ISOLATION AND TESTING THE CHOLESTEROL REDUCTION ABILITY (*in-vitro*) OF *Lactococcus lactis* FROM FERMENTED SMOOTH PIGWEED *(Amaranthus hybridus)* LEAVES.

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A Thesis submitted to the Graduate School in partial fulfilment of the requirements for the Master of Science Degree in Nutritional Sciences of Egerton University.

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DECLARATION AND RECOMMENDATION

Declaration

This thesis is my original work and has not been presented in this or any other University.

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Recommendation

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DEDICATION

I dedicate this work to my daughter Tasha, my parents Mr. and Mrs. Mariga and to my sisters Christine and Karen.

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I acknowledge all who took part in making this work a success. I wish to acknowledge the contribution by the Department of Human Nutrition for accepting my studentship. I would also like to extend my sincere appreciation to the Research and Extension Division, Egerton University and the Department of Dairy and Food Science and Technology for providing me with funds and facilities respectively, for this research. My candid gratitude goes to my supervisors, Prof. Anakalo Shitandi and Prof. Prisca Tuitoek for their undivided guidance and supervision of this work up to the very end. Special thanks go to Mr. Kasangi for his continuous support during and after the laboratory work. I also wish to extend my thanks to the Graduate School and Egerton University as a whole for giving me the opportunity to study in the institution. Lastly, I wish to thank my dear parents Mr. and Mrs. Mariga, my sisters Christine and Karen for their financial, spiritual and material support during the whole period of study.

ABSTRACT

Elevated serum cholesterol levels in humans is generally a risk factor for the development of coronary heart diseases. It has been reported that a culture of *Lactobacillus acidophilus* (probiotic) actively taking up cholesterol from a laboratory medium would function *in vivo* to exert a hypocholesterolemic effect. Thus, the use of probiotics as a biological procedure of cholesterol reduction is increasing rapidly. However, most probiotics in the market are derived from animal products mainly milk which are expensive. Developing probiotics from plant materials and especially amaranthus would consequently be a promising remedy in cholesterol reduction. The present study aimed to isolate and test cholesterol reduction ability (*in vitro*) of *Lactococcus lactis* from fermented smooth pigweed (*Amaranthus hybridus*) leaves. The objectives of the study were to; isolate *L. lactis* bacteria from *A. hybridus* leaves harvested at maturity (30 days), determine cholesterol removal ability of *L. lactis* isolated from the *A. hybridus* leaves and establish whether there were any differences in the amounts of cholesterol removed from the growth media by *L. lactis* and *Lb. acidophilus* ATCC 43121 (the positive control). It further aimed at determining whether fermentation had any influence on protein, mineral and moisture content in amaranthus leaves and the acceptability of the fermented leaves as compared to fresh boiled leaves. To achieve this *A. hybridus* was grown at Kenya Agricultural Research Institute, Njoro. The leaves were harvested at maturity and fermented for five days. After fermentation, *L. lactis* strain was isolated and its ability to remove cholesterol from growth medium tested. This ability was compared with that of *Lb. acidophilus* ATCC 43121. Protein, ash and moisture content of the leaves before and after fermentation was also determined. Acceptability of the fermented leaves was compared to fresh boiled leaves. The experiment was laid in a completely randomized design (CRD) at $P < 0.05$. *Lactococcal* strain was successfully isolated from fermented amaranthus leaves. The strain and *Lb. acidophilus* ATCC 43121 removed 52 and 56 μg/ml of cholesterol, respectively. These amounts were not significantly different at $P < 0.05$. After fermentation protein decreased from 36.07 to 16.65%, ash increased from 19.76 to 36.21% and moisture content increased from 5.44 to 6.22% respectively. There was no significant difference in acceptability whereby, fermented leaves scored 6.90 points compared to 6.83 points scored by fresh boiled leaves at significance level of $P < 0.05$. This study concludes that *L. lactis* isolated from fermented amaranthus leaves has the potential to reduce cholesterol from the growth medium with minimum changes in nutritional content of the leaves.

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LIST OF ABBREVIATIONS

ANOVA	Analysis of Variance
ATCC	American Type Culture Collection
bv	Biovar
$CaCO_3$	Calcium carbonate
cfu	Colony forming unit
CVD	Cardiovascular diseases
d	Day
DHA	Docohexanoic acid
DNA	Deoxyribonucleic acid
EPS	Exocellular Polysaccharides
g	Gravity
GALT	Gut Associated Lymphoid Tissue
GIT	Gastrointestinal tract
H_2O_2	Hydrogen peroxide
HMG	Hydroxymethylglutaryl
KOH	Potassium Hydroxide
L	*Lactococcus*
LAB	Lactic Acid Bacteria
Lb	*Lactobacillus*
LDL	Low-density lipoprotein
MRS	de Mann Rogosa Sharpe
NaCl	Sodium Chloride
NA	Nutrient agar
nm	Nanometre
PCA	Plate count agar
PCR	Polymerase Chain Reaction
PUFA	Polyunsaturated fatty acid
SCFA	Short Chain Fatty Acid
Subsp.	Sub species
TVC	Total viable count

μg	Micro-gram
μg/ml	Micro-gram per millilitre
v/v	Volume by Volume
w/v	Weight by Volume

CHAPTER ONE
INTRODUCTION

1.1 Background information

Green leafy vegetables have for long been recognized as the cheapest and most abundant potential sources of vitamins, minerals and protein (Aletor *et al.,* 2002). This is because of their ability to utilize a wide range of virtually unlimited and readily available primary materials (Klijn *et al.,* 1995). These include water, CO_2, atmospheric nitrogen and sunlight. For example, cassava leaves are, depending on the varieties, rich in protein (14-40% dry matter), minerals, vitamins B1, B2, C and carotenes (Aletor *et al.,* 2002). However, with the advent of modern farming these plants are facing extinction and as a result food insecurity may worsen. Currently, people are concentrating on the exotic vegetables such as cabbages and kales which are sensitive to environmental conditions such as rainfall and soils. This is done at the expense of the more tolerant and nutritious African indigenous vegetables such as black nightshade *(Solanum nigrum)* Smooth Pigweed (*Amaranthus hybridus)* and pumpkin (*Cucurbita maxima*) leaves (Katuriina, 2000; Ndegwa and Aagaard, 2003).

Some of these vegetables are fermented and consumed in meals which make them a potential source of probiotics. A probiotic is a preparation, or a product containing viable and defined microorganisms in sufficient numbers resulting in enhanced health attributes. These alter the intestinal microflora (by implantation or colonization) in a compartment of the host and by that exert beneficial health effects in the host (Marteau *et al.,* 2002; Marteau *et al.,* 2007). For example, the ability to reduce serum cholesterol levels, antimicrobial substrate production and immune modulation, are considered as effective health properties. Lactic acid bacteria (LAB) consist of twenty genera such as *Lactobacilli, Bifidobacteria* and *Lactococci* (Furgus and Michael, 2000). The most widely studied probiotic bacteria are *Lactobacilli* and *Bifidobacteria* and extensive studies on the beneficial effects on human health have been reported (Perdigon *et al.,* 1990). However, information on the probiotic activity of *Lactococci* is scarce since they are traditionally not considered to be natural inhabitants of the human gastrointestinal tract (Grahn *et al.,* 1994). Several studies have showed the possibility of the presence of *Lactococci* in human or animal gastrointestinal tract (GIT) (Klijn *et al.,* 1995).

Lactococci can also be found in milk and milk products, plant materials (fermented vegetables and fruits) and intestines of fish and herbivores. Studies have shown probiotic activities of *Lactococci* isolated from dairy foods which include the ability to inhibit the growth of other bacteria and cholesterol removal from growth media (Grahn *et al.,* 1994). However, few studies have been published concerning the probiotic activity of *Lactococci* from plant materials (Kelly *et al.,* 1998).

