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Probiotic Potential of Bacterial Isolates From 'Amabere amaruranu' Cultured Milk

Blaise B. Boyiri
East Tennessee State University

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Probiotic Potential of Bacterial Isolates From 'Amabere amaruranu' Cultured Milk

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A thesis

presented to

the faculty of the Department of Biological Sciences

East Tennessee State University

In partial fulfillment

of the requirements for the degree

Master of Science in Biology

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by

Blaise Baalayel Boyiri

August 2014

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Edward M. Onyango, PhD, Chair

Bert Lampson, PhD

Victoria R. Palau, PhD

Keywords: Cultured milk, Digestive tract conditions, Lactic acid bacteria, Probiotics,

Mucins

ABSTRACT

Probiotic Potential of Bacterial Isolates From 'Amabere amaruranu' Cultured Milk

by

Blaise Baalayel Boyiri

Probiotics are viable nonpathogenic microbes that positively affect host health. Probiotics inhibit infection, activate immunity, and promote mucosal-barrier development. Many microbes have probiotic activity. Nonetheless, the selection of stable strains and their specific mechanism(s) of action are not fully elucidated. Bacteria from 'Amabere amaruranu' cultured milk from Kenya were isolated and identified by PCR sequence analysis of the 16S rRNA gene. Isolates were examined for stability to acid and bile, antimicrobial activity, mucin production, and degradation and sensitivity to antibiotics, hence their potential for probiotics. *Lactobacillus* isolates were acid unstable, bile-stable, nonmucinolytic, and presented antibacterial activity. *L. rhamnosus* cell fractions increased MUC4 and MUC3 expression in colon cells. *Bacillus* isolates were acid and bile stable, nonmucinolytic and lacked antimicrobial activity. In conclusion, *Lactobacillus* isolates that were nonmucinolytic, stable in bile, demonstrated antibacterial activity, sensitive to antibiotics, and stimulated increase MUC4 and MUC3 levels in colon cells could be potential probiotics.

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DEDICATION

To Boyiri Boyiri Jr. and Britney Boyiri

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Definition and History of Probiotics

The concept of ingesting live microbes for their potential health benefits evolved around the early 20th century (Metchnikoff, 1907). This idea came from observations that were made by Ilya Ilyich Metchnikoff (1845-1916) that the longevity of native Bulgarians could be attributable to their consumption of cultured dairy products (Bibel, 1988). Bacterial cultures have long been used in dairy products to develop a distinctive flavor and increase shelf life (Bruhn et al., 2002). The practice of adding selected viable microbes to food is now referred to as 'probiotics' (O'Sullivan, 2001). Probiotics are viable nonpathogenic microbes that have the potential to confer health benefits on their host when ingested in sufficient amounts (FAO/WHO Joint Working Group, 2002; Fuller, 1991; Shanahan, 2005). Interestingly, both live and dead probiotic cells are efficacious in function, and their cell components can effectively act as biological response modifiers (Adams, 2010). Probiotic activities are not limited to only the gastrointestinal tract but seem to demonstrate significant biological effects on distant organs and tissues as recently reviewed by Adams (2010). A wide variety of bacterial species could provide probiotic activity, but the most common strains in commercial use are the lactobacilli and bifidobacteria groups (Reid, Anukam, & Koyama, 2008; Shah, 2007). Lactic acid bacteria in particular have been consumed safely by all human generations in various fermented dairy foods (Fuller, 1992; van der Kamp, 1996). Fuller (1987) reported an increase interest in the use of live microbial agents for health maintenance

and disease prevention. This report was supported by Brady, Gallaher, and Busta (2000) and Isolauri (2000) that several scientific and medical communities have developed interest in exploring the functionality and application of probiotics in human health.

<div align="center">Sources of Probiotic Bacteria</div>

Collins, Thornton, and Sullivan (1998) pointed out the imperativeness to always establish the source of probiotic bacteria in order to substantiate their health claims. Bacterial strains among human gut commensal microbiota and milk microflora that demonstrate substantial stability and beneficial properties are potential sources of probiotics (Isolauri, Kirjaviainen, & Salminen, 2002a). Many microorganisms including lactobacilli, bifidobacteria, streptococci, and *Saccharomyces* are commonly considered for probiotic preparations (Goldin & Gorbach, 1992).

Cultured-milk as Source of Probiotic Strains

The high nutritional value and water content of milk makes it one of the foods that harbor complex microbial communities (Quigley et al., 2011). The understanding of the microbial communities in milk and other dairy products is important. The microbes determine the quality of the milk products. These microbes may include spoilage bacteria or potential pathogens as well as beneficial micro-organisms (Cousin, 1982). The diversity of microbial communities of raw milk for instance may be influenced by the location of the animal and the season of milking it (Bonizzi, Boffoni, Feligini, & Enne, 2009; Randazzo, Pitino, Ribbera, & Caggia, 2010). Other factors that affect the

microbial composition include handling and the storage procedures. Most commonly

studied microbial communities in milk are the culturable microbes. These include the

Lactobacillus, Streptococcus, Enterococcus, Lactococcus, Leuconostoc, Weisella, and

Pediocococus strains. Strains of other genera such as *Staphylococcus,*

Propionibacterium, Corynebacterium, and *Brevibacterium* as well as *Saccharomyces*

may also be present (Coppola, Blaiotta, & Ercolini, 2008). It is however apparent that

the culture-based method of studying the microbial composition of milk does not usually

account for all strains. This is because the method relies on isolation and cultivation of

the strains for identification and as a result may fail to isolate some species that are

represented in low numbers. Hugenholtz, Goebel, and Pace (1998) reported that

minority species may be out-competed in laboratory media by numerically more

abundant microbial species.

Selection Criteria for Probiotics

The selection of probiotic strains has been in large part based on historical

recognition of safe consumption of cultures. It is essentially based on reports of many

years of consuming a particular culture without obvious harmful side effects (Kopp-

Hoolihan, 2001). Most probiotic strains are similar to the natural gut commensal

microflora. Preferable probiotics strains are those of human origin (Collins et al., 1998).

The definition of probiotics requires adequate verification of the efficacy and safety of

selected strains, thus, assessment of these factors constitutes an important part of the

characterization of probiotics for human use (Isolauri, Salminen, & Ouwehand, 2004).

Among the prerequisites for selecting probiotic bacteria is their ability to show high

capacity to adhere to and colonize the gut (Huis in't Veld, Havenaar, & Marteau, 1994). Additional considerations for selecting suitable probiotics are that the strains should not have the capacity to degrade mucins. This is to avoid possible degradation of the protective mucus layer of the digestive tract (Zhou, Gospal, & Gill, 2001). Furthermore, suitable probiotic strains should be nonpathogenic even in immunocompromised hosts (Collins et al., 1998) and should demonstrate appreciable ability to exert beneficial effects on their host (Kopp-Hoolihan, 2001). The selected strains should show stability to stress, especially that of digestive tract conditions including tolerance to low pH and high concentration of bile acids. This establishes the ability of the strain to survive and proliferate in the *in vivo* conditions of the digestive tract (Collins et al., 1998). Saarela, Alakomi, Puhakka, and Matto (2009) reported that the probiotic isolates usually encounter various stressful conditions during isolation, formulation into food or supplements, storage, and transit along the gastrointestinal tract. Some of the common stress conditions as noted by Antione (2011) included refrigeration storage at 4 ^{o}C, transit along mouth and gastrointestinal tract temperatures of about 25 ^{o}C and 37 ^{o}C respectively, chemical challenges including hyper-acidic gastric environment of about pH1 to 2, and neutralization or detergent effects of bile in the small intestines. However, Saarela et al. (2005) reported that tolerance of cells to stress is strain specific and that cells at stationary phase of growth seemed to be more tolerant to stressful conditions than actively growing cultures **(**Saarela et al., 2009)*.* Furthermore, desirable probiotic strains should not provoke the host immune responses against themselves or their products (Pouwels, Leer, & Posno, 1992). Probiotic strains are also required to demonstrate appropriate antibiogram profiles including sensitivity to antibiotics and

resistance to metronidazole and that to some extend prevents the used of strains that may transfer antibiotic resistance trait to other gastrointestinal tract dwellers. They are also required to possess effective immunostimulatory effects on the mucosal immune system especially in the activation of cytokines (Collins et al., 1998).

Probiotics in Food Synthesis

Microorganisms have been involved in the preparation of cultured foods by many communities around the world. These organisms have demonstrated appreciable potential to improve the quantity, availability, and digestibility of some dietary nutrients (Kopp-Hooligan, 2001). There are reports of increase concentration of folic acid in yogurt and 'bifidus milk' (commercial milk preparation that contains *Bifidobacterium bifium*) through fermentation with lactic acid bacteria (Alm, 1982). Fermentation of food with lactic acid bacteria also increases the content of vitamin B complexes and promotes the production of short-chain fatty acids and the hydrolysis of amino acids (Friend & Shanahan, 1984).

Therapeutic Effects of Probiotics

A growing body of scientific evidence supporting probiotic contributions to human health is available. Modification of gut microbial communities by probiotic therapy has demonstrated therapeutic potentials in clinical conditions associated with barrier dysfunction and inflamed mucosa (Isolauri, 2001). Probiotics aid digestion, inhibit pathogenic infections, promote development of intestinal integrity, reduce pH of the large bowel, activate mucosal immunity, and suppress tumorigenesis and cancer.

Probiotics in Aiding digestion and Improving Bowel Movement

Lactose intolerance is a common complaint for some people when they consume milk or dairy products. It involves the difficulty for people with this condition to digest the lactose component in milk. There are several reports about the ability of some cultures to provide a remedy to this condition through the production of lactase for the hydrolysis of lactose during fermentation and digestion (Savaiano & Levitt, 1987). Probiotics also contribute to improvement of bowel movement especially among the elderly. Significant laxative effect from the consumption of *Bifidobacterium* cultured milk was reviewed by Yaeshima (1996).

Probiotics in Mucosal Barrier Development and Reduction of Inflammation

The mucosal barrier consists of a single cell layer epithelium and an aggregate of other secretory cells and gut immune cells and their products. The epithelial cell layer of the gastrointestinal tract is strategically located between the many microbes and antigens of the intestinal lumen and the inflammatory and immune effector cells of the lamina propria (Mack, Michail, Wei, McDougall, & Hollingsworth, 1999). It is capable of regulating the production of selected chemokines in response to invasive bacteria (Yang, Eckmann, Panja, & Kagnoff, 1997) and as a result may be an important component in the development of the host innate and adaptive immune responses. The interaction of the gut epithelium with resident microbiota also contributes to the development and the function of the mucosal barrier. Isolauri (2001) illustrated the importance of gut microflora in the intestinal defense barrier. She demonstrated in the study that the absence of intestinal microflora resulted in an increase in antigen

transport that led to increased inflammation. Inflammation is mediated by the increased production of proinflammatory tumor necrosis factor (TNF) that contributes to the recruitment and activation of immune cells, the release of cytolytic enzymes and reactive oxygen species (ROS), and the consequent exacerbation of tissue damage at sites of inflammation (Ganesan, Travis, Ahmad, & Jazrawi, 2002; Van Deventer, 1997). This is demonstrated in the rise in levels of proinflammatory cytokines including TNF at the local sites of inflammation and in the peripheral circulation of patients with Crohn's Disease (Borruel et al., 2002; Grip, Janciauskiene, & Lindgren, 2004; MacDermott, Sanderson & Reinecker, 1998; Present et al., 1999). Emerging clinical evidence is reported about the specific beneficial impacts of probiotics in the development of the mucosal barrier and the possible prevention and/or treatment of gastrointestinal inflammatory diseases. The anti-inflammatory action of *Lactobacillus reuteri* has been demonstrated in previous studies with findings supporting its ability to inhibit experimental colitis in transgenic interleukin-10- deficient mice (Madsen, Doyle, Jewell, Tavernini, & Fedorak, 1999). Furthermore, Pena et al. (2004) reported the ability of *L. reuteri* to reduce the levels of proinflammatory cytokines including TNF-alpha in mice with colitis. Probiotics involvement in managing the condition of necrotizing enterocolitis has been reported by Bin-Nun et al. (2005), Hoyos (1999), Lin et al. (2008), and Ruemmele et al. (2000). In premature infants, for instance, probiotics are thought to transiently improve the balance of colonizing bacteria that facilitate the development of mucosal immunity to prevent excessive inflammation associated with necrotising enterocolitis (NEC) (Nanthakumar, Fusunyan, Sanderson, & Walker, 2000). Khailova et al. (2009) recently provided evidence for the protection against NEC in neonatal rat

model through oral administration of *Bifidobacterium bifidum* and suggested that the protection was associated with reduction of ileal inflammation, regulation of mucus layer formation, and improvement of intestinal integrity.

Role of Probiotics in Production of Mucins and the Importance of Mucins

One important structural component that protects the mucosal surface is the mucus layer overlying the mucosal membrane. The mucus layer is a result of the interaction of various mucosal secretions including water, mucin glycoproteins, trefoil peptides, surfactant phosphates, electrolytes, and antibodies (Macfarlane, Woodmansey, & Marfarlane, 2005). Large carbohydrate-rich mucin glycoproteins form the predominant constituents of mucus (Aksoy, Thornton, Corfield, & Sheehan, 1999). The mucins are synthesized, stored, and secreted from cells on the epithelial surface of ducts and lumens and from enterocytes and goblet cells in the underlying mucosa (Aksoy, Corfield, & Sheehan, 2000). Mucins are particularly secreted in the respiratory, gastrointestinal, and genital tracts and in other accessory organs such as the pancreatic glands and gallbladder (Reid & Harris, 1998). The mucus layer provides residence and source of nutrients to the gut microflora (Derrien, Vaughan, Plugge, & Vos, 2004). It however limits the access of these microbes in the lumen from direct interaction with the epithelium through simple steric hindrance (Dai, Nanthkumar, Newberg, & Walker, 2000). These barriers inhibit epithelial cell adherence and, thus, interrupt colonization and possible invasion of pathogens. Mucus also provides hydrophobic properties to the mucosal surface and therefore prevents the delivery and influx of water soluble bacterial product and toxins (Lugea, Salas, Casalot, Gaurner, & Malagelada, 2000; Mack, Ahrne,

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Hyde, Wei, & Hollingsworth, 2003). Furthermore, the mucus layer lubricates and

physically protects the mucosa against dehydration and mechanical injuries

(Lichtenberger, 2000; Matsuo, Ota, Akamatsu, Sugiyama, & Katsuyama, 1997).

Transmembrane mucins remain anchored to the epithelial cell membranes. They

perform functions including trophic, signaling, and adhesive roles in various epithelial

cell processes (Andrianifahanana, Moniaux, & Batra, 2006). Moniaux, Escande,

Porchet, Aubert, and Batra (2001) previously reported some major roles of mucins in

growth, improvement of epithelial integrity, suppression of carcinogenesis and

metastasis, as well as in the promotion of fetal development. There are several reports

in favor of probiotics involvement in the production of various types of mucins and the

specific contributions of each mucin to gastrointestinal health. Fyderek et al. (2009)

reported that mucus constituted an integral part of the mucosal barrier and plays

important roles in hindering the penetration of the mucosa by luminal bacteria. It also

prevents the interaction between bacterial products and host cell receptors, thus

preventing the tendency to trigger inflammatory processes. This was supported by

McAuley et al. (2007) earlier report that surface mucins play the role of targets to

invading pathogens by limiting the interaction of the pathogens with the gut epithelium.

It also supported the suggestion of Gork et al. (1999) that secretory mucin glycoproteins

in the gut seemed to reduce the possibility of bacterial attachment. These findings

further supported a previous study by Pullan et al. (1994) in which they suggested that

reduction in the mucus layer thickness among adult inflammatory bowel disease (IBD)

patients caused an increased exposure of microbes to the gut immune system that led

to sustained inflammation. Mack et al. (1999) reported the role of a specific

Lactobacillus plantarum (strain 299v) to enhance the production and secretion of mucins (MUC2 and MUC3) from human intestinal (HT-29) epithelial cells. Follow-up studies on the role of secreted mucin-3 (MUC3A and MUC3B) by Mack et al. (2003) provided evidence to the effect that MUC3 seemed to inhibit the attachment of enteropathogenic *Escherichia coli* to the epithelium of the gastrointestinal tract. Similar effect of probiotic mixtures in increasing muc2 gene expression and muc2 glycoprotein secretion in rat colon was reported by Caballero-Franco, Keller, De Simone, and Chadee (2007). Therefore, during selection of probiotic strains, it is important to test and confirm that the strain does not degrade mucins. This is to avoid possible disruption of the protective mucus layer by the strains after ingestion (Fernandez, Boris, & Barbes, 2005; Zhou et al., 2001). However, the ability of potential probiotic strains to stimulate the production of mucin glycoproteins production in the gut epithelium is considered as a positive trait.

Probiotics in Improving Gut Microflora and Intestinal Health

The gastrointestinal tract of a newborn infant is devoid of the resident gut microbiota and the colonization of premature infant's gut with the characteristic microflora generally takes longer to establish. As a result of the slow colonization process, premature infants at this stage are susceptible to intestinal infections including ulceronecrotic colitis (Pinegin, Korzhunov, Ivanova, Volodin, & Goncharova, 1983). A rapid establishment of representative intestinal microflora in premature infants was demonstrated by Akiyama et al. (1994) through the administration of a *Bifidobacterium breve* strain.

