isolates are associated with the development of antibiotic resistance. Vet. Microbiol. 165(3-4):410-415.

Yang Y, Liu Y, Ding Y, Yi L, Ma Z, Fan H, Lu C (2013). Molecular characterization of *Streptococcus agalactiae* isolated from bovine mastitis in Eastern China. PLoS One 8(7):e67755.

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*Full Length Research Paper*

# Growth potential of common foodborne bacterial pathogens on some selected vegetables sold in Jimma Town, Southwestern Ethiopia

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**Raw vegetables are major vehicles for the transmission of foodborne infections. In Ethiopia, there is a habit of consuming raw vegetables as salad, particularly tomato *(Solanum lycopersicum L.),* cabbage *(Brassica oleracea L.),* carrots *(Daucus carota L.),* lettuces *(Lactuca sativa L.)* and green peppers *(Capsicum annuum L.)* without adequate treatment. The objectives of this study were to assess the prevalence of some foodborne pathogens and to evaluate the growth potential of common foodborne bacterial pathogens. A total of 180 vegetable samples were purchased from different sites of Jimma town and analyzed following standard microbiological methods. In addition, prevalence of *Salmonella* and *Staphylococcus aureus* were determined. Similarly, different raw vegetables were challenged with test strains *(Salmonella typhimurium ATCC 13311, Escherichia coli ATCC 25922* and *Staphylococcus aureus ATCC 25923).* The growth rates of test strains varied in various vegetables. Tomato samples were the most suitable for the growth of test strains. *Salmonella* and *S. aureus* were isolated from 23 (12.8%) and 18 (10%) vegetable samples, respectively. *Salmonella* were most prevalent in lettuce but *S. aureus* was more prevalent in green pepper. The use of food grade chemicals to kill pathogens and reduce the microbial load before consumption is recommended.**

**Key words:** Challenge study, growth potential, bacterial pathogens, raw vegetables.

## INTRODUCTION

Vegetables can be used as salad mixes, side dishes or ingredients in the meals. Fresh products that contain complex and colorful blends incorporating a wide variety of vegetable mixes and flavors would especially benefit from sales in all market segments. In general, as consumers continue to lead a healthy lifestyle, there are broad product development opportunities in this category. Currently, supermarkets and the food service outlets are the primary retail outlets for these products (Amoah et al., 2009). Thus despite their nutritional and health benefits, outbreaks of human infections associated with the consumption of fresh or minimally processed vegetables

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have increased in recent years (Beuchat, 2002).

Since vegetables are produced in a natural environment, they are vulnerable to contamination by human pathogens. The majorities of diseases associated with fresh vegetables are primarily those transmitted by the fecal oral route, and therefore, are a result of contamination at some point in the process (Johnston et al*.,* 2005). Vegetables could be contaminated with bacterial pathogens from human or animal sources including *Salmonella*, *Shigella*, *E. coli* O157:H7, *Listeria monocytogenes*, *Staphylococcus aureus* and *Campylobacter*, and resistance pathogens to different antimicrobials (Al-Binali et al., 2006; Simon et al., 2007; Allende et al., 2008; Elhariry, 2011). As the result, vegetables have been associated with outbreak of foodborne disease in many countries.

Foodborne bacterial pathogens commonly detected in fresh vegetables were coliform bacteria, *Staphylococcus aureus* and *Salmonella* spp. (Tambekar and Mundhada, 2006). Coli forms are commonly used bacterial indicator of sanitary quality of foods and water and considered as an indicator of microbial pollution and they are common inhabitant of animal and human guts (Tortora, 1995). The presence of these bacteria poses a serious threat to public health with outbreaks arising from food and water that has been contaminated by human or animal feces or sewage. *S. aureus* is the third most common cause of confirmed food poisoning in the world and the illness is due to the ingestion of preformed enterotoxin produced in foods (Acco et al., 2003).

Ethiopia has highly diversified agroecological zones which are suitable for the production of various types of vegetables. Vegetables are mainly grown by traditional farmers in home gardens. About 27% of the vegetable species recorded from home gardens in Ethiopia were consumed as raw or cooked (Asfaw, 1997). Particularly, in the urban parts of the country eating of raw vegetables becomes more common. Vegetable farmers around Jimma town supply vegetables to the local market; but the market place of Jimma town is not well organized. Vegetables are sold in front of shops besides with other goods and on street by street vendors. In addition, vegetables can be stored in poor quality containers and house before sell for at least one day. This can increase potential contamination of vegetables with animals and human's feces, soil, dusts and other postharvest contaminants (Al-Binali et al., 2006). Contamination of vegetables are of special concern, because it is likely to be consumed raw, without any type of microbiologically lethal processing, thus posing a potential food safety problem.

The present study was undertaken to examine the growth potential of common foodborne bacterial pathogens in some fresh vegetables particularly tomato (*Solanum lycopersicum* L.), cabbage (*Brassica oleracea* L.), carrot (*Daucus carota* L.), lettuce (*Lactuca sativa* L.), and green pepper (*Capsicum annuum* L.) samples collected from different sites (Kochi, Agip and Merkato) and markets (shops and street venders) of Jimma town.

## MATERIALS AND METHODS

### Description of the study area

The study was conducted in Jimma town, which is located at 353 km south west of Addis Ababa. The town's geographical coordinates are approximately 7°41' N latitude and 36° 50'E longitude. From a climatic point of view, abundant rainfall makes this region one of the best watered of Ethiopian highland areas, conducive for agricultural production (Alemu et al., 2011).

### Study design and study population

The cross sectional study design was used. The sampling sites were Kochi, Agip, and Merkato. The study periods were covered from September, 2011 to May, 2012.

### Sampling techniques

A simple random sampling technique was used to address representative of the whole population.

### Data collection

As the study has survey and experimental parts, data were collected using structured questionnaires.

### Collection of samples

A total of 180 fresh vegetable samples were purchased at different sampling days from local markets of Jimma town, southwest Ethiopia. The samples consisted of 36 each of tomato (*S. lycopersicum* L.*)*, cabbage (*B. oleracea* L.), carrots (*D. carota* L.), lettuces (*L. sativa* L.), and green peppers (*C. annuum* L.). All samples were collected using sterile plastic bags aseptically and immediately brought to the Postgraduate and Research Laboratory of Biology Department, Jimma University, for analysis. Microbiological analysis was conducted within 3 h of sample collection.

### Sample preparation

For sample preparation, 25 g samples were aseptically removed from each sample, shredded into approximately 2 - 3 cm pieces using a sterile stainless steel knife and vigorously shaken in 225 ml of sterile 0.1% (w/v) buffered peptone water (Oxoid) for 3 min separately to homogenize the samples (Shalini, 2010).

### Detection of pathogens

#### *Salmonella*

For detection of *Salmonella*, 25 g vegetable samples were added to 225 ml buffered peptone water, vigorously shaken and the suspension was incubated at 37°C for 24 h for metabolic recovery and proliferation of cells (Deza et al*.,* 2003). From this, 1 ml of culture was transferred into separate tubes each containing 10 ml of selenite cystein broth. The broth was incubated at 37°C for 24 h.

After secondary enrichment, culture from enrichment broth was separately streaked on plates of Xylose Lysine Desoxycholate (XLD) (Oxoid) medium. Pink colonies with or without black centers from selective medium was picked, purified and tested biochemically (Cheung et al., 2007).

#### *S. aureus*

For detection of *S. aureus*, golden yellow colonies from MSA during staphylococci count were picked, purified and preserved. Coagulase test was done by two ways: slide coagulase test and tube coagulase test (Cheesbrough, 2006).

#### Determination of growth potential of selected pathogens

The growth potential of representative foodborne pathogens (such as *S. typhimurium* ATCC 13311, *S. aureus* ATCC 25923, and *E. coli* ATCC 25922*)* were assessed in tomato, cabbages, carrots, lettuce, and green pepper samples. These vegetable samples were chopped using food processor (NM- 343) to small pieces and homogenized. From the homogenate, 200 g of each fresh sample was steamed at 80°C for 10 min to kill any vegetative cells which may be present in the samples. The test strains were activated for 24 h to give an inoculums level of $10^2$ - $10^3$ CFU $g^{-1}$. Then, 0.5 ml overnight culture of each standard inoculums of test strains were separately and aseptically inoculated to 50 g of each freshly prepared samples. Ten grams of each inoculated samples were homogenized in 90 ml of buffered peptone water (BPW) and 0.1 ml of appropriate dilution were spread plated on xylose lysine desoxycholate (XLD) medium for *S. typhimurium* ATCC 13311, Mannitol salt agar (MSA) for *S. aureus* ATCC 25923 and violet red bile agar (VRBA) for *Escherichia coli* ATCC 25922. The survival and growth pattern of the test pathogens were evaluated through aseptically periodic withdrawal of portion of samples (10 g) aseptically at every 6 h intervals for 0 - 24 h and making of counts after incubation at 37 $^0$C for 24 h (Muleta and Ashenafi, 2001; NACMCF, 2010).

#### Statistical analysis

The mean values of microbial count were analyzed by Microsoft excel 2010. In addition, the data obtained from the respondents were analyzed by SPSS version 16.

#### Ethical consideration

Ethical clearance was obtained from Research Review and Ethical committee of College of Natural Science, Jimma University.

## RESULTS

### General vegetable farm and management conditions

The general characteristics of farm and management conditions of vegetables sold in Jimma town were summarized in Table 1. Vegetables sold in Jimma town were 100% cultivated in traditional farming methods by rural farmers. The preferred cultivation seasons of the vegetables were found out to be during dry season (41.1%) using irrigation. The water sources for irrigation were river (63.3%) and well (36.7%). A large number of vegetable farmers (73.3%) used inorganic fertilizers although 26.7% used animal manure to increase the fertility of the farm land. The vegetable farmers used different materials to harvest the produce including sack (41.1%), hand basket (32.2%) and plastic bags (26.7%). The harvested vegetables were stored at different places before selling. About 62.2% of the vegetable farmers were stored in store room. However, 37.8% of the respondents stored vegetables simply on the floor in the vegetable farms (Table 1).

Vegetables were transported from farm site to market by different means of transportation. Donkey were mostly used (35.6%) followed by horse cart (26.7%), car (23.3%) and humans back (14.4%). Sack and plastic bags were used as transporting containers while 64.4% of the respondents used sack and 35.6% of vegetable farmers and sellers used plastic bags. About 63.3% of the respondents placed vegetables on the bed in front of the shop for sell. On the other hand, 35.6% of vegetable sellers vended vegetables on street without using bed or plastic sheet. However, 1.1% of respondents used plastics to vend vegetables on floor. Vegetables were not availed to the consumers as soon as harvested. Therefore, 77.8% of the sellers stored vegetables for up to three days, whereas 22.2% stored for more than three days before sold to consumers. Over 54% of the respondents consumed vegetables without heat treatment. However, 33.3 and 12.2% of the respondents consumed after heat treatment and treating with food grade chemicals, respectively (Table 1).

### Frequency of isolation of *Salmonella* spp. and *S. aureus*

Among 180 vegetable samples analyzed, 23 (12.8%) samples were positive for *Salmonella* isolates (Table 2). With regard to frequency distribution in each vegetable type, *Salmonella* isolates were highly prevalent in lettuce (16.7%). The frequency distribution of *Salmonella* in both tomato and cabbage were equal (13.9%). On other hand, *Salmonella* were isolated in 11.1% of carrot samples. However, green pepper contained the least *Salmonella* isolates (8.3%) as compared to lettuce, cabbage, tomato, and carrot samples (Table 2).

Of the total 180 vegetable samples, 18 (10.0%) were positive for *S. aureus* (Table 2). *S. aureus* was prevalent in each vegetable type. In most case, the levels of prevalence were different between vegetables. However, the prevalence of *S. aureus* in both cabbage and lettuce were equal (11.1%). *S. aureus* was most frequently isolated from green pepper (13.9%) followed by cabbage and lettuce. In carrot, the prevalence was 8.3% with least prevalence (5.6%) in tomato samples (Table 2).

**Table 1.** General vegetable farm and management conditions, Jimma town, south western Ethiopia, 2011/2012.

| Characteristics | Respondents(n=90) | |
|---|---|---|
| | Frequency | Percent (%) |
| **Methods of cultivation** | | |
| Traditional | 90 | 100 |
| **Water source of irrigation** | | |
| River | 57 | 63.3 |
| Well | 33 | 36.7 |
| **To increase fertility of farm** | | |
| Inorganic fertilizers | 66 | 73.3 |
| Animal manure | 24 | 26.7 |
| **Harvesting equipments** | | |
| Sac | 37 | 41.1 |
| Hand basket | 29 | 32.2 |
| **Storage place before selling** | | |
| In store room | 56 | 62.2 |
| On the floor in vegetable farm | 34 | 37.8 |
| **Transporting containers** | | |
| Sac | 58 | 64.4 |
| Plastic bags | 32 | 35.6 |
| **How long do you store before sell** | | |
| 1 day | 5 | 5.6 |
| 2 days | 29 | 32.2 |
| 3 days | 36 | 40.0 |
| More than 3 days | 20 | 22.2 |
| **Consumption habit** | | |
| Without heat treatment | 49 | 54.4 |
| With heat treatment | 30 | 33.3 |
| With food grade chemicals | 11 | 12.2 |

**Table 2.** Prevalence of *Salmonella* and *S. aureus* in raw vegetables, Jimma town, south western Ethiopia, 2011/2012.

| Sample type | Sample size (180) | Number of *Salmonella* positive samples (%) | Number of *S. aureus* positive samples (%) |
|---|---|---|---|
| Tomato | 36 | 5 (13.9) | 2 (5.6) |
| Cabbage | 36 | 5 (13.9) | 4 (11.1) |
| Carrot | 36 | 4 (11.1) | 3 (8.3) |
| Lettuce | 36 | 6 (16.7) | 4 (11.1) |
| Green pepper | 36 | 3 (8.3) | 5 (13.9) |
| Total | 180 | 23 (12.8) | 18 (10.0) |

## Growth potential of common foodborne pathogens

### *Growth potential of Salmonella typhimurium in some raw vegetables*

The growth potential of *S. typhimurium* ATCC 13311, *E. coli* ATCC 25922 and *S. aureus* ATCC 25923 were analyzed in tomato, cabbage, carrot, lettuce and green pepper over a period of 24 h. In challenge studies, the highest counts of *S. typhimurium* ATCC 13311 were 9.3 log10 CFU $g^{-1}$ with 24 h in tomato samples (Figure 1). Nevertheless, the lowest counts were 5.6 log10 CFU $g^{-1}$ in green pepper. The counts of *S. typhimurium* ATCC 13311 in the remaining vegetable samples including

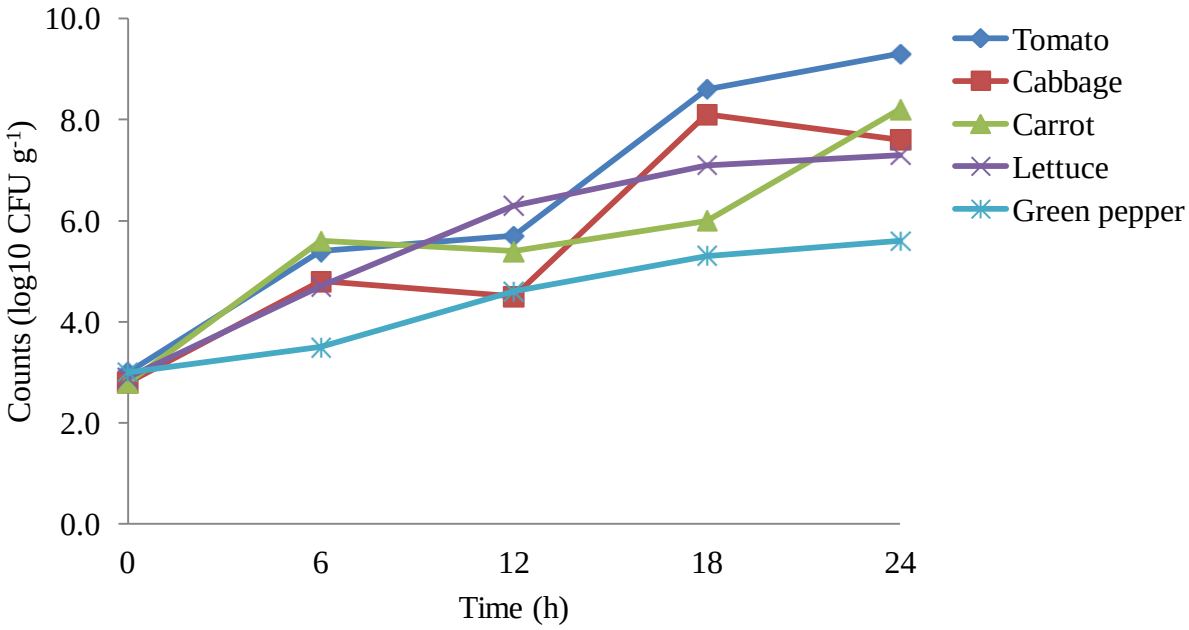


**Figure 1.** Growth potential of *Salmonella typhimurium* ATCC 13311 in selected raw vegetables.

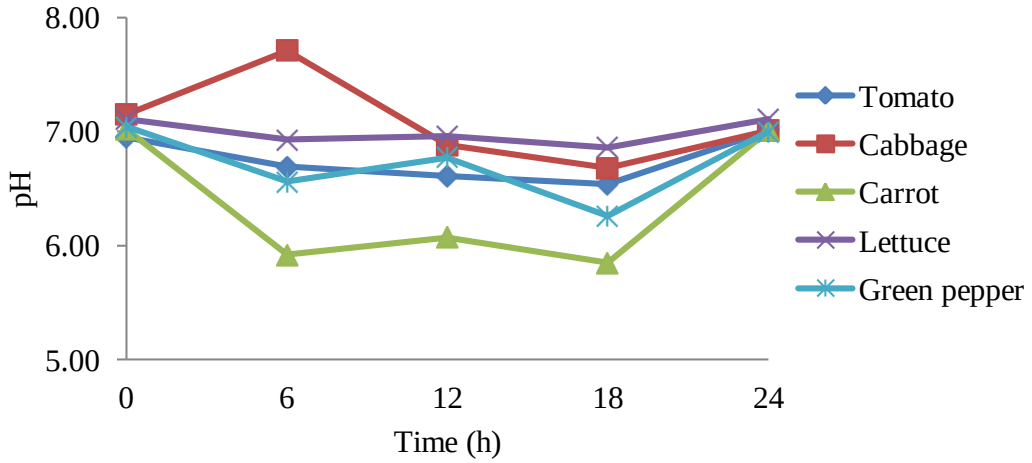


**Figure 2.** Change in pH of selected vegetables challenged with *Salmonella typhimurium* ATCC 13311.

carrot, cabbage and lettuce were 8.2, 7.6 and 7.3 CFU $g^{-1}$, respectively. Counts of the test strain increased by about two log units in the first 6 h in each vegetable tested except in green pepper where it increased by 0.5 log units. In the second 6 h the growth rate was decreased in cabbage, carrot and tomato with drop in pH. However, in the third 6 h the growth rate of *S. typhimurium* ATCC 13311 was increased when pH was relatively unvaried. There were rising tendencies in pH during the last phase of 24 h incubation (Figure 2). Overall, the growth rate of *S. typhimurium* ATCC 13311 was lower in green pepper than other vegetable samples (Figure 1).

**Growth potential of *Escherichia coli* in some raw vegetables**

Growth of *E. coli* ATCC 25922 was fast in the first 6 h in all vegetable samples with different growth rates and the highest counts were recorded in carrot (9.5 log10 CFU $g^{-1}$) followed by lettuce (9.4 log10 CFU $g^{-1}$.) with in 24 h (Figure 3). The growth rate of *E. coli* ATCC 25922 in lettuce was faster throughout 24 h of incubation than other vegetables. In contrast to this, *E. coli* ATCC 25922 grew slowly in green pepper throughout the incubation time and the pH of green pepper decreased in every 6 h interval but increased thereafter in the last part of

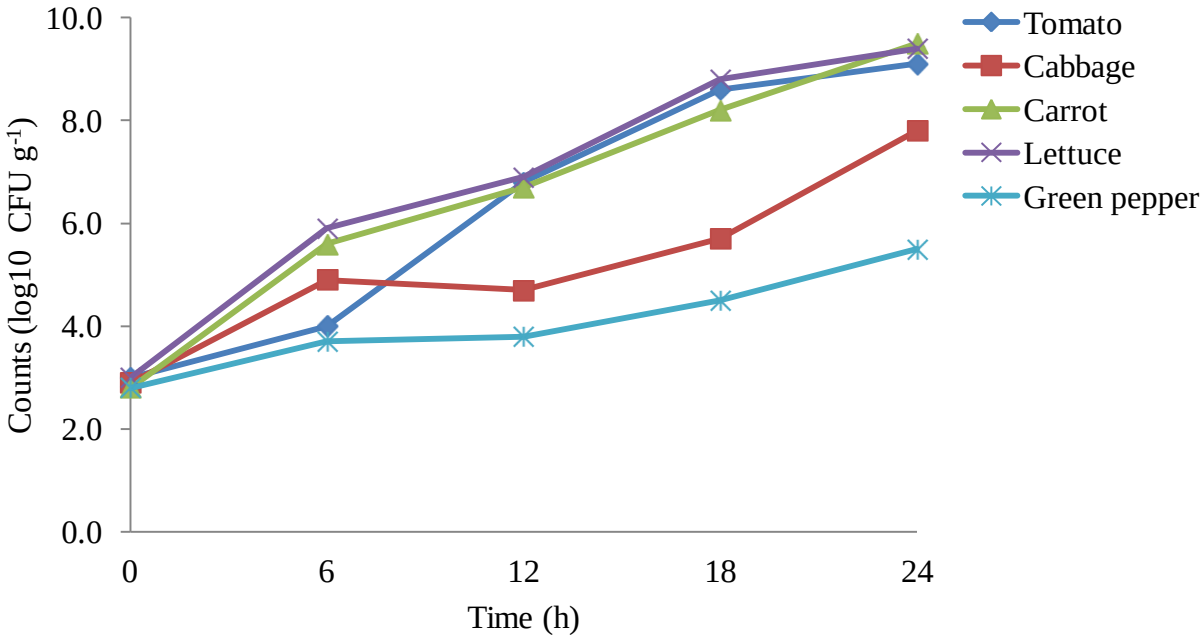


**Figure 3.** Growth potential of *Escherichia coli* ATCC 25922 in some selected raw vegetables.

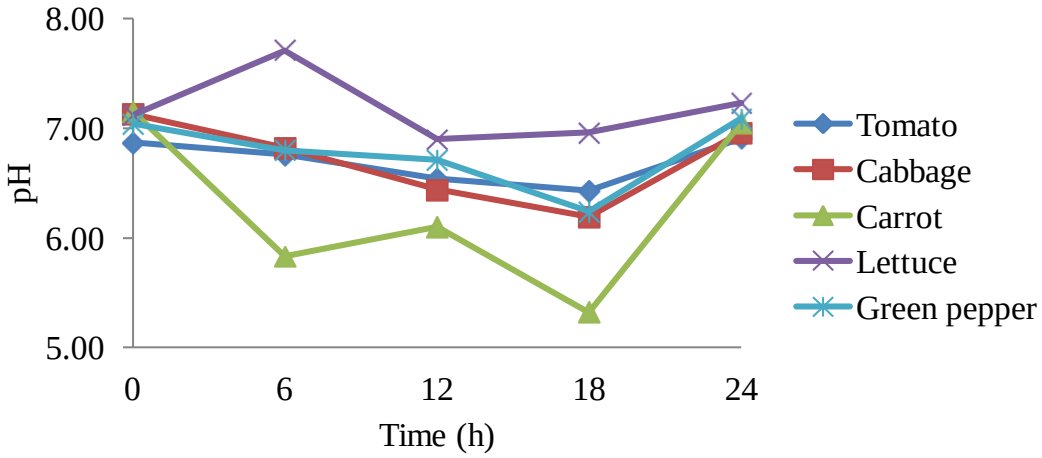


**Figure 4.** Change in pH of selected vegetables challenged with *Escherichia coli* ATCC 25922.

incubation period. The counts of *E. coli* were increased in lettuce and carrot by three log units, in cabbage by two log units, and in tomato and green pepper by one log units at the first 6 h of challenge test. The growth rates of E. *coli* in tomato increased by two log units at the second and third 6 h of incubation period, with decrease in pH. However, the growth of *E. coli* ATCC 25922 in the cabbage decreased in the second 6 h and became increased in third and fourth 6 h in line with its pH. The growth patterns of *E. coli* ATCC 25922 in carrots were increased throughout challenge test while pH was varied with time (Figures 3 and 4).

**Growth potential of *S. aureus* in some raw vegetables**

In the first 6 h *S. aureus* ATCC 25923 grew faster in carrot than other vegetables (Figure 5). However, the growth of *S. aureus* ATCC 25923 decreased in the second and third 6 h with pH. In the last 6 h the counts were increased by two log units and its pH also increased to neutral. The maximum load of *S. aureus* was recorded in tomato (8.3 log10 CFU $g^{-1}$) and the minimum was recorded in green pepper (5.6 log10 CFU $g^{-1}$) with in 24 h of challenge test. The growth rate of *S. aureus* ATCC 25923 was continuously increased until 12 h of challenge

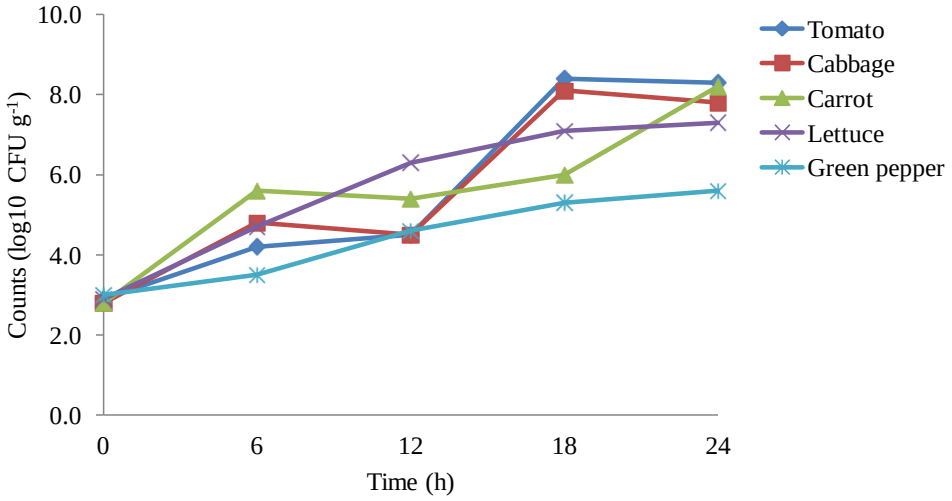


**Figure 5.** Growth potential of *Staphylococcus aureus* ATCC 25923 in some selected raw vegetables

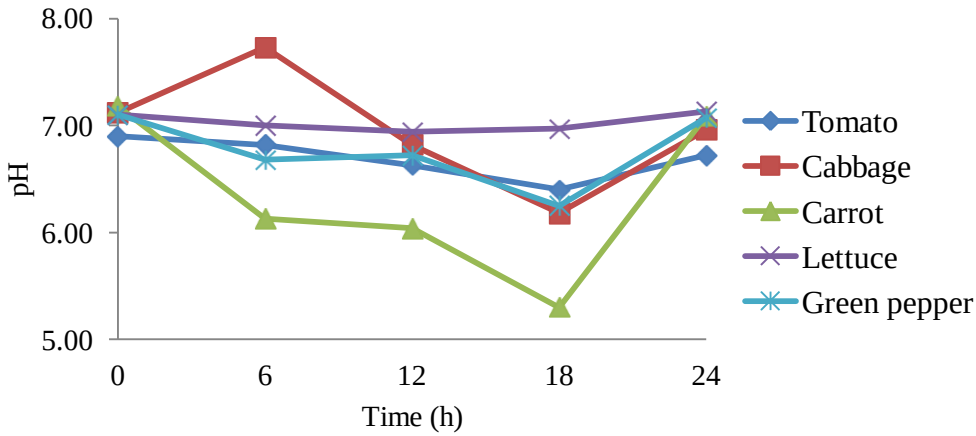


**Figure 6.** Change in pH of selected vegetables challenged with *Staphylococcus aureus* ATCC 25923

tests in lettuce, but became slow as the pH decreased. The *S. aureus* ATCC 25923 counts were increased approximately by two log units for the first two 6 h. *S. aureus* ATCC 25923 was grew faster in cabbage than in tomato in first two 6 h and counts of *S. aureus* ATCC 25923 in tomato became higher than cabbage in the last two 6 h and followed similar patterns. Similarly, the pH of tomato and cabbage were varied with time of challenge test. In contrast to this, the growth rate of *S. aureus* ATCC 25923 in green pepper was very slow compared to other vegetables. In addition, the pH of green pepper was in acidic range for the first, second and third 6 h of challenge test (Figures 5 and 6).