The beneficial health effects related to probiotics include, immune modulation, increase of mineral absorption, detoxification, pathogen inhibition, relief of constipation and their ability to reduce serum cholesterol levels. It has been reported that a culture of *Lb. acidophilus* actively taking up cholesterol from growth media would function *in vivo* to exert a hypocholesterolemic effect (Liong and Shah, 2005a). For example, *Lb. acidophilus* ATCC 43121 can incorporate some of the cholesterol removed from media into the cellular membrane during growth (Noh *et al.,* 1997). This property has beneficially influenced serum cholesterol levels in pigs (De Rodas *et al.,* 1996). This is because cholesterol incorporated into or attached to cells of bacteria in the intestine is likely to be unavailable for absorption into the blood. The ability to incorporate cholesterol into or attach it to cells of bacteria has been equated to the ability to remove cholesterol from media. Many reports have been published on cholesterol removal from laboratory media by *Lactobacilli* and *Bifidobacteria* (Gilliland *et al.,* 1998). However, few such studies have been published concerning this ability in *Lactococci* from plant materials (Kimoto *et al.,* 2002).

1.2 Statement of the problem

The use of probiotics as a biological procedure of cholesterol reduction is increasing rapidly. However, most probiotic products in the market are derived from animal products mainly milk. Apart from these products being expensive, animal products are the main cause of hypercholesterolemia, thus, probiotics from plant materials and especially amaranthus would be a promising remedy in cholesterol reduction. This is because plant probiotics are cheap and readily available in the most parts of the country.

1.3 General objective

The study had the overall aim of isolation and testing of cholesterol reduction ability (*in vitro*) of *L. lactis* from fermented smooth pigweed (*A. hybridus*) leaves and determination of any nutritional changes and acceptability of the leaves.

1.4 Specific objectives

The specific objectives of this study were to:

1. Isolate *L. lactis* bacteria from fermented *A. hybridus* leaves harvested at maturity (30 days).
2. Determine the cholesterol removal ability of *L. lactis* isolated from the *A. hybridus* leaves and test whether there were any significant differences in the amounts of cholesterol removed from the growth media by *L. lactis* and *Lb. acidophilus* 43121 (the positive control).
3. Determine whether fermentation had any influence on protein, mineral and moisture content in amaranthus leaves.
4. Determine the acceptability of the fermented amaranthus leaves as compared to fresh boiled leaves.

1.5 Hypothesis (Ho)

The following null hypotheses were tested at 0.05 significance level:

1. There are no *L. lactis* bacteria in fermented *A. hybridus* leaves harvested at maturity.
2. *Lactococcus lactis* does not remove cholesterol from the growth medium and there is no significant difference in the amounts of cholesterol removed from the growth media by *L. lactis* and *Lb. acidophilus*.
3. Fermentation has no effect on protein, mineral and moisture content in amaranthus leaves.
4. There are no significant differences in the organoleptic quality between the fermented amaranthus leaves and the fresh boiled leaves.

1.6 Significance of the study

Most probiotic food products available in the market are from fermented animal products which are expensive hence not readily available. On the other hand, there are very few probiotic products from plant materials (fermented fruits and vegetables) available. Strains of *L. lactis* bacteria have been found to be present in fermented vegetables in large numbers. Establishing

the availability of this strain in fermented amaranth leaves and determining its ability to remove cholesterol from growth medium as *Lb. acidophilus* is a step towards creation of noble probiotic. This, will lead to increased attention on biological procedures (use of probiotic food products) from fermented vegetables to reduce serum cholesterol, usually associated with increased risk of cardiovascular diseases (CVD).

1.7 Scope of the study

The study focused on isolation and determining the cholesterol removal ability (from growth medium) of *L. lactis* strain and comparing this ability to that of *Lb. acidophilus* (positive control). The strain was isolated from fermented *A. hybridus* leaves harvested at maturity (30 days). The study also sought to establish whether fermentation had any influence on protein, mineral and moisture content in amaranthus leaves. Finally, the acceptability of the fermented amaranthus leaves was compared to that of fresh boiled leaves.

CHAPTER TWO
LITERATURE REVIEW

2.1 Introduction

In this chapter, nutrition profile of *A. hybridus* leaves, eco-climatic requirements and maturation period are discussed. Lactic acid bacteria (LAB) used as probiotics as well as the mechanism by which they exert health effects in their host are also reviewed. Lastly, means of cholesterol reduction by LAB will be discussed.

2.2 Smooth pig weed/African spinach (*A. hybridus*)

The nutritional value of traditional leafy vegetables is higher than several known common vegetables often supplying most of the daily requirements for vitamins A, B complex and C (ascorbic acid) for rural people (James *et al.*, 2003). Most of these traditional leafy vegetables have a potential for income generation but fail to compete with exotic vegetables at present due to lack of awareness. Consumption of traditional diets known to these societies are said to have many beneficial effects such as prevention of some age related degenerative diseases; arteriosclerosis and stroke (James *et al.*, 2003). Despite these advantages, most traditional plant foods are generally uncultivated and underutilized. The production and utilization of these vegetables can make a much-needed contribution to better nutrition and income in Kenya but there is a serious threat that many species will drop out of use in some areas if no appropriate countermeasures are taken.

Knowing the nutritional, medicinal and economic value could definitely add value to the cultivation, consumption, conservation, and regional/international commercialization of native Kenyan vegetables. Such knowledge if well exploited could as well serve as one of the main corridors for hunger and poverty alleviation in Kenya. This study was carried out on the key assumption that while hunger and malnutrition threaten millions of people in Kenya, the value of African traditional leafy vegetables is not fully appreciated, particularly in the urban areas where cardiovascular cases are on the rise. This is because many traditional vegetables are associated with poor rural lifestyles and low socio-economical status. Yet, increased consumption of traditional leafy vegetables can have a positive effect on nutrition, health and economic well being of both urban and rural populations (Dhellot *et al.,* 2006). Vegetables such as Amaranth (*mchicha*), Cowpea (*kunde*), African nightshades (*osuga, managu*), Crotalaria (*mitoo*), Ethiopian kale or Water spinach (*kanzira*), Jute (*mlenda*) and Cat's whiskers or spiderplant (*saga*) are good

sources of micronutrients including iron, folate, iodine, selenium and zinc, as well as vitamins A, B complex, C and E (Dhellot *et al.,* 2006). Knowledge about nutritional needs and food uses is important not only among the urban and rural poor and food-insecure households, but also for more affluent households as well given the rise in diet-related, non-communicable diseases. Several abiotic and biotic stresses limit crop production in Kenya. These range from drought to salinity and from pests to diseases. Exploitation of indigenous leafy vegetables such as amaranthus which are adapted to the local environment will not only overcome these stresses but also improve food security, nutrition and health of the rural poor. Amaranth plant is widely grown as a leafy vegetable in tropical and sub-tropical Africa, Asia, the Pacific Islands, the Caribbean and Central America (Myers, 1998). Leaves of various amaranthus species have been eaten as leafy vegetables for a long time (Mwangi, 2003). The species used as vegetables are; *A. hypochondriacus, A. cruentus* and *A. hybridus. Amaranthus hybridus* leaves have been shown to have the following nutritional profile; dry matter (%) 89.0±2.0, crude protein (g kg^{-1} DM) 36±1.2, crude fibre (g kg^{-1} DM) 12.7±4.2, ether extract (g kg^{-1} DM) 9.6±4.1, ash (g kg^{-1} DM) 19.8±7.0, N-free extract (g kg^{-1}DM) 45.7±0.8 and gross energy (MJ kg^{-1}) 420.0 (Iheanacho and Udebuani, 2009).