Probiotics in Inhibition of Infections

Although the human gastrointestinal tract is sterile at birth, it is rapidly colonized by a dynamic mixture of microbes soon after birth (Tappenden & Deutsch, 2007). The colonization of the gut that begins promptly after birth is affected by the mode of delivery, early feeding strategies, and the hygiene and related conditions around the child (http://chp.sagepub.com/content). Hence the lumen ultimately presents a major entry route to many food and water borne infections as a result of the constant exposure of the gut epithelium to gastrointestinal content (Hansson, 2012). Despite this enormous challenge the gut is hardly overwhelmed by pathogenic infections due to the presence of effective microbial barriers (Kim & Khan, 2013). Several approaches by which the resident microbiota and probiotics impede pathogenic infection have been proposed. Lewus, Kaiser, & Montville (1991) reported the ability of lactic acid bacteria to directly inhibit the growth of pathogens through production of bacteriocins. *In vitro* studies suggested that probiotics potentially act favorably in the host through modification of the resident microbial flora to promote effective competition against pathogens for adhesion to the intestinal epithelium or compete against pathogens for necessary nutrients and growth factors. Others produce antitoxin effects and reverse some of the consequences of infection on the epithelium, such as secretory changes and neutrophil migration (Michail & Abernathy, 2002, 2003). Probiotics are also thought to inhibit infections through reduction of the local gut pH by stimulating the activities of lactic acid producing microflora (Langhendries et al., 1995). *Lactobacillus* derived probiotics for instance may influence the reduction of the pH of the microenvironment of the gut to the detriment of pathogenic microbes (Fayol-Messaoudi et al., 2005). *Lactobacillus rhamnosus GG* in

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particular secretes an antimicrobial substance distinct from lactic acid that has inhibitory

activity against other bacteria in the pH range of 3 to 5 (Silva, Jacobus, & Gorbach,

1987). This is demonstrated in the impaired growth of virulent microbes in a pH-

dependent manner in coculture experiment with *Lactobacillus* species (Fayol-

Messaoudi et al., 2005). Talarico and Dobrogosz (1999) reported that *Lactobacillus*

reuteri residing in the gut inhibited a wide spectrum of microorganisms with the

antibacterial compound reuterin. There are preliminary evidence that probiotic bacteria

may inhibit the gastric colonization and activity of *Helicobacter pylori*, which is

associated with gastritis, peptic ulcers, and gastric cancers (Kopp-Hoolihan, 2001).

Aiba, Suzuki, Kabir, Takagi, and Koga (1998) and Kabir et al. (1997) reported the ability

of *Lactobacillus salivarius* to inhibit *Helicobacter pylori* colonization in *in vitro* studies as

well as in mice. Probiotic secretions have the potential to inhibit growth and attenuate

virulence of some enteric bacterial pathogens (Sherman, Ossa, & Johnson-Henry,

2009).

Probiotics in the Prevention of Diarrhea in Infants and Adults

Diarrheal conditions are caused by pathogenic bacterial and viral overgrowth in

either the small intestines or colon. Regardless of the mechanisms leading to the

diarrheal condition, the end result is always accumulation and expulsion of fluids and

electrolytes (Bezkorovainy, 2001). The most extensively studied gastrointestinal

condition treatable by the administration of probiotics is acute infantile diarrhea (Isolauri

et al., 2002a). In patients hospitalized for acute rotavirus diarrhea, *Lactobacillus* strain

GG (ATCC 53103) as fermented milk or as freeze-dried powder significantly reduced

the duration of the diarrhea compared to a placebo group given pasteurized yogurt (Isolauri et al., 1991).   This was supported by Saxelin's (1997) report that *Lactobacillus rhamnosus GG* mediated the prevention of *Escherichia coli* enterotoxin traveler's diarrhea.

Probiotics in the Treatment of Antibiotic-associated Diarrhea

Antibiotics are microbial metabolites that can inhibit the growth of other microorganisms. Antibiotic-associated gastrointestinal disorders are well recognized and *Lactobacillus rhamnosus GG* has demonstrated effective prophylactic effect against antibiotic-associated diarrhea (Akiyama et al., 1994). Black, Einarsson, Lidbeck, Orrhage, and Nord (1991) reported the reduction in incidence and recolonization time of ampicillin-associated diarrhea through the delivery of *Bifidobacterium longum* and *Lactobacillus acidophilus* probiotic mixture. Similar effect for erythromycin-associated diarrhea through the administration of yogurt containing *Bifidobacterium longum* has also been reported by Colombel, Cortot, Neut, and Romond (1991).

Probiotics in the Activation of Mucosal Immunity

The gut associated lymphoid tissue makes the gastrointestinal tract the largest lymphoid or immune organ in the human body (Targan & Shanahan, 1994). Animal models and human studies have found that probiotic bacteria are able to enhance nonspecific and specific immune responses by activating macrophages, increasing levels of cytokines, elevating natural killer activity, and increasing levels of immunoglobulins (Sanders, 1999). Mallin, Suomalainen, Saaxelin, and Isolauri (1996)

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reported increase in secretory IgA levels through the ingestion of certain strains of

Lactobacillus. This report agreed with the findings by Arunachalam, Gill, and Chandra

(2000) in which a doubled-blind, placebo-controlled study of 12 human subjects were

found to have increased immunity due to ingestion of *Bifidobacterium lactis* strains.

Probiotics in Autoimmune Disorders and Allergies

　　Information provided by Cabana, McKean, Wong, Chao, and Caughey (2007)

highlighted the importance of infant exposure to environmental microbes for appropriate

development of the immune system. This emphasizes the new version of the "hygiene

hypothesis" that proposes reduced exposure to environmental and/or enteric stimuli,

including microbes, underlies the rising incidence of childhood atopic and autoimmune

diseases (http://chp.sagepub.com/content). Preliminary studies are pointing to the

potential for probiotics to modulate allergic reactions through improvement of the

mucosal barrier function (Naidu, Bidlack, & Clemens, 1999). The rationale in probiotics

capacity to alleviate allergy by modulating the intestinal microbiota is supported by

observations in which allergic children were found to have demonstrated a different

microbiota composition than healthy infants. Kalliomaki et al. (2001) reported that the

main changes associated with the allergic trait were less frequent colonization with

lactobacilli and lower counts of bifidobacteria.

Efficacy and Safety of Probiotic Therapy

Although it has been proposed that inactivated microbes and probiotic cell components can significantly act as biological response modifiers on various processes in tissues and organs (Adams, 2010; Isolauri et al., 2002b; Zhang, Nan, Ricardo, & Neu, 2005), probiotics are generally reported to elicit their beneficial effects when the strains were ingested alive and in sufficient quantities (FAO/WHO Joint Working Group, 2002). The maintenance of probiotic shelf life has always been an issue of concern during preparation of probiotic foods and supplements. Moreover, considerable differences exist in the bioavailability, biological activities, doses, and microfloral composition among probiotic preparations (http://journals.lww.com/jpgn 2006). These disparities raise the question of reliability in taking probiotics for their therapeutic effects. In addition to concerns regarding the efficacy of probiotics is also the issue of safety (Champagne, Roy & Roy, 2005; Philips, Kaliasapathy, & Tran, 2006; Vinderola, Prosello, Ghiberto, & Reinheimer, 2000). Snydman (2008) stated three theoretical safety concerns in taking probiotics, including the danger of probiotic sepsis, metabolic effect on the gastrointestinal tract, and possible horizontal transfer of antibiotic resistance to potential pathogenic gastrointestinal dwellers. Regardless of the several promising findings on the safety of probiotics from studies involving immune-compromised patients with HIV (Apostolou et al., 2001) and transplant populations (Rayes et al., 2005), the concerns regarding probiotic safety are still not entirely addressed. There are several reports of bacteremia and fungemia with lactobacilli and saccharomyces organisms, especially in patients with immunocompromised status or

indwelling central venous catheters (Enache-Angoulvant & Hennequin, 2005; Land et al., 2005). These concerns raised the necessity for broader investigation into not only the beneficial effects of probiotics but also the safety of the products in human health (Abe, 2010).

Motivation and Scope of Current Study

Several new findings keep trickling in about novel probiotic strains and their role in various aspects of human health. So far many different bacterial and fungal strains with probiotic activities have been reported. Nonetheless, the identification and selection of probiotic strains, their specific effects, mechanism(s) of actions, and efficacious dosage are not fully elucidated yet. Motivated by the diverse potential sources of novel probiotic strains and the interesting emerging benefits of several strains, I seek to investigate the following questions:

1. Are bacterial isolates from 'Amabere amaruranu' cultured-milk, a traditional cultured-milk preparation of the Kisii people of Southwest Kenya, stable to digestive tract conditions?

2. Do the cultured milk isolates show sensitivity to antibiotics?

3. Do the bacterial isolates show capacity to degrade mucin?

4. Do the bacterial isolates have the potential to stimulate mucin glycoproteins (MUC3 and MUC4) production in human enterocytes?

Hypothesis

Because no deleterious effects from the consumption of 'Amabere amaruranu' cultured-milk (a traditional cultured-milk preparation by the Kisii people of Southwest

Kenya) has been reported, the bacterial isolates in the preparation could be stable to digestive tract conditions and could have probiotic benefits.

Objectives

1. To isolate, identify, and determine the stability of cultured-milk bacterial isolates to digestive tract conditions.

2. To examine the isolates for antimicrobial activity, sensitivity to antibiotics, and mucin degradation activity.

3. To examine the isolates for their effect on human normal colon cells regarding the production of mucin glycoproteins (MUC3 and MUC4).

CHAPTER 2

MATERIALS AND METHODS

Experimental Materials

'Amabere amaruranu' Cultured-milk

The cultured milk was obtained from a native of Kenya in Johnson City, Tennessee. The pH of the milk was measured and isolation of bacteria from it begun immediately after it was received.

Acid Buffer

The pH of 1X Phosphate buffered saline pH7.4 (Gibco Invitrogen, Grand Island, NY 14072) was adjusted with drops of 1M HCl to pH2.0 with the aid of Ultra Basic –10 pH/mV meter (Denver Instrument, Thermo Scientific, Cat number 02-2283).

Culture Media for Milk Isolates

MRS Media: This medium so-named after its inventors; de Man, Rogosa and Sharpe; is an enriched medium that was developed in 1960 for the growth of *Lactobacillus* species. MRS agar plates were prepared by suspending 31 g of Thermo Scientific Oxoid MRS agar (Cat. number CM0361B) in 500mL of dH_2O in a sterile 1000 Pyrex bottle. The suspension was autoclaved for 20 minutes at 121 ^{o}C. The medium was then put in 55 ^{o}C water bath to cool before pouring it into sterile Petri dishes. The dishes were covered immediately to avoid contamination and allowed to solidify at room temperature. The plates were then stored at 4 ^{o}C for later use. The MRS broth was

prepared by suspending 26 g of Thermo Scientific Oxoid MRS broth (Cat. number CM0359B) in 500 mL of dH_2O in a sterile 1000 mL Pyrex bottle and autoclaving it for 20 minutes at 121 ^{o}C. The broth was cooled and stored at room temperature for later use.

M17 media: This medium is an enriched medium for isolation and study of bacteria from milk in the laboratory. It was developed in 1975 by Terzaghi, and Sardine. The M17 broth was prepared by suspending 18.63 g of Thermo Scientific Oxoid M17 broth (Cat. number CM0817B) in 500 mL of dH_2O in a sterile 1000 mL Pyrex bottle and autoclaving it for 20 minutes at 121 ^{o}C. It was cooled and stored at room temperature for later use.

M17 agar plates were made by suspending 18.63 g of Thermo Scientific Oxoid M17 broth (Cat. number CM0817B) and 7.5 g of Bacto agar in 500 mL of dH_2O in a 1000 mL Pyrex bottle and autoclaving it for 20 minutes at 121 ^{o}C. The medium was then put in 55 ^{o}C water bath to cool before pouring it into sterile Petri dishes. The dishes were covered immediately to avoid contamination and allowed to solidify at room temperature. The plates were then stored at 4 ^{o}C for later use.

Bovine Bile Conditioned Medium

Three percent bovine bile (Sigma Aldrich Co. 3060 Spruce Street, St. Louis, MO 63103 USA, Cat. number B-3883) was prepared by dissolving 1.5 g of dried bovine bile in 50 mL of 1X Phosphate buffered Saline pH7.4 (Gibco Invitrogen, Grand Island, NY14072). The suspension was filter sterilized using PVDF membrane; Millipore Millex – GV 0.22µm pore size (Fisher scientific, Cat. number SLGVR04NK). 0.5mL of the 3%

bovine bile solution was then added to 4.5 mL of M17 and MRS sterile broth to prepare

0.3% bovine bile conditioned media.

Mucinized Agarose Medium B

Point-five percent mucinized agarose medium B was prepared with partially purified

porcine gastric mucin type III (Sigma Aldrich Co. 3060 Spruce Street, St. Louis, MO

63103 USA, Cat. number M1778) by dissolving 0.5% (w/v) mucin into 1.5 % (w/v)

medium B (with composition 7.5 g Trypton, 7.5 g Casitone, 3.0 g Yeast extract, 5.0 g

Meat extract, 5.0 g NaCl, 3.0 g $KH_2PO_4.H_2O$, 0.5 g KH_2PO_4, 0.5 g $MgSO_4.7H_2O$, 0.5 g

Cysteine HCl, 0.02 g Rasarurin, 5 g mucin, 15 g agarose) with or without 0.3% D-(+)-

glucose).

Human Colon CCD 841 CoN Cell Line

Human colon CCD 841 CoN cell line was obtained from American Typed Culture

Collections® (Cat. number CRL-1790). Cells were cultured in Dulbecoco's modified

Eagle's medium (DMEM) containing 10% fetal calf serum (FCS) and 5% Penicillin-

streptomycin (Corning cellgro®, Mediatech, Inc, Manassas, VA 20109) until the cells

reached 70% confluence. The cells were passaged two times and stored in aliquots in

complete DMEM storage media in liquid nitrogen.

Cultured-milk Bacterial Extracts

Lactobacillus rhamnosus isolated and identified from 'Amabere amaruranu' cultured

milk was cultured in MRS broth in a 250 mL flask with sterile gauze stopper. The flask

was incubated at 37 °C on shaker in aerobic condition until the culture reached stationary phase of growth (OD$_{600}$ nm of 1.0). One milliliter of culture broth was transferred into a clean microfuge tube and centrifuged at 8000 rpm for 5 minutes at 4 °C. The cell pellets were washed with sterile PBS 1X pH7.4 and resuspended in 1mL PBS 1X pH7.4. The suspension was then sonicated on ice using a sonicator (Fisher Scientific Co., Toronto, ON, Canada) at 40% amplitude for 18 seconds followed by 2 minutes intervals of rest and repeated 5 times to avoid heating and denaturation of proteins. The suspension of sonicated cells was then centrifuged at 125 x g for 5 minutes and the collected supernatant was filter-sterilized using PVDF membrane; Millipore Millex – GV 0.22 μm pore size (Fisher scientific, Cat. number SLGVR04NK) and used as the sample for *L. rhamnosus* cytoplasmic fractions (BCF) for the testing effect of bacterial extracts for MUC4 and MUC3 production in normal colon cells. The bacterial cell extracts were stored at -20 °C and used in testing with the CCD 841 CoN cells at 70% confluence for effect in mucins production.

Experimental Methods

Preparation of 'Amabere amaruranu' Cultured-milk

Cultured-milk preparations are widely eaten in many communities in Kenya and other parts of the world. A sample of cultured-milk from the Kisii region in Southwest Kenya (Figure 1), (Nationsonline.org, 2014) is the source of the bacterial isolates in this study. The milk sample is called 'Amabere amaruranu' (Figure 2). Fresh milk is usually pasteurized and poured into clean gourds (Figure 2), containing a stock culture of 'Amabere amaruranu' cultured milk usually once in about 24-48 hours. The source of

the original stock culture is not known. It is believed to constitute part of the rich heritage

of the Kisii people that has been handed down from many generations and it is

preserved in the community through continuous use by households. Because the milk is

prepared this way, it of necessity contains bacteria. The composition of the bacteria in

this preparation has not been reported, but the bacterial isolates could be potentially

beneficial because no harmful effect from its consumption has ever been reported.

Figure 1. Map of Kenya showing Kisii Region in the Southwest of Kenya. nationsonline.org. (2014) Political Map of Kenya. One World - Nations Online (OWNO). Accessed May 2014: www.nationsonline.org/oneworld/map/kenya_map2.htm

Figure 2. Sample of 'Amabere amaruranu' Cultured-milk and Gourd

Isolation of Bacteria from 'Amabere amaruranu' Cultured-milk

One millimeter of a sample of 'Amabere amaruranu' cultured milk preparation was first homogenized with 9 mL of 1X sterile phosphate buffer saline (PBS) at pH 7.4. The homogenate was serially diluted by 10-fold using 1X sterile PBS at pH 7.4. Then 0.25 mL aliquots of the final dilution were inoculated on MRS agar at pH 6.2 (Thermo Fisher Scientific, Pittsburgh 15275, Cat number CM0361B; De Man, Rogosa, and Sharpe, 1960) and M17 agar at pH 6.2 (Thermo Fisher Scientific, Pittsburgh 15275, Cat number CM0785B; Terzaghi and Sandine, 1975). A set of the plates were incubated in anaerobic conditions (using a standard anaerobic jar chamber (Thermo Fisher scientific,

cat number B-260671) with the anaerobe container system sachet (Fisher scientific, cat number B-260678). A second set of plates was incubated in aerobic condition. However, both sets of plates were incubated at 37 °C for 48 h. Individual colonies on the M17, and MRS agar plates were randomly picked from representatives of all morphologically distinct colonies for five other successive subcultures on fresh plates to purify the colonies (Leisner et al., 1997). Samples of each pure isolate were stored at -80°C in a microbank^TM bacterial and fungal preservation system (Pro-Lab Diagnostic, 20 Mural St., Unit 4, Richmond Hill, OH; Fisher Scientific, Cat number 22286-154). Working cultures were kept on MRS or M17 agar slants at 4 °C and streaked every 4 weeks on agar plates (Herrero, Gonzalez, & Suarez, 1996; Samelis, Maurogenakis, & Metaxopoulos, 1994).

Selection of Bacterial Isolates by Colony Morphology

Colonies of bacterial isolates were selected on agar plates based on distinction in colony morphology. Colonies with distinction in morphology including pigmentation, elevation, margin features, and size were randomly selected and further streaked for purification. The colonies were then gram stained and the slides were examined by microscopy using a compound light microscope.