## DISCUSSION

The current study reveals the possible source of pre- and post-harvest contaminants of vegetables. In Jimma, farmers are cultivating vegetables following traditional farming system. Farmers cultivate vegetables during rainy season, dry season and throughout the year. Most of the time, they used water from river and well as source of water for irrigation purpose. Therefore, river could be the main source for contamination of vegetables during pre-harvest in the field since it could contain sludge from different towns and villages (Aycicek et al., 2006). Pathogens from irrigation water may survive in soil and contaminate vegetable which in turn be transported to consumers with the possibility of causing diseases (Halablab et al., 2011). Other possible source of contamination could be animal manure used by farmers to increase the fertility of farm land. In addition, harvesting equipments, storage place, mechanisms of transportation to the market, placement in the market, and length of

storage before selling could be the source of post-harvest contamination of vegetables (Natvig et al., 2002).

The presence of *S. aureus* and *Salmonella* spp. in vegetables are dangerous to consumers. *Salmonella* spp. was isolated from higher number of lettuce (16.7%) than other vegetable samples. This may be due to having foliar surfaces with many folds and the fragility of leaves (Aycicek et al., 2006). In other report, too, *Salmonella* spp. was isolated from vegetables particularly lettuce samples (Rajkowski and Fan, 2008). The contamination of vegetables with human pathogen could occur during the growth of the produce using animal manure, contaminated water or cross contamination during the cutting as the cut of vegetable can harbor and support the growth of food borne pathogen due to nutrients leakage from plant cellular material (Eni et al., 2010). The presence of *Salmonella* in 25 g of sample examined is regarded as potentially hazardous to consumers, and is unacceptable for consumption (Cheung et al., 2007). In addition, *S. aureus* was isolated from higher number of green pepper (13.9%). In similar study, Eni et al. (2010) from Nigeria reported that *S. aureus* was the most frequently isolated pathogens from vegetable samples. Surface of vegetables may be contaminated by this organism through human handling and other environmental factors and can be able to survive for several weeks. Thus, contamination of vegetables during distribution and handling may allow bacterial growth and subsequently production of toxins which may represent potential risk to humans. Therefore, cleaning and use of the right types and concentrations of food grade chemicals for cleaning should be practiced to make the vegetables fit for consumption. The level of microbial contamination observed in vegetables of our study may be a reflection of poor storage conditions and how long these produce were kept before they were collected.

Challenge studies showed that the pH of all vegetable samples inoculated with test strains before incubation was around neutral. However, it varies from 5.30 (carrot) - 7.73 (cabbage) throughout incubation period and there is a tendency to become neutral in the last phase of 24 h of incubation.

The decrease in pH can be attributed to the production of acids by pathogens after decomposition of carbohydrate. Likewise, the increase in pH may also be due to an increase in bases from the decomposition of nitrogenous compounds by endogenous or microbial enzymes. To our knowledge, there has not been any report on growth dynamics of foodborne pathogens in vegetables; hence, comparison of our results with previous studies difficult.

The growth rate of *S. typhimurium* ATCC 13311 in vegetables were fast in the first 6 h of challenge studies and became slow for the second 6 h as the pH of the samples decrease. However, its growth rate increased in the last phase of incubation period since the pH of the samples raise to neutral. Pathogens grow best around neutral pH; hence the growth patterns of *Salmonella* vary since pH changes of the samples depend on the metabolites produced by microbes. *S. typhimurium* ATCC 13311 multiplied more than 100 folds in the vegetable samples with in 6 h except in green pepper. The infectivity of *Salmonella* varies with strain, the food vehicle, ages and health status of the patients (Elexson et al., 2011). However, in all vegetable samples *S. typhimurium* could attain the infective dose level within 6 to 12 h where fewer than 100 viable cells are needed to be ingested to produce the disease syndrome.

On other hand, the growth rate of *E. coli* ATCC 25922 was steadily increased in tomato, carrot and lettuce. Regardless of pH change, *E. coli* ATCC 25922 was multiplied more than 1000 folds with in 12 h of incubation period and reached the infective dose. Even though the pH of carrot is less than other vegetable samples, *E. coli* ATCC 25922 attained maximum count (9.5 log10 CFU $g^{-1}$) in carrot with in 24 h of incubation period more than other test strains. This showed that *E. coli* ATCC 25922 is more resistant to low pH (Don, 2008).

Moreover, the growth of *S. aureus* ATCC 25923 was enhanced considerably in four vegetable samples such as tomato, cabbage, carrot, and lettuce and reached greater than 6 log10 CFU $g^{-1}$ within 18 h. At this level, *Staphylococcus* could secret staphylococcal enterotoxin (Ghosh et al., 2004; Schelin et al., 2011). In the last phase of incubation period, the growth rate of *S. aureus* ATCC 25923 became nearly stable. The present study shows that, even though the pH range of vegetables were found between 5.30 – 7.73, the maximum count of the test strains were recorded within optimum pH range .In agreement with our study, the growth potential of *E. coli* and *S. typhimurium* were analyzed in fruit juice by Yigeremu et al. (2001). They found that the maximum counts of pathogens were reached at pH ≥ 5.00.

Generally, variation in growth rate of foodborne pathogens in various vegetable samples could be attributed to intrinsic factors including pH and $a_w$. However, tomato is more suitable than other vegetable samples to support the growth of *S. typhimurium* ATCC 13311, *E. coli* ATCC 25922 and *S. aureus* ATCC 25923 as it has high water activity. On other hand, green pepper was less suitable to support the growth of foodborne pathogens. Thus, green pepper could have some inhibitory components that can retard the growth rate of foodborne pathogens.

## Conclusion

There was lack of awareness on feasible sanitation methods to prevent foodborne diseases associated with consumption of fresh vegetables. The possible source of contamination of vegetables could be irrigation water, animal manure used as fertilizers and water used to wash vegetables as most sellers wash or refresh different

vegetables before selling them with the same water again and again.

Out of the total 180 samples of different vegetables, *Salmonella* isolates were found from 23 samples with more prevalence in lettuce than other vegetable samples. Likewise, *S. aureus* were encountered from 18 samples with more prevalence in green pepper. This could be an indication of poor hygienic practice and frequent hand contact at the time of harvesting and in the market.

The growth potential of *S. typhimurium* ATCC 13311, *E. coli* ATCC 25922, and *S. aureus* ATCC 25923 were faster in tomato samples than other vegetables. This could be due to high water activity ($a_w$) of tomato samples.

## Recommendations

To limit the introduction of pathogenic bacteria to vegetables through irrigation, the origin of irrigation water should be known. Where wells are used, such wells should be well maintained, and all irrigation sources should be monitored routinely for human pathogens.

Manure used as fertilizer should be treated by composting to eliminate pathogenic microorganisms and farmers should be educated on the need to allow sufficient amount of time between the final manure application and harvest.

Vegetable processors should be educated on the adverse effect of using untreated or polluted water for food processing as these could serve as sources of contamination.

Consumers should treat raw vegetables with food grade chemicals to kill pathogens and significantly reduce the microbial load before consumption.

The growth rate of foodborne bacterial pathogens was fast in fresh vegetables and can rich the infective dose after 6 h of incubation. Therefore, fresh vegetables should be consumed within less than 6 h of preparation.

In the future, the effect of storage time and minimal processing on microbiological quality and safety of vegetables should be analyzed. Vegetables should reach consumers within short period of time after harvest.

## Conflict of interests

The authors have not declared any conflict of interests.

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## REFERENCES

Acco M, Ferreira FS, Henriques JA, Tondo, EC (2003). Identification of multiple strains of *Staphyloccocus aureus* colonizing nasal mucosa of food handlers. Food Microbiol. 20:489-493.

Al-Binali AM, Bello CS, El-Shewy K, Abdulla SE (2006). The prevalence of parasites in commonly used leafy vegetables in South Western Saudi Arabia. Saudi Med. J. 27:613-616.

Alemu A, Tsegaye W, Golassa L, Abebe G (2011). Urban malaria and associated risk factors in Jimma town, South-west Ethiopia. Malar. J. 10:173-200.

Allende A, Selma MV, López-Gálvez F, Villaescusa R, Gil MI (2008). Role of commercial sanitizers and washing systems on epiphytic microorganisms and sensory quality of fresh-cut escarole and lettuce. Post Harvest Biol. Technol. 49: 155-163.

Amoah P, Drechsel P, Abaidoo RC, Abraham EM (2009). Improving food hygiene in Africa where vegetables are irrigated with polluted water. West Africa Regional Sanitation and Hygiene Symposium, Accra, Ghana.

Asfaw Z (1997). Conservation and Use of Traditional Vegetables in Ethiopia. InTraditional African vegetables, Proceedings of the International Plant Genetic Resources Institute (IPGRI), Guarino, L., Ed., International workshop on genetic resources of traditional vegetables in Africa: Conservation and use. Nairobi. pp. 57-65.

Aycicek H, Oguz U, Karci K (2006). Determination of total aerobic and indicator bacteria on some raw eaten vegetables from wholesalers in Ankara, Turkey. Int. J. Hyg. Environ. Health. 209: 197- 201.

Beuchat LR (2002). Ecological factors influencing survival and growth of human pathogens on raw fruits and vegetables. Microbes Infect. 4:413-423.

Cheesbrough M (2006). District Laboratory Practice in Tropical Countries. Part 2 (2nd ed.). Cambridge: Cambridge University press.

Cheung PY, Kwok KK, Kam KM (2007). Application of BAX system, Tecra UniqueTM *Salmonella* test, and a conventional culture method for the detection of *Salmonella* in ready-to-eat and raw foods. J. Appl. Microbiol. 103:219-227.

Don SM (2008). Optimal conditions for the growth of *Echerchia coli.* www.scribd.com/doc/11337868/Optimal-Conditions-for-the-Growth-of-E-coli. Last accessed April 12th 2012.

Elexson N, Tuan Z, Tunung R, Ubong A, Son R (2011). Time course study on the growth of *Salmonella* Enteritidis on raw vegetables used in sandwiches. Int. Food Res. J. 18:1505-1508.

Elhariry HM (2011). Biofilm formation by *Aeromonas hydrophila* on green leafy vegetables: cabbage and lettuce. Foodborne Patho. Dis. 8:80-95.

Eni A O, Oluwawemitan A I, Solomon U (2010). Microbial quality of fruits and vegetables sold in Sango Ota, Nigeria. Afr. J. Food Sci. 4:291-296.

Ghosh M, Bansal S, Ganguli A (2004). Prevalence and Survival of S. aureus in street vended Indian green chutneys. J. Food Technol. 2:67-70.

Halablab MA, Sheet IH, Holail HM (2011). Microbiological Quality of Raw Vegetables Grown in Bekaa Valley, Lebanon. Am. J. Food Technol. 6: 129-139.

Johnston LM, Jaykus LA, Moll D, Martinez MC, Anciso J , Mora B, Moe CL (2005). A field study of the microbiological quality of fresh produce. J. Food Protect. 68:1840-1847.

Muleta D, Ashenafi M (2001). *Salmonella, Shigella* and growth potential of other food borne pathogens in Ethiopia street vended foods. East Afr. Med J. 78: 576-580.

National Advisory Committee on Microbiological Criteria for Foods (NACMCF). (2010). Parameters for Determining Inoculated Pack/Challenge Study Protocols. J Food Prot. 73: 140-202.

Natvig EE, Ingham SC, Ingham BH, Cooperband LR, Roper TR (2002). *Salmonella* enterica serovar Typhimurium and *Escherichia coli* contamination of root and leaf vegetables grown in soils with

incorporated bovine manure. Appl. Environ. Microbiol. 68:2737-2744.
Rajkowski KT, Fan X (2008). Microbial quality of fresh-cut iceberg lettuce washed in warm or cold water and irradiated in a modified atmosphere package. J. Food Safety. 28:248-260.
Schelin J, Wallin-Carlquist N, Cohn MT, Lindqvist R, Barker GC, Radstrom P(2011).The formation of *Staphylococcus aureus* enterotoxin in food environments and advances in risk assessment. Foodborne Infect. 2:580-592.
Shalini S (2010). Study on microbiological aspects of fresh fruit and vegetables (including green leafy vegetables) in and around National Capital Region (NCR). Bhaskaracharya College of Applied Sciences. Dwarka, New Delhi, India.
Simon SL, Holzapfel WH, Halasz A (2007). Biogenic amine contents and microbial contamination of leafy vegetables during storage at 5°C. J. Food Biochem.17:407-418.
Tambekar DH, Mundhada RH (2006). Bacteriological quality of salad vegetables sold in Amravati city (India). J. Biol. Sci. 6: 28-30.
Tortora G (1995). Microbiology. The Benjamin puplishing co. Ic., New York, USA. pp. 274-278.
Yigeremu B, Bogale M, Ashenafi M (2001). Fate of *Salmonella* species and *E. coli* in Fresh-Prepared Orange, Avocado, Papaya and Pine Apple Juices. *Ethiop.* J. Health Sci. 11:89-95.

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*Full Length Research Paper*

# Effects of plant growth promoting bacterial isolates from Kavango on the vegetative growth of *Sorghum bicolor*

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**Sorghum is an important cereal that is processed into a variety of foods and leisure beverages across the African continent. Low sorghum yields experienced in agriculture is a result of major production constraints such as soil nutrient deficiency and plant disease. It is important that the methods for crop production are of a sustainable nature as the chemical fertilizers in current use are detrimental to the natural environment. The aim of this study was to determine the effects of plant growth promoting (PGP) bacteria on growth of *Sorghum bicolor*. PGP bacteria isolated from the rhizosphere of *Pennisetum glaucum* (*Pseudomonas stutzeri* ACM2-32, *Kosakonia cloacae* FCM2-50, *Bacillus subtilis* ASM1-59 and *Bacillus amyloliquefaciens* LSM1-61) and *S. bicolor* (*Stenotrophomonas maltophilia* LCS2-11) plants in Kavango (Namibia), were used as peat-based inoculants to evaluate their effects on the growth of *S. bicolor*. The combination treatment $T_9$ (*B. amyloliquefaciens* LSM1-61: *K. cloacae* FCM2-50: *P. stutzeri* ACM2-32) significantly ($p = 0.032$) enhanced the biomass of *S. bicolor* as compared to the water control. Single inoculants consisting of *S. maltophilia* LCS2-11, *K. cloacae* FCM2-50 and *B. amyloliquefaciens* LSM1-61 and combination inoculants $T_7$, $T_8$ and $T_9$, enhanced *S. bicolor* root biomass as much as a commercial fertilizer control. These results indicate that the plant growth promoting bacteria induced a beneficial effect on growth of sorghum seedlings. The future work involves testing these promising inoculants on growth of these sorghum plants to maturity stage to determine effects on seed yield over three seasons in multi-location trials.**

**Key words:** Rhizosphere bacteria, peat based inoculants, plant growth promoting bacteria, *Sorghum bicolor*.

## INTRODUCTION

Africa's population might rise by 58% in 2030 (DESA, UN, 2013), consequently the demand for agricultural crops might increase significantly by then. The utilization of sustainable methods for enhancing harvest yield are gaining preference over the environmentally damaging agrochemicals which are currently being used. Solving agricultural concerns identified with the application of ecologically unfavourable fertilizers and the control of

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**Table 1.** Source and plant growth promoting trait profiles of bacterial isolates.

| Bacterial isolate | Site | Plant rhizosphere | $PO_3^{-4}$ solub. | $N_2$ fixation | IAA | Anti-fungal |
|---|---|---|---|---|---|---|
| *S. maltophilia* LCS2-11* | Lukas 2 | Sorghum | - | + | + | - |
| *P. stutzeri* ACM2-32 | ATR | Pearl millet | + | - | + | - |
| *E. cloacae* FCM2-50 | Field E | Pearl millet | - | + | ++ | - |
| *B. subtilis* ASM1-59 | ATR | Pearl millet | - | - | ++ | + |
| *B. amyloliquefaciens* LSM1-61 | Lukas 2 | Pearl millet | - | - | ++ | + |

*Siderophore producing isolate; ATR coordinates - 17°53′49.75″ S 20°09′07.07″ E; Lukas 2 coordinates - 17°53′43.80″ S 20°14′05.26″ E; Field E coordinates - 17°54′04.40″ S 20°14′14.34″ E.

plant diseases can be fulfilled by using plant root associated bacteria (Akhtar and Siddiqui, 2011; Glick, 2012). Various groups of bacteria found in the volume of soil affected by the presence of plant roots (Uren, 2007), also known as the rhizosphere, have been shown to be beneficial for the growth, yield and crop quality of plants (Orhan et al., 2006). These bacteria are able to colonize the rhizosphere and in some instances enter the roots of plants eventually inducing a beneficial effect on the host plant (Kloepper et al., 1980). Mechanisms by the bacteria aimed at inducing plant growth promotion include production of antibiotics against pathogenic microorganisms, production of antifungal compounds, production of plant hormones, increasing the availability of soluble phosphorus, increasing iron availability to plants, nitrogen fixation and regulation of ethylene concentration (Lucy et al., 2004).

The sorghum plant has the capacity to grow in moderately poor semi-arid and sub-tropical conditions of Africa (Taylor, 2003). Sorghum (*Sorghum bicolor* (L.) Moench) is an important crop throughout Western, Eastern and Southern Africa, that is used mainly for food and beverage production. Sorghum meal is used for making porridge and as an added substance to lower consistency and increase supplement and caloric thickness in porridge produced from other grains (Smith and Frederiksen, 2000; Ohiokpehai, 2003). In beer production, sorghum malt is used in the saccharification of the starchy substrate prior to fermentation (Smith and Frederiksen, 2000). Despite the genetic potential, generally low sorghum grain yields are experienced as a consequence of major constraints such as nutrient deficiency, soil water deficiency and plant diseases (Wortmann et al., 2009).

Commercial inorganic fertilizers may offer a short term solution for crop production but the financial component and long term soil fertility concerns labels them as an unfavourable option (Namibia Resource Consultants and Vigne and Associates Consultants, n.d.). For those countries that do not manufacture fertilizers, as opposed to need to import them, bacterial inoculants can be used to produce local cereals and agricultural products at a reasonably less expensive cost. The utilization of rhizosphere related microorganisms offers an appealing option to agrochemicals considering the fact that their plant growth and crop yield enhancing capacities have been shown over the recent decades (Saharan and Nehra, 2011). This study was carried out to assess the effects of treating *S. bicolor* seed with peat based plant growth-promoting rhizobacterial suspensions.

## MATERIALS AND METHODS

### Bacterial isolates

Native bacterial isolates (*Stenotrophomonas maltophilia* LCS2-11 and *Pseudomonas stutzeri* ACM2-32, *Enterobacter cloacae* FCM2-50, *Bacillus subtilis* ASM1-59 and *Bacillus amyloliquefaciens* LSM1-61) exhibiting increasing nutrient availability, plant hormone production and anti-fungal capabilities were obtained from The Department of Biological Sciences, University of Namibia. The bacteria were isolated from the rhizospheres of pearl millet and sorghum plants that were grown in the fields of subsistence farmers along the Kavango River (Table 1).

### Preparation of treatments

Bacterial isolates were grown in VM–ethanol broth at 28 ±2°C for 3 days. The bacterial cell concentration was adjusted to $OD_{660}$ = 0.9 in 50 ml VM–ethanol broth volume, washed with sterile distilled water and resuspended in 50 ml of 0.85% NaCl. This procedure was repeated for some bacterial isolates depending on the number of treatments and number of replicates. Starke Ayres® palm peat was prepared according to the manufacturer's instructions and dried overnight at 60°C. Approximately 50 g dry palm peat was placed into separate aluminium foil containers and sterilized via autoclaving. The palm peat was then aseptically transferred into Ziploc® plastic bags, moistened with 5 ml sterile distilled water per bag and kept at 4°C.

The application of phosphate solublizers alone or in combination with nitrogen fixers is beneficial for the growth of cereal (Zaidi and Khan, 2005). Therefore, combination treatments were made up of one phosphate solubilizing isolate (*P. stutzeri* ACM2-32), a $N_2$–fixer and an isolate with antifungal capability. The inoculum treatments were prepared according to Rose et al. (2011) with slight modifications. Treatments (Table 2) consisted of 50 g palm peat and 20 ml of bacteria solution, that is, 3 ml bacteria-0.85% NaCl suspension + 17 ml sterile distilled water for single bacterial treatments and 3 ml bacteria-0.85% NaCl suspension (×3 different isolates) + 11 ml sterile distilled water for combination bacterial treatments, whereas 3 ml 0.85% NaCl + 17 ml sterile distilled water was the control. After transferring the bacteria solutions to the palm peat enclosed in Ziploc® bags, the treatments were incubated for 3 days at 30°C before applying to soil. The non-inoculum control treatments were a commercial fertilizer, Hygrotech Terra Nova

**Table 2.** Comparisons of treatments for root masses, plant masses and root : shoot ratios.

| Treatment | Root mass (g) | Plant mass (g) | Root : shoot ratio |
|---|---|---|---|
| $T_1$ | $0.09 \pm 0.01^{c}$ | $0.31 \pm 0.04^{b}$ | 0.39 |
| $T_2$ | $0.05 \pm 0.01^{bc}$ | $0.22 \pm 0.06^{b}$ | 0.31 |
| $T_3$ | $0.09 \pm 0.03^{c}$ | $0.29 \pm 0.02^{b}$ | 0.42 |
| $T_4$ | $0.07 \pm 0.05^{bc}$ | $0.17 \pm 0.16^{b}$ | 0.86 |
| $T_5$ | $0.09 \pm 0.02^{c}$ | $0.39 \pm 0.18^{b}$ | 0.33 |
| $T_6$ | $0.07 \pm 0.01^{bc}$ | $0.23 \pm 0.11^{b}$ | 0.50 |
| $T_7$ | $0.08 \pm 0.00^{c}$ | $0.34 \pm 0.08^{b}$ | 0.33 |
| $T_8$ | $0.08 \pm 0.04^{c}$ | $0.34 \pm 0.30^{b}$ | 0.39 |
| $T_9$ | $0.10 \pm 0.03^{c}$ | $0.45 \pm 0.04^{ab}$ | 0.28 |
| $T_{10}$ | $0.14 \pm 0.07^{a}$ | $0.83 \pm 0.16^{ac}$ | 0.20 |
| $T_{11}$ | $0.07 \pm 0.09^{bc}$ | $0.18 \pm 0.23^{b}$ | 0.58 |
| $T_{12}$ | $0.19 \pm 0.05^{a}$ | $0.39 \pm 0.08^{b}$ | 0.95 |

Data is presented as mean ±SD for root and plant masses and as decimal form for root mass : shoot mass. [a] = mean difference between treatment and peat + water is significant at the 0.05 level. [b] = mean difference between treatment and fertilizer is significant at the 0.05 level. [c] = mean difference between treatment and no peat is significant at the 0.05 level. $T_1$ = LCS2-11 (*Stenotrophomonas maltophilia*); $T_2$ = ACM2-32 (*Pseudomonas stutzeri*); $T_3$ = FCM2-50 (*Enterobacter cloacae*); $T_4$ = ASM1-59 (*Bacillus subtilis*); $T_5$ = LSM1-61(*Bacillus amyloliquefaciens*); $T_6$ = ASM1-59: LCS2-11: ACM2-32; $T_7$ = ASM1-59: FCM2-50: ACM2-32; $T_8$ = LSM1-61: LCS2-11: ACM2-32; $T_9$ = LSM1-61: FCM2-50: ACM2-32; $T_{10}$ = Fertilizer; $T_{11}$ = peat + water; $T_{12}$ = no peat.

applied at 200 kg/hectare and a treatment with no peat.

#### Application of treatments and planting sorghum seeds

Plant pots (15 cm diameter x 12 cm depth) containing 1.6 kg of unprocessed arenosol type soil collected from a field (17°53′57.90″ S; 20°14′04.39″ E) were used in this study. Using a sterile trowel, treatments were transferred from the Ziploc® bags and mixed with soil in the plant pots. *S. bicolor* seeds bought from Rundu Open Market were surface sterilized by soaking in 70% ethanol for 5 min, then in 1.5% sodium hypochlorite for 1 min and rinsed three times in sterile distilled water. The seeds were dried for 2 h in sterile conditions and planted into the pots containing treatments. There were two replicates for each treatment with one seed planted per pot. After 25 days, the dry mass was determined by drying plants in an oven (50°C) until the weight remained constant; the length and mass of shoots and roots were recorded.

#### Specifics for greenhouse pot experiments

The pot experiments were carried out at the University of Namibia Main campus' (Windhoek) greenhouse facility for 25 days. The plant pots were arranged in a randomized block manner with two blocks. The plants were watered every day with an average atmospheric pressure of 1006.923 hPa, an average maximum temperature of 34.1°C and an average 13 h 26 m 58 s daylight length per day for the duration of the pot experiments.

#### Statistical analysis

SPSS statistics (SPSS, version 22.0.0.0, 2013) was used to analyse the data. Analysis of variance (ANOVA)\Kruskal-Wallis one-way analysis of variance procedure was performed followed by post hoc Fisher's least significant difference (LSD). All analyses were tested at 5% level of significance.

## RESULTS AND DISCUSSION

Three of the single inoculant treatments ($T_1$, $T_3$ and $T_5$) and three combination treatments ($T_7$, $T_8$ and $T_9$) had comparatively similar growth effects on sorghum root mass as the fertilizer treatment. Treatment $T_9$ was able to enhance sorghum plant growth significantly as compared to the water control. Apart from inoculants $T_2$ and $T_4$, the remaining peat based bacterial suspensions evoked a valuable impact on the development of *S. bicolor*.

### PGP and biocontrol bacteria inoculation effects on sorghum

Three of the single inoculant treatments ($T_1$, $T_3$ and $T_5$) and three combination treatments ($T_7$, $T_8$ and $T_9$) had comparatively similar growth effects on sorghum root mass as the chemical fertilizer treatment. Treatment $T_9$ was able to enhance sorghum plant growth significantly as compared to the water control. Though two of the single inoculants $T_2$ and $T_4$ did not bring about any improved growth on the plants, it was determinable that the peat based bacterial suspensions elicited a beneficial

effect on the growth of *S. bicolor*.

The results showed that single bacterial suspension treatments consisting of *K. cloacae* FCM2-50 (p = 0.089), *S. maltophilia* LCS2-11 (p = 0.089) and *B. amyloliquefaciens* LSM1-61 (p = 0.122) enhanced root growth of *S. bicolor*. The combination bacterial treatments $T_7$ (*B. subtilis* ASM1-59: *K. cloacae* FCM2-50: *P. stutzeri* ACM2-32) and $T_8$ (*B. amyloliquefaciens* LSM1-61: *S. maltophilia* LCS2-11: *P. stutzeri* ACM2-32) also produced enhanced root growth on *S. bicolor*. Treatment $T_9$ (*B. amyloliquefaciens* LSM1-61: *K. cloacae* FCM2-50: *P. stutzeri* ACM2-32) enhanced both *S. bicolor* root growth (p = 0.196) and whole plant biomass (p = 0.032). Unsurprisingly, the difference in mean root dry mass between the fertilizer and the water control was statistically significant (p = 0.044). *K. cloacae* FCM2-50, *B. subtilis* ASM1-59 and *B. amyloliquefaciens* LSM1-61 are described as high producers of IAA, thus enabling root growth stimulation. Additionally, *Kosakonia* spp. are known to promote seedling root elongation via ACC deaminase activity (Li et al., 2000).

The water control and the no peat treatments were significantly different (p = 0.003) with regard to sorghum plant dry mass. Sorghum plants that grew in the no peat control treatment often had greater and at times statistically significant than most of the inoculant treatments. However, the average root-shoot ratio of 0.95 (0.58 for water control) for sorghum plants in the no peat treatment suggests that nitrogen availability was lower in the no peat treatment as compared to the inoculation treatments.

$N_2$-fixing bacteria play a critical role in the accumulation of plant biomass by providing an environment where the plant acquires nitrogen for assimilation (Pilbeam, 2010). The root-shoot ratio of the plant is also determined by nitrogen availability. The average root : shoot ratio of $T_4$ (0.86) and the no peat treatment (0.95) were greater than that of the water control treatment ($T_{11}$ = 0.58). The rest of the treatments had a smaller average root : shoot ratio as compared to the water control. A nitrogen deficiency often causes the growth of an increased root fraction so that the root system is allowed to increase nutrient acquisition (Pilbeam, 2010). As compared to the water control, the lower root-shoot ratios in plants treated with inoculations suggests that there was more nitrogen available as a result of the bacterial treatments.

We can conclude from our data that bacterial treatments were able to enhance sorghum growth, comparable to that of the commercial fertilizer in terms of root biomass. *K. cloacae* FCM2-50: *B. amyloliquefaciens* LSM1-61: *P. stutzeri* ACM2-32 enhanced sorghum plant biomass. Enhancement of sorghum growth in terms of root biomass comparable to the level of commercial fertilizer was accomplished by single inoculants of *S. maltophilia* LCS2-11, *K. cloacae* FCM2-50, and *B. amyloliquefaciens* LSM1-61. Similarly, combination inoculants of *B. amyloliquefaciens* LSM1-61: *K. cloacae* FCM2-50: *P. stutzeri* ACM2-32, *B. amyloliquefaciens* LSM1-61: *S. maltophilia* LCS2-11: *P. stutzeri* ACM2-32 and *B. subtilis* ASM1-59: *K. cloacae* FCM2-50: *P. stutzeri* ACM2-32 promoted sorghum vegetative root growth.