Amaranth is also well balanced in terms of the essential amino acids that humans require (Myers, 1998). Amaranth plant is extremely drought tolerant and requires little amount of water (James *et al.,* 2003). Environmental hardiness and utility as a grain or vegetable has led to the resurgence in amaranth production. It is adaptable to different climates, which allow it to withstand great heat, as well as different types of soil (Ole *et al.,* 2004). This makes it an appealing food crop for the areas that suffer droughts. Amaranth has a short maturity period of only 30 days which means that the production of the food crop would not take as long as many other crops e.g. kales and cabbages which mature in 50 to 60 and 70 to 85 days respectively (Mwangi, 2003; Iheanacho and Udebuani, 2009). Apart from amaranth being consumed as a vegetable, its seeds are used as human food. Amaranth can be cooked as a cereal, ground into flour, popped like popcorn, sprouted, or toasted (Aletor *et al.,* 2002). The seeds can be cooked with other whole grains, added to stir-fry or to soups and stews as a nutrient dense thickening agent (Iheanacho and Udebuani, 2009). Amaranth flour is used in making pastas and baked foods. However, it must be mixed with other flours for baking yeast breads, as it contains no gluten. One part amaranth flour to 3-4 parts wheat or other grain flours may be used (Dhellot *et*

al., 2006). In the preparation of flatbreads, pancakes and pastas, 100% amaranth flour can be used (Iheanacho and Udebuani, 2009). Sprouting the seeds will increase the level of some of the nutrients and the sprouts can be used on sandwiches and in salads. Amaranth seed is high in protein (15-18%) and contains appreciable amounts of lysine and methionine, two essential amino acids that are not frequently found in cereal grains (Dhellot *et al.,* 2006). It is high in fiber and contains calcium, iron, potassium, phosphorus, and vitamins A and C. The fiber content of amaranth is three times that of wheat and its iron content, five times more than wheat. It contains two times more calcium than milk (Iheanacho and Udebuani, 2009).

Amaranth also contains tocotrienols (a form of vitamin E) which has cholesterol-lowering activity in humans (Dhellot *et al.,* 2006). Cooked amaranth is 90% digestible and because of this ease of digestion, it has traditionally been given to those recovering from an illness or ending a fasting period. Amaranth consists of 6-10% oil, which is found mostly within the germ (Iheanacho and Udebuani, 2009). The oil is predominantly unsaturated and is high in linoleic acid, which is important in human nutrition. *Amaranthus hybridus* seed oils can be considered as source of omega-3 polyunsaturated fatty acid (PUFA). The omega-3 polyunsaturate, docosahexaenoic acid (DHA), plays a number of biologically important roles, particularly in the nervous system, where it is found in very high concentrations in cell membranes. In infants, DHA is required for the growth and functional development of the brain, with a deficiency resulting in a variety of learning and cognitive disorders (Dhellot *et al.,* 2006).

2.3 Lactic acid bacteria

The lactic acid bacteria are a group of Gram-positive, catalase-negative, non-respiring, non-spore forming, cocci or rods, which produce lactic acid as the major end product of the fermentation of carbohydrates (Furgus and Michael, 2000). They are the most important bacteria in desirable food fermentations, being responsible for the fermentation of sour dough bread, sorghum beer, all fermented milk and milk products, cassava (to produce *gari* and *fufu*) and most "pickled" (fermented) vegetables (Parvathy and Puthuvallil, 2005). Historically, bacteria from the genera *Lactobacillus, Lactococcus, Leuconostoc, Pediococcus* and *Streptococcus* are the main species involved. Several more bacteria have been identified, but play a minor role in lactic fermentations. Lactic acid bacteria carry out their reactions - the conversion of carbohydrate to lactic acid plus carbon dioxide and other organic acids - without the need for oxygen (Parvathy and Puthuvallil, 2005). They are described as microaerophilic as they do not utilize oxygen.

Because of this, the changes that they effect do not cause drastic changes in the composition of the food. Some of the families are homofermentative, that is, they only produce lactic acid, while others are heterofermentative and produce lactic acid plus other volatile compounds and small amounts of alcohol (Parvathy and Puthuvallil, 2005).

Lactobacillus acidophilus, Lb. bulgaricus, Lb. plantarum, Lb. caret, Lb. pentoaceticus, Lb. brevis and *Lb. thermophilus* are examples of lactic acid-producing bacteria involved in food fermentations. All species of lactic acid bacteria have their own particular reactions and niches, but overall, *Lb. plantarum*, a homofermenter, produces high acidity in all vegetable fermentations and plays the major role (Kimoto *et al.,* 2000). Species of the genera *Streptococcus* and *Leuconostoc* produce the least acid (Sirisansaneeyakul *et al.,* 2007). Homofermenters, convert sugars primarily to lactic acid, while heterofermenters produce about 50% lactic acid plus 25% acetic acid and ethyl alcohol and 25% carbon dioxide (Furgus and Michael, 2000). The lactic acid produced by LABs is effective in inhibiting the growth of other bacteria that may decompose or spoil the food. Despite their complexity, the whole basis of lactic acid fermentation centres on the ability of LABs to produce acid, which then inhibits the growth of other non-desirable organisms. These other compounds are important as they impart particular tastes and aromas to the final product (Parvathy and Puthuvallil, 2005). The heterofermentative *Lactobacilli* produce mannitol and some species also produce dextran.

2.4 *Lactococcus lactis*

Lactococcus lactis is a Gram-positive, catalase-negative, non-respiring, non-spore forming, cocci shaped bacterium used widely for industrial production of fermented dairy products such as milk, cheese, and yoghurt. These are important food supplies for many people. Extensive research has been done on the microorganism's metabolic pathway to increase its efficiency for dairy production (Tanous *et al.,* 2007). Due to its important applications, simple metabolism and limited biosynthetic capabilities, its genome has been sequenced to help researchers understand genes that are responsible for its fermentation pathway (Sean *et al.,* 2006). Furthermore, scientists study DNA recombination to improve its survival and resistance to antibiotics and to manipulate its metabolic pathways for better use in industrial production (Tanous *et al.,* 2007). Besides its high use in industrial application, *L. lactis* can also be found in the wild on plants and within the digestive tract of cows, insects and fish. It is believed that in nature, *L. lactis* stays dormant on plant surfaces awaiting to be ingested along with the plant into

insect or animal digestive tract, where it becomes active and multiplies intensively (Alexander *et al.,* 2001). Not only is it important in dairy production, but also has potential of use as oral vaccine, foreign protein production and metabolite through genetic engineering to manipulate *L. lactis* in the researchers' favour (Sean *et al.,* 2006). *Lactococcus lactis* has two subspecies with few phenotype and genotype differences, *L. lactis* subsp. *lactis* and subsp. *cremoris*, where subsp. *lactis* is preferred for making soft cheese while subsp. *cremoris* is for hard cheese (Alexander *et al.,* 2001). These organisms were originally classified under the genus *Streptococcus*, but in 1985, it was assigned to the current genus (Nakarai *et al.,* 2000).

2.4.1 *Lactococcus lactis* cell structure and metabolism

The metabolic pathway of *L. lactis* can function through aerobic and anaerobic reactions. It requires minimally glucose, arginine, methionine, glutamate and valine for growth (Tanous *et al.,* 2007). The main metabolism of *L. lactis* is through the anaerobic pathway, fermentation, which produces lactic acid from the available carbohydrates and is used for industrial food production. The carbon sources for *L. lactis* include; fructose, galactose, glucosamine, glucose, lactose, maltose, mannitol, mannose, ribose, sucrose and trehalose (Sean *et al.,* 2006). However, the growth rate of the cell with the intake of each carbon source is different. Growth rate on glucose, mannose, galactose, sucrose, lactose and glucosamine are the same, while fructose and mannitol growth rates are lower (Oliveira *et al.,* 2005). Under anaerobic reaction, glycolysis breaks down extracellular carbohydrates to pyruvate, then converts pyruvate to lactic acid (Hols *et al.,* 1999). Besides the anaerobic pathway, *L. lactis* has an aerobic system to assist in its development (Brooijmans, 2007).