Identification of Cultured-milk Isolates by PCR

Cultured-milk isolates were identified by amplifying and comparing the 16S rRNA gene with that of known bacteria as described by Ralitsa, Sheuerman, Chakraborty, and Lampson (2012). The 16S rRNA gene is a preferred target of identification because it

possesses both highly conserved and highly variable domains. The conserved regions provide the site for amplification of the gene with universal PCR primers, while the hypervariable region facilitates the identification of the corresponding micro-organisms (Delbes & Montel, 2005). Bacterial isolates that were identified as pure were grown on MRS or M17 agar plates for 24 hours, after which, for each isolate, a single isolated colony was picked from the plate and suspended in a 1.5 mL Eppendorf tube containing 1µL of dH_2O. The cells were dispersed by forcefully spinning the loop against the bottom of the tube to produce a homogeneous suspension. This suspension was used as a DNA template to perform polymerase chain reaction (PCR). This reaction is used to isolate and amplify the 16S rRNA gene from bacterial cells. The sequence of this gene is commonly used to identify unknown bacterial genera. The following PCR reagents were mixed with the bacterial samples to undergo DNA amplification: dH_2O 22 µL, 10x PCR Buffer (Go Taq Flexi, Promega) 10 µL, Dimethyl sulfoxide (Corning cellgro) 5 µL, 25 mM $MgCl_2$ (Promega) 3 µL, 10 mM Deoxyribonucleotide Triphosphate Mix (Promega) 1 µL, 20 µM Forward Primer (63f) 1.25 µL, 20 µM Reverse Primer (1387) 1.25 µL, single bacterial colony (DNA) template 1 µL and Taq Polymerase (Go Taq Flexi, Promega) 0.5 µL.

The sequence of the forward primer, 63f, used in this reaction is 5'-CAG GCC TAA CAC ATG CAA GTC-3' and the sequence of the reverse primer, 1387r, used is 5'-GGG CGG WGT GTA CAA GGC-3' where 'W' is a code for A or T (http://www.basic.northwest.edu/biotools/oligocalc.html). Fifty microliters of this mixture of reagents was transferred into a 250 µL microfuge tube for each bacterial isolate and placed in a thermocycler in which they were exposed to the following cyclic temperature

changes: 95 °C for 3 minutes, 95 °C for 1 minute (repeated for 29 cycles), 55 °C for 1 minute, 72 °C for 2 minutes (repeated for 29 cycles), 72 °C for 5 minutes, and hold at 4 °C. The samples were then checked after completion of the thermocycle program on a 0.75% agarose gel to determine if DNA was successfully amplified by using gel electrophoresis. The amplified DNA was then purified using GeneClean Turbo kit. The purified samples were sent to the DNA Sequence Service at the University of Tennessee to obtain the whole sequence of the 16S rRNA gene using primers 63f and 1387r. The polygraph of the sequences were then processed using the program Chromas that visualizes the quality of a sequence and allow for the selection of only the best segment of the sequence to be used for identification. The best sequence was then submitted to an online database, Ribosomal Database Project (RDP) (http://rdp.cme.msu.edu) that compares it to a number of bacterial DNA sequences. The program then identifies the genus of the unknown bacterium based on sequence similarity to known 16S rRNA segments and estimates the 20 closest species matches and their percentage similarities. The identified isolates were stored at -80 °C.

Identification of Cultured-milk Isolates by API 50 System

The milk isolates were further identified to the species level by carbohydrate metabolism using the API 50 system (bioMerieux sa 69280 Marcy l'Etoile - France) Colonies on overnight anaerobic culture plates were suspended in 2 mL of distilled water to prepare a heavy suspension. Two milliliters of bacterial suspension of isolates with turbidity equivalent to number 2 McFarland standard was prepared by adding drops of the bacterial suspension to distilled water. An ampule of API 50 medium for

Staphylococcus and *Lactobacillus* was then opened and twice the number of drops that prepared the 2 McFarland suspension were transferred into it and mixed by vortexing. The reference numbers of the isolates were then recorded on the API 50 strips and the tubes filled with the inoculum. The tubes were covered with sterile mineral oil and incubated anaerobically for a period as stipulated on the manufacturer's manual. Positive and negative tests were indicated by yellow and blue colorations respectively.

Stability of Bacterial Isolates to Digestive Tract Conditions in vitro

Effect of Acid Buffer on Viability of Milk Bacterial Isolates

The test in this study was done in vitro in pH2 1M HCl buffer over a period of 3 hours. This was to determine if the cells will survive in the acidic stomach condition because gastric content is reported to measure at pH of about 1.0 to 2.0 (Antione, 2011), with transit period of about 3 to 4 hours (Smith & Morton, 2010). Five milliliters of a 24-hour MRS or M17 broth cultures were centrifuged at 65 x g for 5 minutes. Cell pellets were washed with sterile 1X PBS at pH7.4 and resuspended in 5 mL of 1X PBS.

The suspension of washed cells was divided into aliquots of 1 mL in microfuge tubes and centrifuged at 65 x g for 5 minutes. The supernatant was decanted and the pellet was resuspended in 1 mL of 1X PBS at pH2.0 and mixed by vortexing for 30 seconds. The suspension was then incubated at 37 °C in the oxygen condition similar to which the isolate was originally isolated. One microfuge tube containing 1 mL aliquot of acidified cell suspension was taken at 5 s, 1 h, 2 h, and 3 h intervals and centrifuged at 65 x g for 5 minutes. The cell pellet was washed with 1 mL 1X PBS at pH7.4. The

acidified cell pellet was then resuspended in 1mL of 1X PBS at pH7.4 and serially diluted with 1X PBS at pH7.4 by 6-fold. Then 250 µL of the final dilution was inoculated on agar plates of the medium on which the isolate was grown and incubated at 37 ᵒC for 24 hours to examine effect of low pH on viable cell count.

Effect of Acid Buffer and/or Bovine Bile on Growth of Milk Isolates

This test was conducted in vitro with surviving cells after incubation in pH2 buffer for 3 hours. Cells in 1 mL aliquots of a 3-hour preacidified suspension were spun at 65 x g for 5 minutes and the pellets were then washed once with 1X PBS at pH7.4 and then resuspended in 5 mL of fresh MRS or M17 broths that had been conditioned with or without 0.3% dried unfractionated bile bovine (Sigma Aldrich Co. St. Louis, MO 63103 USA, Cat number B-3883). The broth cultures were then incubated in conditions that were similar to those under which cells were isolated. Cells in similar volumes of untreated culture broths were also washed with 1X PBS at pH7.4 and resuspended in fresh broth as control or in 0.3% bile bovine conditioned broth to test the effect of bile only on their growth. One milliliter of incubating culture of each test was drawn at 0, 1, 3, 6, and 9 hour intervals for determining the optical densities of the culture broth at 600 nm wave lengths with Gene Sys-10uv spectrophotometer (Thermo Electron corporation, Madison, WI 53711, USA) (Gilliland & Walker, 1990; Toit et al., 1998).

Mucin Degradation Activity on Agarose Petri Dish

Mucin provides protection to the epithelium and therefore its degradation is undesirable of probiotic bacteria. Failure to degrade mucin is considered a prerequisite

for selection of probiotics strains (Zhou et al., 2001). The capacity of the isolates to degrade mucin was assayed with partially purified porcine gastric mucin type III (Sigma Aldrich Co. 3060 Spruce Street, St. Louis, MO 63103 USA, Sigma Cat. # M1778). The medium was prepared by adding 0.5% (w/v) mucin to 1.5 % (w/v) medium B (7.5 g Trypton, 7.5 g Casitone, 3.0 g Yeast extract, 5.0 g Meat extract, 5.0 g NaCl, 3.0 g $KH_2PO_4.H_2O$, 0.5 g KH_2PO_4, 0.5 g $MgSO_4.7H_2O$, 0.5 g Cysteine HCl, 0.02 g Rasarurin, 0.5% mucin, 1.5% agarose and with or without 0.3% D-(+)- glucose). Five microliters of a 24-hour viable bacterial culture was inoculated on agarose medium in a petri dish and incubated at 37 °C anaerobically for 72 hours. The plates were then stained with 1% Amido black 10 B (Fair Lawn, New Jersey 07410) in 3.5 M acetic acid for 30 minutes. The plates were then decolorized with 1.2 M acetic acid for 1 minute for the mucin lysis zone (discolored halo) around the colony of positive test and positive control (fecal flora) to appear. Negative control used in this study was *Lactobacillus casei* (ATCC collection, Manassas, VA 20110 USA). The mucin degradation activity was defined as the size of the mucinolytic zone around the colony (Zhou et al., 2001).

Antimicrobial Activity of Cultured-milk Isolates

The capacity of the strains to inhibit growth of representative intestinal pathogens was determined. The culture broths of bacterial isolates at the exponential phase (OD_{600} of 0.5) were pelleted at 10,000 g for 30 minutes at 4 °C. The supernatant fluid was filtered using PVDF membrane; Millipore Millex –GV 0.22µm pore size (Fisher scientific, Cat number SLGVR04NK) to remove any remaining bacterial cells in it. Bacterial test strains including *Escherichia coli, Klebsialla pneumonia*, *Enterococcus*

faecalis, Pseudomonas aeruginosa, and *Enterobacter cloacae* (ATCC collection,

Manassas, VA 20110 USA) were then cultured overnight in Thermo Scientific LB broth (

Fisher Scientific, Cat number BP 9722-500) and samples were inoculated on Thermo

scientific LB agar (Fisher Scientific, Cat R453632) plates using sterile cotton buds. A

sterile paper disc with diameter 8 mm was infused with 50 µL of the filter-sterilized spent

broth of each milk isolate and placed on the surface of agar plates that had been

inoculated with test strains. A disc infused with 50µl of fresh sterile broth was used as

control. The plates were then incubated at 37 °C in aerobic condition for 48 hours and

inspected for zones of inhibition of growth of test strains around the disc. The diameter

of the halo around the disc was measured in millimeters as the amount of antimicrobial

activity of the bacterial isolates.

Sensitivity of Cultured Milk isolates to Antibiotics

The sensitivity of isolates to antibiotics was determined on MRS or M17 agar

plates using eight different antibiotic discs in a sensi-disc dispenser (BBL, Cockeysville,

Maryland 21030, USA). Antibiotics assayed included ampicillin (10 µg), bacitracin (10

IU/IE/UI), chloramphenicol (30 µg), erythromycin (15 µg), kanamycin (30 µg), penicillin

(10 IU/IE/UI), streptomycin (30 µg), and tetracycline (30 µg). The averages of two

readings of the zone of inhibition of growth around the disc were measured in

millimeters as the antibiotic sensitivity of isolates (Delgado, O'Sullivan, Fitzgerald, &

Mayo, 2007). Bacterial strains that harbor antibiotic resistance plasmids are considered

unsuitable for use as human or animal probiotics (Morelli & Wright, 1997; Saarela,

Mogensen, Fonden, Matto, & Mattila-Sandholm, 2000; Salminen et al., 1998).

Effect of Bacterial Extracts on Mucin-3 and Mucin-4 Production in CCD 841 CoN Cells

Human Colon CCD 841CoN Cell Culture

Human colon 841 CoN cell line was purchased from the American Typed Culture Collections and grown in DMEM complete medium with 10% FCS and 5% antibiotics (100 U/mL penicillin G, 100 mg/mL streptomycin sulfate). The culture was seeded in a biological safety II cabinet in sterile T75 flasks and incubated at 37 °C in a humidified atmosphere with 5% CO_2. Culture media were changed after every 72 h until cells reached 70% confluence. The cells were then detached and harvested following trypsinization with 3mL of 1X trypsin-EDTA (Gibco, Grand Island, NY) at 37 °C in about 4 minutes. Fresh complete DMEM medium was added to the detached cells to deactivate the effect of trypsin-EDTA. The cell suspension was then transferred into a 50 mL centrifuge tube and centrifuged at 125 x g for 5 minutes. Cell pellets were rinsed with PBS 1X pH7.4 and resuspended in 5 mL DMEM storage medium containing 40% FCS and 10% DMSO. Cells were counted with haemocytometer and dispensed into aliquots in 1 mL microfuge tubes for storage in liquid nitrogen.

Treatment of Human Colon CCD 841 CoN Cells with Bacterial Extracts

Human colon CCD 841 CoN cells were seeded at cell density of $1X10^5$ per well T75 flask containing 10 mL of complete DMEM media and allowed for 8 days to grow to about 70% confluence. The media were aspirated and the cells were treated with complete DMEM media containing either 10% vehicle (1X PBS pH7.4) or 10% bacterial extracts; (bacterial cytoplasmic fractions (BCF), bacterial cytoplasmic fractions and

culture spent broth mixture (BCM), bacterial spent broth (BSB); for 24 h at 37 °C in 5%

CO_2. The cells were trypsin harvested following microcentrifugation at 125x g for 8

minutes and 4 °C and washed twice with 1X PBS7.4 and used for extraction of proteins.

Human Colon CCD 841 CoN Whole Cell Extract

To prepare the colon whole cell extract, the treated cells were detached by

trypsinization and transferred into microfuge tube and washed twice with 1X PBS pH7.4.

The cells were then suspended in a 50 mL 5% protease inhibitor cocktail lysis buffer

(Calbiochem, EMD Chemicals, Inc. San Diego, CA 92121, Cat. number 539134) and

incubated on ice for 3 h to lyse the cells. The protein concentration of the lysed whole

cell extract determined using BCA^{TM} protein assay kit (Pierce, Lot number HG104261).

The concentration of all the protein extracts were standardized to 2.0 µg/µL and stored

at -20°C.

Western Blot Analysis

Aliquots (15 µg) of equal quantities of homogenized protein extracts were

solubilized in equal volumes of loading buffer (3.55 mL of deionized water, 1.25 mL of

0.5 M Tris-HCl, pH6.8, 2.5 glycerol, 2 mL 10% (w/v) SDS, 0.2 mL 0.5% (w/v)

bromophenol blue, for the stock sample and 50 µL of 2- beta mercaptoethanol added to

950 µL of stock sample to prepare loading buffer each time ready to use it). The

proteins were then loaded into 4 – 20% Mini-protean® TGC^{TM} gel (Bio-Rad, Cat

number 456-1096) and separated by sodium dodecyl sulfate -polyacrylamide gel

electrophoresis in 1X Tris-glycine SDS-PAGE running buffer (30.0 g tris base, 144 g

glycine, 10 g sodium dodecyl sulfate, in 1 liter of double distilled water) using Mini

Protean® Tetra cell electrophoresis tank (Bio-Rad laboratories, Inc., Cat number 165-

8035) and power was supplied from Max Power 100VA (Fisher Scientific, 2555 Keeper

Boulevard Dubuque, Iowa 52001). After 1 h of electrophoresis at 200mV, 40mA, the

protein on the gel was transferred onto immobilon transfer membrane, pore size 0.45

μm (Millipore Corporation, Billerica, MA 01821, Cat. number IPVH00010) in 1X western

blot transfer buffer (30.0 g tris base, 144 g glycine in 1 liter of double distilled water).

The membrane was removed from the assembly and quenched with 5% non-fat dry milk

in 1X Tris-buffered saline tween-20 pH7.4 (88 g NaCl, 2 g KCl, 30 g tris base, 5 mL

tween-20 in 1 liter of double distilled water with pH adjusted to 7.4). The membrane was

then incubated with primary antibodies against Muc-3 (rabbit anti-muc3, Jackson

Immunoresearch lab; www.jacksonimmuno.com) and Muc4 (mouse anti-muc4, Jackson

Immunoresearch laboratory; www.jacksonimmuno.com) at 4 °C on shaker for 6 h. The

membrane was washed three times for 15 minutes each with 1X TBST (10 mM Tris-Cl,

pH 7.4, 150 mM NaCl, 0.05% Tween-20) and immunoblotted with corresponding

secondary antibodies. Donkey antirabbit IgG conjugate was incubate with Muc3 and

donkey antimouse IgG conjugate was used for Muc4. The membranes were incubated

on shaker for 1h at room temperature. The membrane were further washed three times

for 15 minutes each with 1x TBST and prepared for film development. A 1:1 ratio of

Supersignal West Femto reagents were pipetted into microfuge tube and mixed by

hand. The mixture was added to the immobilon transfer membrane on a transparency

and covered by another transparency and then exposed to a radiogragh

(chemiluminescent signals) to develop using Proteinsimple Flour chem M (3040

Oakmead Village Drive, Santa Clara, CA 95051) (Mattar et al., 2002).

CHAPTER 3

RESULTS

Identification of Cultured-milk Isolates by PCR

Fourteen isolates were identified from the PCR sequences of the 16S rRNA gene. They included 4 *Lactobacillus sp.*, 4 *Bacillus sp.*, 4 *Staphylococcus sp.*, and 2 *Acetobacter sp* (Table 1).

Table 1

Identification of Cultured-milk Isolates by PCR

Plate Identification Number	Suggested name for bacterial isolate	Percentage similarity	Number of unique oligos submitted for query
MRS-4AN	*Lactobacillus rhamnosus* 1[a]	0.998	1054
MRS-6AN	*Lactobacillus rhamnosus* 2	0.994	993
M17-3AN	*Lactobacillus paracasei*	0.994	993
M17-4AN	*Lactobacillus rhamnosus* 3	0.935	553
M17-1AE	*Bacillus pumilus* 1	1.0	1135
M17-2AE	*Bacillus pumilus* 2	1.0	1098
M17-3AE	*Bacillus safensis* 1	0.998	986
M17-4AE	*Bacillus safensis* 2	1.0	1201
M17-1AN	*Staphylococcus caprae*	0.996	956
M17-2AN	Uncultured bacterium [b]	0.994	1197
MRS-4AE	*Staphylococcus epidermidis* 1	0.995	1002
MRS-5AE	*Staphylococcus epidermidis* 2	1.0	1193
MRS-1AE	*Acetobacter tropicalis* 1	1.0	986
MRS-2AE	*Acetobacter tropicalis* 2	0.993	993

Cultured milk isolates identified from PCR sequence match for 16S rRNA gene. [a] Numbers represent Isolates that differ in colony morphology but were suggested to have the same genus or species identity. [b] The isolate was identified to the genus level as *Staphylococcus* but no name was suggested for it by the system.

Identification of Cultured-milk Isolates by API 50 System

Eight isolates were identified from the API assay. They included 2 *Lactobacillus rhamnnosus,* 2 *Lactobacillus paracasei*, 1 *Staphylococcus aureus*, 1*Staphylococcus sciuri,* and 2 *Staphylococcus epidermidis* (Table 2).

Table 2

Identification of Cultured-milk Isolates by API System

Plate identification number	Suggested name for bacterial isolate [a]	Percentage similarity
MRS-4AN	*Lactobacillus rhamnosus* 1[b]	99.6
MRS-6AN	*Lactobacillus rhamnosus* 2	99.8
M17-3AN	*Lactobacillus paracasei* 1	99.3
M17-4AN	*Lactobacillus paracasei* 2	95.7
M17-1AN	*Staphylococcus aureus*	100
M17-2AN	*Staphylococcus sciuri*	99.8
MRS-4AE	*Staphylococcus epidermidis* 1	99.7
MRS-5AE	*Staphylococcus epidermidis* 2	99.6

[a] Only *Lactobacillus* and *Staphylococcus* isolates were identified by the API assay.