## Conclusion

From this study, it is concluded that PGP bacteria inoculants improve the growth of sorghum seedlings to level comparable to chemical fertilizers. These findings show the possibility of using bacterial inoculants as an inexpensive, effective and environmentally friendly alternative for increased agricultural crop productivity. An added advantage is that these PGP bacteria are ecologically adapted to the soils of this agro ecological zone as they were originally isolated from there. The eventual goal is to prove that the inoculants facilitate and improve plant growth and increase grain seed yield in sorghum. By developing inoculants consisting of native PGPR and bio-control bacteria, we improve our potential to alleviate challenges of heavily depending on importing fertilizers in countries that do not have chemical fertilizer manufacturing companies like Namibia or where subsistence farmers do not afford the price of the chemical fertilisers. The advancement of field trials at multiple locations is a necessary step towards assuring the accomplishments of effective bacterial inoculants. These inoculants offer a cheap preferential option to support current and future sorghum based industries.

## Conflict of interests

The authors did not declare any conflict of interest.

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### REFERENCES

Akhtar MS, Siddiqui ZA (2011). Role of plant growth promoting rhizobacteria in biocontrol of plant diseases and sustainable agriculture. In Plant growth and health promoting bacteria, Microbiology Monographs Volume 18. Maheshwari DK: Springer Berlin Heidelberg, Berlin.

DESA, UN (2013). World Population Prospects, The 2012 Revision. Department for Economic and Social Affairs, New York.

Glick BR (2012). Plant growth-promoting bacteria: mechanisms and applications. Scientifica 1-15.

Kloepper JW, Leong J, Teintze M, Schroth MN (1980). Enhanced plant

growth by siderophores produced by plant growth-promoting rhizobacteria. Nature 286: 885-886.
Li J, Ovakim DH, Charles TC, Glick BR (2000). An ACC deaminase minus mutant of *Enterobacter cloacae* UW4 No longer promotes root elongation. Curr. Microbiol. 41:101-105.
Lucy M, Reed E, Glick, BR (2004). Applications of free living plant growth-promoting rhizobacteria. Antonie van Leeuwenhoek 86:1-25.
Namibia Resource Consultants and Vigne and Associates Consultants (n.d.). The Commercialisation of Mahangu: Report for Ministry of Agriculture, Water and Rural Development and the Namibian Agronomic Board.
Ohiokpehai O (2003). Nutritional aspects of street foods in Botswana. Pak. J. Nutr. 2:76-81.
Orhan E, Esitken A, Ercisli S, Turan M, Sahin F (2006). Effects of plant growth promoting rhizobacteria (PGPR) on yield, growth and nutrient contents in organically growing raspberry. Sci. Hortic. Amsterdam 111:38-43.
Pilbeam DJ (2010). The utilization of nitrogen by plants: A whole plant perspective. Annual plant reviews volume 42: Nitrogen metabolism in plants in the post-genomic era. pp. 305-351.
Rose MT, Deaker R, Potard S, Tran CKT, Vu NT, Kennedy IR (2011). The survival of plant growth promoting microorganisms in peat inoculant as measured by selective plate counting and enzyme-linked immunoassay. World J. Microbiol. Biotechnol. 27:1649-1659.
Saharan BS, Nehra V (2011). Plant growth promoting rhizobacteria: a critical review. Life Sci. Med. Res. 21:1-30.
Smith CW, Frederiksen RA (2000). Sorghum: Origin, history, technology, and production John Wiley and Sons.
Taylor JRN (2003). Overview: Importance of sorghum in Africa. In Afripro: Workshop on the Proteins of Sorghum and Millets: Enhancing Nutritional and Functional Properties for Africa, Pretoria. (Vol. 2, No. 4).
Uren NC (2007). Types, amounts, and possible functions of compounds released into the rhizosphere by soil-grown plants. In The rhizosphere: Biochemistry and organic substances at the soil-plant interface 2nd ed. CRC Press, Boca Raton, FL.
Wortmann CS, Mamo M, Mburu C, Letayo E, Abebe G, Kayuki KC, Chisi M, Matavavarira M, Xerinda S, Ndacyayisenga T (2009). Atlas of Sorghum Production in Eastern and Southern Africa. The Board of Regents of the University of Nebraska, University of Nebraska-Lincoln.
Zaidi A, Khan MS (2005). Interactive effect of rhizospheric microorganisms on growth, yield and nutrient uptake of wheat. J. Plant Nutr. 28: 2079-2092.

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*Full Length Research Paper*

# Screening of microbial isolates for extracellular fructosyltransferase production

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**Fructooligosaccharides are gaining importance by leaps and bounce every day due to their low caloric value and accompanying health benefits for the consumer. Hence, newer potential sources for the fructosyltransferase (Ftase) enzyme are thoroughly scrutinized. The present work was carried out for preliminary and secondary screening of the microbial isolates for determination of the Ftase producing potential. Three classes of microorganisms' viz. molds, yeasts and bacterial isolates were qualitatively screened. The preliminary screening revealed molds as the most potent group exhibiting greater zone of hydrolysis in the range of 0.30 ± 0.10 to 2.58 ± 0.10 cm. The secondary screening of 20 selected mold isolates was performed in liquid batch culture. Four isolates identified as *Aspergillus niger*, *Aspergillus flavus*, *Aspergillus stallus and Aspergillus versicolor* exhibited higher fructosyltransferase : invertase ratio which is most critical for the synthesis of fructooligosaccharides due to higher transferase activity and low hydrolytic activity. *A. niger* exhibited highest Ftase activity, 36.88 ± 0.23 IU/mg. *A. flavus* produced 21.45 ± 0.33 IU/mg of Ftase. *A. stallus* produced 18.09 ± 0.14 IU/ mg and *A. versicolor* was found to produce 23.78 ± 0.12 IU/mg of Ftase without any cultural medium optimization.**

**Key words:** Prelimnary screening, secondary screening, fructosyltransferase, fructooligosaccharides.

## INTRODUCTION

Functional foods like fructooligosaccharides are gaining prime importance in the health market because they provide multiple health benefits like low caloric value, promoting growth of *Bifidobacteria* in colon (prebiotic property), low glycemic index and low carcinogenicity in aid of basic nutrition (Dominguez et al., 2013; Moore et al., 2003 and Slevin et al., 2014). The most popular functional food coming into the lime light is fructo-oligosaccharides (FOS) obtained from sucrose.

FOS are oligosaccharides of fructose containing single glucose unit. They are produced by action of fructosyl-transferase (Ftase) on sucrose. FOS are mainly composed of 1-kestose, nystose and β- fructofuranosyl nystose (2→1) position of sucrose (Sangeetha et al., 2005).

The sucrose is biologically transformed into FOS either by using microorganism itself (Chien et al., 2001; Sanchez et al., 2010) or by enzymes derived from them (Fawkia et al., 2009).

Scientists are constantly attempting to isolate new strains of microorganisms having higher potential for production of the enzyme. Since screening is an elaborate time consuming and tedious process, very few attempts have been made to indigenously isolate the

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microbe from environment and subject them to screening (Madlova et al., 1999; Reddy et al., 2010; Fernandez et al., 2007). The objective of the present study was to screen the filamentous fungi, yeast and bacteria to enable their usage in industries.

## MATERIALS AND METHODS

### Preliminary screening of the isolates

Thirty one yeasts, forty two bacteria and fifty four molds isolated from previous work (Belorkar et al., 2013) were selected for prelimnary screening on basis of their colony size as compared to their counterparts in the plates containing selective medium (sucrose- 30.0, $NaNO_3$- 2.5, $MgSO_4.7H_2O$-0.5, $KH_2PO_4$-1.5 and Agar agar powder -20 at pH 5.50). The basal medium (20 ml in a 100 ml Erlenmeyer flask) used for cultivation of fungi during prelimnary screening contained (g/L): Sucrose- 10.0, $NaNO_3$- 2.5, $MgSO_4.7H_2O$ -0.5 and $KH_2PO_4$-1.5 at pH 5.50.

#### *Screening for yeast isolates*

The modified method of Maugeri and Heernalsteens (2007) was followed in which the medium was replaced by the above defined basal medium at pH 5.50. Loop full of cells were inoculated into 10 ml of basal medium in test tubes and cultivated at 25°C for 24 h. After adequate development of the cultures, they were transferred into 500 ml flasks containing a further 100 ml of the same medium, and cultivated at 25°C for 72 h .The cells were removed by centrifugation at 6000 ×*g* (10 min, 4°C), and the cell-free supernatants were used to screen for transfructosylating activity by plate method containing selective medium. The conditions maintained were for support of fructosyltransferase production.

#### *Screening for molds isolates*

The molds were cultivated on basal medium at pH 5.5 with sucrose as the substrate. Screening was carried out under static conditions at 28°C for 72 h. All the experiments were conducted in triplicates. The supernatant was centrifuged at 6000 ×*g* (10 min, 4°C). The cell-free supernatant was used to screen for transfructosylating activity by plate method containing selective medium.

### Preliminary screening of Ftase production by fungi

Wells were made aseptically by using cock borer in the Petri plate containing selective media (sucrose- 30.0, $NaNO_3$- 2.5, $MgSO_4.7H_2O$ -0.5, $KH_2PO_4$-1.5 and Agar powder -20 at pH 5.50). Two hundred microliter of cell-free supernatants of the yeast and mold cultures were individually (crude enzyme) loaded into the well and kept for incubation at 28°C for 12 h. Staining was carried out by spraying triphenyltetrazolium chloride (TTC) reagent to the agar plate and kept for incubation of 20 min in the dark. After washing with the 0.1 M acetate buffer (pH-5), the extracellular production of fructosyltransferase was confirmed by the appearance of the red zone (measured in cm) around the well according to the protocol reported by Reddy et al. (2010).

#### *Screening for bacteria*

To select strains of bacteria, the isolates were plated in selective media plates and incubated for 48 h and subjected to TTC staining directly to measure the zone of hydrolysis described by Kim et al. (2000).

### Secondary screening

#### *Assay of fructosyltransferase activity*

Quantitative assay of fructosyltransferase was based on the procedure used by Yun et al. (1997). The filterate was taken as a crude enzyme with 50% sucrose solution as a substrate at 5.50 pH (0.1 M sodium acetate buffer). The mixture was incubated for 1 h at 60°C. The reducing sugars were estimated by Dinitro-salicylic acid reagent. The enzymatic reaction was terminated by keeping the test tube at 100°C in a water bath for 10 min. One unit of enzyme activity was defined as the amount of enzyme producing 1 µmol of glucose under experimental conditions.

#### *Assay of hydrolytic activity*

Sucrose hydrolytic activity was measured according to the method described by Sangeetha et al. (2003). The filtrate was taken as a crude enzyme with 0.50% sucrose solution as a substrate at 5.50 pH (0.1 M sodium acetate buffer). The mixture was incubated for 1 h at 60°C. The reducing sugars were estimated by dinitro-salicylic acid reagent. The enzymatic reaction was terminated by keeping the test tube at 100°C in a water bath for 10 min. One unit of sucrose hydrolytic activity was considered as the amount of enzyme required to produce 1 µmol of glucose under experimental conditions.

#### *Determination of protein concentration*

The protein content was determined following Lowry et al. (1951).

### Biomass concentration

Biomass concentration was determined in g/100 ml volume of fermentation medium. The biomass obtained by filtration of the fermentation broth was washed thrice with distilled water, air dried and weighed.

### Statistical analysis

All the experiments were conducted in triplicate. The results were expressed in Mean ± SE. One-way ANOVA using SPSS 16.0 was performed to analyze the variance in preliminary screening.

### Identification of the selected isolates

These four isolates were then identified morphologically by lactophenol cotton blue staining and found to be SSFM1 as *Aspergillus niger* NFCCI2736, SSFM2 as *Aspergillus flavus* NFCCI2734, SAFM30 as *Aspergillus stallus,* RSCDS48 as *Aspergillus versicolor gr.*

## RESULTS AND DISCUSSION

### Preliminary screening

Thirty one (31) yeast isolates, forty two bacterial isolates

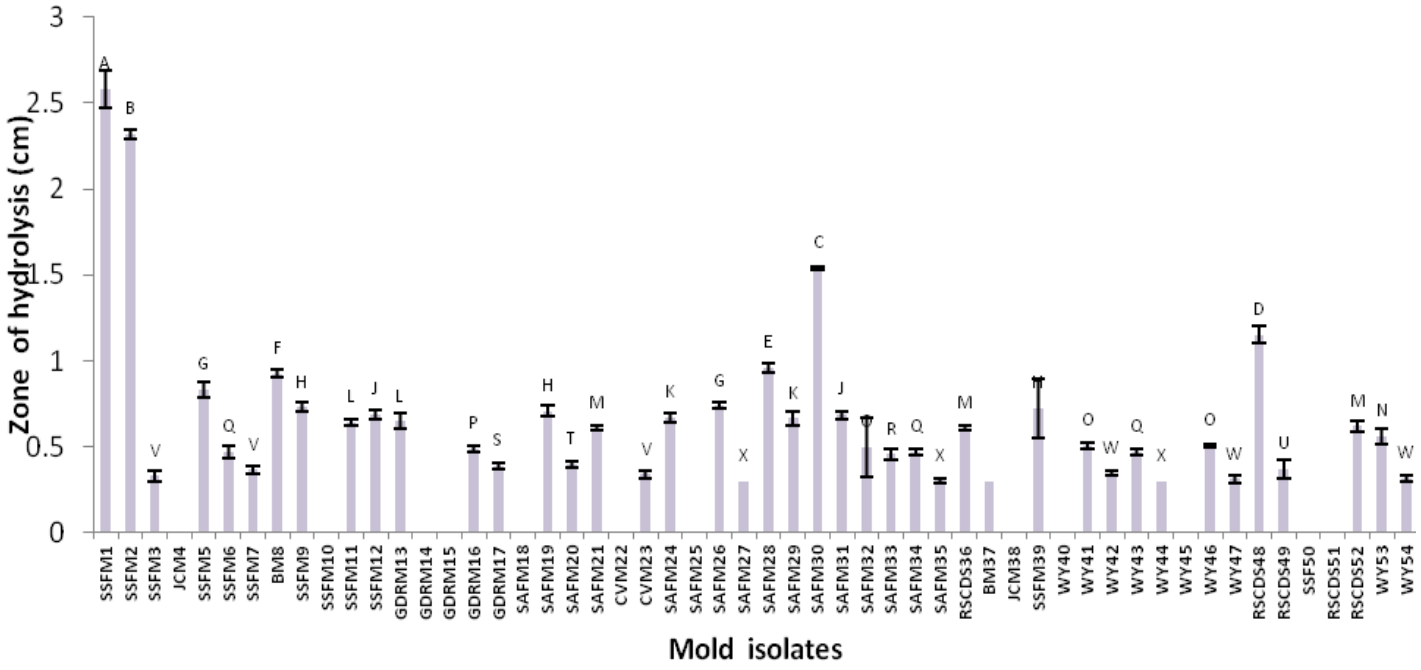


**Figure 1A.** Zone of hydrolysis (cm) for mold isolates. Same legends represent the zone of hydrolysis of the isolates not significantly different.

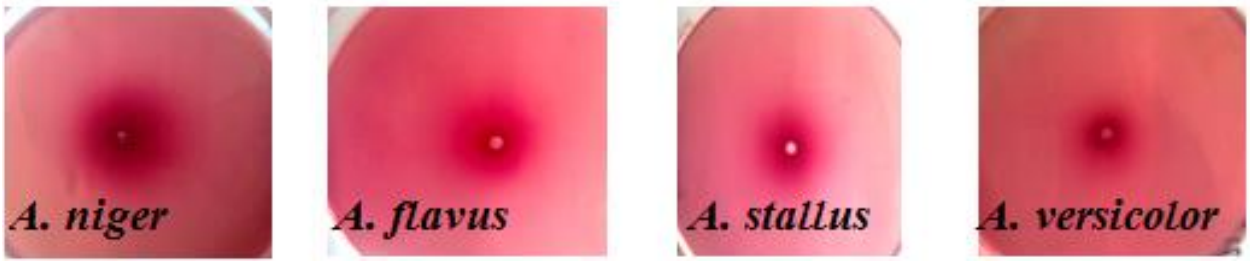


**Figure 1B.** Zone of hydrolysis exhibited by identified isolates during preliminary screening. Same legends represent the zone of hydrolysis of the isolates not significantly different.

and 54 molds isolated during the previous study were subjected to prelimnary screening. The zone of hydrolysis for molds, yeasts and bacteria are presented in Figures 1A, 2 and 3, respectively. The mold isolates proved to be the most potential Ftase producers. The zone of hydrolysis developed after TTC staining is represented in Figure 1B

The mold isolates exhibited highest zone of hydrolysis ranging from 0.30 ± 0.10 to 2.58 ± 0.10 cm. The largest zone of hydrolysis was recorded for isolate SSFM1 (2.58 ± 0.10 cm) and smallest zone was recorded for SAFM 27, SAFM 35, BM 37 and WY 44. The zone of hydrolysis recorded for molds was found to be similar to the findings of Reddy et al. (2010). Yeasts have been consistently reported to be potent producers of Ftase since the pioneering work of Bacon and Edelman (1950) investigating action of invertase on sucrose. Similarly, isolation of potent bacterial strains like *Psuedomonas* sp. No. 65 from soil has been reported by Kim et al. (1997).

The zone of hydrolysis for yeast isolates was found to be in the range of 0.15 ± 0.01 to 0.58 ± 0.01 cm as given in Figure 2. The maximum zone of hydrolysis was exhibited by RSCDS 24, the value being to 0.58 ± 0.01 cm. The zone of hydrolysis for bacterial isolates was found to be in the range of 0.12 ± 0.01 to 0.54 ± 0.10 cm. The maximum zone of hydrolysis exhibited was SSFB2, the value being 0.54 ± 0.10 cm as given in Figure 3. The bacterial and yeast isolates exhibited less potential for fructosyltransferase production as compared to results of Kim et al, (1997) and Maugeri and Hernalsteens (2007), respectively .

In this study, highest range of zone of hydrolysis was found to be of filamentous fungi among the three classes of microbes, similar to the results reported by Yun (1996).

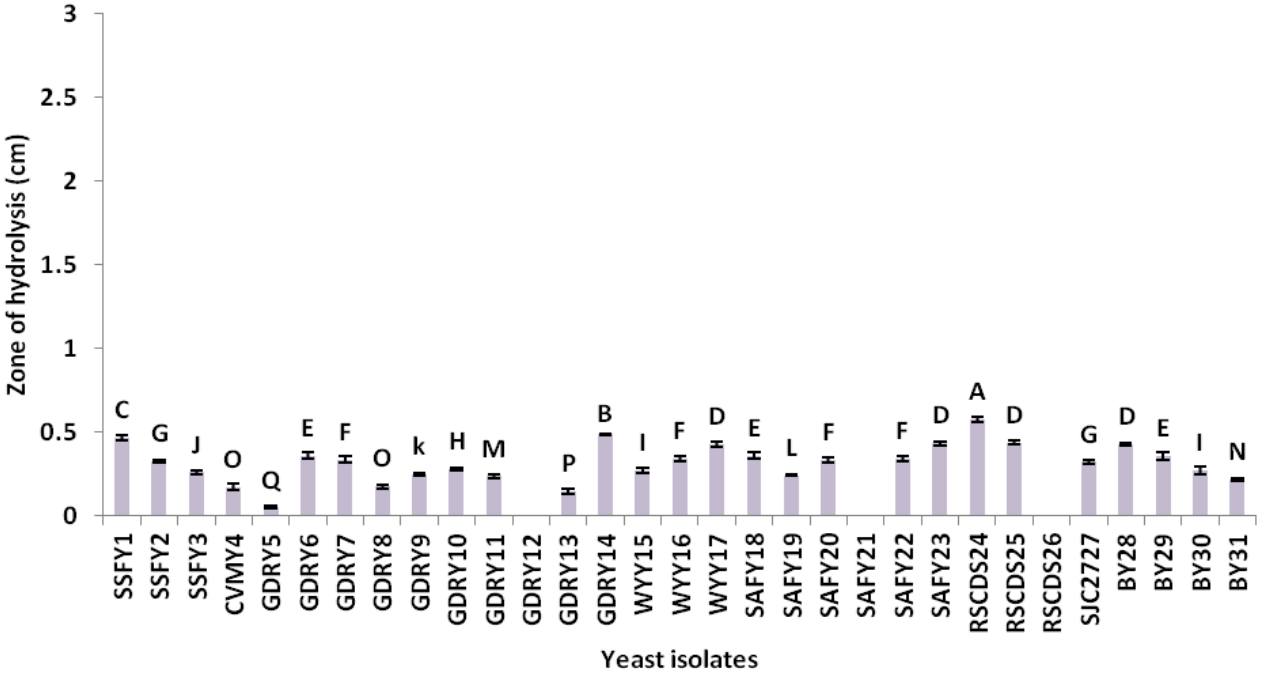


**Figure 2.** Zone of hydrolysis in cm for yeast isolates. Same legends represent the zone of hydrolysis of the isolates not significantly different.

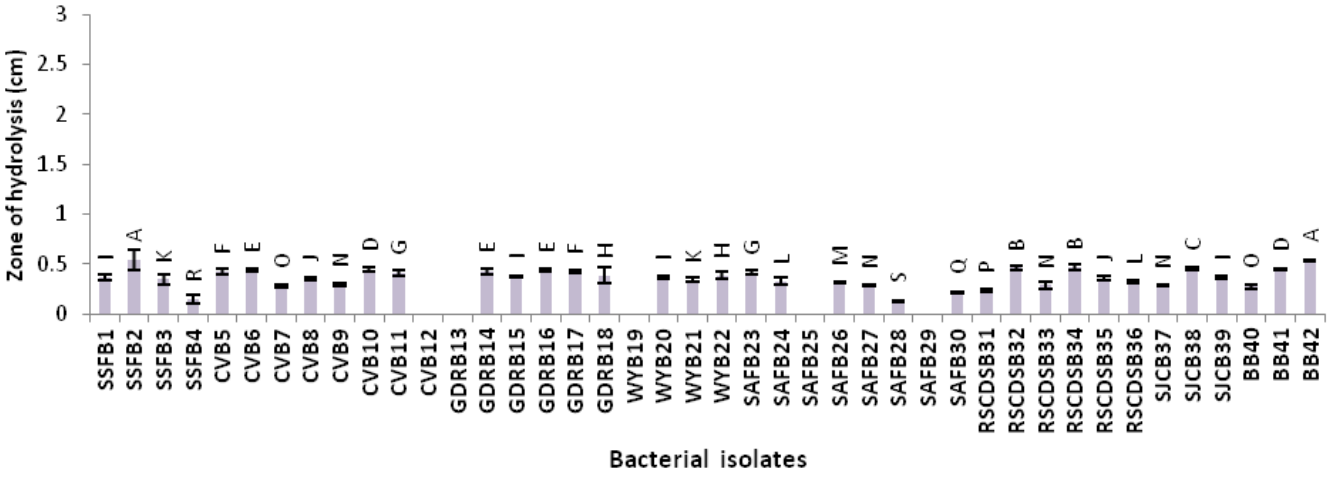


**Figure 3.** Zone of hydrolysis in cm for bacterial isolates. Same legends represent the zone of hydrolysis of the isolates not significantly different.

The filamentous fungi were therefore, selected for secondary screening in this study as well.

## Secondary screening for determination of extracellular Ftase and invertase production

The comparison of zone of hydrolysis of the three microbial groups was subjected to preliminary screening directed in the present study towards selection of molds for secondary screening. Twenty (20) mold isolates were selected for secondary screening in liquid basal medium depending upon their zone of hydrolysis namely SSFM1, SSFM2, SSFM5, BM8, SSFM9, SSFM11, SSFM12, GDRM13, SAFM19, SAFM21, SAFM24, SAFM26, SAFM28, SAFM29, SAFM30, SAFM31, RSCDS36, SSFM39, RSCDS48 and RSCDS52.

The average values of biomass production, Ftase production and invertase (Inv) production by the isolates are presented in Table 1. The most vital parameter in screening experiments for Ftase production is the Ftase :

**Table 1.** Biomass production, extracellular fructosyltransferase (Ftase) and Invertase (Inv) activity reached in liquid batch fermentation under static condition.

| Isolate | Ftase (IU/ml) | Inv (IU/ml) | Protein (mg/ml) | Ftase (IU/mg protein) | Inv (IU/mg protein) | Ftase/Inv ratio | Biomass (g/ 100 ml/ 72 h) |
|---|---|---|---|---|---|---|---|
| SSFM1* | 35.98 ± 0.10 | 22.02 ± 0.10 | 0.98 ± 0.006 | 36.88 ± 0.23 | 22.56 ± 0.26 | 1.63 | 27.60 ± 0.15 |
| SSFM2* | 20.39 ± 0.28 | 13.49 ± 0.10 | 0.95 ± 0.006 | 21.45 ± 0.33 | 14.19 ± 0.07 | 1.51 | 17.85± 0.05 |
| SSFM5 | 7.87 ± 0.10 | 12.40 ± 0.10 | 1.25 ± 0.002 | 6.30 ± 0.07 | 9.92 ± 0.06 | 0.63 | 9.25 ± 0.20 |
| BM8 | 8.53 ± 0.31 | 10.22 ± 0.10 | 1.20 ± 0.002 | 7.12 ± 0.25 | 8.53 ± 0.09 | 0.83 | 9.70 ± 0.15 |
| SSFM9 | 6.90 ± 0.18 | 15.36 ± 0.10 | 1.32 ± 0.004 | 5.21 ± 0.12 | 11.60 ± 0.11 | 0.44 | 8.60 ± 0.15 |
| SSFM11 | 4.42 ± 0.27 | 21.96 ± 0.18 | 1.18 ± 0.002 | 3.71 ± 0.24 | 18.59 ± 0.15 | 0.20 | 7.65 ± 0.55 |
| SSFM12 | 5.63 ± 0.18 | 16.33±0.18 | 1.06 ± 0.004 | 5.33 ± 0.19 | 15.46 ± 0.23 | 0.34 | 7.40 ± 0.15 |
| GDRM13 | 5.81 ± 0.18 | 8.77 ± 0.10 | 1.35 ± 0.006 | 4.31 ± 0.14 | 6.51 ± 0.08 | 0.66 | 7.60 ± 0.15 |
| SAFM19 | 6.78 ± 0.10 | 19.24 ± 0.18 | 1.37 ± 0.006 | 4.93 ± 0.07 | 13.99 ± 0.19 | 0.35 | 7.85 ± 0.15 |
| SAFM21 | 5.14 ± 0.10 | 6.23 ± 0.10 | 0.96 ± 0.006 | 5.36 ± 0.07 | 6.49 ± 0.13 | 0.82 | 7.30 ± 0.20 |
| SAFM24 | 5.69 ± 0.10 | 25.83 ± 0.10 | 1.12 ± 0.002 | 5.07 ± 0.09 | 23.03 ± 0.12 | 0.22 | 7.70 ± 0.05 |
| SAFM26 | 6.96 ± 0.10 | 13.13 ± 0.10 | 1.08 ± 0.004 | 6.42 ± 0.07 | 12.11 ± 0.06 | 0.53 | 8.75 ± 0.25 |
| SAFM28 | 9.74 ± 0.10 | 16.82 ± 0.10 | 1.34 ± 0.004 | 7.27 ± 0.09 | 12.55 ± 0.08 | 0.58 | 9.75 ± 0.20 |
| SAFM29 | 5.38 ± 0.10 | 7.139 ± 0.10 | 1.02 ± 0.006 | 5.29 ± 0.12 | 7.00 ± 0.14 | 0.75 | 7.40 ± 0.20 |
| SAFM30* | 18.57 ± 0.10 | 13.67 ± 0.10 | 1.03 ± 0.006 | 18.09 ± 0.14 | 13.31 ± 0.13 | 1.36 | 21.30 ± 0.10 |
| SAFM31 | 1.86 ± 0.18 | 5.76 ± 0.10 | 1.25 ± 0.004 | 1.50 ± 0.13 | 4.65 ± 0.15 | 0.32 | 8.05 ± 0.40 |
| RSCDS36 | 5.14 ± 0.10 | 10.67 ± 0.10 | 1.23 ± 0.004 | 4.20 ± 0.10 | 8.75 ± 0.09 | 0.47 | 7.30 ± 0.16 |
| SSFM39 | 6.23 ± 0.10 | 15.55 ± 0.10 | 1.09 ± 0.004 | 5.73 ± 0.11 | 14.31 ± 0.08 | 0.40 | 8.25 ± 0.16 |
| RSCDS48* | 29.65 ± 0.10 | 28.37 ± 0.10 | 1.25 ± 0.002 | 23.78 ± 0.12 | 22.76 ± 0.12 | 1.04 | 28.30 ± 0.02 |
| RSCDS52 | 5.20 ± 0.10 | 16.58 ± 0.10 | 1.02 ± 0.002 | 5.12 ± 0.09 | 16.27 ± 0.13 | 0.31 | 7.30 ± 0.20 |

All values presented in the table are mean ± SE. *Identified isolates (SSFM1 identified as *Aspergillus niger*; SSFM2 identified as *A. flavus*; SAFM 30 identified as *A. stallus*; RSCDS 48 identified as *A. versicolor*).