2.4.2 Application to biotechnology

Lactococcus lactis has been researched thoroughly and put into many applications. It has several fermentative pathways, but the most important purpose is its property in the production of dairy products such as cheese (Sean *et al.,* 2006). *Lactococcus lactis* specializes in lactate dehydrogenase excreting lactic acid, which is used to preserve food and extend food shelf life. Dairy industries continue to improve the activities and effectiveness of *L. lactis* by manipulating its environment and cell behavior (Oliveira *et al.,* 2005). Another study utilizes the simple and harmless bacteria as mucosal vaccine. In developing countries where vaccines are limited and not affordable, diseases can spread easily. Researchers attempt to prove that a mucosal vaccine against *Streptococcus pneumoniae* using *L. lactis* is more effective than vaccination with purified

live antigen (Hols *et al.,* 1999). *Lactococcus lactis* is treated to recombine vaccine strains, so the cell can express the vaccine protein PspATIGR4. The result shows that *L. lactis* has more potential and safety in developing vaccine in human and should be considered to be used against more pathogens (Hols *et al.,* 1999).

2.4.3 Gene modification of *L. lactis*

Many researches are being conducted on how to improve the efficiency of *L. lactis* in the production of various products including lactic acid, nisin and L-alanine (a sweetener in dairy products) (Tanous *et al.,* 2007). Recombining genes has been a challenge, so in recent research, scientists demonstrated the parameter requirement for successful transformation of *L. lactis* IL1403 strain with electroporation (Oliveira *et al.,* 2005). A critical factor is that the resistors used in electroporation should be in parallel to shorten the pulse decay time, which should stay between 20 to 40 ms for the best result (Gerber and Solioz, 2007). The concentration of 2-3% glycine in the media also will provide the optimal growth for the transformation efficiency. Many other important factors are also listed in the parameter to provide a better method for *L. lactis* recombination (Brooijmans, 2007). This research will help the dairy industry to manipulate the genome to have better control over the organism's activities (Gerber and Solioz, 2007).

Most current research (Sirisansaneeyakul *et al.,* 2007) deals with improving the industrial use of *L. lactis*. A new method optimizing lactic acid fermentation has been studied recently that deals with immobilizing the cells to provide advantages (Tanous *et al.,* 2007). The cells are first entrapped within beads of alginate or microcapsules of alginate membrane. The advantage from this method includes the ability to re-use the immobilized cells shortening the processing time, elimination of process to remove the bacteria from the final product, high density of cell to increase activity and production, and reduce contamination (Oliveira *et al.,* 2005). As a result, optimal lactic acid production can be achieved with the immobilized group of *L. lactis* and continuous operation with controlled pH. However, the major downsides to this method consist of the cost of creating batch of immobilized cells and the cost of fermentation medium (Sirisansaneeyakul *et al.,* 2007).

Another industrial research on *L. lactis* deals with the production of L-alanine, which is used as sweetener in dairy products (Gerber and Solioz, 2007). Many microorganisms produce L-alanine, but the maximum conversion rate from carbohydrates remain only between 50–60% (Gerber and Solioz, 2007). The alaD gene coding for alanine deydrogenase, an enzyme that

converts pyruvate into alanine, was inserted into *L. lactis* chromosome. To ensure for maximal production, lactate dehydrogenase gene was knocked out to eliminate any competition for pyruvate substrate and co-factors (Sirisansaneeyakul *et al.,* 2007). When this cell is grown under uncontrolled pH, it produces 50% alanine, 35% acetoin, and other substances (Gerber and Solioz, 2007). In addition, controlling the pH to 7.5, the mutant cell improves the production to 75% alanine. Alanine production with change of pH increases drastically over the maximal conversion of other microorganisms (Gerber and Solioz, 2007). The increase in performance of *L. lactis* reveals another industrial advantage for producing amino acid and genetic engineering (Hols *et al.,* 1999).

Nisin is an antibiotic-like substance, called a bacteriocin, produced by the "food grade" starter strain, *L. lactis* subsp. *lactis*. It is a natural antimicrobial agent with activity against a wide variety of Gram-positive bacteria, including food-borne pathogens such as *Listeria*, *Staphylococcus* and *Clostridium* (Sirisansaneeyakul *et al.,* 2007). The primary target of nisin is believed to be the cell membrane. Unlike some other antimicrobial peptides, nisin does not need a receptor for its interaction with the cell membrane, however, the presence of a membrane potential is required (Gerber and Solioz, 2007). Nisin is a natural preservative present in cheese made with *L. lactis* subsp. *lactis*, but it is also used as a preservative in heat processed and low pH foods. Since nisin cannot be synthesized chemically, the nisin-producing *L. lactis* strains are used for its industrial synthesis (Brooijmans, 2007). The first established use of nisin was as a preservative in processed cheese products, but numerous other applications in preservation of foods and beverages have been identified. It is currently recognized as a safe food preservative in many parts of the world (Oliveira *et al.,* 2005). Nisin has been used as a preservative in various pasteurized dairy products and canned vegetables, baked, high-moisture flour products, and pasteurized liquid egg. There is interest in the use of nisin in natural cheese production (Brooijmans, 2007). Considerable research has been carried out on the anti-listerial properties of nisin in foods and a number of applications have been proposed. Uses of nisin to control spoilage lactic acid bacteria have been identified in beer, wine, alcohol production and high acid foods such as salad dressings. Production of highly purified nisin preparations has led to a growing interest in the use of nisin for human ulcer therapy and mastitis control in cattle (Tanous *et al.*, 2007).

2.4.4 *Lactococcus lactis* as a probiotic

One of the important properties of probiotics is the ability to survive in the intestine (Kimoto *et al.,* 2000). There have been few studies on the probiotic property of *Lactococci,* since they are formally not considered to be natural inhabitants of the intestine. *Lactococci* can be found in milk and milk products, plant materials (i.e. fermented vegetables and fruits, etc.) and intestines of cows, fish and insects. To evaluate *Lactococci* as probiotic bacteria, investigations on their ability to survive during gastric transit by *in vitro* and *in vivo* tests have been done. When exposed to an *in vitro* simulated gastrointestinal environment, such as low pH and bile, only *L. lactis* subsp. *lactis biovar diacetylactis N7* showed a moderate survival rate among the strains tested (Kimoto *et al.,* 1999). The tested strains were orally administered to mice and intestinal passage of the ingested strains was monitored by two methods: antibiotics and Polymerase Chain Reaction (PCR). Viable cells of strain N7 were recovered from feces within 24-48 h after administration but not at 72 h. *Lactococcus lactis* subsp. *cremoris* ATCC 19257, which had a poor survival rate *in vitro* test, was also detected at 12 h but not at 24 h (Kimoto *et al.,* 2000). Tolerance to bile was tested in MRS, glucose-supplemented M17 agar and M17 broth and this varied among the tested strains and growth media.

Addition of Tween 80 to the medium enhanced tolerance to bile in some *Lactococci.* The most promising strain, *L. lactis* subsp. *lactis bv. diacetylactis* N7, was selected for its resistance to the conditions of gastrointestinal tract and its ability to inhibit enteric bacteria using a spot-on-the-lawn technique (Kimoto *et al.,* 2000). Strain N7 had good survival in the low pH value and in the presence of lysozyme. Strain N7 clearly inhibited growth of enteric bacteria such as *Escherichia coli* and *Enterococcus faecalis*, but not *Lb. acidophilus* (Kimoto *et al.,* 2000). These results indicate a possibility that strain N7 reaches the intestine alive after ingestion and displays probiotic activity to inhibit certain harmful enteric bacteria. These results therefore indicate that *Lactococci* can reach the mouse intestine alive, but not colonize it. If administered daily, viable strain N7 may exist continuously in the intestine (Kimoto *et al.,* 2004). The effect of strain N7 on intestinal microbial balance and on animal health will be the subject of a further study. Studies have also shown probiotic activities of *Lactococci* isolated from dairy foods which include cholesterol removal from growth media (Grahn *et al.,* 1994; Kimoto *et al.,* 2004). However, few studies have been published concerning the probiotic activity in *Lactococci* from plant materials.