[b] Isolates differ in colony morphology but were suggested to have the same species identity.

pH of Cultured-milk Isolates Broth Culture

When the pH of broth cultures of the bacterial isolates was measured at 12 h and 24 h after incubation, the pH of the *Lactobacillus rhamnosus* isolates was in the range

of 3.9 to 4.2. *Lactobacillus paracasei* isolates maintain a slightly acidic pH around

pH6.7 to 6.9. *Bacillus spp* maintained almost neutral pH condition of about pH7.0 to

7.1. *Staphylococcus aureus* maintained slightly acidic pH of ranging 6.7 to 6.8.

Staphylococcus sciuri maintained its culture pH around 6.8 to 6.9. *Staphylococcus*

epidermidis 1 had its pH ranging from 4.9 to 5.9. *Staphylococcus epidermidis* 2 had its

pH ranging from 5.7 to 5.9 and the *Acetobacter* isolates had their pH ranging from 5.6 to

6.2 (Table 3).

Table 3

pH of Broth Culture of Bacterial Isolates

Bacterial isolates [a]	pH of broth culture after 12 h incubation	pH of broth culture after 24 h incubation
Lactobacillus rhamnosus 1	4.2	3.9
Lactobacillus rhamnosus 2	4.2	4.0
Lactobacillus paracasei 1	6.8	6.9
Lactobacillus paracasei 2	6.7	6.9
Bacillus pumilus 1	7.1	[b]
Bacillus pumilus 2	7.0	7.1
Bacillus safensis 1	7.1	7.1

Table 3 (continued)

Bacillus safensis 2	7.0	7.1
Staphylococcus aureus	6.8	6.7
Staphylococcus sciuri	6.8	6.9
Staphylococcus epidermidis 1	5.9	4.9
Staphylococcus epidermidis 2	5.9	5.7
Acetobacter sp 1	6.2	5.8
Acetobacter sp 2	6.2	5.6

[a] Two milliliters of broth culture was taken for measurement of pH at each time.

[b] pH of the isolate was not measured at 24 h because the flask containing the culture got broken.

Effect of Acid Buffer on Viability of Bacterial Isolates

The viability of *Lactobacillus, Staphylococcus*, and *Acetobacter* isolates greatly diminished when the cultures were exposed to pH2 acid buffer and grown on Petri dish. *Bacillus* isolates were stable when the cells were exposed to pH2 acid buffer. Viability was examined by inoculating sample of the treated culture at 1 hour interval on Petri dishes. The plates were incubated for 24h and the colonies that form on the plates were counted as viable colony forming units (cfu). All colonies that seemed to have overlapped in space were counted as one colony (Table 4).

Table 4

Effect of Acid Buffer on Viability of Bacterial Isolates

Bacterial isolates	Viability of cells (x10^6 cfu/mL)			
	0 h	1h	2 h	3 h
Lactobacillus rhamnosus 1[a]	478	13	12	0
Lactobacillus rhamnosus 2	598	16	10	0
Lactobacillus paracasei 1	786	2	0	0
Lactobacillus paracasei 2	688	6	0	0
Bacillus pumilus 1	978	914	918	908
Bacillus pumilus 2	721	691	701	614
Bacillus safensis 1	488	394	379	408
Bacillus safensis 2	892	789	778	698
Staphylococcus aureus	456	38	0	0
Staphylococcus sciuri	374	12	0	0
Staphylococcus epidermidis 1	152	14	8	0
Staphylococcus epidermidis 2	116	20	11	0
Acetobacter sp 1	764	0	0	0
Acetobacter sp 2	144	0	0	0

[a] Isolates differ in colony morphology but were suggested to have the same species identity.

Effect of Acid Buffer and/or Bovine Bile on Growth of <u>Lactobacillus</u> Isolates

The growth of *Lactobacillus paracasei* isolate 1 that had been exposed to pH2 acid buffer for 3h then grown in fresh media declined greatly (Figure 3). The growth of the isolate also declined when it was exposed to pH2 acid buffer for 3 h and then grown in 0.3% bile-conditioned media. However, when the untreated culture of *Lactobacillus paracasei* isolate 1 was grown in 0.3% bile-conditioned media, the growth of the isolate gently increased through the 9 h period.

Similarly, the growth of *Lactobacillus paracasei* isolate 2 that had been exposed to pH2 acid buffer for 3 h and then grown in 0.3% bile-conditioned media declined through the 9 h period (Figure 4). The growth of the isolate remained static when the cells were exposed to pH2 acid buffer for 3 h and then grown in fresh broth. However, when the untreated *Lactobacillus paracasei* isolate 2 was grown in 0.3% bile-conditioned media, the growth of the isolate increased gently through the 9 h period.

The growth of *Lactobacillus rhamnosus* isolate 1 and 2 that had been exposed to pH2 acid buffer for 3 h and then grown in fresh media remained static (Figure 5 and 6 respectively). However, the growths of the isolates declined when they were exposed to pH2 acid buffer for 3 h and then grown in 0.3% bile-conditioned media. When the untreated *Lactobacillus rhamnosus* isolates 1 and 2 were grown in 0.3% bile-conditioned media, their growths remained static through the 9 h period. However, the growth of untreated control *Lactobacillus paracasei* and *Lactobacillus rhamnosus* isolates increased rapidly when the isolates were cultured in fresh broth.

Figure 3. Effect of Acid Buffer and/or Bovine Bile on Growth of *L. paracasei* Isolate 1.
Bacteria were exposed to different treatments (pH2 acid buffer then untreated broth;
pH2 acid buffer then 0.3% bovine bile conditioned broth; 0.3% bovine bile conditioned
broth; unconditioned broth for control) and grown for 9 h in conditions similar to which
the isolate had been isolated.

Figure 4. Effect of Acid Buffer and/or Bovine Bile on Growth of *Lactobacillus paracasei*

Isolate 2. Bacteria were exposed to different treatments (pH2 acid buffer then untreated

broth; pH2 acid buffer then 0.3% bovine bile conditioned broth; 0.3% bovine bile

conditioned broth; unconditioned broth for control) and grown for 9 h in conditions

similar to which the isolate had been isolated.

Figure 5. Effect of Acid buffer and/or Bovine Bile on Growth of *Lactobacillus rhamnosus*

Isolate 1. Bacteria were exposed to different treatments (pH2 acid buffer then untreated

broth; pH2 acid buffer then 0.3% bovine bile conditioned broth; 0.3% bovine bile

conditioned broth; unconditioned broth for control) and grown for 9 h in conditions

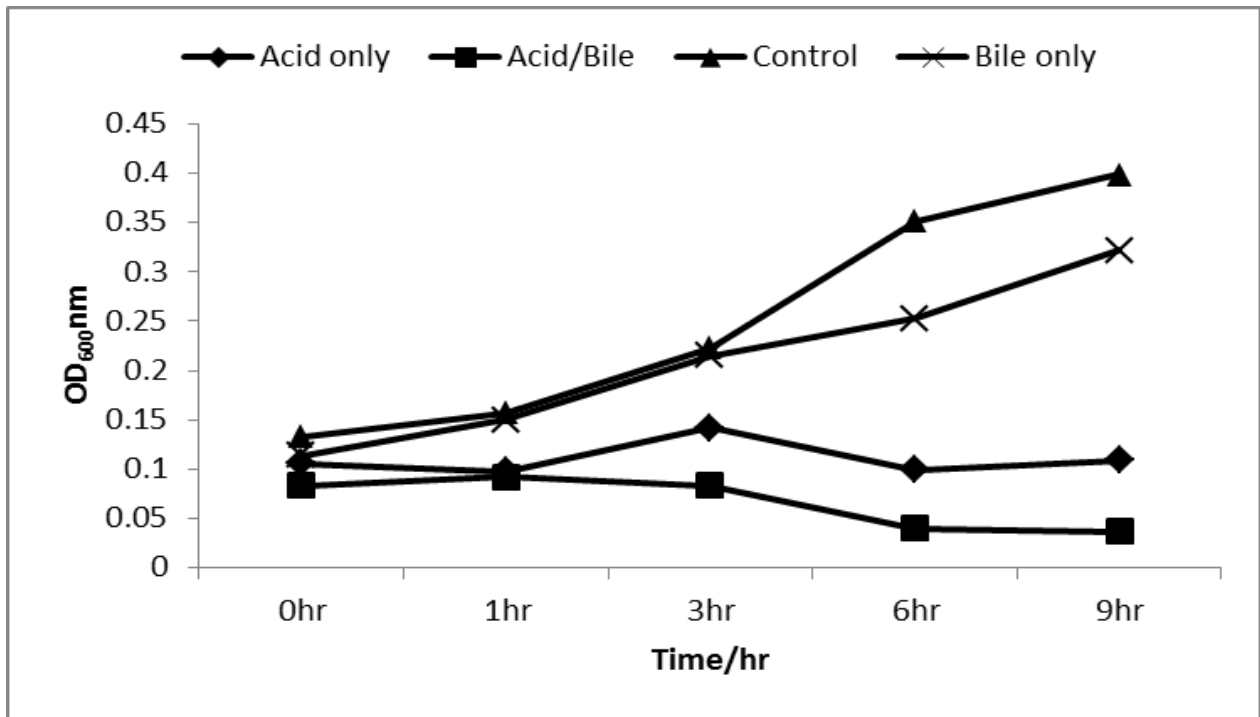similar to which the isolate had been isolated.

Figure 6. Effect of Acid Buffer and/or Bovine Bile on Growth of *Lactobacillus rhamnosus*

Isolate 2. Bacteria were exposed to different treatments (pH2 acid buffer then untreated

broth; pH2 acid buffer then 0.3% bile conditioned broth; 0.3% bile conditioned broth;

unconditioned broth for control) and grown for 9h in conditions similar to which the

isolate had been isolated.

Effect of Acid Buffer and/or Bovine Bile on Growth of <u>Bacillus</u> Isolates

The growth of *Bacillus pumilus* isolate 1 that had been exposed to pH2 acid buffer

for 3 h and then grown in 0.3% bovine bile-conditioned media declined rapidly in the first

1 h and increased gently through the next 8 h period (Figure 7). When the isolate was

exposed to pH2 acid buffer for 3h and then grown in fresh broth, its growth increased

gently through the 9h period. The growth of untreated *Bacillus pumilus* isolate 1

declined gently in the first 1 h and increased gently through the next 8 h when the isolate was grown in 0.3% bovine bile-conditioned media.

The growth of *Bacillus pumilus* isolate 2 that had been exposed to pH2 acid buffer for 3 h and then grown in 0.3% bovine bile-conditioned media declined gently in the first 1 h and then increased gently through the next 8 h period (Figure 8). When the isolate was exposed to pH2 acid buffer for 3 h and then grown in fresh broth, its growth increased gently through the 9 h period. However, the growth of untreated *Bacillus pumilus* isolate 2 declined gently in the first 1 h and increased gently through the next 8 h period when the isolate was grown in 0.3% bovine bile-conditioned media.

The growth of *Bacillus safensis* isolate 1 that had been exposed to pH2 acid buffer for 3 h and then grown in 0.3% bovine bile-conditioned media remained static through the 9 h period (Figure 9). When the isolate was exposed to pH2 acid buffer for 3h and then grown in fresh broth, its growth increased gently in the first 1 h and declined gently through the next 8 h period. The growth of the untreated *Bacillus safensis* isolate 1 remained static when it was grown in 0.3% bovine bile-conditioned media.

The growth of *Bacillus safensis* isolate 2 that had been exposed to pH2 acid buffer for 3 h and then grown in 0.3% bovine bile-conditioned media decreased gently in the first 1 h and then remained static through the next 2 h and begun to increase gently through the next 6 h period (Figure 10). When the isolate was exposed to pH2 acid buffer for 3 h and then grown in fresh broth, the growth of the isolate increased gently through the 9 h period. The growth of the untreated *Bacillus safensis* isolate 2 also

decreased gently in the first 1 h and begun to increase in the following hours when it was grown in 0.3% bovine bile-conditioned media. The growth of untreated control *Bacillus pumilus* and *Bacillus safensis* isolates increased rapidly when the isolates were cultured in fresh media.
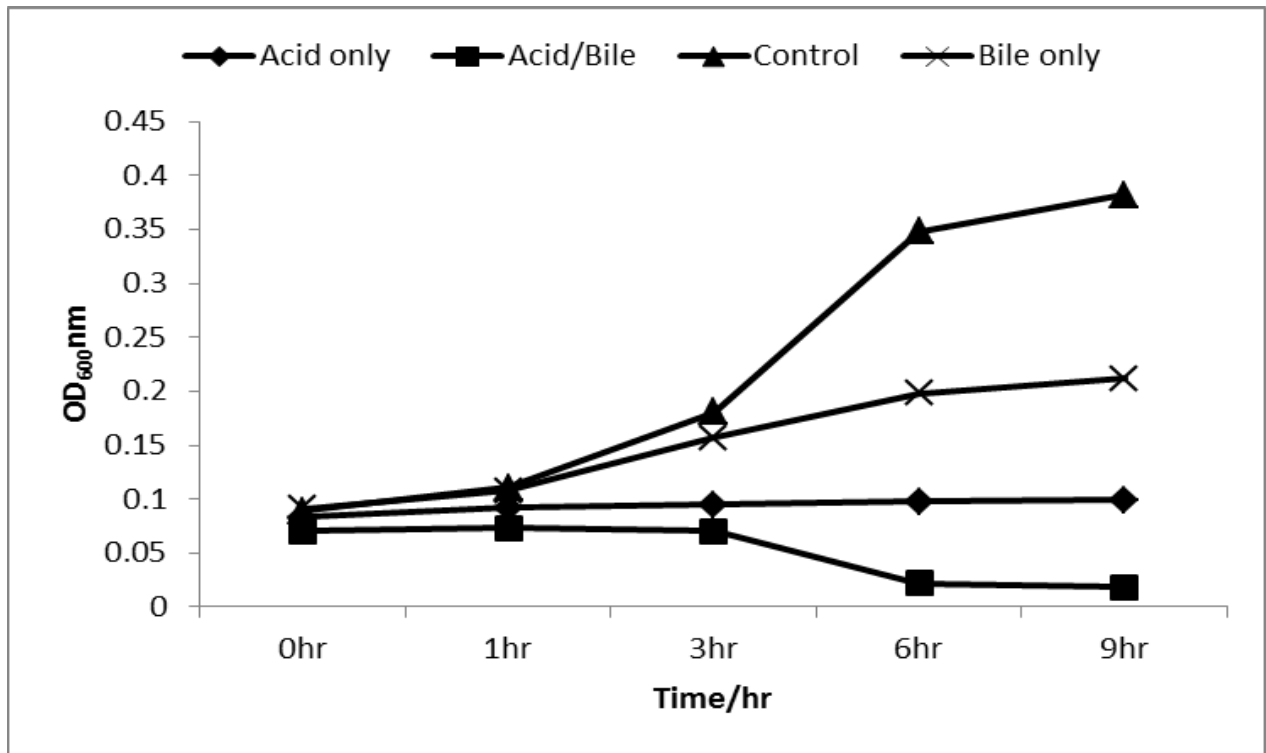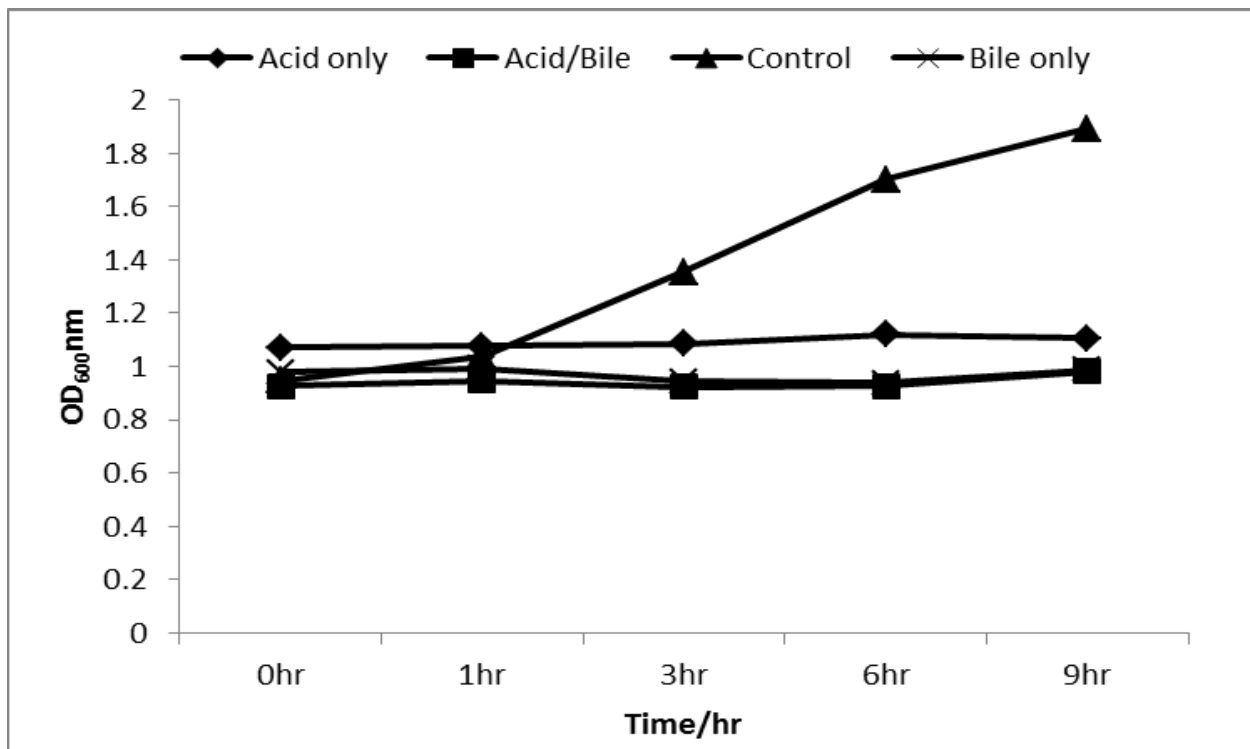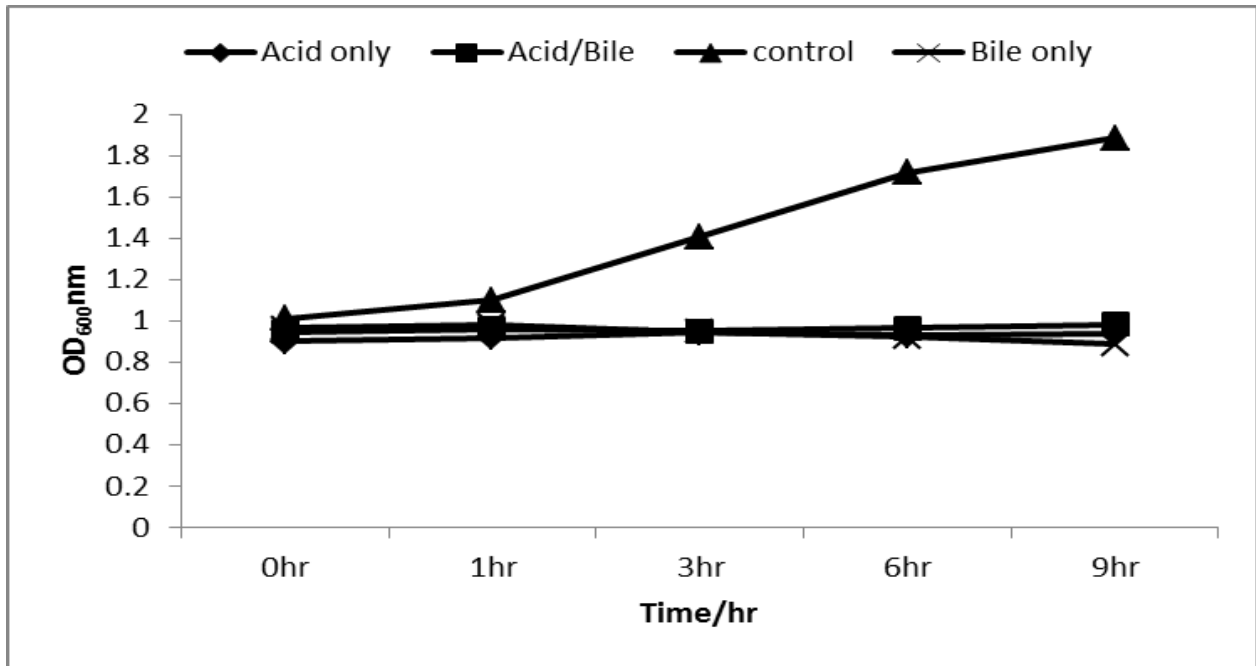