Inv ratio (Hidaka et al., 1988; Hidaka 1988). The Ftase : Inv ratio was also determined for each mold and was found to be desirable in SSFM1 (1.63), SSFM2 (1.51), SAFM30 (1.36) and RSCDSM48 (1.04). In the earlier screening experiments, the ratio was reported between 0.3-15.9 (Fernandez et al., 2007; Patel et al., 1994; Reddy et al., 2010). The ratio determines the efficacy of transferase activity for synthesis of FOS. The extracellular Ftase production has been reported to be in the range of 0.053 IU to 660 IU/ml (Maiorano et al., 2008).

The four molds selected from the twenty molds subjected to secondary screening which exhibited high Ftase/Inv ratios was recorded for SSFM1 (1.63), SSFM2 (1.51), SAFM30 (1.36) and RSCDS48 (1.04).

All the 20 mold isolates selected exhibited Ftase production to varying degree ranging from 3.71 ± 0.24 to 36.88 ± 0.23 IU/mg. *A. niger* exhibited highest Ftase activity, 36.88 ± 0.23 IU/mg. *A. flavus* produced 21.45 ± 0.33 IU/mg of Ftase. *A. stallus* produced 18.09 ± 0.14 IU/ mg and *A. versicolor* was found to produce 23.78 ± 0.12 IU/ mg of Ftase.

## Conclusion

As FOS has already been given GRAS status, the molds will be promising tool for production of Ftase enzyme for synthesis of FOS. The results of the present screening experiments revealed microbes especially molds to be source exhibiting potential for Ftase production which is gaining importance in food industry for synthesis of prebiotics. The urge of researchers to search for newer Ftase producing microbes can only be quenched with such screening experiments. The present study revealed higher Ftase : Inv ratio of the isolates which can be even enhanced upon optimization of cultural medium. Further optimization experiments will be carried out to boost the Ftase production levels for maximization of FOS synthesis.

## Conflict of interests

The authors have not declared any conflict of interests.

### REFERENCES

Bacon JSD, Edelman J (1950). The action of invertase preparations. Arch. Biochem. 28: 467-468.

Belorkar SA, Rai V, Gupta AK (2013). Isolation of potential microbial producers of Fructosyl transferase from Baggasse and selected soil sites of Chhattisgarh. Asian. J. Microbiol. Biotech. Environ. Sci.15:785-788.

Chien CS, Lee WC, Lin TJ (2001). Immobilization of *Aspergillus japonicus* by entrapping cells in gluten for production of fructooligo-

saccharides. Enzyme Microb. Technol. 29: 252-257.
Dominguez AL, Rodrigues LR, Lima NM, Teixeira JA (2013). An overview of the recent developments on fructooligosaccharide production and applications. Food Bioprocess Technol .DOI 10.1007/s11947-013-1221-6.
Fawkia M, El B, Azza M, Abdel F, Doaa A, Mostafa FA, Ahmed FA (2009). Production and some properties of fructosyl transferase from *Bacillus subtilis*. J. Appl. Sci. Res. 5: 1132-1141.
Fernandez RC, Ottoni CA, Da Silva ES, Matsubara RMS, Carter JM (2007). Screening of β-fructofuranosidase-producing microorganisms and effect of pH and temperature on enzymatic rate. Appl. Microbiol. Biotechnol. 75:87-93.
Hidaka H (1988). Fructooligosaccharides. In: The Amylase Research Society of Japan (ed) Handbook of amylases and related enzymes. Pergamon. pp. 228-230.
Hidaka H, Hirayama M, Sumi N (1988). A fructooligosaccharide producing enzyme from *A. niger* ATCC 20611. Agric. Biol. Chem. 52:1181-1187.
Kim BW, Kwon HJ, Park HY, Nam SW Park JP, Yun JW (2000). Production of novel transfructosylating enzyme from *Bacillus macerans EG -6*. Biopro. Eng. 23:11-16.
Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951). Protein measurement with the folin phenol reagent. J. Biological. Chem.19:265-275.
Madlova A, Antosova M, Barathova M, Polakovic M, Stefuca V, Bales V (1999). Screening of microorganisms for transfructosylating activity and optimization of biotransformation of sucrose to fructo-oligosaccharides. Chem. Pap. 53:366-369.
Maugeri F, Heernalsteens S (2007). Screening of yeast strains for transfructosylating activity. J. Mol. Cat. B: Enzy. 49:43-49.
Moore NCC, Yang LP, Storm H, Oliva HM, Saavedra JM (2003). Effect of fructooligosaccharide-supplemented infant cereals a double minded randomized trial. Br. J. Nutr. 90:581-587.
Patel V, Saunders G, Bucke C (1994). Production of fructooligosaccharides by *Fusarium oxysporum*. Biotechnol. Lett.16:1139-1144.
Reddy PP, Reddy GSN, Sulochana MB (2010). Screening of β-fructofuranosidase producers with high transfructosylating activity and its $3^2$ experimental run studies on reaction rate of enzyme. J. Biol. Sci.10:237-241.
Sanchez OF, Rodriguez AM, Silva E, Caicedo LA (2010). Sucrose biotransformation to fructooligosaccharides by *Aspergillus sp.* N74 free cells. Food Bioprocess Technol. 3:662-673.
Sangeetha PT, Ramesh MN, Prapulla SG (2003). Microbial production of Fructooligosaccharide. Asian. J. Microbiol. Biotech. Environ. Sci. 5:313-318.
Sangeetha PT, Ramesh MN, Prapulla SG (2005). Fructooligosaccharides production using fructosyltransferase obtained from recycling culture of *Aspergillus oryzae* CFR 202. Pro.Biochem. 40:1085-1088.
Slevin MM, Allsopp PJ, Magee PJ, Bonham MP, Naughton VR, Strain JJ, Duffy ME, Wallace JM, MacSorley EM (2014). Supplementation with calcium and short-chain fructooligosaccharides affects markers of bone turnover but not bone mineral density in postmenopausal women. J. Nutr.144: 297-304. Doi: 10.3945/jn.113.188144
Yun JW (1996). Frutooligosaccharides - occurrence, preparation and application. Enzyme Microb. Technol.19: 107-117.
Yun JW, Kim DH, Moon HY, Song CH, Song SK (1997). Simultaneous formation of fructosyltransferase and glucosyltransferase in *Aureobasidium pullulans*. J. Microbiol. Biotechnol. l7: 204-208.

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African Journal of Microbiology Research

*Full Length Research Paper*

# Biochemical characteristics and microbial association of Isabgol (*Plantago ovata* Forks.) growing soils in Western Arid region of India

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**Isabgol (*Plantago ovata* Forks.) is one of the important cash crops in arid and semi-arid regions of India. The husk of Isabgol seeds are primarily used as laxative in medicinal preparations. The cultivation of Isabgol crop is very much dependent on soils and weather conditions as this crop is highly susceptible to many biotic and abiotic stress parameters. Soil microbial population is involved in many direct and indirect interactions with crop plants. The type of microbes present in the soils also affects the plant health. Soil samples from the rhizospheric zone of the plants were collected from Barmer, Jalore and Ajmer Districts of Rajasthan State in western arid region of India. The soil EC and pH (1:2.5) recorded were in the range of 0.12 to 0.46 dS/m and 7.4 to 8.9, respectively, depicting neutral to alkaline soils, the macro nutrient viz; N, P, K were found to be in the range of 128.0 to 192.54, 19.4 to 80.4 and 149.8 to 338.8 kg/ha, respectively. The DTPA extractable micronutrients Cu, Zn, Mn and Fe were in the range of 0.29 to 3.50, 0.26 to 1.5, 0.51 to 4.51 and 1.0 to 5.38 ppm, respectively, in the soil samples of Isabgol growing regions of Rajasthan. Total viable count (TVC) gives a quantitative idea about the presence of microorganism such as bacteria and fungi in samples. The total bacterial count of soil microorganisms varied from $0.8x10^7$ to $1.96x10^7$ cfu/g whereas, total fungal count varied from $1.52x10^6$ to $2.85x10^6$ cfu/g. Beneficial microorganism population in terms of total *Azotobacter* count ($0.1x10^5$ to $1.0\ x10^5$ ) psuedomonads counts ($3.33x10^5$ to $5.8x10^5$ cfu/g) and phosphate solubilizing bacterial count ($0.1x10^3$ to $1.0x10^3$ cfu/g) varied highly in different soils.**

**Key words:** Isabgol (*Plantago ovata* Forks.), bacterial count, fungal count, N, P, K, macronutrients, micronutrients.

## INTRODUCTION

Isabgol (*Plantago ovata* and *Plantago psyllium*) is grown as a cash crop in Gujarat, Punjab and Rajasthan. India is the largest producer and exporter of this crop in the world. The seed husk is used to cure inflammation of the

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mucus membrane of gastrointestinal and genito-urinary tracts, chronic constipation, dysentery, duodenal ulcers, gonorrhea and piles. It is also used in calico printing, setting lotions and food industry (Dhar et al., 2005). Isabgol is an irrigated rabi crop which remains in the field for about four months. The crop is grown in marginal, light, well-drained sandy-loam to loamy soils having pH between 7 and 8. It requires a cool climate and dry sunny weather as light showers cause seed shedding. Isabgol makes a moderate demand for nutrients. Usually, 25 kg of each N and P per hectare is given at plantation. The crop is given 1 or 2 hand-weedings during the entire growing period. The plants are about 50 cm high and each plant gives out between 25 to 100 tillers, depending upon the fertility of soil and weather conditions and a good crop may yield about 800-1000 kg of seeds per hectare (Aishwath and Ram, 2008). The plant bears the flowering spikes in about 60 days after sowing and matures in the next 2 months. The yellowing of the lower leaves is an indication of maturity, confirmed by pressing a spike between two fingers when the mature seeds come out. The crop is harvested close to the ground in the early morning hours to avoid loses owing to seed shedding. The harvested material is stacked for 1 or 2 days, made to be trampled by bullocks, winnowed and the separated seed crop is collected. Seeds are processed through a series of grinding mills to separate the husk. About 30% husk by weight is thus recovered. The current level of production in the country is estimated at 90,000 tonnes. Isabgol production is primarily limited to Rajasthan and Gujarat. Some areas in Haryana, Bihar and Madhya Pradesh are also reported to be under Isabgol cultivation, though production in these areas is negligible. In India, Rajasthan's share is 61,000 tonnes (67%) and Gujarat accounts for 29,000 tonnes (33%) for total national Isabgol production and Rajasthan state accounts for 95,000 hectares area under this crop with Jalore, Barmer and Jaisalmer districts being the major production centers (Maiti and Mandal, 2000).

Application of biofertilizers leads to sustainability of our cropping system that is under threat due to continuous and excessive use of chemical fertilizers and other agrochemicals. Beneficial rhizospheric microorganisms for plants that are better known as plant growth promoting rhizobacteria (PGPR) favours plant growth by fixation or solubilization of plant nutrients and the production of growth stimulants. PGPR may facilitate plant growth and development both directly and indirectly. Direct stimulation may include providing plants with fixed nitrogen, phytohormones, iron that has been sequestered by bacterial siderophores, and soluble phosphate, while indirect stimulation of plant growth includes preventing phytopathogens (biocontrol) and thus, promote plant growth and development (Bashan, 1998; Glick, 1995). Isolating of native strains adapted to the environment and their study may contribute to the formulation of inoculants to be used in region specific crops. The present investigation was undertaken with a view to study the physical and chemical properties, its microbial diversity and isolation of rhizospheric bacteria and to work out the microbiological profile of rhizospheric soils.

## MATERIALS AND METHODS

### Estimation of biochemical characteristics of soil samples

Thirteen (13) soil samples (P1 to P13) from two Agro-eco sub regions viz. 2.1 and 2.3 (NBSS Publ. 51) under Western plain hot arid ecosystem desert soils (Jaiselmer and Barmer) with 100-300 mm rainfall, 1700-2000 potential evapo-transpiration, 25-30°C mean temperature, LGP <60 days and Western plain (Jalore) hot arid ecosystem desert soils with 300-450 mm rainfall, 1800-1900 potential evapo-transpiration, 24-27°C mean temperature and LGP 60-90 days, respectively were collected during the crop growth stage of pre-flowering in rabi season so that maximum microbial activity under plant root influence could be observed from the rhizospheres of Isabgol growing soils of Barmer, Jalore and Jaiselmer districts of Rajasthan (Table 1). Soil samples were analyzed for electrical conductivity (EC) and pH using aqueous soil extract (2:1), organic carbon (Walkley and Black, 1934), $NH_4^+$-N (Keeney and Nelson, 1982); 0.5M $NaHCO_3$-P (Olsen et al., 1954) neutral 1 N $NH_4OAc$-K (Hanway and Heidel, 1952) and DTPA-extractable Fe, Zn, Cu and Mn (Lindsay and Norvell, 1978). The soils of Jalore, Barmer and Jaiselmer were classified taxonomically at suborders as Typic Torripsamments, Typic Calciorthids and Typic Camborthids (Soil Survey Staff, 2010). Location of sample sites under Isabgol cultivation in different districts of Rajasthan and their soil classification are presented in Table 1.

### Estimation of microbial characteristics of soil samples

The rhizospheric soil samples collected from Isabgol fields were used for making serial dilutions upto $10^{-7}$. Ninety milliliters of the diluents was measured into bottles used for serial dilution containing 10 g soil of each sample. The mixture was shaken using a horizontal shaker (Remi Make, India) for 30 min. Further dilutions were made and desired dilutions were pour plated in Petri dishes containing selective and non-selective growth media. The total aerobic mesophilic bacterial count and total mesophilic fungal count was determined using nutrient agar and Rose Bengal agar medium using standard microbiological methods. The isolation of rhizospheric bacteria on selective nutrient agar medium and other media were carried out for total viable bacterial count and total viable fungal count. Different beneficial microbial population selective and specific growth media such as Rose Bengal dextrose agar media for fungal population, Pikovskaya's agar medium for total phosphate solubilizing (PSB) bacteria, King's B medium for Pseudomonas and Azotobacter medium for Azotobacter were employed. Total viable microbial count of theses soil samples were estimated by serial dilution technique using nutrient agar medium for bacterial count and Rose Bengal Agar medium for fungal count. All the bacterial growth media employed in this study were procured from Hi-media, India.

## RESULTS AND DISCUSSION

Plant rhizosphere soils are known to be the preferred

**Table 1.** Location of sample sites under Isabgol cultivation in different districts of Rajasthan.

| Sample no. | District | Village/location | Agro-ecological region | Soil taxonomy |
|---|---|---|---|---|
| P1 | Jalore | Kharwa, Bhinmal-A | 2.3* | Typic Camborthids |
| P2 | Jalore | Kharwa, Bhinmal-B | 2.3 | Typic Camborthids |
| P3 | Jalore | Kharwa, Bhinmal-C | 2.3 | Typic Calciorthids |
| P4 | Jalore | Ghaseri, Bhinmal | 2.3 | Typic Camborthids |
| P5 | Jalore | Hadetar, Sanchoor-A | 2.3 | Typic Torripsamments |
| P6 | Jalore | Hadetar, Sanchoor-B | 2.3 | Typic Torripsamments |
| P7 | Jalore | Hadetar, Sanchoor-C | 2.3 | Typic Calciorthids |
| P8 | Barmer | Sajitada-A | 2.1* | Typic Camborthids |
| P9 | Barmer | Sajitada-B | 2.1 | Typic Camborthids |
| P10 | Barmer | Nmibli | 2.1 | Typic Calciorthids |
| P11 | Barmer | Bhukha | 2.1 | Typic Camborthids |
| P12 | Jaiselmer | Fatehgarh, Sam | 2.1 | Typic Torripsamments |
| P13 | Jaiselmer | Pokharna | 2.1 | Typic Torripsamments |

2.1*- Western plain hot arid ecosystem desert soils (Jaiselmer and Barmer) with 100-300 mm rainfall, 1700-2000 potential evapo-transpiration, 25-30°C mean temperature, LGP <60 days; 2.3* Western plain (Jalore) hot arid ecosystem desert soils with 300-450 mm rainfall, 1800-1900 potential evapo-transpiration, 24-27°C mean temperature, LGP 60-90 days.

ecological niche for various types of soil microorganisms due to rich nutrient availability. Physio-chemical properties like pH, EC, are indictors of soil quality for understanding the nutrient status of soil and also its correlation with prevailing microbial population. The Isabgol cropping areas of Rajasthan falls under two agro-ecological regions viz; Western plain hot arid ecosystem desert soils (Jaiselmer and Barmer) with 100-300 mm rainfall, 1700-2000 potential evapo-transpiration, 25-30°C mean temperature, LGP <60 days and Western plain (Jalore) hot arid ecosystem desert soils with 300-450 mm rainfall, 1800-1900 potential evapo-transpiration, 24-27°C mean temperature, LGP 60-90 days (Table 1). Majority of soil samples under the present investigation falls under Typic Camborthids followed by Typic Torripsamments. Biochemical properties of soils under Isabgol cultivation in different districts of Rajasthan are presented in Table 2. Soil properties of the samples (P1 to P13) exhibited variations with respect to different soil sample sites. The electrical conductivity of the soils are in the range 0.12-0.46 dS/m and the hydrogen ion concentration between 7.4 - 8.9 thereby depicting neutral to slightly alkaline soils which is in confirmity with earlier findings for soils of coriander crops in Rajasthan (Mishra et al., 2013). Organic carbon content of the soils ranged from 1.6 to 4.1 g/kg across the soils (P1 to P13). In the surface soil samples from Jalore districts (P1 to P7) it was in the range 1.8 to 3.3 g/kg, 1.6 to 4.1 g/kg in the soils of Barmer district and 1.8 to 3.3 g/kg in Jjaiselmer district soils. Conclusively the soil organic carbon content was low at all the sites being the inherent characteristic of the soils of the arid and semi-arid regions. The soil texture varied between sandy loam to loamy sand. The available nitrogen content was low in all the soils with the lowest value in P13 (128.0 kg/ha) and highest in P5 (192.5 kg/ha). The $P_2O_5$ content in the soil samples P1, P2, P3, P5, P7 and P12 was in the lower range, medium in P4, p10 and P11 whereas slightly high in P8, P9 and P13. The $K_2O$ content in soils P1, P2 and P3 was low (149.8-160.2 kg/ha) and medium in the rest of the soils (P4 to P13; 209.4 to 338.8). The slightly higher values for $K_2O$ content was obtained in the soils of Barmer and Jaiselmer districts.

The concentration of DTPA extractable micronutrients was in the medium to higher range in almost all the soil samples. DTPA-Cu concentration in the soils was medium for Jalore district (P1 to P4) and higher in rest of the soils from Barmer and bordering areas of Jaiselmer district. Highest DTPA-Cu content was observed in the Typic Camborthids of Barmer district, the DTPA-Zn concentration in the soil samples varied from 0.26 to 1.5 ppm, the lowest being in Typic Torripsamments of Jaiselmer (P13) and highest in the Typic Camborthids of Barmer (P11). The soils from Jalore (P1 to P4) and Jaiselmer district (P13) were low in DTPA-Zn content, whereas rest of the soils has medium values for DTPA-Zn concentration (Hand Book of Agriculture, ICAR). Rest of the soils can be grouped under marginally deficient category in terms of DTPA-Mn and DTPA_Fe. Similar results were also reported by Chattopadhyay et al. (1996) and Shyampura and Sehgal (1996) for the arid and semi-arid soils of Rajasthan, India.

Electrical conductivity is an important factor in determining the salinity/sodicity or both in the soils. It

**Table 2.** Biochemical properties of soils under Isabgol cultivation in different districts of Rajasthan.

| Sample no. | Soil EC (dS/m) | Soil pH (1:2.5) | Organic carbon (g/kg) | Surface texture | Soil taxonomy | Macro nutrients (kg/ha) | | | Micronutrients (ppm) | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | N | $P_2O_5$ | $K_2O$ | Cu | Zn | Mn | Fe |
| P1 | 0.21 | 8.2 | 3.30 | Sandy loam | Typic Camborthids | 163.1 | 27.2 | 160.2 | 0.31 | 0.48 | 3.83 | 3.83 |
| P2 | 0.33 | 8.3 | 2.80 | Sandy loam | Typic Camborthids | 189.1 | 23.1 | 158.7 | 0.29 | 0.42 | 3.21 | 3.80 |
| P3 | 0.24 | 8.9 | 3.00 | Loamy sand | Typic Calciorthids | 155.7 | 19.4 | 149.8 | 0.30 | 0.45 | 3.27 | 3.74 |
| P4 | 0.19 | 8.2 | 3.00 | Sandy loam | Typic Camborthids | 188.4 | 63.1 | 241.1 | 0.32 | 0.53 | 3.55 | 3.79 |
| P5 | 0.38 | 8.1 | 1.80 | Loamy sand | Typic Torripsamments | 192.5 | 34.6 | 212.9 | 1.55 | 0.84 | 2.63 | 5.38 |
| P6 | 0.17 | 8.0 | 2.10 | Sandy loam | Typic Torripsamments | 189.2 | 29.4 | 209.4 | 1.45 | 0.78 | 2.32 | 5.29 |
| P7 | 0.46 | 8.3 | 2.70 | Sandy loam | Typic Calciorthids | 187.5 | 31.4 | 231.1 | 1.63 | 0.84 | 2.68 | 4.98 |
| P8 | 0.38 | 7.4 | 2.30 | Sandy loam | Typic Camborthids | 163.8 | 80.1 | 222.1 | 1.26 | 0.86 | 2.41 | 5.12 |
| P9 | 0.12 | 8.1 | 2.40 | Loamy sand | Typic Camborthids | 178.4 | 79.8 | 214.9 | 1.56 | 0.91 | 2.37 | 5.34 |
| P10 | 0.17 | 8.3 | 4.10 | Sandy loam | Typic Calciorthids | 165.6 | 43.1 | 338.8 | 1.23 | 0.81 | 2.18 | 5.76 |
| P11 | 0.26 | 8.1 | 1.60 | Loamy sand | Typic Camborthids | 153.4 | 35.6 | 315.2 | 3.50 | 1.50 | 4.51 | 2.21 |
| P12 | 0.20 | 8.2 | 1.80 | Sandy loam | Typic Torripsamments | 135.2 | 33.2 | 274.2 | 0.44 | 0.31 | 3.65 | 3.47 |
| P13 | 0.36 | 8.2 | 2.30 | Sandy loam | Typic Torripsamments | 128.0 | 61.1 | 299.8 | 0.51 | 0.26 | 0.51 | 1.00 |

represents the availability of salts in the soil. Increase in electrical conductivity of soil, increases the availability of soluble salts to the plants and thus effect on soil fertility status of the soil which in turn may affect plant health. The pH of soil plays an important role in the occurrence and dominance of a particular group of micro-organism. Soil microorganisms, just like higher plants depends entirely on soil for their nutrition, growth and activity. The major soil factors which influence the microbial population, distribution and their activity in the soil are nutrients, moisture, temperature, aeration, pH (H-ion concentration) and salt concentration (Knight et al., 1997). All these factors play a great role in determining not only the number and type of organism but also their activities. Variations in any one or more of these factors may lead to the changes in the activity of the organisms which ultimately affect the soil fertility level.

Microorganism requires a favorable nutritional and physical environment to grow and multiply. Isolation of microorganisms was done by using serial dilution methods followed by purification using Gram's staining and repeated streaking on nutrient agar media. These steps are essential to obtain well separated discrete colonies in different selective media. Total viable aerobic bacterial count of the samples ranged from $0.8x10^7$ to $1.96x10^7$ cfu/g soil while the total aerobic fungal population varied from $1.52x10^6$ to $2.85x10^6$ cfu/g soil. The maximum soil bacterial population was observed from soil sample of P8 locality in district Barmer. Similarly, maximum fungal population was recorded from Isabgol field soil samples of Nibla locality in Barmer district while minimum was observed from Gasedi, Bhinmal locality in Jalore district (Table 3).

Total viable count (TVC) gives a qualitative idea about the presence of microorganism such as bacteria, yeast and mold in a sample on different agar media. To isolate plant growth promoting rhizobacteria specific growth media such as Azospirillum semi-solid agar media Azotobactor agar media, King's B Media and Pikovaskaya agar media were used to enumerate the rhizobacteria on the basis of their ability to grow in the given media. The colonies grown on these specific growth media were enumerated and bacterial cultures were selected and isolated on the basis of different colony morphologies through subculture technique. Microorganism requires a favorable nutritional and physical environment to grow and multiply. Isolation of microorganisms was done by using serial dilution methods followed by purification using Gram's staining and repeated streaking on nutrient agar

**Table 3.** Microbial properties of Isabgol growing soils from Rajasthan.

| Sample no. | District | Village/location | Total viable microbial count (CFUs/g) | | | | |
|---|---|---|---|---|---|---|---|
| | | | Bacteria | Fungi | Azotobactor | Pseudomonads | Phosphate solubilizing bacteria |
| P1 | Jalore | Kharwa, Bhinmal-A | $1.1X10^7$ | $1.62X10^6$ | $0.3X10^5$ | $4.6X10^5$ | $0.7X10^3$ |
| P2 | Jalore | Kharwa, Bhinmal-B | $0.9X10^7$ | $1.28X10^6$ | $0.7X10^5$ | $5.2X10^5$ | $0.4X10^3$ |
| P3 | Jalore | Kharwa, Bhinmal-C | $1.2X10^7$ | $1.70X10^6$ | $0.1X10^5$ | $5.6X10^5$ | $1.0X10^3$ |
| P4 | Jalore | Ghaseri, Bhinmal | $1.2X10^7$ | $1.52X10^6$ | $0.8X10^5$ | $3.8X10^5$ | $0.1X10^3$ |
| P5 | Jalore | Hadetar,Sanchoor-A | $1.3X10^7$ | $2.12X10^6$ | $1.0X10^5$ | $3.3X10^5$ | $0.8X10^3$ |
| P6 | Jalore | Hadetar,Sanchoor-B | $1.8X10^7$ | $1.78X10^6$ | $0.6X10^5$ | $3.8X10^5$ | $0.9X10^3$ |
| P7 | Jalore | Hadetar,Sanchoor-C | $1.9X10^7$ | $1.58X10^6$ | $0.3X10^5$ | $4.3X10^5$ | $0.3X10^3$ |
| P8 | Barmer | Sajitada-A | $1.96X10^7$ | $2.40X10^6$ | $0.8X10^5$ | $5.8X10^5$ | $0.7X10^3$ |
| P9 | Barmer | Sajitada-B | $0.96X10^7$ | $2.40X10^6$ | $0.5X10^5$ | $3.4X10^5$ | $0.6X10^3$ |
| P10 | Barmer | Nmibli | $1.9X10^7$ | $2.85X10^6$ | $0.7X10^5$ | $4.5X10^5$ | $0.9X10^3$ |
| P11 | Barmer | Bhukha | $0.96X10^7$ | $1.53X10^6$ | $0.6X10^5$ | $4.3X10^5$ | $0.1X10^3$ |
| P12 | Barmer | Fatehgarh, Sam | $0.90X10^7$ | $2.25X10^6$ | $0.4X10^5$ | $3.5X10^5$ | $1.0X10^3$ |
| P13 | Jaiselmer | Pokharna | $1.0X10^7$ | $2.10X10^6$ | $0.9X10^5$ | $4.1X10^5$ | $0.7X10^3$ |

media. These steps are essential to obtain well separated discrete colonies in different selective media. Pure cultures were preserved on nutrient agar slants stored at 4°C in a refrigerator and further used for estimation of various plant growth promotion characteristics. Microbial count on nitrogen free Azotobacter medium revealed the population of Azotobacter in Isabgol field soil samples which varied from $0.1x10^5$ to $2.0x10^5$ cfu/g. The Pikovskaya medium containing tricalcium phosphate as sole source of phosphorus for bacterial growth provided the rough estimate of phosphate solubilizing bacterial (PSB) population and it ranged from $0.2x10^3$ to $4.8x10^3$ cfu/g and Pseudomonads population on Kings B medium varied from $3.3x10^6$ to $5.8x10^6$ cfu/g soil among the collected soil samples of Isabgol crop (Table 3).

It is well established that inoculation with diazotrophic bacteria like *Rhizobium*, *Azotobacter* and *Azospirillum* enhances the plant growth as a result of their ability to fix nitrogen. Growth promotion may be attributed to other mechanisms such as production of plant growth promoting hormones in the rhizosphere and other PGP activities (Bhashan and Bashan, 2005).

Sakthivel and Karthikeyan (2012) had studied thirty rhizospheric soil samples collected from commercially grown *Coleus forskohlii* from Perambalur and Salem districts of Tamil Nadu. The results obtained showed that among the 30 isolates of Perambalur and Salem districts the range was from 4.00-$9.22x10^6$ and 4.66-$10.00x10^6$ for *Azospirillum* spp., 3.00-$7.66x10^6$ and 3.88-$8.00x10^6$ for *Bacillus* spp, 4.66-$12.00x10^6$ and 4.88-$13.00x10^6$ for *Pseudomonas* spp. and 2.22-$8.00x10^6$ and 3.66-$9.00x10^6$ for *Azotobacter* spp., respectively, for the two districts. In the present investigation, the population of Isabgol plant beneficial rhizobacteria was found lower than reported by Sakthivel and Karthikeyan (2012) which may be due to different agro-ecological condition prevailing in the Rajasthan as compared to Tamilnadu. In addition to plant growth promoting traits, these bacterial strains must be rhizospheric competent, able to survive and colonize in the rhizosphere soil (Grover et al., 2009). During the past couple of decades, the use of plant growth promoting rhizobacteria (PGPR) for sustainable agriculture has increased tremendously in various parts of the world. Significant increases in growth and yield of agronomical important crops in response to inoculation with PGPR have been repeatedly reported (Biswas et al., 2000; Asghar et al., 2002; Vessey, 2003).