2.5 Probiotics

Within the functional foods, is the small but rapidly expanding arena of probiotics (Oliveira *et al.,* 2005). A functional food is a food that can be satisfactorily demonstrated to affect beneficially one or more target functions in the body, beyond adequate nutritional effects. This implies an improved state of health and well-being and/or reduction of risk of disease (Catherine *et al.,* 2005). Probiotics are found in fermented foods and they produce lactic acid as the main end product (Parvathy and Puthuvallil, 2005). They can also be found in capsules which contain very high concentration of one or more microorganisms. This rather diverse group of bacteria is often collectively called the lactic acid bacteria (LAB). Some of the fermented foods for which lactic acid fermentation plays a major role include; cheese, yoghurt, *kefir* (goat, sheep, cow's milk), *fil*, *viili* (ropy, sour milk) *mursik,* sourdough bread, *idli* (rice), *ogi* (maize, sorghum), *enjeraa* (tef, barley, wheat), *gari* (cassava), soy sauce, pickles, sauerkraut, *kimchi* (cabbage, raddish), fish sauce, *rakfisk* (traut), *balao balao* (rice shrimp) and dry fermented sausage.

Lactic acid bacteria (LAB) were first thought to be present in milk only, where they produced milk acid (lactic acid) from milk sugar (lactose) (Furgus and Michael, 2000). Other scientists, however, noted that similar bacteria could be found in other habitats e.g. human gastrointestinal tract (GIT) (McNaught and MacFie, 2001). This has led to therapeutic manipulation of the GIT flora, for treatment of several common clinical disorders (Mainville *et al.,* 2005). The concept was given attention by Elie Metchnikoff who was manipulating the GIT flora among Bulgarians to treat colitis (Shanahan and O'Sullivan, 2000). The human GIT contains approximately 10^{14} bacteria representing more than 500 different strains. The number increases progressively down the GIT. The colon has more than 100 times more species and 100 000 times more organisms than any other intestinal area (Catherine *et al.,* 2005; Peter *et al.,* 2006). *Helicobacter pylori,* may be the only organism that truly colonizes the stomach (Klaenhammer and Kullen, 1999). Colonization of the gastric mucosa by *H. pylori* is the main cause of gastritis and ulcers, and is strongly associated with gastric lymphoma and cancer (Marteau *et al.,* 2007). Studies have shown that some LAB, for example *Bifidobacteria* may kill or inhibit attachment of *H. pylori* to the gastric mucosa (Amy and Ana, 2004). The duodenum and proximal small bowel (jejunum) has been shown to contain few microorganisms mainly aerobes and facultative anaerobes (Liong and Shah, 2005b). In the ileum, bacteria increase markedly and there is a shift from aerobic to anaerobic organisms (Peter, 2002). The number

increases in the colon and the flora consists almost entirely of strict anaerobes such as *Bacteroides* species, anaerobic *Lactobacilli* and *Clostridia.*

To survive in the GIT bacteria must overcome physical and chemical barriers such as acid and bile (Liong and Shah, 2005b). The acidic environment kills most bacteria that enter the stomach (Peter, 2002). Gruzza *et al.* (1992) and Kimoto *et al.* (2000) studied the ability of *L. lactis* to survive GIT passage using animal models (rat). The study showed that *Lactococci* bacteria could withstand the GIT barriers but their numbers decreased with time. This led to the conclusion that supplementation is necessary to maintain *Lactococci* bacteria in sufficient numbers so as to exert health effects. Grahn *et al.* (1994) also illustrated that *L. lactis* strain can withstand acid and bile in the GIT of humans. There is still much controversy as to how probiotics work but the following mechanisms are considered important:

2.5.1 Adherence and colonization of the gut

The ability of probiotics to adhere to intestinal cells is a desirable quality, as this is the first step in colonization and may enable modification of the host immune system (Kimoto *et al.,* 1999). A number of probiotics have been shown to strongly adhere to human cell lines. Examples are, *Lb. casei* GG, *Lb. acidophilus* LA1, *Lb. plantarum* and a variety of *Bifidobacteria* (Kimoto *et al.,* 1999; Laura and Glenn, 2002). Studies have also demonstrated the ability of probiotic organisms to inhibit adherence by pathogenic organisms, such as enteropathogenic *Escherichia coli* and *Salmonella typhimurium* (Kimoto *et al.,* 2000). However, the mechanisms of adherence are still under investigation (Catherine *et al.,* 2005). Kimoto *et al.* (2000) investigated the ability of *Lactococcus* strain to adhere to human enterocyte-like Caco-2 cells. The study showed that the strains had excellent adherence properties which are important characteristics for any microorganism to be used as a probiotic.

2.5.2 Competition for nutrients and production of antimicrobial substances

Probiotic strains also inhibit pathogenic organisms by competing for the limited substrates required for fermentation and by secreting antimicrobial products called bacteriocins (Hurst *et al.,* 1983). For example, *Lb. acidophilus* has been shown to produce two compounds, bacteriocin lactacin B and Acidolin (Laura and Glenn, 2002). Lactacin B inhibits other *Lactobacilli in vitro*, whereas Acidolin inhibits enteropathogenic organisms (Shanahan and O'Sullivan, 2000). *Lactobacillus casei GG* also produces an inhibitory substance, with similar broad-spectrum activity (Jeanne *et al.,* 2005). Various studies have also illustrated the capability

of *L. lactis* bacteria strains to produce nisin (Linda *et al.,* 1992; Sirisansaneeyakul *et al.,* 2007). This is a broad-spectrum bacteriocin with enhanced antimicrobial activity against enteric pathogens. The bacteriocins produced by various bacteria give them a competitive advantage which therefore means that bacteriocin producers can colonize the gut easily and exhibit their probiotic effects.

2.5.3 Stimulation of mucosal and systemic host immunity

There is considerable evidence from animal studies that probiotic organisms can modulate the mucosal and systemic immune systems (Marteau *et al.,* 2007). This stimulation of host immunity is shown to relate to the ability of microorganisms to adhere to intestinal cells and interact with the gut-associated lymphoid tissue (GALT) (Perdigon *et al.,* 1990). Oliveira *et al.* (2005) gave 16 volunteers fermented milk supplemented with *Lb. acidophilus*, *Bifidobacterium BB12* and *Streptococcus thermophilus* for three weeks (wks), during which time they ingested attenuated *Salmonella typhi* Ty21a vaccine. They found that the specific serum IgA titre rise was significantly higher than in controls. This denoted an enhancement of the humoral immune response. Carmen *et al.* (2007) demonstrated stimulation of the innate immune system in 13 healthy subjects. They were given *Bifidobacterium lactis*, whereby their polymorphnuclear cell phagocytic capacity was enhanced significantly as compared to 12 controls. James *et al.* (2003) also studied the ability of probiotics to stimulate the immune system. In the study, 39 children with acute rotavirus diarrhoea were randomly assigned to receive *Lactobacillus GG* or a placebo milk product. They found an increased IgA specific antibody secreting cell response to rotavirus in the probiotic group, associated with a reduction in diarrhoea.

Microorganisms commonly used as probiotics include; *Lb. acidophilus, Lb. casei, Lb. delbruckii, Lb. crispatus, Lb. johnsonii, Bifidobacteria adolescentis, B. animalis, B. bifidum, B. breve, B. infantis, B. lactis, B. longum, Bacillus subtilis, Enterococcus, Leuconostoc mesenteroides, Pediococcus acidilactici and Saccharomyces boulardii* (O'May and MacFarlane, 2005).