Figure 7. Effect of Acid Buffer and/or Bovine Bile on Growth of *Bacillus pumilus* Isolate 1. Bacteria were exposed to different treatments (pH2 acid buffer then untreated broth; pH2 acid buffer then 0.3% bovine bile conditioned broth; 0.3% bovine bile conditioned broth; unconditioned broth for control) and grown for 9h in conditions similar to which the isolate had been isolated.

Figure 8. Effect of Acid Buffer and/or Bovine Bile on Growth of *Bacillus pumilus* Isolate

2. Bacteria were exposed to different treatments (pH2 acid buffer then untreated broth;

pH2 acid buffer then 0.3% bovine bile conditioned broth; 0.3% bovine bile conditioned

broth; unconditioned broth for control) and grown for 9 h in conditions similar to which

the isolate had been isolated.

Figure 9. Effect of Bile acid Buffer and/or Bovine Bile on Growth of *Bacillus safensis*

Isolates 1. Bacteria were exposed to different treatments (pH2 acid buffer then

untreated broth; pH2 acid buffer then 0.3% bovine bile conditioned broth; 0.3% bovine

bile conditioned broth; unconditioned broth for control) and grown for 9 h in conditions

similar to which the isolate had been isolated.

Figure10. Effect of Acid Buffer and/or Bovine Bile on Growth of *Bacillus safensis* Isolate

2. Bacteria were exposed to different treatments (pH2 acid buffer then untreated broth;

pH2 acid buffer then 0.3% bovine bile conditioned broth; 0.3% bovine bile conditioned

broth; unconditioned broth for control) and grown for 9 h in conditions similar to which

the isolate had been isolated.

Effect of Bcid Buffer and/or Bovine Bile on Growth of Staphylococcus Isolates

The growth of *Staphylococcus aureus* that had been exposed to pH2 acid buffer for

3 h and then grown in 0.3% bovine bile-conditioned media remained static over the first

3 h and decreased gently through the next 6 h period (Figure 11). When the isolate was

exposed to pH2 acidic buffer for 3 h and then grown in fresh broth, the growth remained static through the 9 h period. The growth of untreated *Staphylococcus aureus* increased rapidly when the isolate was cultured in 0.3% bovine bile-conditioned media.

The growth of *Staphylococcus sciuri* isolate that had been exposed to pH2 acid buffer for 3 h and then cultured in 0.3% bovine bile-conditioned media remained static for the first 3 h and begun to decrease gently through the next 6 h period (Figure 12). When the *Staphylococcus sciuri* isolate was exposed to pH2 acid buffer for 3 h and then cultured in fresh broth, its growth remained static through the 9 h period whereas the growth of its untreated culture increased rapidly when it was grown in 0.3% bovine bile-conditioned media.

The growth of *Staphylococcus epidermidis* isolates 1 and 2 that had been exposed to pH2 acid buffer for 3 h then cultured in 0.3% bile-conditioned media increased rapidly through the 9 h period (Figure 13 and 14 respectively). When the isolates were exposed to pH2 acidic buffer for 3h and then cultured in fresh broth, their growth also increased rapidly through the 9-h period and the growth of untreated *Staphylococcus epidermidis* isolate 1 and 2 also increased rapidly when they were grown in 0.3% bile-conditioned media. The growths of untreated control *Staphylococcus aureus*, *Staphylococcus sciuri* and *Staphylococcus epidermidis* isolates 1 and 2 all increased rapidly when the isolates were cultured in fresh media.
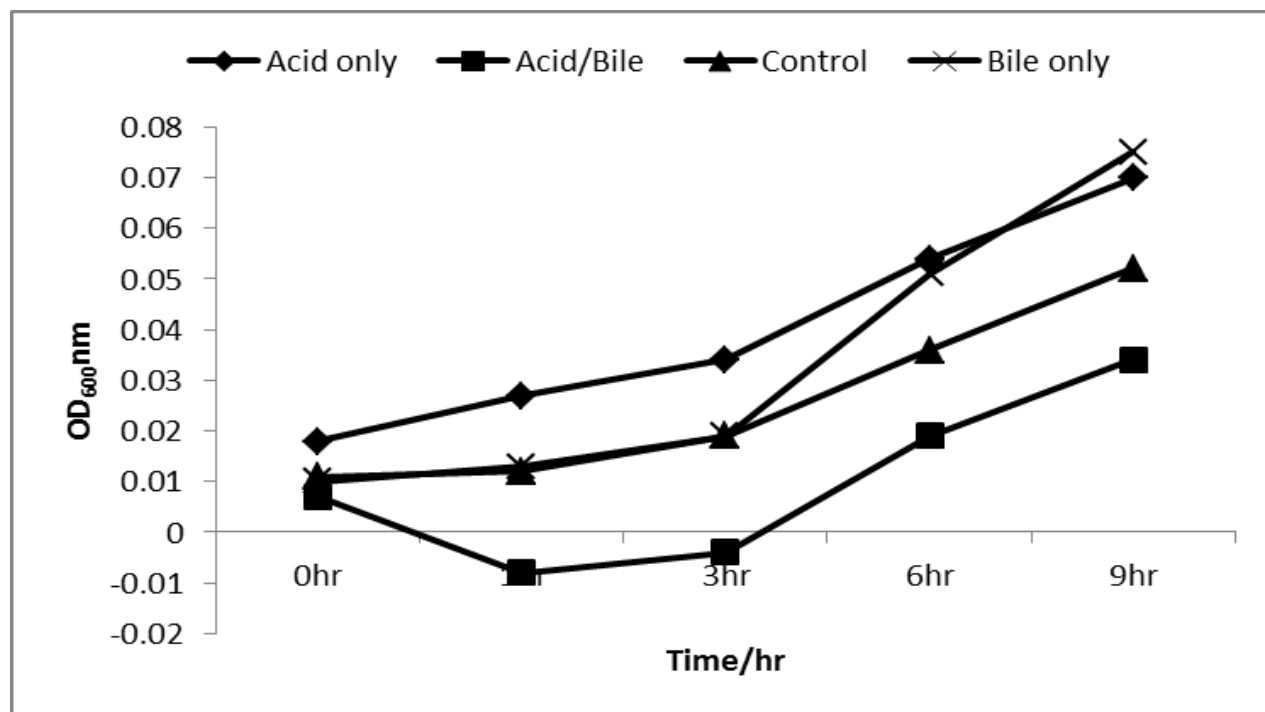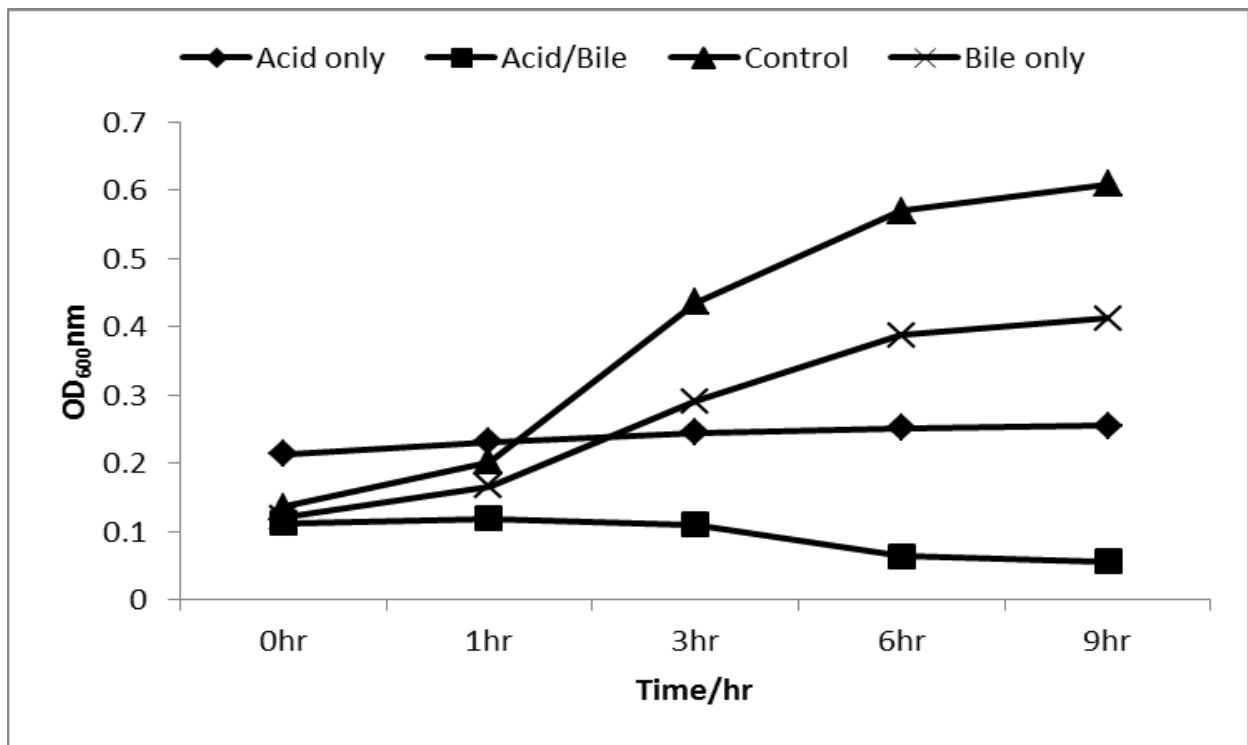
Figure 11. Effect of Acid Buffer and/or Bovine Bile on Growth of *Staphylococcus aureus*.

Bacteria were exposed to different treatments (pH2 acid buffer then untreated broth;

pH2 acid buffer then 0.3% bovine bile conditioned broth; 0.3% bovine bile conditioned

broth; unconditioned broth for control) and grown for 9 h in conditions similar to which
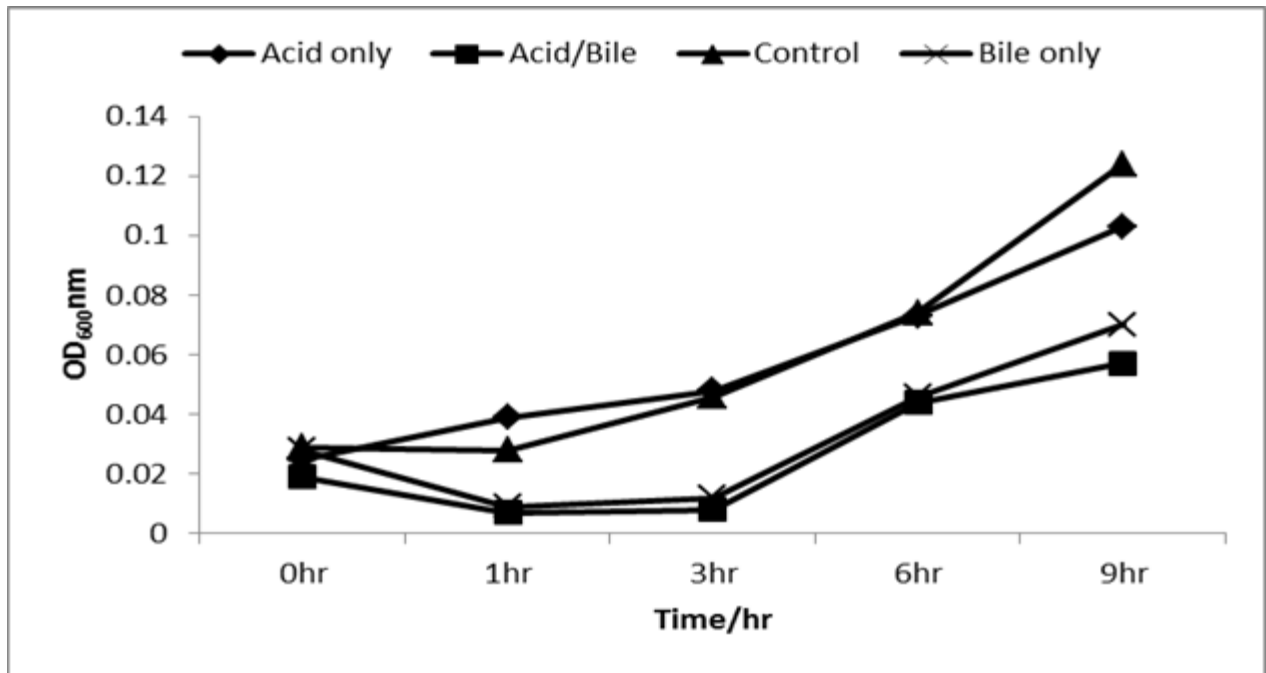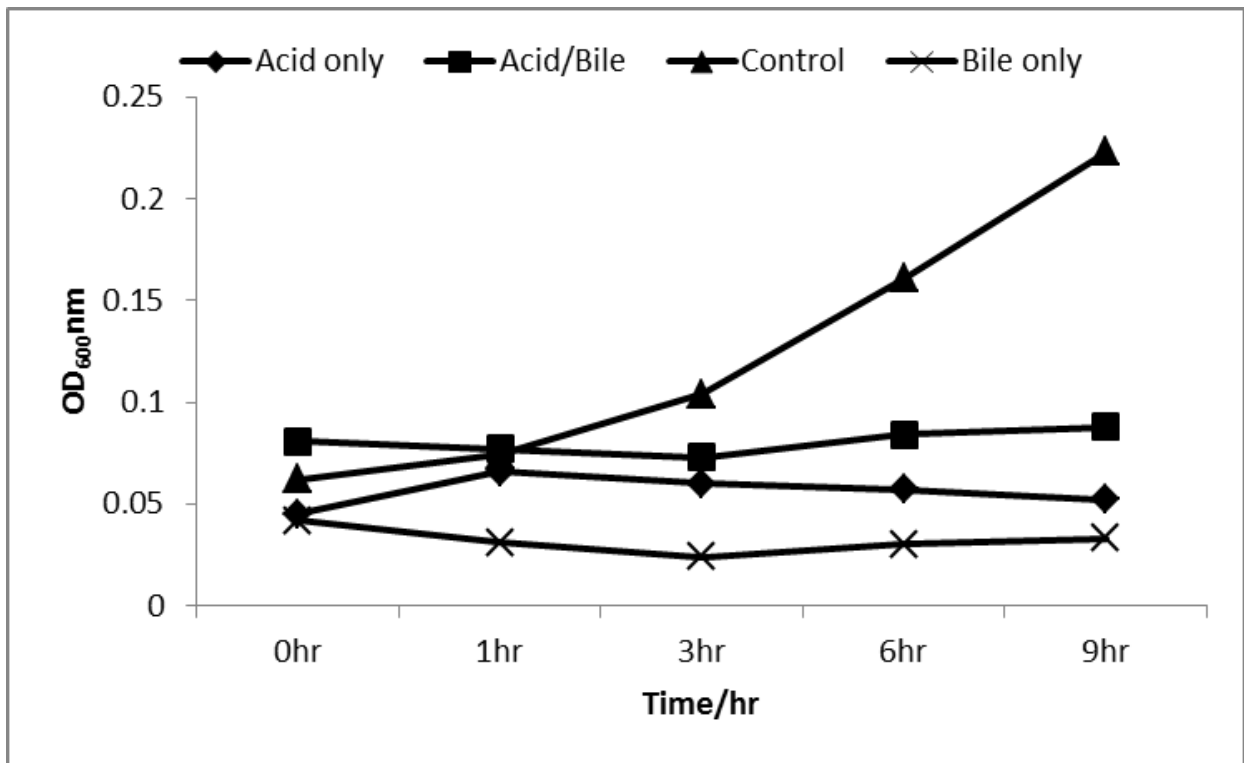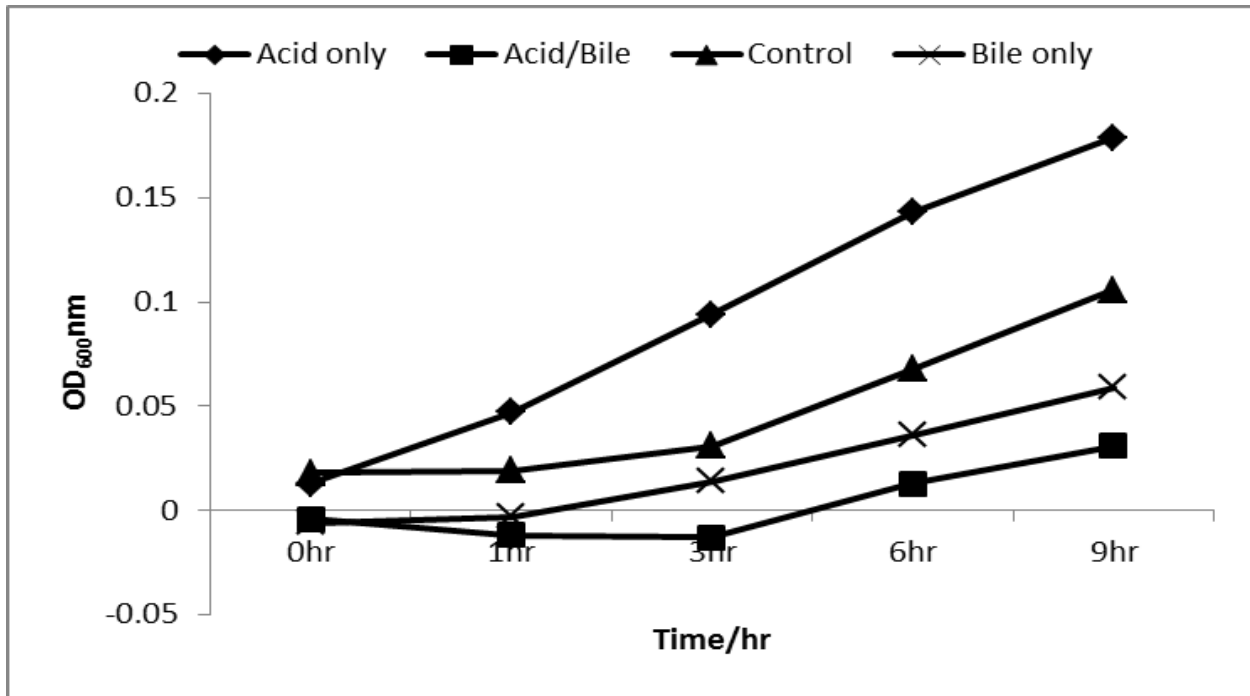
the isolate had been isolated.
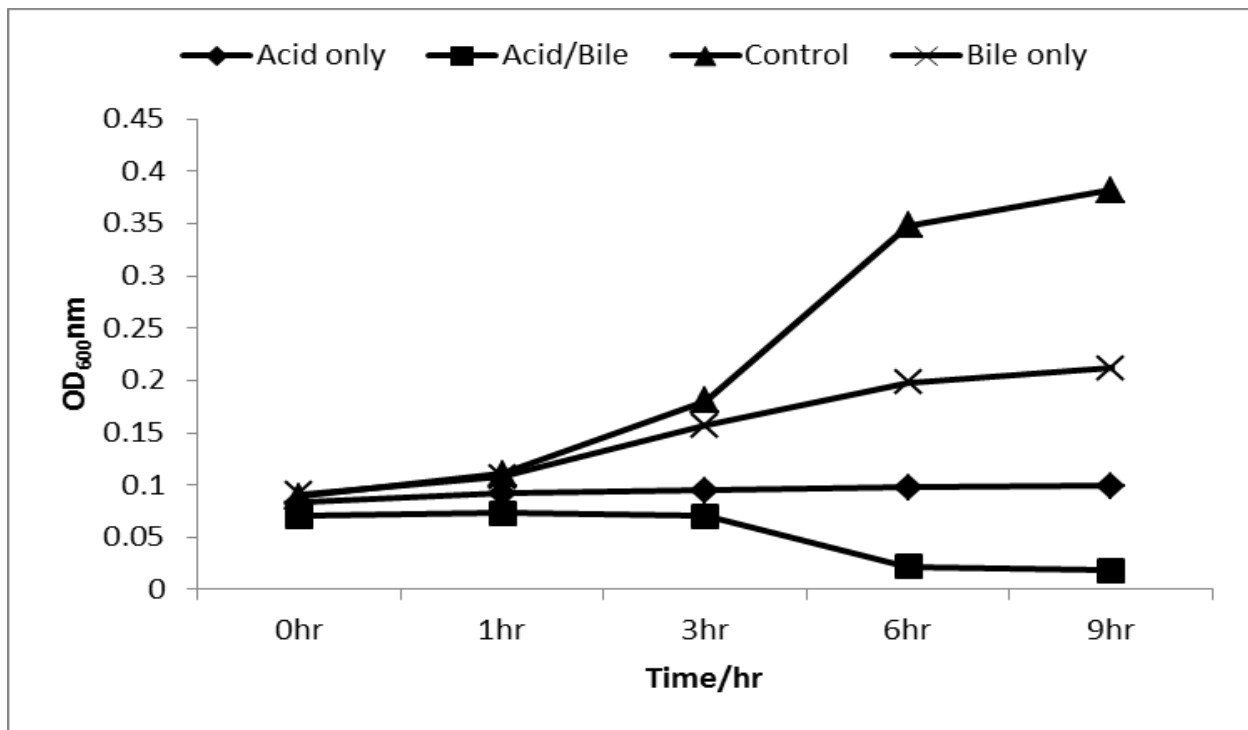
Figure 12. Effect of Acid Buffer and/or Bovine Bile on Growth of *Staphylococcus sciuri*.