A general concept has been developed, where bacterial strains were identified that were able to colonize plant roots, stimulate plant growth, and/or reduce the incidence of plant disease. Importance of native strains and ecological specificity while selecting the microbial inoculants for a specific environment is also realized. Isolation of microorganisms, screening for desirable characters, and selection of efficient strains and production of inoculums are important steps for making use of this microbe-based technology.

## Conclusions

Though the soils of Jalore, Barmer and Jaiselmer fall under the arid region of India and are poor to medium in terms of major and minor nutrients availability, the crop of Isabgol thrives well. The nutrient requirement of Isabgol crop can still be fulfilled by these soils and thus Isabgol cultivation is a good proposition for these areas. Plant growth promoting rhizo-bacterial population can also con-

tribute significantly towards nutrient availability. Total count of phosphate solubilizing bacteria, free living nitrogen fixing bacteria and Pseudomonads estimated for different soil samples recorded significant differences among the samples of different locations. Thus it can be concluded that the diverse agro-climatic conditions of this semi-arid Rajasthan offers a great potential of further microbiological exploration for novel microbes and especially nitrogen fixers and phosphate solubilizing bacteria for application in Isabgol and other agricultural crops.

## Conflict of interests

The authors did not declare any conflict of interest.

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### REFERENCES

Aishwath OP, Ram C (2008). Response of blond psyllium (*Plantago ovata*) to split application of nitrogen on growth, yield and quality, Indian J. Agric. Sci. 78:323-327.

Asghar HN, Zahir ZA, Arshad M, Khalig A (2002). Plant growth regulating substances in the rhizozphere: microbial production and functions. Adv. Agron. 62:146-151.

Bashan Y (1998). Inoculants of plant growth-promoting bacteria for use in agriculture. Biotechnol. Adv. 16:729-770.

Bhasan Y, Bashan de LE (2005). Bacteria. In: Encyclopedia of Soils in the Environment, Elsevier, V K 1:103-115.

Chattopadhyay T, Sahoo AL, Singh RS, Shypmpura RL (1996). Available micronutrient status in the soils of Vindhyan scraplands of Rajasthan in relation to soil characteristics. J. Indian Soc. Soil Sci. 44:678-680

Dhar MK, Kaul S, Sareen S, Koul AK (2005). *Plantago ovata*: Genetic Diversity, Cultivation, Utilization and Chemistry. Plant Genet. Resour. 3(2):252-263.

Glick BR (1995).The enhancement of plant growth by free living bacteria. Can. J. Microbiol. 41:109-117.

Grover M, Pandey AK, Mishra BK, Lata, Roy RC (2009). Sugarcane crops: plant growth promoting bacteria in growth, yield and productivity. In: Sugar beet crops: Growth, fertilization and yield (Hertsburg Claus T. ed). Nova Science Publishers Inc., New York, pp. 135-151.

Knight BP, McGrath SP, Chaudri AM (1997). Biomass carbon measurements and substrate utilization patterns of microbial populations from soils amended with cadmium, copper, or zinc. Appl. Environ. Microbiol. 63:39-43.

Lindsay WL, Norvell WA (1978). Development of DTPA soil test for Fe, Mn, Zn and Cu. Soil Sci. Soc. Am. J. 42:421-427.

Maiti S, Mandal K (2000). Cultivation of Isabgol. National Research Center for Medicinal and Aromatic Plants, Anand, India, Bulletin. pp. 1-8.

Mishra BK, Ashutosh S, Aishwath OP, Sharma YK, Krishna K, Vishal MK, Saxena SN, Ranjan JK (2013). Microbiological profile of Isabgol (Coriandrum sativum L.) crop rhizosphere in Rajasthan and screening for auxin producing rhizobacteria. International J. Seed Spices, 3(2):59-64.

Sakthivel U, Karthikeyan B (2012). Isolation and characterization of plant growth promoting rhizobacteria (pgpr) from the rhizosphere of *Coleus forskohlii* grown soil. Int. J. Recent Sci. Res. 3(5):288-296.

Shyampura RL,Sehgal JL (1996). Soils of Rajasthan for optimizing land use. NBSSLUP publication No. 51, Nagpur, India.

Soil Survey Staff (2010). Keys to Soil Taxonomy. 11th Edition, USDA, Natural Resource Conservation Service, Washington DC, USA.

Vessey JK (2003) Plant growth-promoting rhizobacteria as biofertilizers. Plant Soil 255:571-586.

Walkley A, Black IA (1934). An examination of the Degtjareff method for determining soil organic matter, and a proposed modification of the chromic acid titration method. Soil Sci. 37:29-38

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# Screening of Amazon fungi for the production of hydrolytic enzymes

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**The use of fungi for the production of enzymes has been widely investigated, but there are few reports concerning the use of these organisms isolated from the Amazon Region for the production of hydrolytic enzymes. Considering the importance of hydrolases for different industrial applications, this work aimed to select Amazon fungi for the production of cellulase, protease, pectinase, amylase and xylanase. In order to verify the potential of the Amazon isolates for the production of these five hydrolytic enzymes, growth on solid media containing specific substrates was performed. The qualitative assay revealed that among 40 Amazon fungi, 16 produce cellulase; 13 present proteolytic activity; 13 have pectinase activity, 27 produce amylase and 16 present xylanolyitc activity. Two amylase producers were grown in liquid media in order to access their amylolytic activity. Fungi isolated from aloe vera presented higher amylase production within 48 h of cultivation on potato starch broth.**

**Key words:** Hydrolytic enzymes, Amazon fungi, solid media assays, amylase activity.

## INTRODUCTION

The enormous impact of fungi on biotechnology has been well-established. These organisms have been used for the production of foods, antibiotics, alcohols, enzymes, organic acids, and numerous pharmaceuticals. Its morphological and physiological diversity includes microscopic molds and yeasts, as well as macroscopic mushrooms and truffles. The microscopic species are, however, best known for their biotechnological applications (Bennett, 1998).

Besides their importance as decomposing agents on forestry ecosystems, fungi are responsible for degrading a wide variety of wood products and providing all kinds of

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food and industrialized goods, as well as petroleum derivatives (Selosse et al., 2004). These abilities result from the fact that these organisms produce several degradative enzymes, which are secreted into the environment. Due to its absorptive mode of nutrition, a large amount of powerful hydrolytic enzymes are synthesized, and thus, various fungi species are used for the production of industrially-important hydrolases (Alves et al., 2002).

The food, feed, agriculture, paper, leather, and textile industries are well-suited for enzyme technology because products, as well as raw materials, consist of biomolecules, which can be produced, degraded, or modified by enzymatic processes (Mussato et al., 2007). The detergent and starch industries consume the greatest amounts of enzymes (Abrahão Neto, 2007).

The industrial importance of hydrolases exceeds that of other classes of enzymes. Hydrolytic enzymes can be produced by different species, either in liquid or solid media (Jecu, 2000). These degrading proteins can act on various substrates, depending on its industrial application. Cellulases, proteases, glucoamylases, xylanases, pectinases, hemicellulases, lipases, and amylases are the hydrolytic enzymes most applied in industrial processes (Silva et al., 2005a).

Fungal extracellular enzymes may be produced in liquid or solid media. The use of solid media permits a fast screening of large populations of fungi, allowing the detection of specific enzymes (Alves et al., 2002).

Considering the enormous microbiological potential of the Amazon Region and the growing applicability of fungal hydrolytic enzymes, the screening of Amazon appears to be an interesting approach to finding novel hydrolase producers. Hence, the aim of this study was to select Amazon fungi for the production of five industrially-important hydrolytic enzymes: cellulase, protease, pectinase, xylanase and amylase using solid media assays, as well as to access the enzymatic activity of most promising isolates.

## MATERIALS AND METHODS

### Amazon fungi

Endophytic and phytopathogenic isolates belonging to the Graduate Program in Biotechnology and Natural Resources from UEA were used in this investigation. Endophytic fungi isolated from the tucumã palm (*Astrocaryum aculeatum*), spiked pepper (*Piper aduncum*), uxi (*Endopleura uchi*), and manioc (*Manihot esculenta*) were investigated. Phytopathogenic isolates were obtained from aloe vera (Aloe vera) and peach palm (*Bactris gasipaes*). Wood-degrading fungi were kindly provided by Professor Ademir Castro e Silva of UEA. The coded isolates and the information relating to their origin are in Table 1.

**Table 1.** Identification code for Amazon fungi evaluated for hydrolytic enzymes production and their respective origin.

| Strain Code | Type[a] | Host | Tissue |
|---|---|---|---|
| UEA_018 | PT | Aloe vera | leaf |
| UEA_025 | PT | Aloe vera | leaf |
| UEA_033 | PT | *Bactris gasipaes* | fruit |
| UEA_042 | EP | *Astrocaryum aculeatum* | seed |
| UEA_064 | EP | *Pipper aduncum* | root |
| UEA_076 | EP | *Endopleura uchi* | fruit |
| UEA_094 | WD | Amazon wood | stems |
| UEA_097 | WD | Amazon wood | stems |
| UEA_099 | WD | Amazon wood | stems |
| UEA_102 | WD | Amazon wood | stems |
| UEA_105 | WD | Amazon wood | stems |
| UEA_107 | EP | *Manihot esculenta* | stems |
| UEA_108 | FT | Aloe vera | leaf |
| UEA_116 | WD | Amazon wood | stems |
| UEA_121 | WD | Amazon wood | stems |
| UEA_122 | WD | Amazon wood | stems |
| UEA_123 | WD | Amazon wood | stems |
| UEA_128 | WD | Amazon wood | stems |
| UEA_129 | WD | Amazon wood | stems |
| UEA_130 | WD | Amazon wood | stems |
| UEA_131 | EP | *Manihot. esculenta* | stems |
| UEA_140 | WD | Amazon wood | stems |
| UEA_143 | WD | Amazon wood | stems |
| UEA_155 | EP | *Pipper aduncum* | stems |
| UEA_165 | EP | *Pipper aduncum* | stems |
| UEA_166 | EP | *Pipper aduncum* | leaf |
| UEA_204 | WD | Amazon wood | stems |
| UEA_206 | WD | Amazon wood | stems |
| UEA_207 | WD | Amazon wood | stems |
| UEA_208 | WD | Amazon wood | stems |
| UEA_212 | WD | Amazon wood | stems |
| UEA_214 | WD | Amazon wood | stems |
| UEA_219 | WD | Amazon wood | stems |
| UEA_220 | WD | Amazon wood | stems |
| UEA_221 | WD | Amazon wood | stems |
| UEA_229 | WD | Amazon wood | stems |
| UEA_233 | WD | Amazon wood | stems |
| UEA_235 | WD | Amazon wood | stems |
| UEA_237 | WD | Amazon wood | stems |
| UEA_239 | WD | Amazon wood | stems |

[a]EP = Endophytic; PT = Phytopathogenic; WD = Wood-degrading.

### Fungi maintenance

Stock cultures of the isolates were maintained on PDA (potato dextrose agar) dishes at 4°C. To maintain the cells' viability, fungi were periodically transferred to new PDA dishes, incubated at 30°C for 5-7 days and stored at 4°C, being used as required.

### Inoculum

The inoculum was prepared in PDA dishes, from the stock culture,

being incubated at 30°C for five to seven days. The Petri dishes containing specific substrates for the enzyme assays were inoculated with a disc of around 7.0 mm diameter from the PDA medium culture.

#### Solid media composition

For assessing the cellulase production of Amazon fungi, the solid media was prepared with 1.8% (v/w) agar, 1.0 % (v/w) carboxymethylcellulose, and 0.1 M sodium acetate buffer solution pH 5.0. For protease assay a solid media containing 1.8% (v/w) agar, 1.0 % (v/w) gelatin, 1.0 % (v/w) skin milk, and 0.1 M citrate-phosphate buffer solution pH 5.0 was prepared. For identifying pectinase-producing-fungi the solid media was prepared with 1.8% (v/w) agar, 1.0 % (v/w) citric pectin, and 0.1 M sodium acetate buffer solution pH 5.0. Amylase production was verified using a solid media containing 1.8% (v/w) agar, 1.0 % (v/w) corn starch, and 0.1 M citrate-phosphate buffer solution pH 5.0 (Teixeira, 1994). Xylanase activity was evaluated on a solid media prepared with 1.8% (v/w) agar, 1.0 % (v/w) birch wood xylan, and 0.1 M sodium acetate buffer solution pH 6.0 (adapted from Silva et al., 2005b). After autoclaving at 121°C for 15 min, the culture media was cooled to approximately 60°C and transferred to Petri dishes for rapid cooling.

#### Solid media enzymatic assays

The Petri dishes containing the specific substrates were inoculated with the Amazon isolates. The dishes were incubated at 30°C for 5-10 days and periodically observed for the evaluation of fungal growth. Before the isolate colonizes one-third of the Petri dish, the presence of a translucent halo around the fungi, which confirms the hydrolytic activity, was verified. A control dish was also prepared where there was no fungal inoculation. All assays were carried out in triplicate.

#### Halo detection on solid media

The halo formation around the fungi indicates the production of hydrolytic enzymes. Protease production could be directly identified, through the presence of a translucent halo in the solid media. For detecting the halo to confirm the production of cellulase, pectinase, amylase, and xylanase, a revealing solution was used, according to the methodology described by Teixeira (1994). In order to verify the halo related with the pectinase production, a 5.0 M hydrochloric acid (HCl) solution was used. For amylase halo detection, a 0.1 M iodide solution was employed, and to verify cellulase and xylanase activities, a 0.1% (v/w) Congo red solution was used.

#### Amylase activity assay

For amylolytic activity detection, two fungi isolates that presented positive results for amylase production were cultivated in liquid media containing potato (200 g/L) and sucrose (20 g/L). PDA fragments (6 x 6 mm) containing fungi mycelia were inoculated on liquid media and kept at 30°C, 150 rpm, during 7 days. Aliquots were withdrawn from the culture broth in 24 h intervals and used for the enzymatic activity assay. Amylolytic activity was accessed through the methodology described by Miller (1959), using maltose as the standard. One unit of enzymatic activity was defined as the amount of enzyme capable to release one μmol of maltose per minute.

## RESULTS AND DISCUSSION

### Growth of Amazon fungi on solid media containing the specific substrates

In general, the Amazon isolates presented sufficient growth on the five specific substrates used on the composition of solid medium, reaching the edge of the Petri dish. Phytopathogenic fungi have shown the fastest growth, while wood-degrading isolates took longer to reach one-third of the plate. The mycelium of phytopathogenic fungi reached the desired distance (7.5 cm) from the center of the Petri dish after 5 days of culture, while wood-degrading isolates took twice this time. The ability of Amazon fungi to grow on different carbon sources indicates that these isolates are a versatile microorganism, which suggests that they are capable of producing the investigated hydrolytic enzymes.

### Hydrolytic enzymes production

The results for the qualitative assays performed on solid media for the production of hydrolytic enzymes by Amazon fungi are presented in Table 2. The 40 fungal isolates were submitted to the hydrolytic tests to verify the production of cellulase, protease, pectinase, amylase and xylanase. Of these, 16 fungi demonstrated cellulase activity, 13 produced protease, 13 are capable of producing pectic enzymes, 27 presented amylase activity, and 16 were shown to be xylanase producers. The percentual distribution of the hydrolytic enzyme production among the 40 tested Amazon fungi is presented in Figure 1.

It can be observed in Figure 1 that of the 40 Amazon fungi evaluated in this work, 32% presented amylolytic activity. Of these, isolates UEA_018, UEA_025, UEA_097, UEA_099, UEA_105, UEA_107, UEA_130, UEA_131, UEA_155, UEA_208, UEA_214, UEA_221, UEA_235, and UEA_239 presented larger halos, which suggest that these fungi may have higher amylase activity than the other isolates (Alves et al., 2002). Amylase (EC 3.2.1.1) are extracellular endo-enzymes that randomly catalyze the hydrolysis of internal α-1,4-glucosidic linkages in starch and related glucans. This enzyme is widely used in various industrial processes, including the production of isomerized sugar and biofuel from starch, bakery applications, textiles and paper industries, pharmaceuticals, and detergents (Tamamura et al., 2014; Sahnoun et al., 2015). Therefore, seeking new sources of this hydrolytic enzyme has appeared as an interesting research approach.

Regarding the xylanase production, 19% of the Amazon isolates showed positive results. UEA_033, UEA_042, UEA_130, UEA_204, and UEA_206 formed larger halos in the solid media, indicating higher xylano-lyitc activity of these isolates. Xylanases (EC3.2.1.8)

**Table 2.** Production of hydrolytic enzymes by Amazon fungi[a].

| Isolate | Cellulase | Protease | Pectinase | Amylase | Xylanase |
|---|---|---|---|---|---|
| UEA_018 | + | + | + | + | + |
| UEA_025 | + | + | + | + | + |
| UEA_033 | - | - | - | + | + |
| UEA_042 | + | + | - | - | + |
| UEA_064 | - | - | - | - | - |
| UEA_076 | - | - | - | - | - |
| UEA_094 | - | - | - | + | - |
| UEA_097 | - | - | - | + | - |
| UEA_099 | - | + | - | + | + |
| UEA_102 | - | - | + | + | - |
| UEA_105 | - | + | - | + | + |
| UEA_107 | - | - | - | + | + |
| UEA_108 | - | - | - | + | - |
| UEA_116 | - | - | - | - | + |
| UEA_121 | + | - | - | + | - |
| UEA_122 | + | - | - | - | - |
| UEA_123 | + | - | - | + | - |
| UEA_128 | + | - | + | - | - |
| UEA_129 | + | + | + | + | + |
| UEA_130 | - | + | + | + | + |
| UEA_131 | + | + | + | + | + |
| UEA_140 | + | - | - | + | + |
| UEA_143 | - | - | - | - | - |
| UEA_155 | - | - | + | + | - |
| UEA_165 | + | - | + | - | - |
| UEA_166 | - | - | - | - | - |
| UEA_204 | + | - | - | + | + |
| UEA_206 | + | + | - | + | + |
| UEA_207 | - | - | - | - | - |
| UEA_208 | - | - | - | + | - |
| UEA_212 | - | + | + | - | - |
| UEA_214 | - | - | - | + | - |
| UEA_219 | - | - | - | - | - |
| UEA_220 | - | - | + | + | - |
| UEA_221 | - | - | - | + | - |
| UEA_229 | + | + | - | + | + |
| UEA_233 | + | - | - | + | + |
| UEA_235 | - | - | - | + | - |
| UEA_237 | - | + | + | - | - |
| UEA_239 | + | + | + | + | - |

[a]Positive tests indicate halo formation.

act on β-1,4 linkages of xylan, the most abundant constituent of hemicellulose (Collins et al., 2005). A great variety of microorganisms have been reported as xylanolytic enzymes producers, among which fungi are the most interesting ones (Silva and Carmona, 2008). Applications of xylanases with or without concomitant use of cellulase include the bioconversion of lignocelluloses to sugar, ethanol and other useful substances, clarification of juices and wines, cellulose pulp production for paper industry, extraction of coffee and vegetable oil, and nutritional value improvement of silage and green feed (Collins et al., 2005).

Fungi isolated from Amazon forest have been assessed for xylanase production. Medeiros et al. (2003)

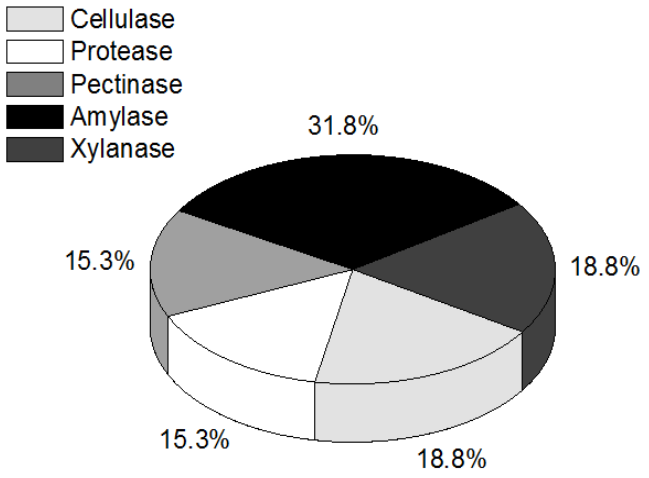


**Figure 1.** Percentual distribution of fungal isolates obtained from Amazon region according to its hydrolytic activity.

isolated and evaluated 10 fungal species from the Amazon forest for their capacity to produce xylan-degrading enzymes. The authors found that the best producing strains of β-xylanase were *Penicillium corylophilum*, *Aspergillus niger* and *Trichoderma longibrachiatum*. These strains were used in bleaching of eucalyptus kraft pulps and showed promising results (Medeiros et al., 2007).

Cellulase detection assays demonstrate that 19% of the Amazon fungi assessed can be considered as a source of this enzyme. Of these, UEA_042, UEA_122, UEA_128, UEA_129, UEA_131, UEA_165, UEA_204, UEA_206, UEA_229, and UEA_239 provided larger halos when compared to the other cellulase producing-fungi. Cellulase represents a complex mixture of hydrolytic enzymes with different specificities, which act over cellulolytic substrates, hydrolyzing glycosidic linkages, and converting them into glucose (Castro and Pereira Jr, 2010).

The most important source of microbial cellulases for industrial processes includes the fungal genera *Aspergillus*, *Penicillium* and *Trichoderma*. Cellulases and other enzymes capable to degrade plants cell wall are often used on clarification of fruit juices and wines, as well as on oil and fruit extraction. The feed industry employs cellulases for improving the digestibility of plants used for animal feed, and the textile industry uses these enzymes for removing the excess of indigo dye from jeans (Silva, 2008). Delabona et al. (2012) used a new strain of *Trichoderma harzianum*, isolated from the Amazon rainforest for the on-site production of cellulases to hydrolyze pretreated sugar cane bagasse. The Amazon strain showed outstanding results on cellulase production, when compared to *Trichoderma reesei*.

Proteolytic and pectolytic activity was verified within up to 15% of the assessed isolates. Larger halos indicating higher protease activity were verified for the isolates UEA_018, UEA_025, UEA_129, UEA_130, UEA_131, UEA_212, UEA_237, and UEA_239. Proteases break peptide linkages, a process named proteolytic cleavage, which is a common mechanism involved at digestion and blood coagulation. Proteinases have different industrial applications, especially on food, leather, detergent and textile industries (Ladeira et al., 2010). On leather industry proteases are used for removing the fur and for the partial degradation of keratin and elastin. Peptidases are also used on pharmaceutical formulations, such as antibiotics and analgesics. (Abrahão Neto, 2007). Fungi and yeast have been reported as protease producers for industrial applications. Ito et al. (2007) evaluated the protease production of a *Beauveria bassiana* Brazilian strain, which presented stability at 60°C. Neves et al. (2006) isolated 50 yeasts from Amazon Region and performed a screening for protease production. An Amazon *Candida intermedia* strain exhibited the higher proteolytic activity and did not present pathogenesis.

Among the pectinase producers, Amazon fungi UEA_018, UEA_025, UEA_102, UEA_128 and UEA_130 formed larger halos, suggesting the higher hydrolytic activity of these isolates. Pectinase are the enzymes responsible for the degradation of pectin, a complex molecule that occurs as structural polysaccharide in the middle lamella and the primary walls of young plant cells (Yadav et al., 2009; Pedrolli and Carmona, 2010). Pectic enzymes have a wide range of applications in food industry, especially in fruit juice extraction, and clarification (Pedrolli et al., 2008). Other applications of pectinases include wine making, oil extraction, pharmaceutical, paper, feed and textile fields (Martínez-Trujillo et al., 2011). It has been reported that microbial pectinases account for 25% of the global food enzymes sales (Jayani et al., 2005). Pectic enzymes used in the fruit juice industries and wine making often come from fungal sources, especially from *Aspergillus niger* (Pedrolli et al., 2008). Therefore, the isolation of new efficient pectinase producing microorganisms, the selection of optimum conditions for its production, and the biochemical characterization of these proteins represent an essential research field for the development of more competitive industrial processes (Marchi et al., 2006).

As shown in Table 2, the Amazon isolates UEA_018 and UEA_025, phytopathogenic fungi isolated from aloe vera, as well as the isolate UEA_129, a wood-degrading fungus, and the isolate UEA_131, endophytic fungi isolated from manioc, presented positive results for all five hydrolytic assays. These fungi showed to be the most versatile among the investigated Amazon isolates. On the other hand, the isolates UEA_064, UEA_076 UEA_143, UEA_166, UEA_207, and UEA_219 do not produce the tested hydrolytic enzymes (Table 2). The halo formed by the isolate UEA_239 in four enzymatic assays can be seen in Figure 2.

Among the five hydrolytic enzymes tested here, the isolates UEA_094, UEA_097, UEA_108, UEA_208,

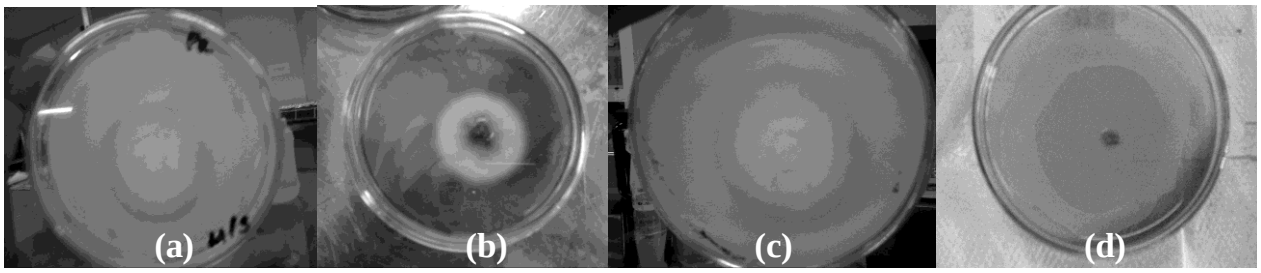


**Figure 2.** Hydrolytic activity of Amazon fungi UEA_239. Presence of the halo indicating the production of (a) protease, (b) amylase, (c) pectinase and (d) cellulase.

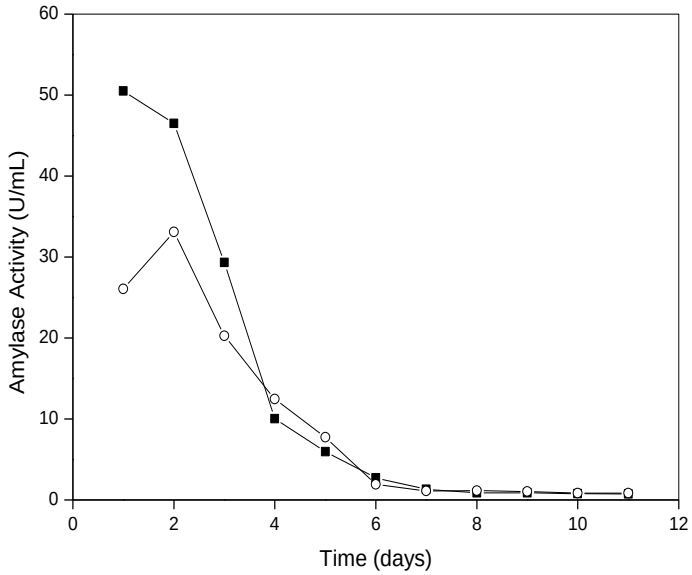


**Figure 3.** Amylolytic activity of Amazon fungi UEA_018 (○) and UEA_025 (■) during liquid media culture on potato starch broth.

UEA_214, UEA_221, and UEA_235 showed only amylolytic activity. The isolate UEA_116 presented only xylanase activity and the fungus UEA_122 produced only cellulase. These Amazon isolates demonstrate a hydrolytic specificity, which can be very interesting for industrial applications, since its extracellular enzymes would act specifically over the desired substrate. It is worth mentioning that the amylase producing isolates UEA_097, UEA_208, UEA_214, UEA_221, and UEA_235 presented higher enzymatic activities, which suggest that these Amazon fungi are potential candidates for use as amylase sources. The same can be observed for the cellulase-producing isolate UEA_122, which presented high enzymatic activity.

The Amazon fungi assessed in this work for cellulase, protease, pectinase, amylase, and xylanase activities were previously tested for lipase production (Zanotto et al., 2009). The isolates that presented the higher hydrolytic activities in the present study did not present lipase synthetic activity in the former work. However, these isolates presented positive results in degrading tributyrine, indicating the presence of lipases and/or esterases with hydrolytic activity.

## Amylase activity

Since most of the tested fungi (31.8%) presented amylolytic activity, the isolates UEA_018 and UEA_025, phytopathogenic fungi from aloe vera, which presented the most intense starch degrading halos, suggesting a prominent amylase production, were selected for amylase activity detection. The results for the quantitative assay performed on liquid media containing potato starch are presented in Figure 3.