2.6 Prevention of hypercholesterolemia

A number of microorganisms have been shown to bring hypocholesterolemia effects in both human and animal studies. These include *Lb. acidophilus* ATCC 43121, some strains of yeast, some *L. lactis* strains (from dairy products) and some strains of *Bifidobacterii* (Fabian and Elmadfa, 2006). Mann and Spoerry (1974) observed that men from the tribes of Samburu,

Rendile and Maasai warriors in Kenya showed a reduction in serum cholesterol levels. This was despite the fact that their main food is meat and milk. This was attributed to consumption of large amounts of milk fermented by a wild *Lactobacillus* strains. The warriors move over long distances in search of pasture and water. They are also known to chew various herbs as they eat meat and also genes might play a role in the low cholesterol levels apart from the consumption of the large contents of fermented milk. Several human studies have suggested a moderate cholesterol-lowering action of dairy products fermented with a certain strains of probiotic bacteria (Larsen *et al.,* 2000). However, the role of fermented milk products as hypocholesterolemic agents in humans is still equivocal, as the clinical studies performed have given variable data and no firm conclusions can be drawn. From several *in vitro* and *in vivo* studies a number of mechanisms have been proposed for the purported cholesterol-lowering action of probiotic bacteria;

2.6.1 Physiological actions of the end products of fermentation

Dietary carbohydrates, especially resistant starches and dietary fiber, are substrates for fermentation (Dora and Glenn, 2002). These produce short chain fatty acids (SCFAs) primarily acetate, propionate and butyrate, as end products (Jeanne *et al.,* 2005). The rate and amount of SCFA production depends on the species and amounts of microflora present in the colon, the substrate source and gut transit time (Ljungh and Wadstrom, 2006). Short chain fatty acids (SCFAs) are readily absorbed in the gut. Butyrate is the major energy source for colonocytes (Tanous *et al.,* 2007). The liver largely takes up propionate, while acetate enters the peripheral circulation to be metabolized by peripheral tissues (Nicole and Martin, 2006). Specific SCFA may reduce the risk of developing cancer and cardiovascular disease (Ingrid *et al.,* 2001). Acetate is the principal SCFA in the colon, and after absorption it has been shown to increase cholesterol synthesis but propionate inhibits cholesterol synthesis as shown in Figure 2.1 (Catherine *et al.,* 2005). Therefore, substrates that can decrease the acetate: propionate ratio may reduce serum lipids and possibly cardiovascular disease risks (Kim *et al.,* 2002). Butyrate has been studied for its role in nourishing the colonic mucosa and in the prevention of cancer of the colon, which is achieved by its ability to promote cell differentiation (Nicole and Martin, 2006). Therefore, a greater increase in SCFA production and potentially a greater delivery of SCFA, specifically butyrate, to the distal colon may result in a protective effect. Butyrate irrigation (enema) has also been suggested in the treatment of colitis (Joseph, 2004). Lactic acid bacteria

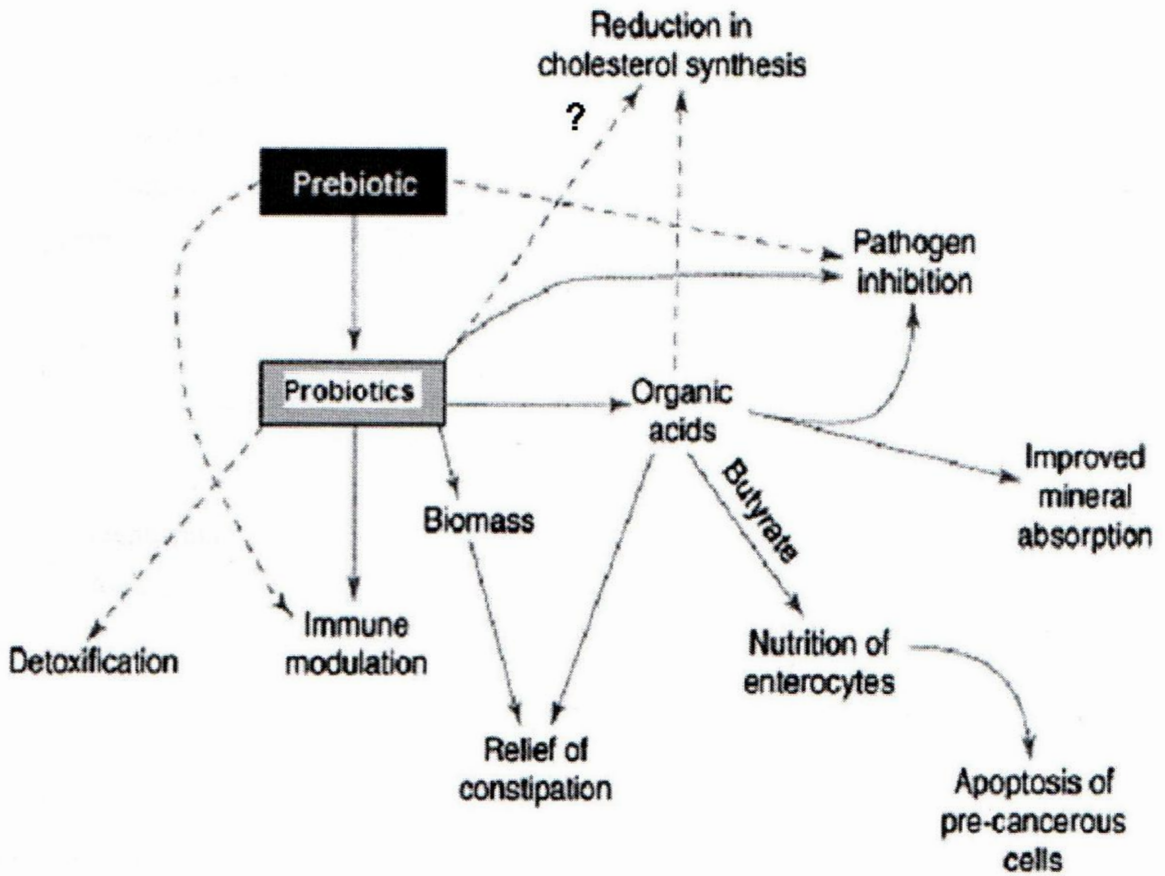
also produce exocellular polysaccharides (EPS) which are either excreted into the growth medium as free EPS or they adhere to the cell as capsular EPS (Kim *et al.,* 2002). These bind bile and cholesterol just like the plant polysaccharides making it unavailable for absorption into the blood (Gilliland and Buck, 1994).

2.6.2 Cholesterol assimilation by lactic acid bacteria

The bacterial degradation of cholesterol has been known to occur by a cholesterol oxidase (cholesterol: oxygen oxidoreductase, EC 1.1.3.6) (Pigeon *et al.,* 2002). This enzyme catalyses the oxidation of cholesterol to 4-cholesten-3-one, along with the reduction of oxygen to hydrogen peroxide (Gilliland and Buck, 1994). A variety of microorganisms producing cholesterol oxidase have been reported (Kimoto *et al.,* 1999). Some of the assimilated cholesterol is incorporated into cellular membrane (Gilliland and Buck, 1994). The ability of a strain of *Lb. acidophilus* to incorporate cholesterol from growth medium into cellular membranes during growth has been demonstrated (Noh *et al.* 1997). Cholesterol incorporated into or adhered to the bacterial cells is less available for absorption from the intestine into the blood (Kimoto *et al.,* 2002).