Bacteria were exposed to different treatments (pH2 acid buffer then untreated broth;

pH2 acid buffer then 0.3% bovine bile conditioned broth; 0.3% bovine bile conditioned

broth; unconditioned broth for control) and grown for 9 h in conditions similar to which

the isolate had been isolated.
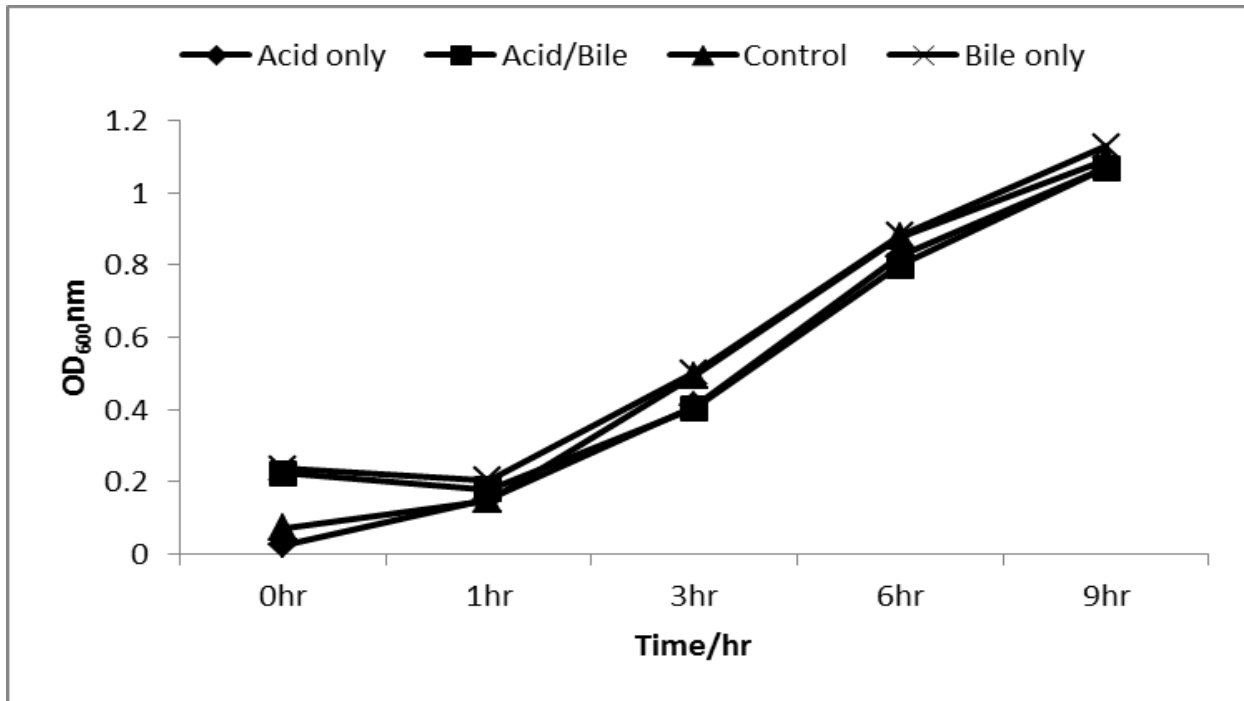
Figure 13. Effect of Acid Buffer and/or Bovine bile on Browth of *Staphylococcus epidermidis* Isolate 1. Bacteria were exposed to different treatments (pH2 acid buffer then untreated broth; pH2 acid buffer then 0.3% bovine bile conditioned broth; 0.3% bovine bile conditioned broth; unconditioned broth for control) and grown for 9 h in conditions similar to which the isolate had been isolated.
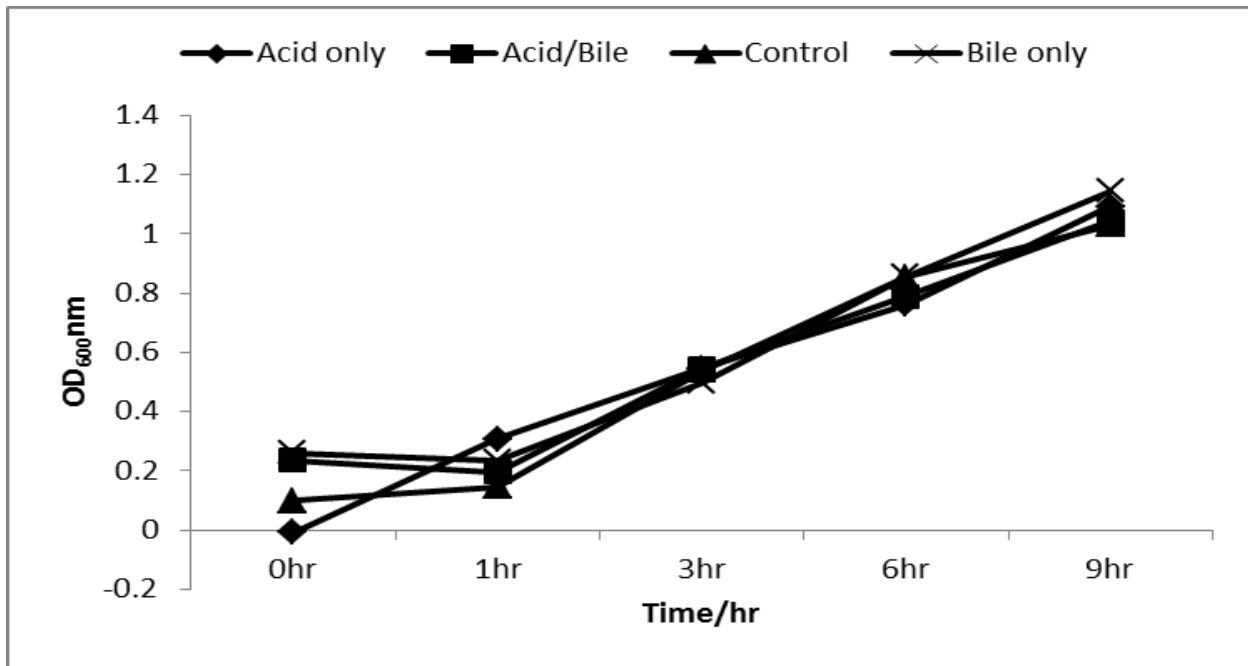
Figure 14. Effect of Acid Buffer and/or Bovine Bile on Growth of *Staphylococcus*

epidermidis Isolate 2. Bacteria were exposed to different treatments (pH2 acid buffer

then untreated broth; pH2 acid buffer then 0.3% bovine bile conditioned broth; 0.3%

bovine bile conditioned broth; unconditioned broth for control) and grown for 9 h in

conditions similar to which the isolate had been isolated.

Viability of <u>Lactobacillus</u> Isolates After a 9 h Period of Exposure to Acid Buffer and/or

Bovine Bile

    Lactobacillus isolates were further examined for viability after exposing the cells

to pH2 acid buffer and/or 0.3% bovine bile over a 9 h period. Cultures of the different

treatments were heavily inoculated on four quadrants of agar plates using sterile cotton

swabs and incubated in conditions similar to those under which the isolates had been

isolated. The identities of the quadrants were: culture exposed to PBS pH7.4 and grown

in fresh broth for control; culture exposed to PBS pH7.4 and grown in 0.3% bovine bile conditioned broth; culture exposed to pH2 acid buffer and grown in fresh broth and culture exposed to pH2 acid buffer and then grown in 0.3% bovine bile conditioned broth. The *Lactobacillus paracasei* isolates that had been exposed to PBS pH7.4 and cultured in 0.3% bovine bile conditioned broth maintained their viability. However, when the isolates were exposed to pH2 acid buffer and then cultured in fresh broth or 0.3% bovine bile conditioned broth, their viabilities diminished greatly. Similarly the viability of *Lactobacillus rhamnosus* isolates that had been exposed to pH2 acid buffer and cultured in 0.3% bovine bile conditioned broth diminished greatly. The viability of untreated *Lactobacillus rhamnosus* isolates that had been cultured in 0.3% bovine bile conditioned media also diminished greatly (Figure 15).

Figure 15. Viability of *Lactobacillus* Isolates After a 9 h Period of Exposure to Acid

Buffer and/or Bovine Bile. PBS is control.

Viability of <u>Acetobacter</u> Isolates After a 9 h Period of Exposure to Acid Buffer and/or

Bovine Bile

Acetobacter isolates were further examined for viability after the exposure of the

cells to pH2 acidic buffer and/or 0.3% bovine bile over a 9 h period. Cultures of the

different treatments were heavily inoculated on four quadrants of agar plates using

sterile cotton swabs and incubated in conditions similar to those under which the

isolates had been isolated. The identities of the quadrants were: culture exposed to PBS

pH7.4 and grown in fresh broth for control; culture exposed to PBS pH7.4 and grown in

0.3% bovine bile conditioned broth; culture exposed to pH2 acid buffer and grown in

fresh broth and culture exposed to pH2 acid buffer then grown in 0.3% bovine bile

conditioned broth. The *Acetobacter* isolates that had been exposed to PBS pH7.4 and

grown in 0.3% bovine bile conditioned broth maintained their viability. When

Acetobacter isolates were exposed to pH2 acid buffer and then cultured in fresh broth,

the viability of cells completely diminished. When exposed cells were culture in 0.3%

bovine bile conditioned broth, the viability of the cells also diminished greatly (Figure

16).
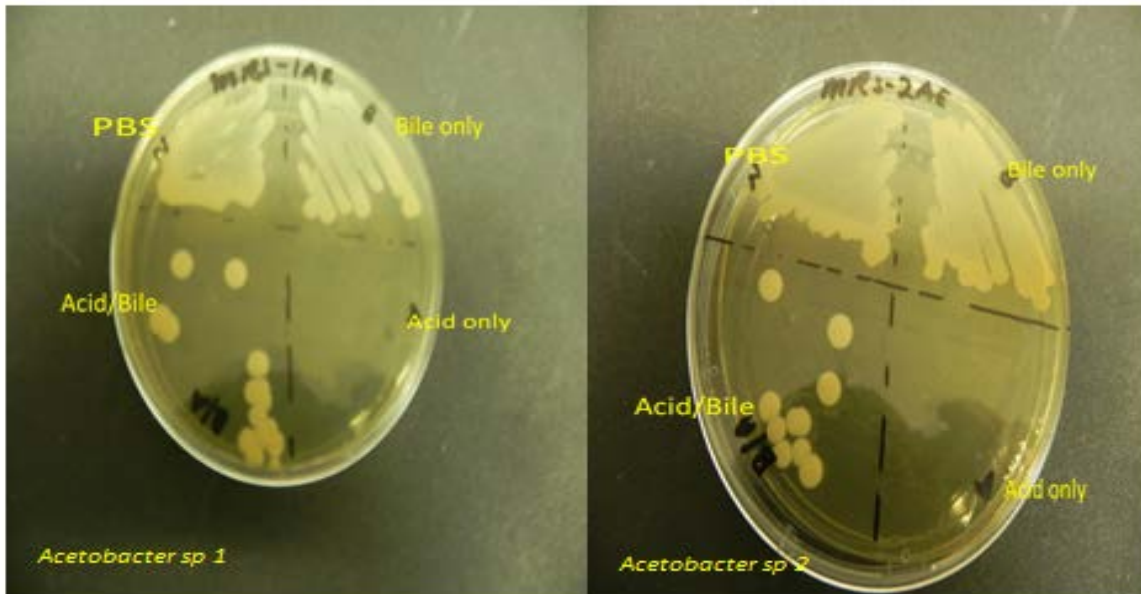
Figure 16. Viability of *Acetobacter* Isolates After a 9 h Period of Exposure to Acid Buffer and/or Bovine Bile. PBS is control.

Viability of <u>Bacillus</u> Isolates After a 9 h Period of Exposure to pH2 Acid Buffer and/or Bovine Bile

Bacillus isolates were further examined for viability after exposing the cells to pH2 acid buffer and/or 0.3% bovine bile over a 9 h period. Cultures of the different treatments were heavily inoculated on four quadrants of agar plates using sterile cotton swabs and incubated in conditions similar to those under which the isolates had been isolated. The identities of the quadrants were: culture exposed to PBS pH7.4 and grown in fresh broth for control; culture exposed to PBS pH7.4 and grown in 0.3% bovine bile conditioned broth; culture exposed to pH2 acid buffer and grown in fresh broth and

culture exposed to pH2 acid buffer then grown in 0.3% bovine bile conditioned broth.