It can be noticed in Figure 3 that UEA_018 presented the highest amylolytic activity after two days of cultivation (33.1 U/mL). After that, the enzymatic activity dropped and

reached a minimum value within eight days of growth. The same behavior was observed for isolate UEA_025, which presented a higher value of amylolytic activity (50.5 U/mL in one day of growth). The lowering of amylase production was observed probably due to the consumption of potato starch (inductive carbon source used for amylase production).

According to Hashemi et al. (2015), the microbial production of α-amylase is greatly influenced by the components of the culture medium, especially the carbon and nitrogen sources. Saleem and Ebrahim (2014) verified the maximum production of *Rhizopus stolonifer* amylase after six days of incubation on starch and a decreased enzymatic production with further incubation, as observed in this study. Chimata et al. (2010) reported that *Aspergillus* MK07 produced most amylase after 5 days of incubation on wheat bran. Both studies were carried out at 30°C culture, the same temperature used here, and therefore, suggesting that the Amazon fungi have a faster amylase production, a very interesting characteristic for industrial enzyme production.

## Conclusion

With this study it was possible to demonstrate the great potential of the Amazon fungi as hydrolytic enzymes suppliers for a large range of industrial applications. Most of the tested isolates produced amylase and the most promising amylolytic producer showed rapid enzyme production in liquid media. Further investigations are necessary in order to establish optimum parameters for the production of these enzymes in a larger scale.

## Conflict of interests

The authors did not declare any conflict of interest.

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### REFERENCES

Abrahão Neto J (2007). Algumas aplicações de enzimas. In: Lima UA, Aquarone E, Borzani W, Schmidell W (Eds.) Biotecnologia Industrial. São Paulo: Edgard Blücher.

Alves MH, Campos-Takaki GM, Porto ALF, Milanez AI (2002). Screening of *Mucor spp.* for the production of amylase, lipase, polygalacturonase and protease. Braz. J. Microbiol. 33:325-330.

Bennett JW (1998). Mycotechnology: the role of fungi in biotechnology. J. Biotechnol. 66:101-107.

Castro AM, Pereira Jr. N (2010). Production, properties and application of cellulases in the hydrolysis of agroindustrial residues. Quim. Nova 33:181-188.

Chimata MK, Sasidhar P, Challa S (2010). Production of extracellular amylase from agricultural residues by a newly isolated *Aspergillus* species in solid state fermentation. Afr. J. Biotechnol. 9(32):5162-5169.

Collins T, Gerday C, Feller G (2005). Xylanases, xylanase families and extremophilic xylanases. FEMS Microbiol. Rev. 29:3-23.

Delabona PS, Farinas CS, Silva MR, Azzoni SF, Pradella JGC (2012). Use of a new *Trichoderma harzianum* strain isolated from the Amazon rainforest with pretreated sugar cane bagasse for on-site cellulase production. Bioresour. Technol. 107:517-521.

Hashemi M, Shojaosadati SA, Razavi SH, Mousavi SM (2015). Different catalytic behavior of α-amylase in response to the nitrogen substance used in the production phase. J. Ind. Eng. Chem. 21:772-778.

Ito ET, Varéa-Pereira G, Miyagui DT, Pinoti MHP, Neves PMOJ (2007). Production of extracellular protease by a Brazilian strain of *Beauveria bassiana* reactivated on coffee berry borer, *Hypothenemus hampei*. Braz. Arch. Biol. Technol. 50:217-233.

Jayani RS, Saxena S, Gupta R (2005). Microbial pectinolytic enzymes: a review. Process Biochem. 40:2931-2944.

Jecu L (2000). Solid state fermentation of agricultural wastes for endoglucanase production. Ind. Crop. Prod. 11:1-5.

Ladeira SA, Andrade MVV, Delatorre AB, Perez VH, Martins MLL (2010). Protease production using agroindustrial residues by thermophilic *Bacillus* sp in submerged fermentation: optimization of the culture medium using an experimental design approach. Quim. Nova 33:324-328.

Marchi CE, Borges MF, Mizubuti ESG (2006). Atividades amilolítica e pectinolítica de *Alternaria solani* e a relação com a agressividade em tomateiro. Summa Phytopathol. 32:345-352.

Martínez-Trujillo A, Arreguín-Rangel L, García-Rivero M, Aguilar-Osorio G (2011). Use of fruit residues for pectinase production by *Aspergillus flavipes* FP-500 and *Aspergillus terreus* FP-370. Lett. Appl. Microbiol. 53:202-209.

Medeiros RG, Hanada R, Ferreira Filho EX (2003). Production of xylan-degrading enzymes from Amazon Forest fungal species. Int. Biodeterior. Biodegradation 52:97-100.

Medeiros RG, Silva Junior FG, Báo SN, Hanada R, Ferreira Filho EXF (2007). Application of xylanases from Amazon forest fungal species in bleaching of *Eucalyptus* kraft pulps. Braz. Arch. Biol. Technol. 50:231-238.

Miller GL (1959) Use of dinitrosalicilic acid reagent for determination of reducing sugar. Anal. Chem. 31:426-428.

Mussato SI, Fernandes M, Milagres AMM (2007). Enzimas: poderosa ferramenta na indústria. Ciencia Hoje. 41:28-33.

Neves KCS, Porto ALF, Teixeira MFS (2006). Screening of yeasts from Amazon Region for extracellular protease production. Acta Amazon. 36: 299-306.

Pedrolli DB, Carmona EC (2010). Purification and characterization of the exopolygalacturonase produced by *Aspergillus giganteus* in submerged cultures. J. Ind. Microbiol. Biotechnol. 37:567-573.

Pedrolli DB, Gomes E, Monti R, Carmona EC (2008). Studies on productivity and characterization of polygalacturonase from *Aspergillus giganteus* submerged culture using citrus pectin and orange waste. Appl. Biochem. Biotechnol. 144: 191-200.

Sahnoun M, Kriaa M, Elgharbi F, Ayadi D-Z, Bejar S, Kammoun R (2015). *Aspergillus oryzae* S2 alpha-amylase production under solid state fermentation: Optimization of culture conditions. Int. J. Biol. Macromol. 75:73-80.

Saleem A, Ebrahim MKH (2014). Production of amylase by fungi isolated from legume seeds collected in Almadinah Almunawwarah, Saudi Arabia. J. Taibah Univ. Sci. 8:90-97.

Selosse M-A, Faccio A, Scappaticci G, Bonfante P (2004). *Chlorophyllous* and *Achlorophyllous* specimens of *Epipactis microphylla* (*Neottieae, Orchidaceae*) are associated with ectomycorrhizal septomycetes, including truffles. Microb. Ecol. 47:416-426.

Silva LAD (2008). Produção e caracterização de enzimas celulásicas por *Aspergillus phoenicis*. Master of Science Thesis, Universidade Federal do Rio Grande do Sul, Porto Alegre, Brasil.

Silva LAO, Carmona EC (2008). Production and characterization of cellulase-free xylanase from *Trichoderma inhamatum*. Appl. Biochem. Biotechnol. 150:117-125.

Silva MF, Lacerda PSB, Jones Junior J (2005a). Sustainable development and green chemistry. Quim. Nova 28:103-110.

Silva R, Lago ES, Merheb CW, Macchione MM, Park YK, Gomes E (2005b). Production of xylanase and CMCase on solid state fermentation in different residues by *Thermoascus aurantiacus* miehe. Braz. J. Microbiol. 36:235-241.

Tamamura SW, Mukai A, Morimoto N, Takehana T, Koike S, Matsui H, Mori H (2014). Enhancement of hydrolytic activity of thermophilic alkalophilic -amylase from *Bacillus* sp. AAH-31 through optimization of amino acid residues surrounding the substrate binding site. Biochem. Eng. J. 86:8-15.

Teixeira MFS (1994). Obtenção de espécies de *Arpergillus* e *Penicillium* termofílicas e termotolerantes na Amazônia e caracterização de suas enzimas de interesse na indústria de alimentos. Master of Science Thesis, Instituto Nacional de Pesquisas da Amazônia, Manaus, Brasil.

Yadav S, Yadav PK, Yadav D, Yadav KDS (2009). Purification and characterization of pectin lyase secreted by *Penicillium citrinum.* Biochemistry (Mosc). 74:800-806.

Zanotto SP, Romano IP, Lisboa LUS, Duvoisin Jr. S, Martins MK, Lima FA, Silva SF, Albuquerque PM (2009). Potential application in biocatalysis of mycelium-bound lipases from Amazonian fungi. J. Braz. Chem. Soc. 20:1046-1059.

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# Bacterial pathogens associated with hand-dug wells in Ibadan city, Nigeria

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**The supply of pipe borne water in Nigeria has over the years become grossly inadequate or totally non-existent. This has led many people to seek alternative sources of water from streams, springs and especially hand-dug wells. This study was carried out to investigate the bacteriological quality of water from hand–dug wells in Ibadan city. This was done by investigating bacterial indicator of water quality in samples collected from 50 hand-dug wells and three bore holes from five different local government areas within Ibadan Municipal city and examined for total coliform and faecal coliform. Water samples from 96% of the wells were found to be contaminated with both total coliform and faecal coliform. The total coliform counts and fecal coliform counts ranged between 0 to 160x10$^3$/100 ml and 0 to 22x10$^3$/100 ml, respectively. The predominant bacterial isolates include *Escherichia coli* (38.5%), *Klebsiella* spp. (16.9%), *Staphylococcus aureus (13.8%)* and *Enterobacter* spp. (9.2%). While antibiotic susceptibility to nitrofurantoin (96.9%), gentamycin (93.6%) and streptomycin (93.3%) was high, it was low in ampicillin (37.8%) and to a lesser extent in Cotrimoxazole(66.7%). 24.1% of the isolates were multidrug resistant with about seven patterns observed. Hand-dug wells in the area of study were highly contaminated with pathogenic bacteria and this exposes consumers of water drawn from these wells to the risk of contracting various waterborne diseases.**

**Key words:** Bacterial pathogens, coliforms, hand dug well, water borne, disease.

## INTRODUCTION

Water is indispensable to the human life for drinking and for domestic purposes. A decrease in the availability of fresh water is fast becoming a global problem especially in the developing nations due to urbanization, industrialization and rapid population growth. The reality is that as world population rapidly increases, necessitating an increase in demand for water, the amount available per person are getting smaller (Lamikanra, 1999).

About half of the Nigerian population is without access to potable water supply (NWSR, 2011). Apart from shortages in supply of potable water, absence of basic sanitary and hygiene practices has led to frequent outbreaks of water borne diseases with high incidences of morbidity and mortality. In many urban cities in Nigeria, pipe-borne water supply is grossly irregular or totally absent.

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In Ibadan, the largest city, West Coast of Africa, only about 22.6% of the population was being served by the municipal water system (ADB, 2008; Ince et al., 2010). This situation has led to many homes relying on alternative sources of water such as hand dug well to meet domestic needs. Most of the wells are poorly constructed due to poverty and lack of proper technology. Since there is really no formal guideline or regulations for the construction of wells in the city, it is not unexpected that water obtained from these wells will vary in quality. Contamination of ground water may be due to faulty well construction and improper well location (Osho and Fagade, 2000).

Besides, overcrowding, poor sanitary practices and the unhygienic methods of drawing water from wells exposes those who depend on wells for their domestic water source especially for drinking to health risks associated with consumption of non-potable water. The major source of water pollution is faecal matter of human or animal origin through which intestinal pathogens such as coliforms and faecal Streptococci gain entrance into the water. The consumption of untreated contaminated water can result in waterborne diseases, which include cholera, typhoid, amoebic and bacillary dysentery and other diarrhoeal diseases. The WHO estimates that water-related diseases are responsible for 80% of all illnesses and death in the developing world with 1.8 million deaths annually from water borne diseases; 90% being children less than 5 years old (Prüss-Üstün et al, 2008)

According to Nigeria Water Sector Roadmap (NWSR) an estimated 194,000 of children under 5 years old die annually in Nigeria due to cholera, diarrhoea and other related water borne diseases (NWSR, 2011). Results from studies carried out in different parts of the country (Akinyemi et al., 2006; Idowu et al., 2011; Odeyemi et al., 2011) show that water sources for domestic purposes have disturbing levels of microbial pollution and this has implications for the prevalence of waterborne diseases.

In order to meet the target 7C of the MDG and her long term development goals of becoming one of the top 20 economies by the year 2020, the government has identified improvement in adequate water supply and sanitation as key areas. According to the finding in a recent study, 63.74% of the population in Moniya, a suburb of Ibadan gets water from hang-dug well, 10.68% from pipe borne water and 25.58% from borehole suggesting that hand-dug well is the main source of water supply in the city (Owoeye, 2013). Since hand-dug well has become a major source of water supply in Ibadan municipality, it is necessary to carry out frequent evaluation of well water quality to investigate the health risk (in terms of microbial contamination by pathogens) to which the consumers are subjected with a view to formulate policies and design strategies that would improve its water quality.

The present study investigates the presence of coliforms and faecal streptococci in hand-dug wells in Ibadan city, Nigeria as well as the susceptibility of resulting bacterial isolates to commonly prescribed antibiotics.

## MATERIALS AND METHODS

### Study sites

Ibadan is located within the southwestern Nigeria between the coordinate 7° 23′ 47″ N, 3° 55′ 0″ E. The capital city of Oyo State and the third largest metropolitan area by population in Nigeria after Lagos and Kano with a population of over 3 million, Ibadan is also the largest metropolitan geographical area located 128 km inland northeast of Lagos and 530 km southwest of Abuja, the federal capital. The principal inhabitants of the city are the Yorubas. Ibadan is densely populated with many unplanned neigbourhoods and is poorly drained leading to flooding during periods of heavy rains (Ajayi et al., 2012). Due to absence of sewage systems and sewage treatment facilities, untreated sewage are indiscriminately discharged into the environment. Dug wells are the main source of water in the study areas. To ensure geographical spread 5 zones based on the five local government areas within Ibadan municipality were selected as sampling areas. The local government areas are Ibadan North; Ibadan North-West; Ibadan North-East; Ibadan South-East; Ibadan South-West. The depth and the sanitary condition of the surrounding of each well were noted. Two methods of raising water from the wells by users were either by rope and bucket or pump (electric or hand-pump).

### Study design

Water from a total of 50 dug wells randomly selected (10 from each local government area) geographically spread across five local government areas in Ibadan city and 3 bore holes were sampled and investigated for their bacteriological quality. The antibiotic susceptibility of the isolates was also determined to ascertain effective drugs that could be used for treatment of waterborne diseases.

### Sample collection

Water samples were collected into 200 ml bottles by dipping sterile bottle into the well with the aid of previously disinfected sinker and rope. Samples were stored in ice box immediately after collection and were processed in the laboratory within 1-2 h after collection.

### Bacteriological analysis

#### *Determination of bacterial counts*

The standard plate count method was used to determine the total viable bacterial population. Nutrient agar was used as culture medium and colonies for total viable counts were counted using a Stuart scientific colony counter. The five tubes most probably number (MPN) technique (APHA, 2006) was used to estimate the coliform density. Differential tests were carried out in brilliant green lactose bile broth (BGLBB) incubated at 44°C for 18-24 h.

#### *Isolation of bacteria*

Samples were inoculated onto nutrient agar, MacConkey agar, mannitol salt agar and eosine methylene blue (EMB) agar, and plates incubated at 37°C for 24 h. Pure cultures were subjected to

**Table 1.** Viable Bacterial Counts of well water samples.

| Sample | Total viable count cfu/ml x $10^3$ | | | | |
|---|---|---|---|---|---|
| | Ibadan North | Ibadan North-West | Ibadan North-East | Ibadan South-East | Ibadan South- West |
| 1 | 0.01 | 0.41 | ND | 0.66 | 0.05 |
| 2 | 0.23 | 0.20 | ND | 0.10 | 0.30 |
| 3 | 0.12 | 9.30 | ND | 0.05 | 0.27 |
| 4 | 0.04 | 43.00 | ND | 0.23 | 6.50 |
| 5 | 5.50 | 1.00 | ND | 0.02* | 3.70 |
| 6 | 2.10 | 54.00 | ND | 13.00 | 0.002* |
| 7 | 0.90 | 31.00 | ND | 1.10 | 0.21 |
| 8 | 0.006 | 0.06 | ND | 0.60 | 1.50 |
| 9 | 0.15 | 0.22 | ND | 1.20 | 1.40 |
| 10 | 0.01 | 100 | ND | 4.00 | 0.20 |
| 11 | 0.03 | - | ND | 0.20 | 5.00 |

*Borehole sample; ND, not determined.

**Table 2.** Coliform count of well water samples.

| Sample | Total viable count cfu/ml x $10^3$ | | | | |
|---|---|---|---|---|---|
| | Ibadan North | Ibadan North-West | Ibadan North-East | Ibadan South-East | Ibadan South- West |
| 1 | 0.28 | 9.00 | 17.00 | 16.00 | 0.07 |
| 2 | 1.60 | 2.20 | 0.00 | 2.40 | 0.52 |
| 3 | 1.60 | 5.00 | 0.70 | 0.70 | 0.13 |
| 4 | 0.35 | 5.00 | 0.20 | 5.00 | 0.50 |
| 5 | 16.00 | 16.00 | 0.22 | 0.00* | 1.70 |
| 6 | 0.04 | 50.00 | 0.04 | 16.00 | 1.10 |
| 7 | 0.50 | 20.00 | 16.00 | 1.10 | 0.02* |
| 8 | 0.17 | 4.00 | 3.00 | 8.00 | 3.00 |
| 9 | 5.00 | 30.00 | 0.00* | 16.00 | 2.30 |
| 10 | 2.40 | 50.00 | - | 160.00 | 0.20 |
| 11 | 1.30 | - | - | 0.00 | 2.30 |

*Borehole sample.

Gram staining, oxidase test, catalase test, motility test, coagulase test (for suspected Staphylococcus) and growth on Triple Sugar Iron (TSI) agar. Bacterial pathogens were then characterized by colonial growth on suitable media and standard biochemical tests as described by Cowan and Steel (1993)

#### Antibiotic susceptibility testing (AST)

The Kirby-Bauer agar disc diffusion technique was used to test the isolates for antibiotic sensitivity. Antibiotic discs tested were from Abtek Biological Ltd, Italy and included Colistin (25 µg), nitrofuratoin (200 µg), tetracyclines [tetracycline (25 µg) , β-lactams [ampicillin (25 µg) folic acid synthesis inhibitor [cotrimoxazole (25 µg)], quinolone [nalidixic acid (30 µg)], aminoglycoside [gentamicin (10 µg)] and streptomycin 25 (µg).

## RESULTS

Water samples from the wells in the 5 Local government areas were grossly contaminated with bacterial pathogens. The total viable counts ranged from 0.002 to 100 x$10^3$ cfu/ml (Table 1). There was no significant difference in counts between study locations ($p$=0.073). The indicator organisms were totally absent in one well each from location 3 and 4 and 2 of the 3 boreholes. The total coliform counts ranged from 0 to 160x$10^3$/100 ml (Table 2) while the faecal coliform counts ranged from 0 to 22x$10^3$/100 ml (Table 3). WHO recommends total absence of coliform and fecal coliform in 100 ml of drinking water (WHO, 2010).

While the difference in total coliform counts between the locations were not significant ($p$= 0.243) the fecal counts between the locations were significantly different ($p$=0.03). Both total viable count and faecal coliform count were highest in location 2 (mean counts of 23.92x$10^3$ and 8.41x$10^3$ respectively) and lowest in location 5 (mean counts of 1.41x$10^3$ and 1.02x$10^3$ respectively), while the total coliform count was highest in location 4 (mean count of 22.52x$10^3$) and lowest in location 5 (Mean count of

**Table 3.** Faecal Coliform count of well water samples.

| Sample | Faecal Coliform count/100 ml X $10^3$ | | | | |
|---|---|---|---|---|---|
| | Ibadan North | Ibadan North-West | Ibadan North-East | Ibadan South-East | Ibadan South- West |
| 1 | 0.14 | 9.00 | 17.00 | 9.00 | 0.02 |
| 2 | 1.60 | 2.20 | 0.00 | 1.30 | 0.52 |
| 3 | 0.23 | 0.70 | 0.70 | 0.70 | 0.08 |
| 4 | 0.17 | 1.70 | 0.20 | 5.00 | 0.50 |
| 5 | 1.70 | 3.50 | 0.14 | 0.00* | 0.80 |
| 6 | 0.04 | 22.00 | 0.04 | 16.00 | 1.10 |
| 7 | 0.22 | 20.00 | 3.50 | 1.10 | 0.00* |
| 8 | 0.07 | 4.00 | 3.00 | 8.00 | 2.30 |
| 9 | 2.20 | 8.00 | 0.00* | 3.30 | 2.30 |
| 10 | 2.40 | 13.00 | - | 22.00 | 0.20 |
| 11 | 0.80 | - | - | 0.00 | 0.20 |

*Borehole sample.

**Table 4.** Bacterial counts with respect to site.

| Parameter | Study site | Mean counts (CFU/ml) | SE mean | StDev | Range (CFU/ml) |
|---|---|---|---|---|---|
| Total viable counts | | | | | |
| | Ibadan North | 1.56X103 | 0.74 | 2.33 | 0.01-6.00x$10^3$ |
| | Ibadan North West | 23.92X103 | 10.59 | 33.49 | 0.06-100 x103 |
| | Ibadan North East | - | - | - | - |
| | Ibadan South East | 2.10x103 | 1.27 | 4.01 | 0.02-13x$10^3$ |
| | Ibadan South west | 1.41X103 | 0.67 | 2.12 | 0.002-6.5x$10^3$ |
| Total coliform counts | | | | | |
| | Ibadan North | 2.79X$10^3$ | 1.54 | 4.88 | 0.04-16.00$10^3$ |
| | Ibadan North West | 19.12X$10^3$ | 5.82 | 18.42 | 2.2-50.00x$10^3$ |
| | Ibadan North East | 3.72X$10^3$ | 2.15 | 6.80 | 0.0-17x$10^3$ |
| | Ibadan South East | 22.52X$10^3$ | 15.42 | 48.75 | 0.0-160x$10^3$ |
| | Ibadan South West | 0.95X$10^3$ | 0.33 | 1.05 | 0.07-3.00x$10^3$ |
| Faecal coliform counts | | | | | |
| | Ibadan North | 0.88 X103 | 0.31 | 0.97 | 0.04-2.4 x$10^3$ |
| | Ibadan North West | 8.41 X103 | 2.42 | 7.65 | 0.7-22.00 x$10^3$ |
| | Ibadan North East | 2.46 X103 | 1.67 | 5.27 | 0.0-17.00 x$10^3$ |
| | Ibadan South East | 6.64 X103 | 2.32 | 7.33 | 0.0-22.00 x$10^3$ |
| | Ibadan South West | 1.02X103 | 0.39 | 1.23 | 0.0-3.5 x$10^3$ |

0.95x$10^3$) (Table 4)

A total of 65 isolates were obtained with fifty-eight (58) fully identified and grouped into seven genera. The most predominantly isolated organisms are *Escherichia coli* (38.5%), *Klebsiella spp* (16.9%), *Staphylococcus aureus* (13.8%), and *Enterobacter spp* (9.2%) (Table 5).

Table 6 shows the susceptibility of the isolates to common antibiotics. The most active antibiotics were nitrofurantoin (93.6%) and gentamicin (93.3%). Streptomycin (89.9%), tetracycline (86.1%) and nalidixic acid (79.2%) were also highly effective. Ampicillin (37.8%) was the least potent antibiotics against the pathogens. 14 (24.1%) of the 58 isolates used for the antibiogram were multidrug resistant (resistance to 3 or more antibiotics). *E. coli* showed 3 multidrug resistance patterns Amp, Col, Nal ,Nit, Cot, Str, Tet; Amp, Cot ,Str ,Tet and Amp ,Cot, Str while *Klebsiella spp.* showed 2 resistance patterns Amp ,Cot ,Str,Tet and Amp ,Cot, Tet (Table 7).

## DISCUSSION

Water quality issues are health related issues because it

**Table 5.** Frequency of isolation of bacteria pathogens from study locations

| Isolate | Ibadan North | Ibadan North-West | Ibadan North-East | Ibadan South-East | Ibadan South-West | Total | % |
|---|---|---|---|---|---|---|---|
| *Escherichia coli* | 4 | 5 | 6 | 6 | 4 | 25 | 38.5 |
| *Klebsiella spp.* | 2 | 3 | 4 | 2 | 0 | 11 | 16.9 |
| *Staphylococcus aureus* | 2 | 0 | 2 | 4 | 1 | 9 | 13.8 |
| *Enterobacter* spp. | 1 | 1 | 2 | 1 | 1 | 6 | 9.2 |
| *Proteus spp* | 0 | 1 | 0 | 1 | 1 | 3 | 4.6 |
| *Flavobacterium* spp. | 0 | 1 | 0 | 1 | 0 | 2 | 3.1 |
| Unidentified | 2 | 1 | 1 | 1 | 2 | 7 | 10.8 |
| *Pseudomonas aeruginosa* | 1 | 0 | 0 | 0 | 1 | 2 | 3.1 |
| Total | 12 | 12 | 15 | 16 | 10 | 65 | 100 |

**Table 6.** Susceptibility of isolates to antibiotics (in %).

| Isolate | Antibiotics | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | AMP | COL | GEN | NAL | NIT | COT | STR | TET |
| *Esherichia coli* | 50 | 95.5 | 100 | 96 | 68.2 | 68.2 | 77.3 | 90.1 |
| *Klebsiella* spp. | 55.6 | 100 | 100 | 100 | 100 | 66.7 | 88.9 | 66.7 |
| *Enterobacteria* spp. | 33.3 | 100 | 100 | 100 | 100 | 66.7 | 83.3 | 100 |
| *Proteus* spp. | 0 | 0 | 100 | 0 | 100 | 0 | 100 | 100 |
| *Pseudomonas aeruginosa* | 50 | 50 | 100 | 100 | 100 | 100 | 100 | 100 |
| *Staphylococcus aureus* | ND | ND | 60 | ND | ND | 100 | ND | 60 |
| Total | 37.8 | 69.1 | 93.3 | 79.2 | 93.6 | 66.9 | 89.9 | 86.1 |

ND, Not determined.

**Table 7.** Resistance patterns of isolates.

| Isolate | resistance patterns | Number of isolates with resistance patterns |
|---|---|---|
| *E. coli* | Amp,Col, Nal, Nit, Cot, Str, Tet | 1 |
| | Amp, Cot, Str, Tet | 2 |
| | Amp, Cot, Str, | 5 |
| *Klebsiella* spp. | Amp, Cot, Str, Tet | 1 |
| | Amp, Cot, Tet | 3 |
| *Enterobacteria* spp. | Amp, Cot, Str, | 1 |
| *Proteus* | Amp, Col,Nal ,Cot, | 1 |
| Total | | 14 |

is important in promoting health and reducing global disease burden. This study reports the bacteriological quality of hand dug well- a major source of water supply in Ibadan city. The information from this report would assist in strengthening efforts to improve the quality of water available in the study areas.

Out of the 50 hand dug wells studied, 48 (96%) were found to be contaminated with both total and faecal coliforms. Total viable bacterial counts (0.002-100 × $10^3$ CFU/mL), total coliform counts (0-160 × $10^3$ CFU/mL),

**Table 8.** Depth of well-studied in meters.

| Sample | Ibadan North | Ibadan North-West | Ibadan North-East | Ibadan South-East | Ibadan South-West |
|---|---|---|---|---|---|
| 1 | 9.6 | 9.6 | 9.0 | U | 9.0 |
| 2 | 8.4 | 8.4 | 12.6 | 15.0 | 7.2 |
| 3 | 6.3 | 6.0 | 6.3 | 12.0 | 8.1 |
| 4 | 9.0 | 6.6 | 7.2 | 4.5 | 7.2 |
| 5 | 5.7 | U | 13.5 | Borehole | 9.0 |
| 6 | 7.5 | 4.8 | 12.6 | U | 8.1 |
| 7 | 9.0 | U | 9.9 | 11.7 | Borehole |
| 8 | 5.4 | 9.0 | 6.3 | 3.60 | 13.5 |
| 9 | 10.2 | U | Borehole | 6.3 | 5.7 |
| 10 | 8.1 | U | U | 5.4 | 2.1 |

U= Unknown.

and fecal coliform (0-22 × $10^3$ CFU/mL) were high (Table 1). TVBC levels in 40% of the wells studied exceeded the recommended limit of 1x$10^2$ CFU/ml. Water sample from all the wells did not meet the WHO standard set for potable water that recommends total absence of coliform and fecal coliform in 100 ml of drinking water (WHO, 2010).

High TVBC, total coliforms and fecal coliforms counts in water sample from hand dug well have also been reported in recent studies within Nigeria (Osho and Fagade, 2000; Akinyemi et al., 2006; Idowu et al., 2011; Odeyemi et al., 2011) and in other developing countries (Akoachere et al., 2013; Gwimbi, 2011). High counts of indicator bacteria suggest heavy pollution of water with fecal matter. This finding has great implications for public health. Using water from these wells for domestic purposes such as drinking, cooking and washing of fruits and vegetables could predispose users to water borne diseases such as diarhoea, cholera and dysentery. A number of interplaying factors could be responsible for the poor bacterial quality of water from the wells in the study area of this work. The potential reasons for the high presence of microbiological contaminants in the wells may be poor sanitation conditions and practices such as washing laundry near water sources, using dirty containers to collect water from the wells or plunging of bucket directly from the ground into the well. The use of bucket and rope in fetching water from hand dug wells and sitting wells close to sanitary facilities have been reported to contribute significantly to high pollution of wells resulting in deterioration of its water quality (Ayantobo et al., 2012).