2.6.3 Enzymatic deconjugation of bile acids

Some strains of *Lb. acidophilus* have been found to secrete bile salt hydrolase (cholylglycine hydrolase; EC 3.5.1.24) (Gilliland *et al.,* 1998). This catalyzes the hydrolysis of glycine- or taurine-conjugated bile salts into amino acid residues and free bile salts. Free bile salts are less soluble than conjugated bile salts, resulting in lower absorption in the intestinal lumen (Liong and Shah, 2005a; Liong and Shah, 2005b). Deconjugation of bile acids reduces serum cholesterol levels by increasing the formation of new bile acids that are needed to replace those that have escaped the enterohepatic circulation (Corzo and Gilliland, 1999). Many researchers have found bile salt hydrolase activity in strains of lactic acid bacteria, including *Lb. acidophilus* (Oliveira *et al.,* 2005). Some microorganisms have been shown to produce enzymes that inhibit the production of Hydroxymethylglutaryl Co-enzyme A (HMG Co A) reductase which is required for the synthesis of cholesterol. These include various strains of *lactobacilli, Bifidobacterii* and *lactococci* (Psomas *et al.,* 2003). Figure 2.1 below gives a brief summary of the health effects derived from probiotics. The question mark (?) on the figure shows the gap that this study sought to solve.



Legend

? The problem the study sought to solve.

—► Health effects that have been satisfactorily studied and shown to be true.

┈► Need for more studies for sufficient scientific evidence.

Figure 2.1. Health effects of probiotics

Source: Catherine *et al.* (2005).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study area

Amaranthus hybridus was grown at Njoro Kenya Agricultural Research Institute (KARI) Research Station, Njoro District, Rift Valley Province, Kenya (Appendix 4). This is located between longitudes 35° 28' and 35° 36' East and latitudes 0° 1' and 1° 10' South, with daily temperatures ranging from 5° C to 28° C. The rain is eratic with an average of 600 to 900 mm annualy. The soil is sandy and volcanic with an average depth of 0.6 m.

3.2 Experimental Design

A completely randomized design (CRD) replicated two times at $\alpha = 0.05$ was used. For analysis, the data was subjected to Statistical Package for Social Sciences (SPSS).

3.3 Fermentation

Amaranth leaves were harvested at maturity (30 days) washed in distilled water and chopped into small pieces (1 cm^2). Then, 2.5% (w/w) NaCl (table salt) was added to the leaves, mixed thoroughly and packed gently into 3 2-kg fermentor jars (Appendix 5). The jars were then covered tightly to provide anaerobic conditions and fermentation went on at room temperature for 5 days. Fermentation progress was monitored by measuring the pH using a pH meter (Ezodo PL-500).

3.4 Isolation of *L. lactis* strain

The fermenting vegetables were used as a source to isolate LAB. Ten grams (10g) of each sample was homogenized with 90 ml of 0.85% (w/v) sterilized NaCl solution using a blender (Waring Blendor) for 5 min. Serial dilutions were prepared by transferring 1 ml of the suspension using a 1 ml pipette into a stoppered test tube containing 9 ml of buffered peptone water to make the first dilution with 10^{-1} concentration of the sample (dilution one). After thorough shaking of dilution one, 1 ml of the solution was again pipetted into the second test tube containing 9 ml buffered peptone water (Himedia) to make the second dilution (10^{-2} concentration). This was repeated up-to dilution 7 (10^{-7} concentration). Using a 1 ml pipette, 0.1 mls was drawn from the appropriate dilutions as shown in Table 3.1 below and pour plated using MRS (Himedia) agar and plate count agar (Himedia).

Table. 3.1 Dilutions plated

Day	Dilutions plated
0.	10^{-1} to 10^{-5}
1.	10^{-2} to 10^{-6}
2.	10^{-3} to 10^{-7}
3.	10^{-4} to 10^{-7}
4.	10^{-5} to 10^{-7}
5.	10^{-6} to 10^{-7}

The media was sterilized in an autoclave (2 ATO PROVET) at 121±1 ^{0}C for 15 minutes. For total viable count (TVC), PCA was used where incubation was done aerobically in an aerobic incubator (Carbolite pin30 (201)) for 24 hours at 30 °C. For the LAB the plates were incubated in an anaerobic incubator (Leec) at 30°C for 48 hours (hrs) and colonies that dissolved $CaCO_3$ forming clear zones around their own colonies on the medium plate were isolated randomly using a sterile wire loop. They were inoculated randomly into 10 ml M17 broth (Sigma). The broths inoculated with each colony were cultivated at 30°C and tested for catalase reaction. Briefly, the cultures were centrifuged in a centrifuge for 10 min at 5,400 x *g* and 4°C, and 2 mls 3% (v/v) H_2O_2 solution added to the pellets. Isolates were also identified through Gram staining, KOH reaction, and gas production from glucose, trehalose, sucrose, mannitol, fructose, maltotriose, arabinose, lactose and raffinose. *Lactobacillus (Lb.) acidophilus* ATCC 43121 was used as a positive control for cholesterol removal ability. Isolated *lactococcal* strains were maintained by subculturing into M17 broth (Sigma) supplemented with 0.5% (w/ v) glucose (GM17) for *Lactococci* or MRS broth for *Lb. acidophilus* and incubating them anaerobically at 30°C for *Lactococci* or at 37°C for *Lb. acidophilus* respectively for 18 hrs. The cultures were stored at 4°C between transfers in a fridge (Kelvator) and subcultured once before experimental use.

3.5 Cholesterol removal ability of *L. lactis* strains

Isolates identified as Gram-positive, catalase-negative cocci were tested for their cholesterol removal ability. Freshly prepared broth (10 ml) was put in stoppered test tubes. For *Lactococci* M17-THIO broth (M17 broth with 0.2% sodium thioglycollate-oxygen scavenger) was used and MRS-THIO broth for *Lb. Acidophilus*. The broth was then supplemented with

0.3% ox gall (as a bile salt). A filter-sterilized cholesterol solution was added to the broth to a final concentration of 70 µg/ml. The broth was then inoculated with 1% (v/v) of each strain's culture and incubated anaerobically by using GasPak anaerobic System for 24 hrs at 37°C. Although the optimum temperature of *Lactococci* is 30°C, these experiments were carried out at 37°C to simulate the conditions of the intestine. After every 3-hour interval, the cells were removed by centrifugation using a centrifuge for 10 min at 5,400 × *g* and 4°C. The remaining cholesterol concentration in each spent broth was then determined using colorimetric method.

The broth samples (0.5 ml) were placed into clean test tubes (duplicates for each sample), 3ml of 95% ethanol added to each tube, followed by 2 ml of 50% potassium hydroxide. The contents of all tubes were mixed thoroughly after addition of each component. Tubes were heated for 10 min in a 60°C water bath, and after cooling, 5 ml of hexane was dispensed into each tube. After mixing thoroughly and on setting for 20 s, 3 ml of distilled water was added, and the mixing repeated. The tubes were then allowed to stand for 15 min at room temperature to permit phase separation after which 2.5 ml of the hexane layer was transferred into a clean test tube. The hexane was evaporated from each tube at 60°C under the flow of nitrogen gas, followed by addition of 4 mls of O-Phthalaldehyde reagent into each tube. The tubes were allowed to stand at room temperature for 10 min, and then 2 ml of concentrated sulphuric acid was pipetted slowly down the inside of each tube. The contents of each tube were immediately mixed thoroughly. After standing at room temperature for an additional 10 min, the absorbance was read at 620 nm against a reagent blank using a spectrophotometer. Using the absorbance obtained, cholesterol amount was then determined from the standard curve. Uninoculated sterile broth was also analyzed.

Standard curve

The same procedure described above was used for the standard curve except that the following amounts of cholesterol were assayed in place of the samples: 0, 10, 20, 30, 40, 50, 60 and 70µg. The absorbance values (at 620 nm) were plotted against micrograms of cholesterol to get a straight-line graph (Appendix 2). This was used for determination of the cholesterol amounts in the spent broths as indicated above.

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3.6 Determination of proximate composition of *A. hybridus* leaves.

The proximate compositions of *A. hybridus* leaves before and after fermentation were determined in triplicates as given below on dry weight basis; sundrying was done by constantly exposing the leaves to sunlight for 3 days and turning of the vegetable leaves to avert fungal growth.