Bacillus pumilus and *Bacillus safensis* isolates that had been exposed to PBS pH7.4

and cultured in 0.3% bovine bile conditioned broth maintained their viability. The

isolates also maintained their viability when the cells were exposed to pH2 acid buffer

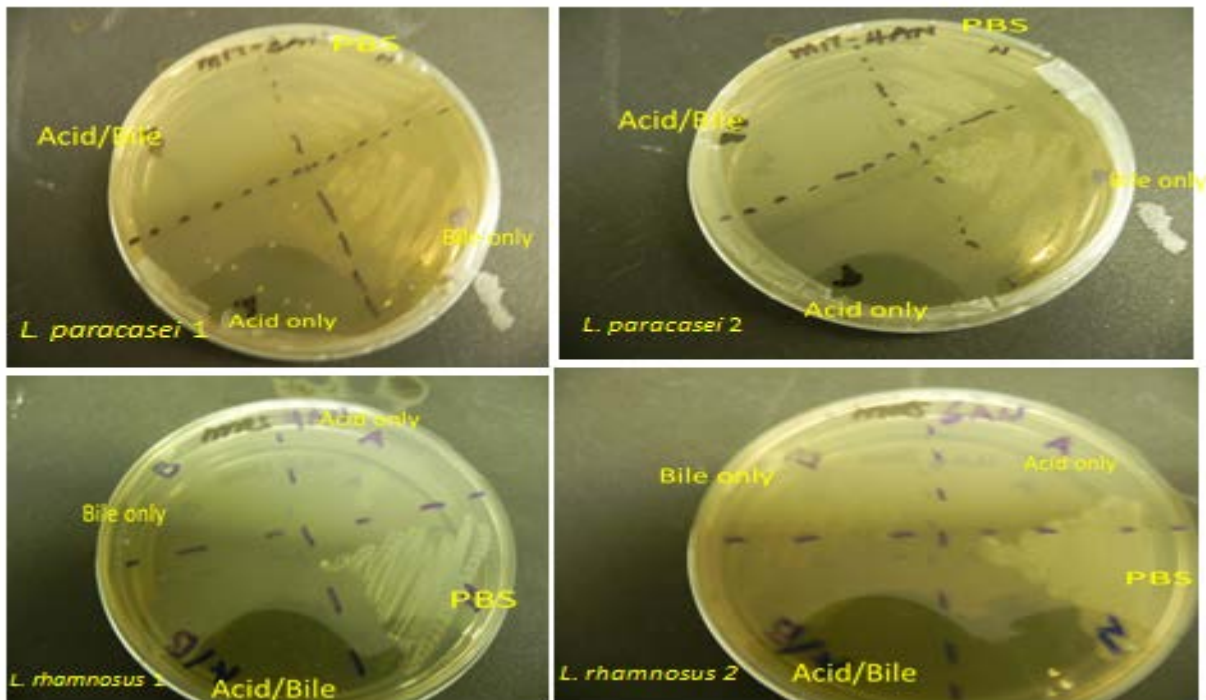and then cultured in fresh broth or 0.3% bovine bile conditioned broth (Figure 17).
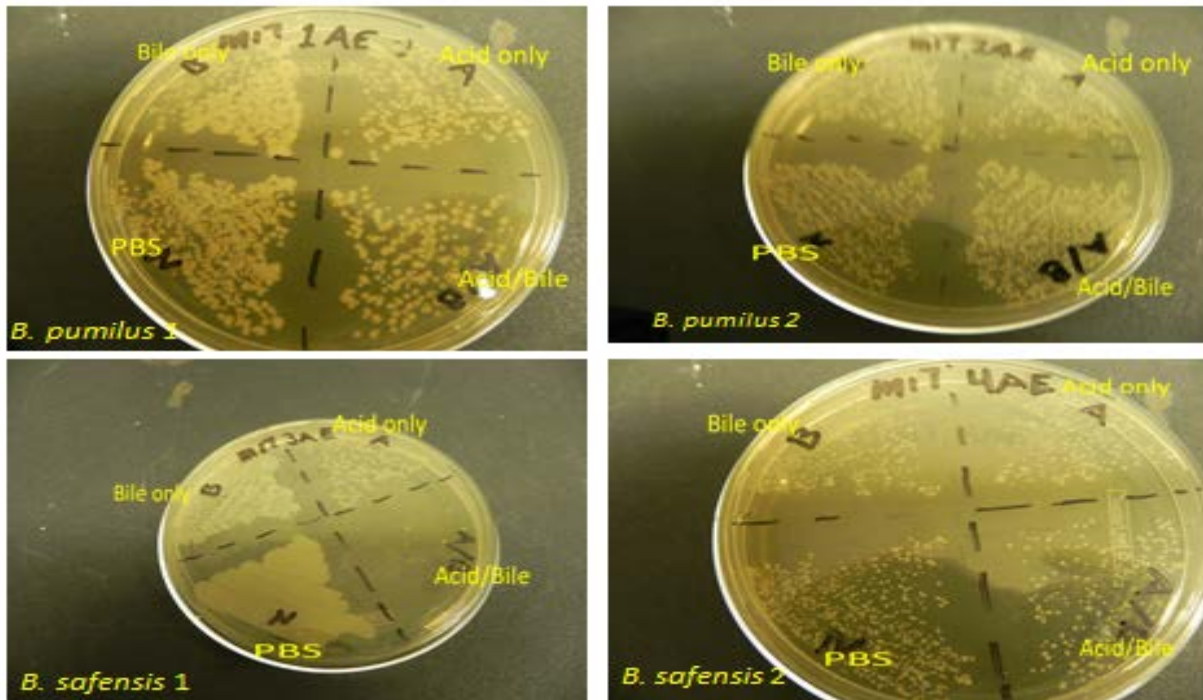
Figure 17. Viability of *Bacillus* Isolates After a 9 h Period of Exposure to Acid Buffer

and/or Bovine Bile. PBS is control.

Viability of Staphylococcus Isolates After a 9 h Period of Exposure to Acid Buffer and/or

Bovine Bile

The viability of *Staphylococcus* isolates were further examined after exposing the

cells to pH2 acid buffer and/or 0.3% bovine bile over a 9-h period. Cultures of the

different treatments were heavily inoculated on four quadrants of agar plates using sterile cotton swabs and incubated in conditions similar to those under which the isolates had been isolated. The identities of the quadrants were: culture exposed to PBS pH7.4 and grown in fresh broth for control; culture exposed to PBS pH7.4 and grown in 0.3% bovine bile conditioned broth; culture exposed to pH2 acid buffer and grown in fresh broth and culture exposed to pH2 acid buffer then grown in 0.3% bovine bile conditioned broth. The *Staphylococcus epidermidis* isolates had been exposed to PBS pH7.4 and grown in 0.3% bovine bile conditioned broth maintained their viability. The isolates also maintained their viability when the cells were exposed to pH2 acid buffer and then cultured in fresh broth or 0.3% bovine bile conditioned broth (Figure 18).

The viability of *Staphylococcus aureus* and *Staphylococcus sciuri* isolates remained stable when their cells were exposed to PBS pH7.4 and cultured in 0.3% bovine bile but greatly diminished when the cells were exposed to pH2 acid buffer and then cultured in fresh broth or in 0.3% bovine bile conditioned broth (Figure 18).
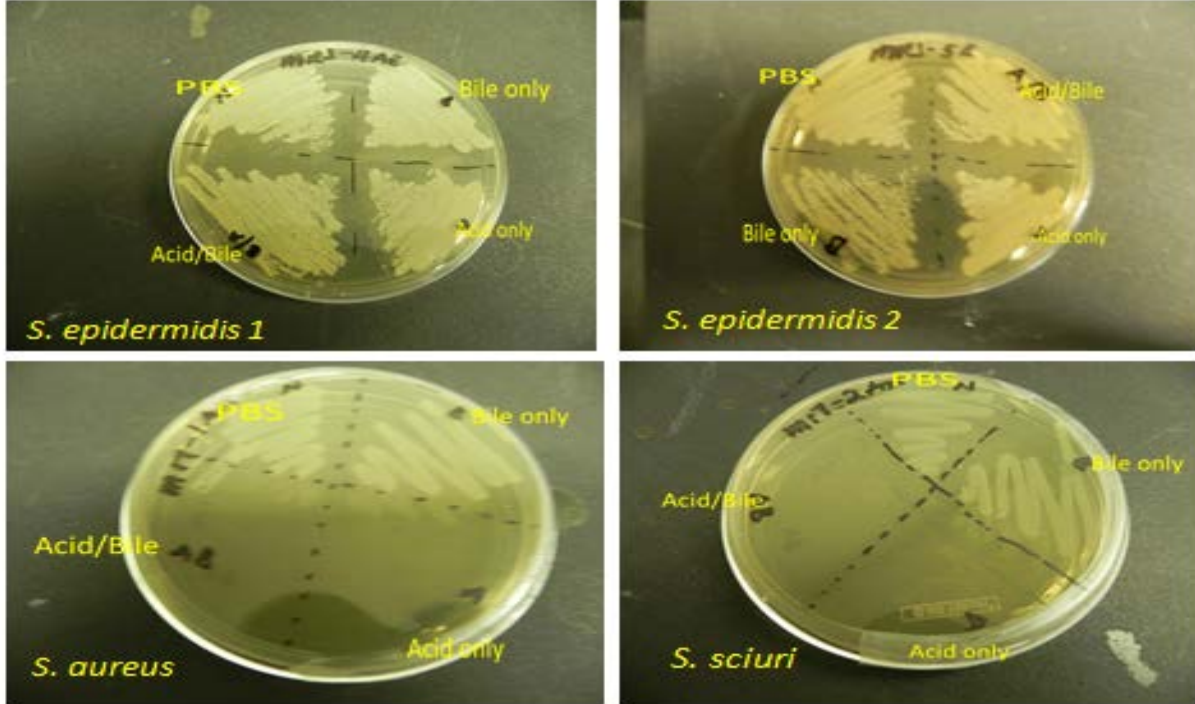
Figure 18. Viability of *Staphylococcus* Isolates After a 9 h Period of Exposure to Acid

Buffer and/or Bovine Bile. PBS is control.

Mucin Degradation Assay on Agarose Petri Dish

When 5µL of a 24-hour culture of milk isolates were spotted on agarose medium

B containing 0.5% partially purified mucin from porcine stomach and without glucose,

only *Staphylococcus sciuri* isolate degraded the mucin. A clear zone around the colony

after staining the agarose plate with 1% Amido black 10 B and washing with 2.5 M

acetic acid for 1 minute was considered positive for mucin degradation activity (Figure

19). The amount of mucin degraded was measured in terms of the size of the clear

zone around the colony of positive test in millimeters. The zone of mucin degradation

measured was14 mm for *Staphylococcus sciuri* isolate compared to 13 mm for the fecal

flora positive control.
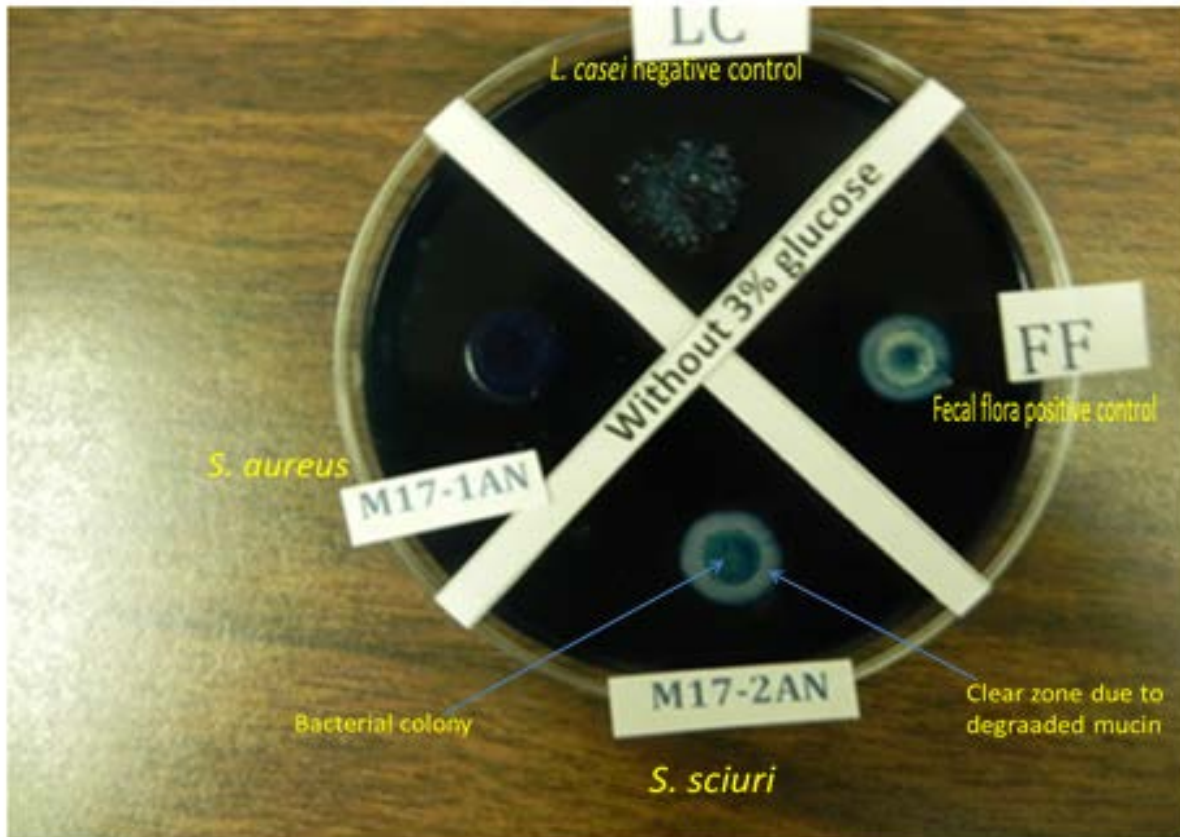
Figure 19. Mucin Degradation Activity on Agarose Medium B Petri Dish. Clear zone

around colonies were considered positive for mucin degradation.

Antimicrobial Activity of Filter-sterilized Spent-broth

Lactobacillus rhamnosus isolate 1 inhibited the growth of *Escherichia coli*,

Enterococcus faecalis, and *Psuedomonas aeruginosa* bacterial test strains. The

Lactobacillus rhamnosus isolate 2 inhibited the growth of *Enterococcus faecalis* and

Pseudomonas aeruginosa. *Lactobacillus paracasei* isolate 1 did not inhibit the growth of any of the indicator strains and the *Lactobacillus paracasei* isolate 2 inhibited the growth of *Escherichia coli* and *Pseudomonas aeruginosa*. *Acetobacter* isolates presented antimicrobial activity against only *Enterococcus faecalis*. All *Bacillus spp* isolates did not inhibit the growth of any of the bacterial indicator strains. *Staphylococcus epidermidis* isolate 1 inhibited the growth of *Enterococcus faecalis* and *Pseudomonas aeruginosa.* However, the zone of inhibition produced by the *Staphylococcus epidermidis* isolate 1 appeared as a hazy zone around the disc indicating that some of the cells of the indicator strains were alive. The *Staphylococcus epidermidis* isolate 2 did not show sign of inhibiting the growth of any of the indicator strains. *Staphylococcus sciuri* isolate inhibited the growth of *Escherichia coli* and *Staphylococcus aureus* isolate did not inhibit the growth of any of the indicator strains (Table 5).

Table 5

Antimicrobial Activity of Filter-sterilized Spent-broth of Isolate

Milk isolates	Antimicrobial activity of milk isolates against bacterial test strains measured as diameter of zone of inhibition in millimeters [a]				
	E. coli	*E. cloacae*	*E. faecalis*	*K. pneumonia*	*P. aeruginosa*
Acetobacter 1	0	0	12	0	10[c]
Acetobacter 2	0	0	10	0	10
Staphylococcus aureus	0	0	0	9	0
Staphylococcus sciuri	11	10	0	9	0
*Staphylococcus epidermidis*1	9	0	14	0	18[b]
*Staphylococcus epidermidis*2	0	0	0	0	0
Bacillus pumillus 1	0	0	0	0	0
Bacillus pumillus 2	0	0	0	0	0
Bacillus safensis 1	0	0	0	0	0
Bacillus safensis 2	0	0	0	0	0
Lactobacillus rhamnosus 1	12	9	14	9	14
Lactobacillus rhamnosus 2	10	9	12	9	12
Lactobacillus paracasei 1	0	0	0	10	10
Lactobacillus paracasei 2	12	10	10	0	12

Sensitivity of Cultured-milk Isolates to Antibiotics

Lactobacillus rhamnosus, Staphylococcus aureus and Bacillus safensis isolates showed susceptibility to all the eight antibiotics tested. *Lactobactocillus paracasei* showed sensitivity to only kanamycin, bacitracin and streptomycin. *Bacillus pumilus* and *Staphylococcus epidermidis* demonstrated resistance to all the eight antibiotics tested. *Acetobacter sp* showed susceptibility to only ampicillin, kanamycin, and streptomycin (Table 6).

Table 6

Sensitivity of Cultured-milk Isolates to Antibiotics

Milk isolates	Sensitivity of milk isolates to antibiotics measures as zone of inhibition of isolates around antibiotic infused disc in millimeters [a][b]							
	A	B	C	E	K	P	S	T
Acetobacter 1	0	0	0	0	20	0	10[c]	26
Acetobacter 2	0	0	0	0	15	0	12	24
Staphylococcus aureus	25	12	30	25	23	20	10	28
Staphylococcus sciuri	20	18	21	21	0	25	0	30
*Staphylococcus epidermidis*1	0	0	0	0	0	0	0	0
*Staphylococcus epidermidis*2	0	0	0	0	0	0	0	0
Bacillus pumillus 1	0	0	0	0	0	0	0	0

Table 6 (continued)

Bacillus pumillus 2	0	0	0	0	0	0	0	0
Bacillus safensis 1	27	9	22	30	18	32	13	25
Bacillus safensis 2	25	9	20	27	17	30	15	26
Lactobacillus rhamnosus 1	28	15	27	32	10	28	9	32
Lactobacillus rhamnosus 2	30	12	28	30	9	30	9	32
Lactobacillus paracasei 1	25	0	25	15	0	20	0	20
Lactobacillus paracasei 2	20	0	29	22	0	20	0	26

Sensitivity was measured as zone of inhibition of growth of the isolate around antibiotic-infused disc in millimeters. [a] Diameter of disc was 8mm. [b] Antibiotics are represented as: A= ampicillin; B = Bacitracin; C = Chloramphenicol; E = Erythromycin; K = Kanamycin; P = Penicillin; S = Streptomycin and T = Tetracycline. [c] Zone sizes of 9 and 10mm are considered as resistant.