The use of electric pump, apart from eliminating contamination from use of bucket, is almost always accompanied with proper sealing of the well. The fecal coliform counts between the locations were significantly different ($p$=0.03). This may indicate that there is a wide variation in the hygiene behaviours by the household in the study locations.

Another factor that could be of importance to the purity of well water is the depth of the well (Table 8). The fact that no form of microbial contaminant was obtained from the three borehole samples in this study supports the view that very deep wells would yield water with better bacterial purity than shallow wells.

*E. coli* (38.5%) the predominant isolate is a major public health concern as its presence is not only an indication of recent contamination with fecal matter but the possible presence of other dangerous intestinal pathogens. Certain pathogenic strains of the organism such as the enteropathogenic O157:H7 implicated in several out breaks of bloody diarrhea (Geldreich et al., 1992) have been detected in water. Other major pathogens present were enteric organisms such as Klebsiella spp. (16.9%), *Enterobacter spp.* (9.2%), *P. aeruginosa* (3.1%), *Proteus spp.* (4.6%) and *S. aureus* (13.8%). Their isolation further confirms the contamination of water with fecal material and the possible presence of other waterborne enteric pathogens such as viruses and protozoa. The high number of pathogenic enteric bacteria associated with water samples from the hand dug wells studied contradicts the widely held believe that underground water is relatively free of microorganism and is thus fit for consumption. The most effective of the antibiotics tested was nitrofurantoin (96.3%). Other agents with high activity against the isolates are Gentamicin (93.6%) and Streptomycin (93.3%) which is aminoglycosides antibiotics available only as injections. This factor and the relative high cost compared to other antibiotics might have discouraged indiscriminate purchase and misuse. High sensitivity of Gentamicin in this study compares with findings from another study where it has also been reported to be strongly active against bacterial pathogens isolated from well water samples. (Akoachere et al., 2013; Odeyemi et al., 2011).

However, susceptibility to commonly use antibiotics such as ampicillin (37.8%) and to a lesser extent cotrimoxazole (66.9%) was low indicating that most of the isolates are resistant to them. This may not be unconnected

with indiscriminate use of these drugs without prescription because they are easily accessible. This finding corroborates previous reports of low susceptibility to ampicillin, penicillin and cotrimoxazole in enteric pathogens isolated from water samples (Akoachere et al., 2013; Odeyemi et al., 2011). Seven patterns of multidrug resistance were observed in about 24.1% of the isolates. This compares with a similar report of about 33.4% multidrug resistance isolates from well water sample in another study( Akoachere et al., 2013). This high level of multidrug resistance may be an indication of rampant antibiotic misuse among the population due to self-medication and relative access to the drugs without prescription. The isolation of many of this multidrug resistance isolates from sources that serves as drinking water to so many communities is of public health significance because it can easily serve as reservoir through which antibiotic resistance traits can spread to other bacterial population.

However, the study confirms previous reports of a relatively high prevalence of bacteriological contamination of private wells the world over. Specifically, the study has revealed that majority of the people in Ibadan city who depend on well water for drinking and cooking are at risk of contracting waterborne diseases. There is need for public awareness campaign to encourage routine disinfection of hand dug wells being used as water sources and to educate the general populace about the importance of good sanitary practices. This will reduce the risk of exposure among the population to waterborne infections from the use of well water.

## Conflict of interests

The authors did not declare any conflict of interest.

### REFERENCES


African Development Bank Group (ADB) (2008). Project: urban water supply and sanitation for Oyo and Taraba States. Country: Nigeria. Project appraisal report. Available from: http://www.afdb.org/fileadmin/uploads/afdb/Documents/Project-and-Operations/AR%20Nigeria002En.pdf [accessed 12 November 2014].

Ajayi O, Agbola SB, Olokesusi BF, Wahab B, Taiwo OJ, Gbadegesin M, Taiwo DO, Kolawole O,Muili A, Adeola MA,Olutade OG, Shiji F, Abiola NA (2012). Flood management in an urban setting: a case study of Ibadan Metropolis, In Martins O. Idowu OA, Mbajiorgu CC, Jimoh OD, Oluwasanya GO, editors. Hydrology for disaster management. Nigerian Association of Hydrological Sciences; 65-81. Available from: http://journal.unaab.edu.ng/index.php/NAHS/article/download/914/882 [accessed 21 December, 2014].

Akinyemi OK, Oyefolu AOB, Salu OB, Adewale OA, Fasure AK (2006). Bacterial associated with Tap and well waters in Lagos, Nigeria. East Cent. Afr. J. Surg. 2(1):110-117.

Akoachere JKT, Omam L, Massalla TN (2013). Assessment of the relationship between bacteriological quality of dug-wells, hygiene behaviour and well characteristics in two cholera endemic localities in Douala, Cameroon. BMC Public Health 13:692.

APHA (2006).Standard methods for the Examination of Water and Waste water. American Public Health Association, American Water Works Association and Water Pollution Control Federation. Washington 21st Ed.

Ayantobo OO, Olusanya GO, Idowu OA, Eruola AO (2012). Water quality evaluation of hand-dug wells in Ibadan, Oyo state, Nigeria In Martins O. Idowu OA, Mbajiorgu CC, Jimoh OD, Oluwasanya GO, editors. Hydrology for disaster management. Nigerian Association of Hydrological Sciences. pp. 231-239.

Cowan ST, Steel S (1993). Manual for identification of medical bacterial. Edition by Barrow G I, Fellthan R KA. Cambridge University. p. 32.

Geldreich EK, Fox JG, Rice RE, Swerdlow D (1992). Searching for a water supply connection in the Cabool, Missouri Disease outbreak of Escherichia coli O157:H7. Water Res. J. 26:8.

Gwimbi P (2011). The microbial quality of drinking water in Manonyane community: Maseru District (Lesotho). Afr. Health Sci. 11(3):474-480

Idowu AO, Oluremi BB, Odubawo KM (2011). Bacteriological analysis of well water samples in Sagamu, Nigeria. Afr. J. Clin. Exp. Microbiol. 12(2): 86- 91

Ince M, Basir D, Oni OOO, Awe EO, Ogbechie V, Korve K, Adeyinka MA, Olufolabo AA, Ofordu F, Kehinde M(2010). Rapid assessment of drinking-water quality in the Federal Republic of Nigeria: country report of the pilot project implementation in 2004–2005. Geneva & New York: World Health Organization & United Nations Children's Fund

Lamikanra A (1999).Essential Microbiology. Amkra Books, 2nd ed. pp. 229-249.

NWSR (2011). Nigerian Water Sector Roadmap. Federal Ministry of Water Resources. pp. 14-15

Odeyemi AT, Akinjogunla OJ, Ojo MA (2011). Bacteriological, physicochemical and mineral studies of water samples from artesian bore-hole, spring and hand dug well located at Oke-Osun, Ikere-Ekiti, Nigeria. Arch. Appl. Sci. Res. 3(3):94-108.

Osho A, Fagade OE (2000). Occurrence of Coliform and Faecal Streptococcus in Ago-Iwoye Well Water. Niger. J. Sci. 34(4):323-329.

Owoeye, JO (2013). Determination of housing and environmental quality for Moniya community in Ibadan, Nigeria. Am. J. Res. Commun. www.usa-journals.com, ISSN: 2325-4076.

Prüss-Üstün A, Bos R, Gore F, Bartram J. (2008) Safer water, better health: costs, benefits and sustainability of interventions to protect and promote health. World Health Organization, Geneva. pp. 11-18. Retrieved from http://www.whqlibdoc.who.int/publications/2008/9789241596435_eng.pdf

World Health Organization (WHO) (2010). Guidelines for Drinking-water Quality. Recommendation, Geneva, p: 1-6.Retrievefrom http://www.who.int/water_sanitation_health/WHS_WWD2010_guidelines_2010_6_en.

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# Prevalence of *Salmonella*, *Escherichia coli* and coliforms on bell peppers from the field to the packing house process

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**The presence of *Salmonella* spp., *Escherichia coli* and coliforms from the field to the packing process of bell pepper was evaluated. A total of 900 samples including bell pepper, worker hands, surfaces, and hand-washing fruits were collected from 11-sampling sites of a farm. From the total samples analyzed, 2.6, 3.0, and 53.7% contained *Salmonella* spp., *E. coli* and coliforms, respectively. The highest percentage of positive samples with *Salmonella* and *E. coli* occurred at the field and packing house, respectively; while coliforms levels increased, as it approached to the final packing process. *E. coli* O157:H7 was not detected in any of the analyzed samples, however *Salmonella* enterica serovar Typhimurium was found during the packing process of the produce. Good agricultural practices from the field to the packinghouse should be implemented; it is also imperative to emphasize hand washing and contact surface disinfection to ensure the safety of the final produce.**

**Key words:** Microbial tracking source, *Salmonella*, hand washing, surface contact, packinghouse, bell pepper (*Capsicum annuum*).

## INTRODUCTION

Fresh produce production, particularly from Latin American countries, has the potential to meet most of the growing global demand for fruit and vegetable products. There is a need to increase food production to feed an

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ever-growing world population, as FAO has predicted that global food production will need to increase by 50% over current levels by 2050 (FAO, 2009). However, the globalization of the food supply may introduce new food safety risks and the potential widespread dissemination of contaminated food. Even thought, produce-related outbreaks in the United States declined by 42% over a 10–year period from 2002-2011, a sharp increase in produce-related foodborne illnesses occurred in 2008, due to a large multi-state *Salmonella* outbreak involving peppers and tomatoes that sickened over 1,535 people (CSPI, 2014). *Salmonella* enterica serovar Saintpaul was the causative agent of this outbreak, and was isolated from serrano and jalapeño peppers from two packinghouses in Tamaulipas, Mexico (Mody et al., 2011). Since then, the FDA has documented different commodities in Mexico contaminated with *Salmonella* spp. including cucumber, jalapeño peppers, serrano peppers, papaya, spinach, Mangoes and coriander (CDC, 2012; FDA, 2012). Additionally, two more documented outbreaks in which cantaloupes and peppers were implicated proved that *Salmonella* could have originated from field and farm operations in Mexico (CDC, 2002; CDC, 2008). Several others tracking-type investigations have located *Salmonella* and *E. coli* O157:H7 in different points of the food production chain such as water, contact surfaces, fresh produce (melon, orange, parsley and bell pepper), worker hands, animals, and soil (Castillo et al., 2004; Mukherjee et al., 2004; Duffy et al., 2005; Gallegos-Robles et al., 2008; Cooley et al., 2014).

As the overall consumption of fresh produce has increased, the amount of produce imported into the U.S. market has also increased. In 2008, nearly 3 million metric tons of fresh vegetables were imported from Mexico to the USA, representing more than one-half of all revenues from US vegetable imports (ERS, 2009; FAS, 2009). Culiacan is the capital of Sinaloa and is located in the Northwestern part of Mexico. This particular region is rich in vegetable produce farms that export several crops to the United States each year, particularly tomatoes and bell peppers (CIAD, 2006; Estrada-Acosta et al., 2014). In an effort to examine microbial contamination in this region, a comprehensive study to determine the presence of bacterial pathogens and the identification of critical points during the production chain is needed. Therefore, to address these data needs, the goals of this study were: 1) to evaluate the incidence of *E. coli*, coliforms, and *Salmonella* spp. from the harvesting to the final packaging process of bell pepper, and 2) to characterize the isolated strains of *E. coli* and *Salmonella* spp.

## MATERIALS AND METHODS

### Sampling collection

Samples were weekly collected during the growing season of November 2006 to December 2007 from an agricultural packing-house operation in the Northwestern region of Mexico. The packinghouse owner in the study gave its consent to participate and to collect samples during the growing season. Samples were collected from the field and the packinghouse facility including fresh fruits, food contact surfaces, hand washing water, and worker hands. Table 1 shows the number of samples collected and locations from each commodity. Fresh produces were collected directly from the plant during harvesting, and from the field containers at the field. In the packinghouse, produce samples were collected from the unloading ramp, brushing, sorting, and waxing. Finally, produces were also collected from the packing bins and packing containers. Samples from worker hands (picker, sorter and packer) were collected both before and after 3h of continue labor. The contact surface areas of the packinghouse equipment were taken from the unloading ramp, roller, conveyor belts, and packing bin. All samples were stored at 5°C and transported to the Centro de Investigacion en Alimentacion y Desarrollo Laboratory in Sinaloa, Mexico for immediate processing. Microbial analyses were initiated within 24h of sample collection.

### Microbiological sampling procedure

Each sample was collected and analyzed as follows: fresh produces from each location were obtained using sterile, disposable gloves and were individually placed into Ziploc® bags previously sterilized with UV light. 195 mL of buffer peptone water 2% (BPW) (Difco) were added to each 100 g sample of bell pepper. Each worker rinsed their hands during 30 s in 200 mL of sterile phosphate buffered saline (PBS) at pH 7. Then, 10 mL of BPW (Difco), were added to 40 mL of PBS and stored at 5°C until analysis. Approximately, 2,500 $cm^2$ of each contact surface was swabbed using a sterile sponge (Whirl Pack® Hydrated Speci-Sponge® Bag) of 3.8 x 7.6 cm in 5 mL of BPW (Difco), followed by addition of 195 mL of BPW 2%. Finally, 800 mL of hand washing water were collected in a 1 L sterile plastic bottle, and 2mL of thiosulphate at 10% were added to neutralize any chlorine presence. All samples were manually homogenized and processed for enumeration of *E. coli*, coliforms and *Salmonella* spp. as described by APHA (2001).

### *Escherichia coli* and coliforms

Quantification of *E. coli* and coliforms was performed using the membrane filtration technique (APHA, 2001). Aliquots of 1 and 10 mL of BPW from each bell pepper produce and sponge samples were added to 50 mL of sterile distilled water and filtered by a cellulose membrane of 47 mm in diameter and 0.45 µm pore size (GN-6 Metricel®, Pall Corp., NY, USA). For each hand washing water sample 100 mL were filtered under the same conditions. After filtration, each membrane was placed on ECC agar (CHROMagar™ECC, Paris, France) and the plates were incubated at 42.5°C for 24 h. Colonies were quantified according to its morphological characteristics: *E. coli* and Coliforms-like colonies (blue and mauve colonies, respectively). A recovery efficiency of ≤1 CFU/mL was calculated for this experiment. Additionally, from the medium selective plate, two or more *E. coli* colonies were transferred onto tryptic soy agar (TSA) (Difco) and incubated at 35°C for 24 h. The isolates were stored at -20°C in glycerol (10%) for further molecular analysis.

### *Salmonella* spp. enrichment and isolation

The same BPW culture used for *E. coli* isolation was used for *Salmonella* culturing according to Castillo et al. (2004) with some modifications. Briefly, 70 mL of the bell pepper produce pre-enrich-

**Table 1.** Number of samples classified by locations.

| Sample type | Source | Number | Sample location |
|---|---|---|---|
| Bell peppers (N=568) | Field | 66 | Plant |
| | | 63 | Harvest |
| | | 61 | Field containers |
| | Packinghouse | 63 | Unloading ramp |
| | | 62 | Brushing |
| | | 61 | Sorting |
| | | 60 | Waxing |
| | | 66 | Packing bin |
| | | 66 | Packing container |
| Workers hands (N=186) | After washing hands (N=98) | 36 | Picker |
| | | 31 | Sorter |
| | | 31 | Packer |
| | After 3 h of labor (N=88) | 38 | Picker |
| | | 27 | Sorter |
| | | 23 | Packer |
| Surface contact area (N=132) | Packinghouse equipment | 33 | Unloading ramp |
| | | 33 | Roller |
| | | 33 | Conveyor belts |
| | | 33 | Packing bin |
| Hand washing water (N=14) | Packinghouse | 14 | Water |
| Total | | 900 | |

ment broth was incubated at 37°C for 24 h. For each surface contact and hands sample, 10 mL BPW was added to 90 mL of universal pre-enrichment broth (UPB) (Difco) and incubated at 37°C for 24 h. For each water sample, a volume of 100 mL was filtered through a cellulose membrane of 47 mm diameter and 0.45 µm pore size (GN-6 Metricel®, Pall Corp., NY, EUA), and placed in 8 mL of UPB, stirred for 1 min and incubated at 37°C for 24 h. For enrichment, 1 mL of the pre-enriched BPW or UPB were added to 10 mL tetrathionate broth (TTB) (Difco) and incubated at 42.5°C for 6 h followed by post-enrichment adding 1 mL of enriched TTB to 10 mL of M broth (Difco) and incubated at 37°C for 24 h. The post-enriched M broth-PCR positive samples were streaked on xylosa desoxycholate (XLD) Agar (Bioxon) and incubated at 37°C for 24 h. The method showed a recovery efficiency of ≤1 CFU/g and ≤1 CFU/mL.

### *Salmonella* spp. detection and confirmation

Two or more *Salmonella* spp. presumptive colonies were isolated and identified by molecular analysis (a total of 50 isolates). PCR was performed according to Chiu and Ou (1996) with some modifications noted below. The DNA template was produced as follows: 1.5 mL post-enrichment M broth was centrifuged (13,200 x *g* for 5 min). The pellet was suspended twice in 400 µL sterile nanopure water and centrifuged under the same conditions listed above. The pellet was suspended in 200 µL of sterile nanopure water and heated for 5 min in boiling water to lyse bacterial cells and release DNA. The presence of *Salmonella* spp. was evaluated in lysed cells using the PCR, described as follows, the primers used were INVA-1 (5'-ACAGTCCTCGTTTACGACCTGATT-3´) and INVA-2 (5'-AGACGACTGGTACTGATCGATATT-3') that corresponds to a specific region of virulence *inv*A gene of *Salmonella* spp. of 244 bp. The PCR mixture (25 µL) consisted of 1X PCR amplification buffer (Promega, Madison WI), 1.5 mM $MgCl_2$, 400 µM dNTPs, primers INVA-1 and INVA-2 1 µM each, 1.25 U *Taq* polymerase (Promega), 13.875 µL of nanopure water and 1 µL of lysed cells. The PCR amplification conditions was produce as follows: one cycle at 94°C for 10 min, 30 cycles consisting of 95°C for 30 s to denature DNA, 56°C for 0.5 min to align the DNA primers and 72°C for 2 min for DNA extension. Amplification was performed in an Eppendorf™ thermocycler. PCR products were visualized by electrophoresis with tris-acetate-EDTA buffer in 1% agarose gel stained with ethidium bromide. Finally, the PCR-positive isolates for *Salmonella* spp. were sent to the Bacteriology Department at National Institute of Epidemiological Diagnosis and Reference in Mexico City for serotyping.

### Detection of pathogenic *Escherichia coli*

Isolates were examined and screened by PCR according to López-Saucedo et al. (2003) as follows: DNA template was extracted from suspended cells grown on TSA, cleaned in nanopure water and lysed at 100°C. The primers used to identify pathogenic strains of *E. coli* were *lt* and *st* for enterotoxigenic *E. coli* (ETEC), *bfp*A and *eae*A for enteropathogenic *E. coli* (EPEC), *eae*A and *stx*1 for enterohemorrhagic *E. coli* (EHEC) and, *stx*2 and *ial* for enteroinvasive *E. coli* (EIEC), which originated DNA fragments of 450, 190, 324, 384, 150, 255 and 650 pb, respectively (López-Saucedo et al*.,* 2003). The PCR mixture consisted of 1X buffer, $MgCl_2$ 1.85 mM, dNTPs 184 µM, primers *lt* (5.0 pM), *st* (6.47 pM), *bfp*A (2.5 pM), *eae*A (3.88 pM), *stx*1 (3.88 pM), *stx*2 (2.5 pM), *ial* (10.25 pM), 0.625 U of DNA *Taq* polymerase (Promega), 2 µL of

**Table 2.** Presence of *Salmonella* spp., *Escherichia coli* and coliforms on samples collected in a bell pepper packinghouse.

| Source | No. of positive samples (%)† | | |
|---|---|---|---|
| | ***Salmonella* spp.††** | ***Escherichia coli*** | **Coliforms** |
| Bell peppers | 15 (2.6%) | 17 (3.0%) | 305 (53.7%) |
| Worker hands | 25 (13.4%) | 24 (12.9%) | 121 (65.1%) |
| Surface contact area | 5 (3.8%) | 9 (6.8%) | 92 (69.7%) |
| Hand washing water | 0 (0.0%) | 2 (14.3%) | 5 (35.7%) |
| Total (N=900) | 45(5.0%) | 52 (5.8%) | 523 (58.1 %) |

†Percentage of positive samples based on total samples analyzed; ††Positive samples by PCR.

**Table 3.** Presence of *Salmonella* spp., *Escherichia coli* and coliforms on bell peppers collected from the field and packinghouse.

| Source of bell pepper sample | No. of samples analyzed | No. of positive samples (%)† | | |
|---|---|---|---|---|
| | | ***Salmonella* spp.††** | ***Escherichia coli*** | **Coliforms** |
| Plant | 66 | 0 (0.0%) | 0 (0.0%) | 14 (21.2%) |
| Harvest | 63 | 6 (9.5%) | 1 (1.6%) | 18 (28.6%) |
| Field containers | 61 | 5 (8.2%) | 1 (1.6%) | 26 (42.6%) |
| Unloading ramp | 63 | 2 (3.2%) | 3 (4.8%) | 38 (60.3%) |
| Brushing | 62 | 0 (0.0%) | 3 (4.8%) | 39 (62.9%) |
| Sorting | 61 | 1 (1.6%) | 3 (4.9%) | 38 (62.3%) |
| Waxing | 60 | 0 (0.0%) | 4 (6.7%) | 41 (68.3%) |
| Packing bin | 66 | 1 (1.5%) | 2 (3.0%) | 48 (72.7%) |
| Packing container | 66 | 0 (0.0%) | 0 (0.0%) | 43 (65.2%) |
| Total | 568 | 15 (2.6%) | 17 (3.0%) | 305 (53.7%) |

†Percentage of positive samples based on total samples analyzed; †† Positive samples by PCR.

lysed cell and nanopure water to reach a total volume of 25 µL. The amplification was performed in an Eppendorf™ thermocycler with the following cycling conditions: 1 cycle at 50°C for 2 min, 1 cycle at 95°C for 5 min, 45 cycles to denature DNA at 95°C for 45 s, primers aligning at 50°C for 45 s and DNA extension at 72°C for 45 s; and a final extension step at 72°C for 10 min. The PCR products were visualized by electrophoresis through 2.5% agarose gel stained with ethidium bromide (López-Saucedo et al., 2003).

### Statistical analysis

Descriptive statistics were performed to quantify the presence of *E. coli*, coliforms, and S*almonella* spp. using Minitab version 14 (MINITAB version 14.1, 2003).

## RESULTS AND DISCUSSION

A total of 900 samples were collected from the field to the packinghouse during the growing season of November 2006 to December 2007. Among the 900 samples, 568 were bell pepper produces, 186 worker hands, 132 surface contact areas and 14 hand washing water. Table 1 specifies the number of samples classified by location. From the total samples analyzed. 5.0, 5.8, and 58.1% showed contamination with *Salmonella* spp., non-pathogenic *E. coli* and coliforms, respectively.

*E. coli* and coliforms were found in 14.3 and 35.7%, of the hand washing water samples (Table 2), with concentrations ranging from 7.1±1.6 to 17.2±41.2 log CFU/100mL, respectively. The study showed higher levels of coliforms than the one specified by the Mexican Official Norm (NOM-127-SSA-1994), which establishes 0 CFU/100mL. Thus, making the water neither acceptable for human consumption nor for hand washing due to possible cross contamination. On the other hand, *Salmonella* was not detected in any of the hand washing water samples analyzed.

In order to identify critical points of contamination on fresh produces, microbial levels on specific sampling locations from the field to the packinghouse were analyzed (Table 1). Coliforms were detected in 53.7% (Table 2) of the fresh produce sample locations with means ranging from 1.29 to 2.24 log CFU/100g. It was shown that coliform levels on fresh produce increased from the field and throughout packing, with 21.2% positive samples in the fresh produces obtained before detached from the plant, 42.6% in the container from harvest to field, and 60.3% from the field containers to the unloading ramp (Table 3). Coliforms levels showed an increased in the brushing and waxing step (62.9 and

**Table 4.** Presence of *Salmonella* spp., *Escherichia coli* and coliforms on worker hands from the field and packinghouse.

| Source of worker' hands sample | | No. of positive samples (%)[†] | | |
|---|---|---|---|---|
| **Sampling time** | **Operator** | ***Salmonella* spp.[††]** | ***Escherichia coli*** | **Coliforms** |
| Washing hands before labor | Picker | 5 (13.9%) | 0 (0.0%) | 16 (44.4%) |
| | Sorter | 6 (19.3%) | 10 (32.3%) | 27 (87.1%) |
| | Packer | 6 (19.3%) | 5 (16.1%) | 27 (87.1%) |
| | Subtotal (N=98) | 17 (17.3%) | 15 (15.3%) | 70 (71.4%) |
| After 3 h of labor | Picker | 0 (0.0%) | 0 (0.0%) | 6 (15.8%) |
| | Sorter | 4 (14.8%) | 4 (14.8%) | 23 (85.2%) |
| | Packer | 4 (17.4%) | 5 (21.7%) | 22 (95.7%) |
| | Subtotal (N=88) | 8 (9.1%) | 9 (10.2%) | 51 (58.0%) |
| Total (N=186) | | 25 (13.4%) | 24 (12.9%) | 121 (65.1%) |

[†]Percentage of positive samples based on samples analyzed. [††]Positive samples by PCR.

68.3 percentage of samples, respectively). Johnston et al. (2005) coincide with our study since they also found an increase in coliforms levels from harvest through packing, with an increase occurring also at the rinse step.

Only 3% of the fresh produce was positive for *E. coli* (Table 2). Fresh produce sampled at the field (plant, harvest and field containers) contained 1.06% of *E. coli*, and the produce collected at the packinghouse had 4.0% being the waxing operation one of the most contaminated (Table 3). The levels of *E. coli* increased at the uploading ramps in the packinghouse with a range of 0.3 log to 1.6 log CFU/100g. *Salmonella* spp. levels remained consistently low with 2.6% of the positive samples among the harvest, field container, unloading ramp, sorting, and packing bins (Table 3). Unlike *E. coli*, *Salmonella*-positive samples were distributed at the field and only a few on the packinghouse points. The results of this study coincide with that of Mukherjee et al. (2004) that reported numbers of 2.3% of produce contaminated with *Salmonella*, however, some studies such as Gallegos-Robles et al. (2008) have reported higher levels (37%) of bell pepper produces contaminated with *Salmonella*. One drawback is the fact that both studies used a smaller amount of samples as the one reported in this study, thus large number of samples need to be sampled to determine the behavior of the pathogen.

A total of 186 worker hands were analyzed as follows: 98 samples after workers washed their hands before labor started and 88 samples were taken after 3 h of labor (Table 4). The results showed the presence of 15.3 and 71.4% of *E. coli* and coliforms before labor, respectively. The levels of *E. coli* and coliforms after washing hands were 1.76±0.73 and 2.77±0.94 log, respectively. Copper sulfate was used at the field as a hand washing disinfectant solution to reduce the presence of microorganisms from hands, however, *E. coli* and coliforms remained present after washing hands. This can be related to inadequate hygienic practices, including the use of contaminated water, lack of or insufficient sinks and manual faucets infrastructure. According to Montville et al. (2002) it is imperative to apply an efficient technique for washing hands before starting working with fruits and vegetables. This technique may include a continuous training by the food safety staff (FDA, 1998). A hand washing technique recommended by Jimenez et al. (2007) consist in washing hands with an antibacterial soap for 30 s, rinsing with water for 15 s, drying with paper towels and rubbing with an alcohol-based gel to reduce at least 3.5 log of *Salmonella* on hands. On the contrary, contamination during the first 3 h of work showed 10.2 and 58% of contaminated hands with *E. coli* and coliforms, respectively. This may be due to the transfer of bacteria through direct contact with contaminated surfaces and fresh produce. Additionally, around 20% of worker hands before starting to work were contaminated with coliforms with a concentration of 1.8±0.9 log CFU/hands but showed no coliforms after 3h of work; while 53% were contaminated with coliforms at a higher concentration 3.0±1.0 log CFU/hands and the contamination continued after 3 h of labor (data not shown). This may be due to the carryover of the bacteria from hands to workers, fresh produce, or surface contact areas. Additionally, the residual bactericidal effect of copper sulfate applied during hand washing before starting the labor can also be related to the absence of coliforms after 3 h of labor.

The presence of *Salmonella* spp. was detected in 17.3% of worker hands before starting to work, including hands of pickers, sorters and packers (Table 4). After 3 h of work, only 9.1% of the sample hands were contaminated; the pickers hands were not included (Table 4). *Salmonella* showed a slightly increase throughout postharvest handling, suggesting a potential transfer of the pathogen from contaminated hands to

**Table 5.** Presence of *Salmonella* spp., *Escherichia coli* and coliforms on contact surface areas of packinghouse equipment.

| Surface contact | No. of positive samples (%)† | | |
|---|---|---|---|
| | ***Salmonella* spp.††** | ***Escherichia coli*** | **Coliforms** |
| Unloading ramp | 0 (0.0%) | 3 (9.1%) | 29 (87.9%) |
| Roller | 0 (0.0%) | 2 (6.1%) | 17 (51.5%) |
| Conveyor belts | 3 (9.1%) | 0 (0.0%) | 21 (63.6%) |
| Packing bin | 2 (6.1%) | 1 (3.0%) | 25 (75.8%) |
| Total (N=132) | 5 (3.8%) | 6 (4.5%) | 92 (69.7%) |

†Percentage of positive samples based on samples analyzed; ††Positive samples by PCR.

fresh produce. Jimenez et al. (2007) reported that about 0.21% of the bacteria are transferred from contaminated hands to pepper produce. *S. enterica* serovar Typhimurium was confirmed by serotyping in 50 of the isolated strains.

Microbial contamination on surface contact areas, including *E. coli* and coliforms increased from harvest throughout packing. *E. coli* and coliforms were found in 4.5 and 69.7% of the total surface contact areas analyzed, respectively (Table 4). The levels of contamination ranged from 1.45±1.15 log CFU/400cm$^2$ and 1.23±0.83 log CFU/400cm$^2$ for *E. coli* and coliforms, respectively. The highest frequency of *E. coli* (9.1%) and coliforms (87.9%) was found in the unloading ramp with concentrations of 2.05±1.02 and 1.63±0.98 log CFU/600cm$^2$, respectively. *E. coli* and coliforms were constant from unloading ramp through the boxes ready for distribution. *Salmonella* spp. was present in 9.1 and 6.1% of conveyor belts and packing bins sample (Table 5). Nine of the isolated strains were confirmed as *S. enterica* serovar Typhimurium.

Duffy et al. (2005) also found the presence of *Salmonella* and *E. coli* in unloading ramps and conveyor rollers at fresh produce packinghouse in Texas. Additionally, Montville and Shaffner (2003) demonstrated the transfer of bacteria from surface contact areas to lettuce (*Lactuca sativa* L.). The findings of the present study suggest that surface contact areas are an important source of microbiological contamination of bell pepper produce in the packinghouse.

Pathogenic *E. coli* was not detected in any of the 900 samples tested. However, *S. enterica* serovar Typhimurium was confirmed in worker hands and surface contact areas. *S. enterica* serovar Typhimurium is one of the most frequently isolated strains worldwide, specifically in Mexico (Gutiérrez -Cogco et al., 2000; Wasyl et al., 2006). López-Cuevas et al. (2009) found *Salmonella* Typhimurium, as well as Infantis, Anatum, Agona, Oranienburg, Minnesota in agricultural water in the Culiacan region. Similarly, Estrada-Acosta et al. (2014) found *Salmonella* Oranienburg in the Culiacan river. These findings highlight the needs for microbial determination numbers to determine the impact of the pathogen in water. The presence of *S. enterica* serovar Typhimurium in worker hands and contact surface areas eventually causes illness to consumers. Infections and outbreaks with *S. enterica* serovar Typhimurium have been linked to consumption of contaminated food such as vegetable salads (Doré et al., 2004; Wasyl et al., 2006).

## Conclusions

Microbiological contamination of bell pepper produce gradually increased during handling in the production system from the time fruit is harvested in the field to its packaging process, being the surface contact areas the main vehicles of transmission of microorganisms to the produce. This study shows that water used for washing hands, worker hands, and surface contact areas were the main source of contamination. Procedures such as good agricultural practices, good Manufacturing practices, and HACCP can minimize but not eliminate foodborne pathogens in agricultural products. Thus, the study demonstrates the presence of contamination that suggests the need for both the improvement of the good agricultural practices and the evaluation of alternative disinfectants. Further studies including a larger number of participating farms assessing the presence and persistence of other bacterial indicators and virulence, as well as fingerprints data of each of the microorganism detected to determine the relationship and source of contamination are needed.

## Conflict of interests

The authors did not declare any conflict of interest.

### REFERENCES

APHA (2001) American Public Health Association. Compendium of Methods for the Microbiological Examination of Foods. 4th Edition. Washington, D. C.

Castillo A, Mercado I, Lucia LM, Martínez-Ruiz Y, Ponce de León J, Murano EA, Acuff GR (2004). *Salmonella* contamination during production of cantaloupe: A binational study. J. Food Prot. 67(4):713-720.

CDC (2002). Center for Disease Control and Prevention. Multistate outbreaks of *Salmonella* serotype Ponna infections associated with eating Cantaloupe from México – United States and Canada, 2000-2002. Morbidity Mortality Weekly Report. 51:1044-1047.

CDC (2008). Center for Disease Control and Prevention. Outbreak of *Salmonella* serotype Saint Paul infections associated with multiple raw produce items - United States, 2008. Morbidity Mortality Weekly Report 57:929-934.

CDC (2012). Center for Disease Control and Prevention. Multistate outbreak of *Salmonella* Braenderup infections associated with Mangoes.http://www.cdc.gov/salmonella/braenderup-08-12/Accessed October 2013.

Chiu C, Ou J (1996). Rapid identification of *Salmonella* serovars in feces by specific detection of virulence genes, *invA* and *spvC*, by an enrichment broth culture-multiplex PCR Combination Assay. J. Clin. Microbiol. 34(10):2619-2622.

CIAD (2006). Research Center for Food and Development. Overview of the Mexican fresh produce industry. Food Safety Workshop, Culiacan Research Station, February 2006.

Cooley MB, Quinones B, Oryang D, Mandrel ER, Gorsky L (2014). Prevalence of shiga toxin producing *Escherichia coli*, *Salmonella* enterica, and *Listeria monocytogenes* at public access watershed sites in a California Central Coast agricultural region. Front. Cell Infect. Microbiol. 4(30):1-13.

CSPI (2014). Center for the Science in the Public Interest. A review of foodborne illness in America from 2002-2011. Center for Science in the Public Interest. http://cspinet.org/reports/outbreakalert2014.pdf Accessed 23 November 2013.

Doré K, Buxton J, Henry B, Pollari F, Middleton D, Fyfe M, Ahmed R, Michel P, King A, Tinga C, Wilson JB (2004). Risk factors for *Salmonella* Typhimurium DT104 and non-DT104 infection: a Canadian multi-provincial case-control study. Epidemiol. Infect. 132(3):485-493.

Duffy EA, Lucia LM, Kells JM, Castillo A, Pillai SD, Acuff GR (2005). Concentrations of *Escherichia coli* and genetic diversity and antibiotic resistance profiling of *Salmonella* isolated from irrigation water, packing shed equipment, and fresh produce in Texas. J. Food Prot. 68(1):70-79.

ERS (2009). Economic Research Service Foreign agricultural trade of the United States. http://www.ers.usda.gov/Data/FATUS/. Accessed 13 March 2009.

Estrada-Acosta M, Jiménez M, Chaidez C, León-Félix J, Castro-del Campo N (2014). Irrigation water quality and the benefits of implementing good agricultural practices during tomato (*Lycopersicum esculentum*) production. Environ. Monit. Assess. 186(7):4323-4330.

FAO (2009). Food and Agricultural Organization. How to feed the world in 2050. http://www.fao.org/fileadmin/templates/wsfs/docs/expert_paper/How_to_Feed_the_World_in_2050.pdf. Acceded September 2014.

FAS (2009). Foreign Agricultural Service. U.S. Trade Internet System. http://www.fas.usda.gov.Accessed March 2009.

FDA (1998). Food and Drug Administration. Guidance for industry: Guide to minimize microbial food safety hazards for fresh fruits and vegetables.www.fda.gov/Food/GuidanceComplianceRegulatoryInformation/GuidanceDocuments/ProduceandPlanProducts/ucm064574.htm. Acceded October 2006.

FDA (2012). Food and Drug Administration. Import Alert 99-23. Detention without physical examination of raw fresh fruits and vegetables due to the presence of pathogenic contamination. http://www.accessdata.fda.gov/cms_ia/importalert_266.html.Acceded January, 2013.

Gallegos-Robles MA, Morales-Loredo A, Alvarez-Ojeda G, Vega PA, Chew MY, Velarde S, Fratamico P (2008). Identification of *Salmonella* serotypes isolated from cantaloupe and chile pepper production systems in Mexico by PCR–Restriction fragment length polymorphism. J. Food Prot. 71(11):2217–2222.

Gutiérrez-Cogco L, Montiel-Vázquez E, Aguilera-Pérez P, González-Andrade MC (2000). Identification of *Salmonella* serotypes in the health service of Mexico. Salud Pública de Mex. 42(6):490-495.

Jiménez M, Siller JH, Valdez JB, Carrillo A, Chaidez C (2007). Bidirectional *Salmonella* enterica serovar Thyphimurium transfer between bare/glove hands and green bell pepper and its interruption. Int. J. Environ. Health Res. 17(5): 381-388.

Johnston LM, Jaykus LA, Moll D, Martha CM, Anciso J, Mora B, Moe CL (2005). A field study of the microbiological quality of fresh produce. J. Food Prot. 68(9):1840-1847.

López-Cuevas O, León-Félix J, Jiménez-Edeza M, Quiroz C (2009). Detection and antibiotic resistance of Escherichia coli and Salmonella in water in agricultura soil. Rev Fitotecnia Mex 32(2):119-126.

López-Saucedo C, Cerna JF, Villegas-Sepulveda N, Thompson R, Velazquez FR, Torres J, Tarr PI, Estrada-García T (2003). Single multiplex polymerase chain reaction to detect diverse loci associated with diarrheagenic *Escherichia coli*. Emerg. Infect. Dis. 9(1):127-130.

Mody RK, Greene SA, Gaul L, Sever A, Pichette S, Zambrana I, Dang T, Gass A, Wood R, Herman K, Cantwell LB, Falkenhorst G, Wannemuehler K, Hoekstra RM, McCullum I, Cone A, Franklin L, Austin J, Delea K, Behravesh CB, Sodha SV, Yee JC, Emanuel B, Al-Khaidii SF, Jefferson V, Williams IT, Griffin PM, Swerdlow DL (2011). National outbreak of *Salmonella* serotype *Saintpaul* infections: Importance of Texas restaurant investigations in implicating jalapeno peppers. PLos One 6(2). E16579.

Montville R, Schaffner DW (2003). Inoculum size influences bacterial cross contamination between surfaces. Appl. Environ. Microbiol. 69(12):7188-7193.

Montville R, Yuhuan C, Schaffner DW (2002). Risk assessment of hand washing efficacy using literature and experimental data. Int. J. Food Microbiol. 73(2-3):305-313.

Mukherjee A, Speh D, Dyck E, Diez-Gonzalez F (2004). Preharvest evaluation of coliforms, *Escherichia coli*, *Salmonella*, and *Escherichia coli*O157:H7 in organic and conventional produce grown by Minnesota farmers. J. Food Prot. 67(5):894-900.

Wasyl D, Sandvang D, Skov MN, Baggesen DL (2006). Epidemiological characteristics of *Salmonella* Typhimurium isolated from animals and feed in Poland. Epidemiol. Infect. 134(1):179-185.

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*Full Length Research Paper*

# Effect of *Eichhornia crassipes* on coliforms load in small water bodies within Lake Victoria basin, Kenya

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**The study investigates the effects of *Eichhornia crassipes* (water hyacinth) infestation based on coliform loads which are pollution indicator organisms. These dams have fish and the waters are commonly used for domestic purposes without any treatment hence it is necessary to check their status. Sampling was done on 25 small water bodies and from which water samples were taken for microbial determination. Membrane filtration method was used to enumerate fecal coliforms by use lauryl sulphate broth and incubated at 44 ± 0.5°C for 18 to 24 h. The results show that water bodies infested by water hyacinth harbored higher levels of fecal coliforms compared to those devoid of the weed. The high fecal coliform levels have negative impacts on the lives of communities using these water bodies. In addition, it reduces native species and disrupts food chains and nutrient cycle. The small water bodies within the Lake Victoria basin need frequent monitoring in order to give the relevant authorities concrete information for proper sensitization to the communities. Water hyacinth should also be properly managed so that it does not chock dams that are used for domestic and fishing activities.**

**Key words:** Coliform load, infestation, pollution, local community, *Eichhornia crassipes.*

## INTRODUCTION

Most of the small water bodies in the Lake Victoria basin, Kenya, were constructed by colonial government in the far flung areas to provide water for the adjacent population and their animals (Kaufman, 1992). Currently, others have risen after excavation pits left over after quarry activities especially during road constructions, and then filled with water during rainfall seasons. Besides provision of water, recent studies have revealed that these ecosystems also form important refugia for some biota, some of which are not found in Lake Victoria. Currently, water hyacinth has found its way into a number of these habitats either chocking them or displacing the

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native macrophytes. Water hyacinth (*Eichhornia crassipes*) has been grouped as one of the top 10 worst weeds in the world (Shanab et al., 2010; Gichuki et al., 2012; Patel, 2012). It is characterized by rapid growth rates, extensive dispersal capabilities, large and rapid reproductive output and broad environmental tolerance (Gichuki et al., 2010; Zhang et al., 2010). In Africa for example, water hyacinth is listed by law as a noxious weed in several countries, it is the most widespread and damaging aquatic plant species. The economic impacts of the weed in seven African countries have been estimated at between US$20 to 50 million every year. Across Africa costs may be as much as US$100 million annually (UNEP, 2012). *E. crassipes* is considered a health hazard because it is associated with several pathogenic organisms and vector diseases (Sitoki, 2001). The ecological status of an aquatic body is a function of the type and diversity of aquatic biota, the water quality and pollution while the high counts of fecal coliforms reflect the presence of other dangerous pathogenic micro-organisms that may threaten lives of the water users (Getabu et al., 1999). Since these dams are great importance to the local communities, their status in terms of portability needs to be well known. This study thus was a step towards providing baseline data on the quality of these waters and the potential health problems that may await the consumers. In doing so, total feacal coliform which is the most basic test for bacterial contamination in water pollution (Gram, 1997) was used.

## MATERIALS AND METHODS

### Study area and Sampling stations

All the small water bodies (SWBs) sampled were within Lake Victoria basin and were carefully selected to represent both high and low altitude. They were found at both southern and northern parts of the basin (Figure 1).

### Sampling

During the expedition, a total of 25 small water bodies were surveyed and their representative samples collected for microbiological analysis. A dinghy was used to access the water bodies for the sampling of various parameters.

### Microbial determination

The membrane filtration method was used during sample analysis for enumeration of coliform bacteria. A standard amount of sample/aliquot of 1 to 100 ml depending on the water turbidity was filtered through a 0.45 µm pore size sterilized membrane filter pad and the filter placed face up on the culture plate containing lauryl membrane sulphate broth and incubated at 18 to 24 h at 44°C with the use of Del agua water testing kit (OXFAM Delagua). The bacterial colonies growing on the cultured membranes were counted, and bacteriologically characterized. The coliform counts were expressed in colony forming units/ 1000 ml.

### Data analysis

Statistical analysis was performed by using Minitab version 14 and PAST statistical softwares. Descriptive statistics was first done to determine the central tendency and dispersion of the data. The results for descriptive statistics were represented in table and charts. The coliform counts per station were then log transformed and subjected to one way analysis of variance (ANOVA) to test if the variation between the dams was significant at 95% confidence level. Duncan's Multiple Range Test was further done for the purpose of pairwise comparison and to test if water hyacinth actually caused a variation.

## RESULTS AND DISCUSSION

Out of the 25 sampled small water bodies, 4 SWBs had water hyacinth with feacal coliform counts >300 cfus $1^{-1}$, 8 SWBs had other water weeds with feacal coliform counts < 100 cfus $1^{-1}$ and the rest had neither water hyacinth nor weeds with no feacal coliform counts recorded. The four water hyacinth infested SWBs with high bacterial counts were, Korowe (1075 cfus), Kobodo (1795 cfus), Stella (845 cfus), and Kachila (TNTC). The 8 SWBs infestated with other water weeds and low bacterial counts were, Yenga, Mauna, Mwer, Kalenyjuok, Oyombe, Bande, Kosiga and Achune. The remaining thirteen non-infestated SWBs did not record any bacterial count (Table 1).

None of the dams without water hyacinth recorded a count exceeding 200 $cful^{-1}$. While all dams with hyacinth recorded counts above 800 $cful^{-1}$ (Figure 2). The low Coliform count dams were either infested with other water weeds or reeds with exception of Achune dam that did not have any buffer zone and had very high infiltration and siltation effect.

One way ANOVA on the log transformed data showed significant variation between the stations with water hyacinth ($F = 18.33$, $P = 0.0001$). Post hoc test grouped the SWBs with water hyacinth (Kobodo, Korowe, and Stella dams) in one sub-set while the rest were scattered in other sub-sets. This is possibly an indication that there is a common factor influencing the three. The variation in numbers between hyacinth infested and non-infested dams can be a clear indication of hyacinth influence together with other factors like surface run-off for the case of non-buffered ones. According to the World Environmental Protection Agency, surface water bodies infested by water hyacinth are at risk of being contaminated by pathogenic and non-pathogenic micro-organisms. The results obtained from the study agrees with EPA's (1989), research findings, because the calculated mean results from the tested bacteriological parameters (Korowe, Kobodo, Stella and Kachila) produced > 300 cfus in the mentioned dams (Figure 2), thus unfit for human drinking.

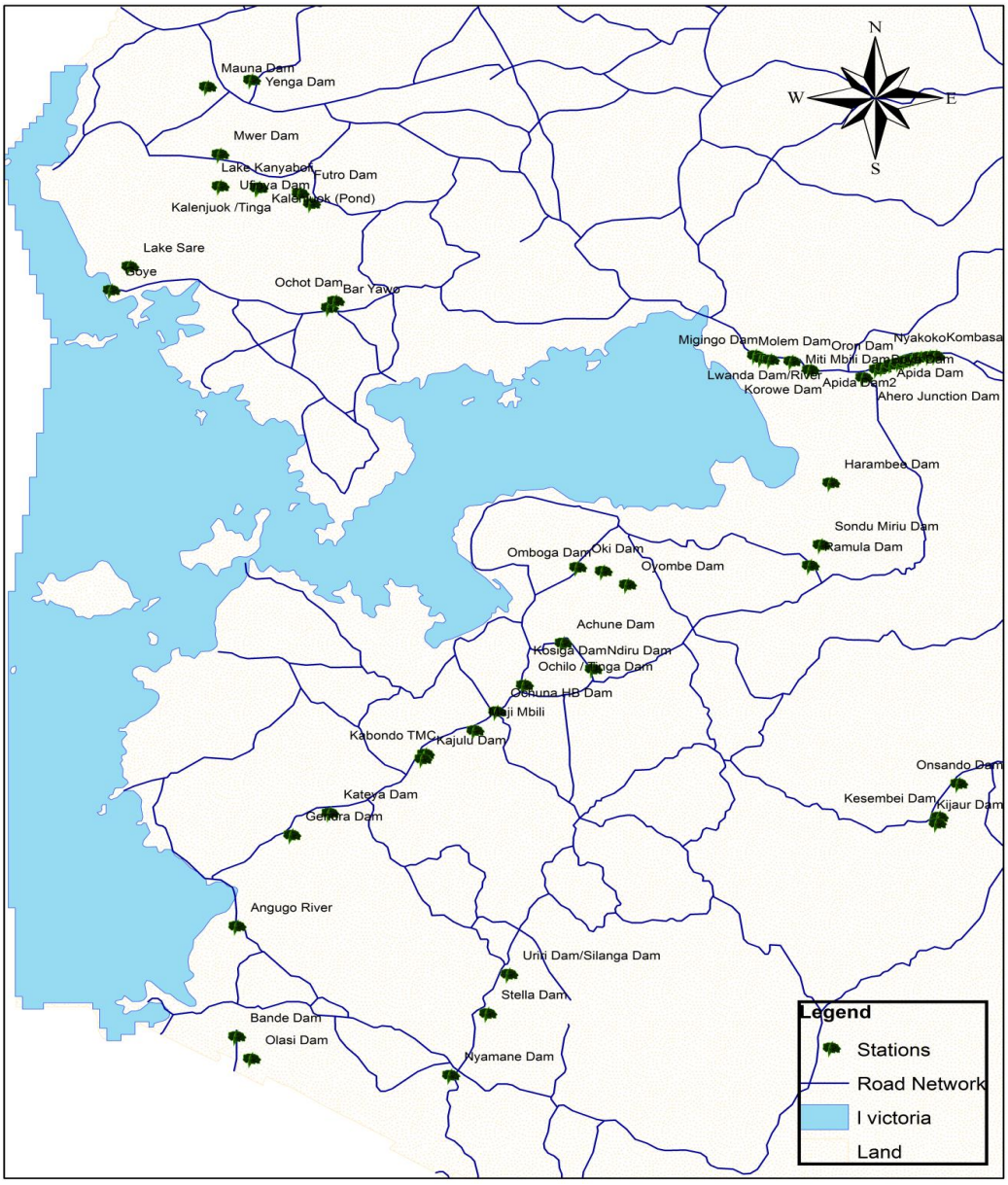


**Figure 1.** Map of Lake Victoria basin showing the small water bodies (SWBs) surveyed.

Based on human drinking water specifications (FAO and WHO), the values in all the dams that recorded the bactarial counts were on the higher side because coliform bacteria in drinking water should be zero coliform per 100ml of water.

The low bacteriological counts in samples from other dams (Achune, Oyombe, Bande, Kosiga, Mwer, Mauna Kalenyjuok and Yenga) infested by other aquatic plants was attributed to several factors. Some of the possible factors include presence of algae and absence of water hyacinth. According to Villamagna (2009) and Parhad and Rao (1974), water hyacinth causes decrease in algae in the system and inturn decreases pH and dissolved oxygen concentration thus, resulting in the increase of fecal coliforms. The absence of water hyacinth therefore leads to increase in algae which inturn reduces coliform loads. The presence of feacal coliforms in these dams could have thus been attributed to lots of pollutants brought in through anthropogenic activities such as domestic washing and crop cultivation. Other possible sources are very high infiltration and siltation effect**,** and lack of buffer zones observed around some of these water bodies. The results concur with findings reported by Mujingni (2012), FAO, (2009), and Narayan and Parveev (2000) that water hyacinth infested water bodies harbour higher number of microorganisms than

**Table 1.** Number of coliforms in each dam sampled (Yes and No means presence or absence of hyacinth respectively while TNTC, means the growth was too numerous to count).

| Site | Status/hyacinth infestation | Mean cfu./1000 ml |
|---|---|---|
| Yenga Dam | No | 55 |
| Mauna Dam | No | 65 |
| Mwer Dam | No | 50 |
| Kalenjuok Dam | No | 155 |
| Ufinya Dam | No | 0 |
| Lake Kanyaboli | No | 0 |
| Lake Sare | No | 0 |
| Ochot Dam | No | 0 |
| Opoda Dam | No | 0 |
| Korowe Dam | Yes | 1075 |
| Oyombe Dam | No | 75 |
| Kobodo Dam | Yes | 1795 |
| Bande Dam | No | 10 |
| Olasi Dam | No | 0 |
| Kokech Dam | No | 0 |
| Stela Dam | Yes | 845 |
| Uriri Dam | No | 0 |
| Kosiga Dam | No | 15 |
| Achune Dam | No | 110 |
| Gesibei Dam | No | 0 |
| Kijauri Dam | No | 0 |
| Damside Dam | No | 0 |
| Harambee Dam | No | 0 |
| Ahero Dam | No | 0 |
| Kachila Dam | Yes | TNTC |

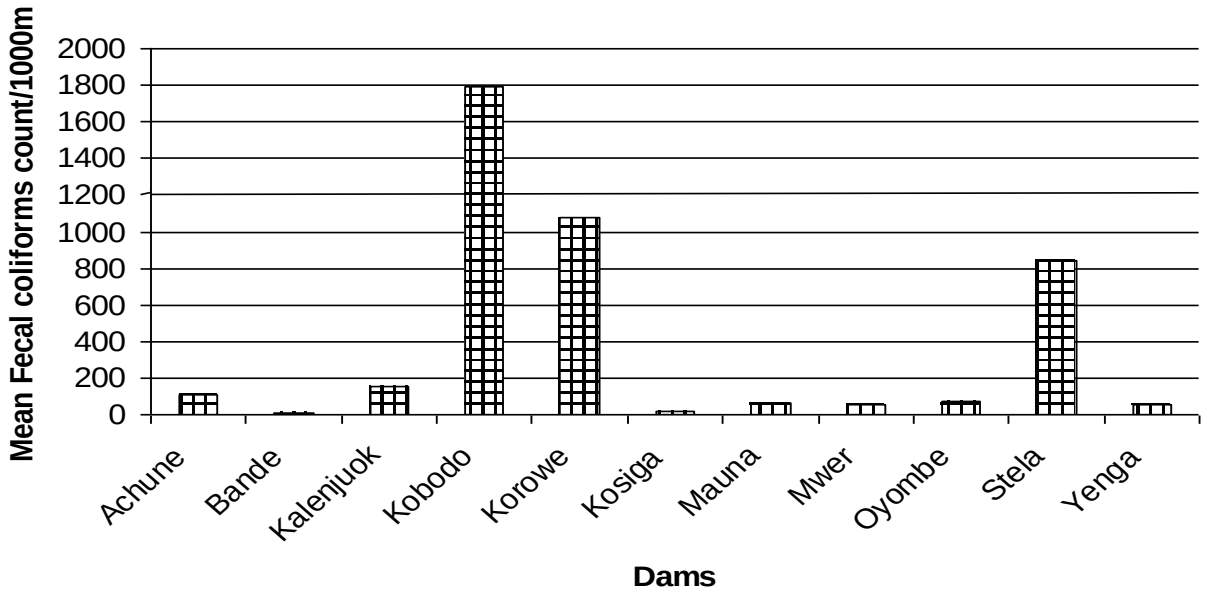


**Figure 2.** Coliform counts of some dams sampled within Lake Victoria basin (Dams not represented in the figure did not have any Colony Forming Units).

non- infested waters.

## Conclusion and Recommendation

From the results, it is clearly seen that water hyacinth infestation had a direct influence on coliforms levels. It is therefore recommended that:

1. Water hyacinth in these four dams be removed and water treated for the safety of the consumers,
2. An experimental survey be done to check colonization pattern, rate and microbial diversity shifts.

### REFERENCES

EPA (1989). Drinking water's total coliforms (including fecal coliforms and *E. coli*), National drinking water regulation; environmental Protection Agency final rule. Federal register 54:27544-27568

FAO (2009). Informal food trading. http://www.capetown.gov.za/EN/CITYHEALTH/ENVIROHEALTH/FOODQUALITYANDSAFETY/Pages/InformalFoodTrading.aspx

Getabu A and Nyaundi J (1999). Relative abundance and distribution of fish in Kenyan waters of Lake Victoria. 16pp, In D. Tweddle and I.G.Cowx (Eds). Report on the fourth FIDAWOG workshop, 29 March-1 April 1999, Jinja, Uganda, LVFRP/TECH/99/05, 122pp.

Gichuki J, Lewis S, L, Nyaundi J (2010). Limnological report of regional hydroacoustic survey of L. Victoria From 19th march to 16th April, 2010.

Gichuki J, Omondi R, Boera P, Okorut T, SaidMatano A, Jembe T, Ofulla A (2012). Water hyacinth *Eichhornia crassipes* (Mart.) Solms-Laubach dynamics and succession in the Nyanza Gulf of Lake Victoria (East Africa): Implications for Water Quality and Biodiversity Conservation. Sci. World J. 12:10.

Gram L (1997). Fish Handling, Preservation and Processing along the Winam gulf, Lake Victoria, Kenya.

Kaufman L (1992). Catastrophic change in species-rich freshwater ecosystems: the lessons of Lake Victoria. Bioscience 42: 846–858.

Mujingni C (2012). Quantification of the impacts of Water Hyacinth on riparian communities in Cameroon and assessment of an appropriate method of control: The case of the River Wouri Basin: The Case of the Wouri River Basin. Msc disseratation. World Maritime University, Malmö, Sweden.

Parhad NM, Rao NU (1974). Effect of pH on survival of *Escherichia coli.* J. WPCF 46(5):980-986.

Patel S (2012). Threats, management and envisaged utilizations of aquatic weed Eichhornia crassipes: anoverview. Rev. Environ. Sci. Biotechnol. 11:249-259. Omondi R (2014). Distribution of water hyacinth and other macrophytes in small water bodies (SWBs) in Lake Victoria, Kenya (LVMP II Report).

Sitoki L (2001). Water quality status and phytoplankton in the Nyanza Gulf and adjacent small water bodies (SWB) within the catchment. MSc.Thesis. UNESCO IHE Delft :Netherlands

UNEP (2012). Fifth Global Environment Outlook (GEO5): Environment for the future we want. United Nations Environment Programme, Nairobi.

Villamagna AM (2009). Ecological effects of Water hyacinth (*Eichhornia crassipes*) on Lake Chapala. Faculty of the Virginia Polytechnic Institute and State University, Blacksbury Virginia, Mexico.

Zhang Y, Zhang D, Barrett S (2010). Genetic uniformity characterises the invasive spread of water hyacinth (*Eichhornia crassipes*), a clonal aquatic plant. Mol. Ecol. 19:1774-1786.

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