Effect of Bacterial Extracts on MUC4 and MUC3 Production in CCD 841 CoN Cells

MUC4 in human colon CCD 841 CoN cells increased when the cells were treated with 10% bacterial cell fractions (BCF) of a *Lactobacillus rhamnosus* isolated from cultured milk compared to the cells that were treated with control 1x PBS pH7.4. MUC4 was expressed in two bands a 250kD and 100kD subunits. The 250kD MUC4 band was similar for both the control and experiment. The 100kD Muc4 band however showed about six-fold increased expression in the cells that were exposed to the BCF as compared to the cells that were exposed to control PBS. MUC 3 was expressed as a single 75kD band and showed increased in level of about two-fold in the CDD 841 CoN cells that were exposure to the 10% BCF (Figure 20).

Figure 20. Western Blot Analysis of MUC4 and MUC3 Expression Levels in Normal Colon Cells. Total proteins (15 μg/lane) from human colon CCD 841 CoN cells treated with 10% *L. rhamnosus* bacterial cytoplasmic fractions (BCF), or 10% Phosphate buffered saline control (PBS) were separated by SDS-PAGE and transferred onto immobillon transfer membrane. The membranes were immunoblotted with mouse anti-muc4 and donkey antimouse IgG for Muc4 and rabbit anti-muc3 with donkey antirabbit IgG for muc3 and exposed to radiogragh (chemiluminescent signals) to develop using proteinsimple flour chem M.

CHAPTER 4

DISCUSSION AND CONCLUSION

Discussion

This study was an examination of the bacterial composition of a Kenyan traditional 'Amabere amaruranu' cultured milk preparation and determined the stability of its isolates to digestive tract conditions, antimicrobial activity, sensitivity to antibiotics and capacity to degrade mucins. The effect of bacterial cell fractions of a *Lactobacillus rhamnosus* isolate that was isolated from the milk was also examined on human colon 841 CoN cells model for the production of MUC4 and MUC3 proteins. The goal of this study was to determine the bacterial communities in the cultured milk and their probiotic potentials. Although a wide variety of bacterial species and genera could have probiotic effects, almost only lactobacilli belonging to Lactic acid bacterial (LAB) and Bifidobacterial groups are mostly found in commercial use (Reid et al, 2008; Shah, 2007). Other bacterial communities in milk could also possibly have probiotic potentials. Bacteria in dairy products and gut microbiota are common sources of screening for probiotic strains. Because the bacterial composition of 'Amabere amaruranu' cultured milk preparation has not been reported, it was a suitable source of screening for potential novel probiotics strains.

Fourteen bacteria isolates with distinct colony and cell morphology were isolated from the cultured milk sample. The isolates were identified by comparing the sequences of the 16S rRNA gene of isolates to that of known bacteria species. The isolates

included 4 *Lactobacillus* isolates, 2 *Acetobacter spp*, 4 *Bacillus isolates,* and 4 *Staphylococcus isolates.* The program used for comparing the gene sequences of bacterial isolates for the identification could reliably identify the organisms to only the genus level. In view of that, some of the isolates were further identified using a biochemical method through API 50 system that compared the biochemical characteristics of the isolates in various chemical indicators to that of known bacterial strains. The bacterial isolates identified by this method included 2 *Lactobacillus rhamnosus* isolates, 2 *Lactobacillus paracasei* isolates*,* 2 S*taphylococcus epidermidis* isolates, 1 *Staphylococcus aureus* isolate, and 1 *Staphylococcus sciuri* isolate*.* The *Bacillus* and *Acetobacter* isolates were not identified by the API assay due to lack of kits specific for identifying the two genera.

Lactobacillus rhamnosus* and *Lactobacillus paracasei* isolates were among the dominant colonies in the milk sample, and that agreed with the reports by Abriouel, Martin-Platero, Maqueda, Valdivia, and Martinez-Bueno (2008), Aponte, Fusco, Andolfi, and Coppola (2008), and Randazzo et al. (2009) that they are among the common bacterial strains in cheese and raw milk from cow, goat, and sheep. Other dominant colonies that were found in the cultured milk sample were *Acetobacter sp, Bacillus pumilus*, *Bacillus safensis,* and *Staphylococcus epidermidis* isolates. The *Bacillus* isolates were found growing on only M17 agar plates that were incubated under aerobic conditions. The *Staphylococcus epidermidis* isolates were found on only MRS agar plates that were incubated under aerobic conditions. *Bacillus pumilus* has been reported by Delbes et al. (2007) as a common component of cow milk and cheese and

Stapylococcus epidermidis was reported by Callon et al. (2007) in goat's milk.

Staphylococcus aureus and *Staphylococcus sciuri* isolates formed very few colonies on

the plates. Aponte et al. (2008) reported the isolation of *Staphylococcus aureus* in cow

milk cheese. *Bacillus safensis*, *Acetobacter sp,* and *Staphylococcus sciuri* isolates were

the bacterial populations that have not been reported in other milk or milk products but

were found in the 'Amabere amaruranu' cultured milk sample. The *Staphylococcus*

aureus and *Staphylococcus sciuri* that were found to have developed very few colonies

could possibly be contaminant species.

It was observed that the viability of *Lactobacillus paracasei and Lactobacillus*

rhamnosus isolates that were exposed to acidic buffer at pH2 greatly diminished after

the first 1 h of exposure. However, unexposed cells of the same isolates were viable

and very stable in 0.3% bovine bile conditioned media. Meanwhile, the pH of

unconditioned broth cultures of the *Lactobacillus rhamnosus* isolates taken at 12 and 24

hours after incubation was observed to be in the range of 3.9 to 4.2. This observation

suggested that *Lactobacillus rhamnosus* isolates could possibly withstand low pH

conditions in spite of the diminished viability and static growth pattern we observed for

the cells that had been exposed to pH2 conditions. Saarela et al. (2009) reported that

the viability of cells is growth phase dependent and that stationary phase cultures

seemed to be more tolerant to stress than cells at log phase. It is therefore possible that

the viability and growth of the *Lactobacillus rhamnosus* isolates could present a different

pattern depending on the growth phase at which the cultures were taken for the study.

Moreover, pH2 is an extreme pH condition of the human stomach that is usually

attained during fasting and starvation (Antione, 2011). Therefore, it is possible that the *Lactobacillus rhamnosus* isolates could survive the low pH conditions of the stomach with appreciable rise in its pH during meals. It has also been mentioned that the resistance of bacteria to bile salts varies among the lactic acid bacteria species and strains (Xanthopoulos, 1997) and because the cultured milk isolates were not identified to the strain level, we could not generalize that the growth responses to bile we observed were representation of all *Lactobacillus species*.

Filter-sterilized spent broth of *Lactobacillus rhamnosus* and *Lactobacillus paracasei* isolates were observed to have presented antimicrobial activity. They inhibited the growth of *Enterobacter faecalis*, *Escherichia coli,* and *Pseudomonas aeruginosa* indicator strains slightly. This is a positive trait for probiotic selection. Because sterilized spent broths of the isolates were used for the test and not live cultures, it is reasonable to suggest that the inhibitory effect could be one of the suggestions that were proposed by Lewus et al. (1991) that resident microbiota and probiotics may impede infection by directly antagonizing the growth of pathogens through production of antimicrobial and antibacterial compounds such as bacteriocins. Moreover, the antimicrobial activity of the isolates in this study, particular that of the *Lactobacillus rhamnosus* isolates, could have been due to their inherent ability to lower the pH of the spent broth to pH around 3.9 and 4.2 as we observed in this study. This suggestion also agreed with the findings of Langhendries et al. (1995) that probiotics may inhibit infections through reduction of the gut local pH by production of lactic acid.

Testing and selecting bacterial strains that do not degrade mucins for probiotic preparation is a check to avoid the use of bacterial strains that have the capacity to degrade the protective mucus layer of the digestive tract (Zhou et al., 2001). *Lactobacillus paracasei* and *Lactobacillus rhamnosus* isolates did not degrade mucin and that made them suitable for probiotic preparation. We also observed that *Lactobacillus rhamnosus* isolate were sensitive to all the eight antibiotics tested and the *Lactobacillus paracasei* isolates were resistant to kanamycin, bacitracin, and streptomycin. It is so appropriate that the *Lactobacillus* isolates in the cultured milk demonstrated sensitivity to antibiotics. This is because the sensitivity of potential probiotic strains to antibiotics has always been considered a positive trait. The use of bacterial strains that show sensitivity to antibiotics for probiotics eliminates the possibility of horizontal transfer of antibiotic resistance traits to potential pathogenic intestinal dwellers (Collins et al., 1998).

The *Staphylococcus aureus* and *Staphylococcus sciuri* isolate that were exposed to acidic buffer at pH2 greatly diminished in viability after the first 1 h of exposure. However, unexposed cells of the same isolates were viable and showed stable growth in 0.3% bovine bile conditioned media. The pH of culture broths of the isolates at 12 h and 24 h after incubation was around 6.7 to 6.9. The *Staphylococcus aureus* isolate did not show antibacterial activity and demonstrates sensitivity to all the antibiotics tested and also did not degrade mucin. The *Staphylococcus sciuri* isolate however degraded mucin and demonstrated resistance against bacitracin and streptomycin. It also did not inhibit the growth of any of the indicator strains. Both

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exposed and unexposed *Staphylococcus epidermidis* isolates to pH2 acidic buffer exhibited stability in fresh broth and in 0.3% bovine bile conditioned media. The pH of culture broths of the isolates at 12 h and 24 h after incubation was around 4.9 to 5.9. They showed resistance to all the 8 antibiotics tested and did not degrade mucin. One of the *Staphylococcus epidermidis* isolates inhibited the growth of *Enterococcus faecalis* and *Pseudomonas aeruginosa,* although the halo around the disc appeared hazy indicating that some growth of the indicator strains was still possible. The bacteriostatic effect observed could have been due to the ability of the isolate to lower its pH to 4.9. Although the *Staphylococcus* isolates were stable to digestive tract conditions and demonstrated some amount of antimicrobial activity against some of the indicator strains, the strains could not recommended for probiotic preparation due various reasons. For instance, the *Staphylococcus sciuri* isolate would be suitable for probiotic preparations because of its mucinolytic ability and *Staphylococcus epidermidis* may not also be recommended because its antibiotic resistance. The *Acetobacter* isolates that had not been exposed to pH2 acidic buffer showed stability in 0.3% bovine bile conditioned media but the growth of the exposed cells in fresh media declined greatly and their viability also completely diminished. *Acetobacter* isolates were nonmucinolytic; showed sensitivity to ampicillin, kanamycin, and streptomycin. One *Acetobacter* isolate inhibited the growth of *Enterococcus faecalis* slightly. Both exposed and unexposed *Bacillus isolates* demonstrated high viability and stability in fresh broth or in 0.3% bovine bile conditioned media. This observation agreed with Sanders et al.'s (2003) report that *Bacillus sp* are stable to stressful conditions due to an inherent ability for them to produce metabolically inactive spores that are resistant to heat, drying, freezing, toxic

chemicals, and radiations. The bacilli did not degrade mucin and did not present antimicrobial activity against any of the bacterial indicator strains. However, *Bacillus pumilus* demonstrated resistance against all the eight antibiotics tested but *Bacillus safensis* was susceptible to all of them. Although the bacilli were very stable to digestive tract conditions and maintained mucins, they may not be suitable probiotics because they lacked antimicrobial activity and showed resistance against antibiotics.

The effect of cell fractions of one of the *Lactobacillus* isolates was examined in human colon CCD 841 CoN cells for production of MUC4 and MUC3. This was done by western blot analysis using whole cell extracts and antibodies specific for MUC4 and MUC3. The treatment of CCD 841 CoN cells with10% cytoplasmic fractions of *Lactobacillus rhamnosus* isolate brought about two-fold increase in the MUC3 protein production in the cell models. MUC3 protein was expressed in low amount in the normal colon cells model and as a single protein band with molecular weight of about 75kD. The low amount of MUC3 protein expression as observed in the study agreed with reports by Ho et al. (1993) and Van Klinken et al. (1995) that MUC3 is not highly expressed in the colon. MUC4 protein was observed in normal colon cells model as two protein bands with molecular weights of 250kD and 100kD bands. This observation is similar to the findings of Choudhury et al. (2000) that mature forms of human MUC4 protein may exist at the cell surface as two associated protein subunits, a large extracellular mucin type subunit (MUC4alpha) and a smaller, membrane-associated growth factor-like subunit (MUC4beta). The treatment of CCD 841 CoN cells with 10% cytoplasmic fraction of *Lactobacillus rhamnosus* resulted in an increase in the smaller

MUC4 protein band that has molecular weight of 100kD. It is interesting that cytoplasmic fraction of the *Lactobacillus rhamnosus* isolate could stimulate the increase in MUC4 protein levels in the normal colon cells. This observation agreed with Adams (2010) that probiotic cell components can act as biological response modifiers. In this regard, it might be suggestive that the administration of probiotic cytoplasmic fractions in place of live cells in probiotic preparations may offer a safe alternative application of the product and hopefully will resolve the concern for the possibility of probiotic sepsis that was raised by Syndman (2008). The ability of cytoplasmic fractions of *Lactobacillus rhamnosus* to up-regulate the expression of MUC4 protein in normal colon cell model could be an essential therapeutic contribution of probiotic cell components to the reduction of colorectal cancers. This hypothesis is informed by the findings made by Moniaux et al. (2007) that MUC4 protein is implicated in the induction of ultrastructural changes in the transformation of normal epithelium. It is also in line with the report of Komatsu, Yee, and Carraway (2002) that MUC4 is implicated in reducing accessibility of tumor cell surface antigens to cytotoxic immune cells, thus aiding in the evasion of the host immune response. The characteristics of the bacterial isolates are summarized in Table 7.

Table 7

Summary of bacterial isolates characteristics

Bacterial Isolates	Characteristics of bacterial isolates					
	Stability to acidic buffer	Stability to bile	Sensitivity to antibiotics	Antimicrobial activity	Non-mucinolytic	Ability to stimulate mucins production
L. rhamnosus 1	-	+	++++++++	+++	+	+
L. rhamnosus 2	-	+	++++++++	++	+	*[a]
L. paracasei 1	-	+	+++++	-	+	*
L. paracasei 2	-	+	+++++	++	+	*
B. pumilus 1	+	+	-	-	+	*
B. pumilus 2	+	+	-	-	+	*
B. safensis 1	+	+	++++++++	-	+	*
B. Safensis 2	+	+	++++++++	-	+	*
S. aureus	-	+	++++++++	-	+	*
S.sciuri	-	+	++++++	+	-	*
S. epidermidis 1	-	+	-	++	+	*
S. epidermidis 2	-	+	-	-	+	*
Acetobacter sp 1	-	+	+++	+	+	*
Acetobacter sp 2	-	+	+++	-	+	*

[a] The isolates were not examined for ability to stimulate mucins production in normal colon cells. *Lactobacillus rhamnosus* isolates have the most positive characteristics.

Conclusion

The results obtained in this study identified the bacterial composition in the stock culture of 'Amabere amaruranu' cultured milk. The isolates included *Lactobacillus rhamnosus*, *Lactobacillus paracasei*, *Bacillus pumilus*, *Bacillus safensis*, *Acetobacter sp*, *Staphylococcus aureus*, *Staphylococcus sciuri,* and *Staphylococcus epidermidis.* *Lactobacillus rhamnosus* isolates did not degrade mucin and showed antimicrobial

effect against indicator strains. They were susceptible to eight antibiotics that are commonly used in human clinical therapy. They were stable to bile but unstable to acidic buffer at pH2. They showed some positive traits for consideration for probiotic food production. *Lactobacillus paracasei* isolates were resistant to five antibiotics and unstable to digestive tract conditions. Treatment of human CCD 841 CoN cells with filter-sterilized *Lactobacillus rhamnosus* cell fractions demonstrated an increase in MUC4 level by six-fold and MUC3 level by two-fold. *Bacillus* isolates demonstrated stability to digestive tract conditions but demonstrate antibacterial activity. *Bacillus pumilus* isolates were found to be resistant to all of the antibiotics tested. *Acetobacter* and *Staphylococcus* isolates were unstable to *invitro* tests for stability to digestive tract conditions*. Staphylococcus sciuri* was the only isolate that degraded mucin. Therefore, *Staphylococcus aureus*, *Staphylococcus sciuri*, and *Acetobacter* isolates cannot be considered suitable for probiotic. We concluded that *Lactobacillus rhamnosus* isolates that did not degrade mucin and showed stability in acid and bile conditions, demonstrated antimicrobial activity against indicator strains, were sensitive to antibiotics, and showed capacity to stimulate increase in MUC4 and MUC3 levels in human CCD 841 CoN cell model could be potential probiotic candidates.

Future Research

It is our hope to examine the antimicrobial activity of the *Lactobacillus* isolates in details in order to determine the mechanism(s) by which they inhibited the growth of the indicator strains. We are interested in studying the optimum concentration of the

Lactobacillus rhamnosus extracts that gives the highest MUC4 and MUC3 levels in

normal colon cells.

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VITA

BLAISE B. BOYIRI

Education: East Tennessee State University, Johnson City, TN

                  MS Biology (Microbiology), 2014

                  University of Cape Coast (Ghana): BS Human Biology, 2006

                  Nandom Senior High School (Nandom, Ghana): WASSCE 1998

Professional

Experience: Graduate Assistant, Department of Health Sciences,

                  Johnson City, TN. Aug, 2012 - May 2014

                  Teacher, Ghana Senior High Sch. (Tamale, Ghana) August 2006-

                  July 2012

Awards: Graduate Assistant Award, Department of Health Sciences, ETSU

                  Dean's Biological Sciences Student Award, Univ of Cape Coast

                  Graduate & Professional Student Association Travel Award (GPSA)

Publications: Boyiri, B.B. Lampson, B. and Onyango, E. M. 2014. *Bacterial*

isolates from 'Amabere amaruranu' cultured-milk Survived Well in

Digestive Tract Conditions and could have Probiotic Potentials.

Experimental Biology, poster.

Boyiri, B. B. and Onyango, E. M. 2014. *Lactobacilli bacterial*

isolates from 'amabere amaruranu' cultured milk have probiotic

potential. Appalachian Student Research Forum, poster*.*