3.6.1 Crude proteins

Micro-Kjeldhal method (AOAC, 1995)

Micro-Kjeldhal method was used where 0.2 g of dried sample was weighed and placed into micro-kjeldhal digestion tubes. Into each tube of the sample, 10 ml of concentrated nitrogen free sulphuric acid were added and one selenium tablet used as catalyst for each case. The samples were then digested in a digester (Gallenhamp digester) at 445^{0}C for 3 hours. The digested samples were cooled to room temperature then distilled using kjeldhal distillation unit (Velp scientifica). The distillate was collected in a 15 ml 0.1M HCL in which a mixed indicator of methyl red and methylene blue had been added. The HCL was titrated against 0.1M NaOH. The calculations were as follows:

% crude protein = $\{\{(V_1 - V_2) \times (M \times 1.4 \times 6.25)\}/W\} \times 100$.

Where V_1 is volume of HCL used for blank test, V_2 is volume of HCL used for test portion, M is molarity of acid and W is weight of test portion.

3.6.2 Moisture content

Oven method (Method 14.003, AOAC, 1984)

The oven method was used where 2.0 g of samples were accurately weighed and transferred into aluminium dishes. The samples were dried in a dry air oven (Electrolux) at 105°C for 6 hours and cooled in a desiccator (Shandon) for 10 minutes. Weights were taken at intervals of 1 hour until a constant weight was achieved then calculations done as percentage for weight loss.

% Moisture content = {{(weight of original sample) – (weight of dry sample)}/{(weight of original sample)}} x 100

3.6.3 Ash content

Gravimetric method (Method 13.002, AOAC, 1995)

Gravimetric method was used where 2.0 g of sample were accurately weighed and placed into silica crucibles. The samples were ashed in a muffle furnace (Bie & Bertsen) at 550^{0}C for 3

hours. The ash was cooled in a desiccator to room temperature and weighed. Ash was calculated as a percentage of the dry sample. That is;

% Ash = {(weight of crucible + ash) – (weight of crucible)}/(weight of original sample) x 100

3.7 Sensory evaluation.

Sensory evaluation was done in a well lit room. Sixty three (63) untrained panelists who were potential consumers of the product (randomly selected) were recruited from the Dairy and Food Science and Technology Department. Potential panelists who were sick, below 15 years or above 55 years or were pregnant were excluded from the study. For comparison purposes, fresh boiled *A. hybridus* leaves were presented together with the fermented sample in identical containers coded with a three-digit random number. The panelists were asked to evaluate the general acceptability on a 9-point hedonic scale ranging from like extremely to dislike extremely, where 1 represented dislike extremely and 9 represented like extremely (Appendix 3). Samples were randomized for each panelist. Clean water at room temperature was provided to the panelist so that they could rinse their mouths before and between samples.

3.8 Statistical analysis

All the data was subjected to Statistical Package for Social Sciences (SPSS) computer package for analysis. A two way analysis of variance (ANOVA) was used to analyze the data at significance level $P \leq 0.05$. Completely randomized design (CRD) replicated two times at $\alpha = 0.05$ was used.

Table 3.2 Summary of data analysis

	Hypothesis	Independent variable	Dependent variable	Statistical analysis
Ho_1	There are no *L. lactis* bacteria in fermented *A. hybridus* leaves harvested at maturity.	*L. lactis bacteria* counts	Fermented *A. hybridus* leaves.	T-test
Ho_2	*L. lactis* does not remove cholesterol from the growth medium and there is no significant difference in the amounts of cholesterol removed from the growth media by *L. lactis* and *Lb. acidophilus*.	Probiotic activity	*L. lactis and Lb. acidophilus*	T-test ANOVA
Ho_3	Fermentation has no effect on protein, mineral and moisture content in amaranthus leaves	Protein, Mineral and moisture	Fermentation	T-test
Ho_4	There are no significant differences in the organoleptic quality between the fermented amaranthus leaves and the fresh boiled leaves.	Organoleptic quality	Fresh and fermented *A. hybridus* leaves	T-test

CHAPTER FOUR

RESULTS AND DISCUSSION

4.1 Microbial and physicochemical changes during fermentation

Lactic acid fermentation involves utilization of fermentable sugars by lactic acid bacteria with subsequent production of lactic acid and carbon dioxide among other by-products. This phenomenon is as a result of multiplication of the lactic acid bacteria facilitated by availability of fermentative substrate. Various physico-chemical changes also occur during fermentation. These changes in the microbial population and physical chemical properties in the fermentation of amaranthus leaves are summarized below (Figure 4.1);

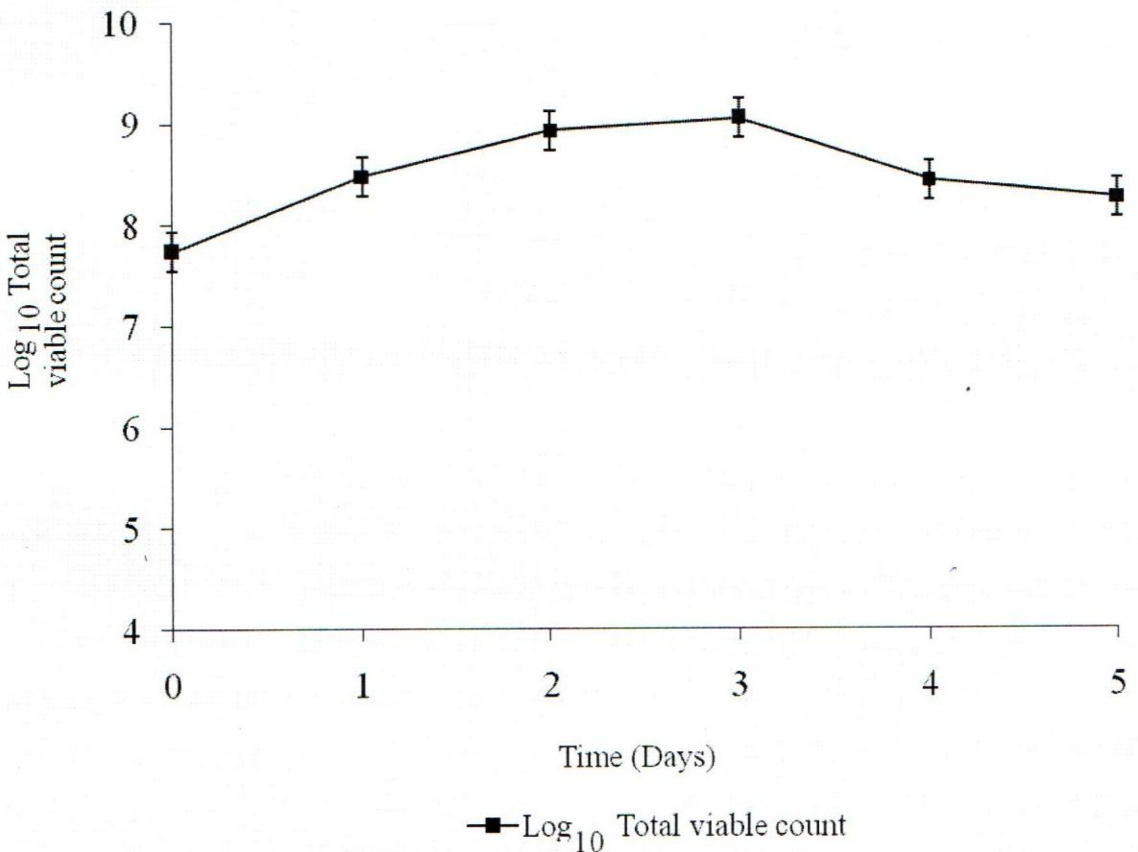


Figure 4.1. The growth of microorganisms during fermentation of *A. hybridus* leaves.

The maximum count for the study was 1.1 x 10^9 cfu/ml which was reached on the third day after which reduction was witnessed up to the last day of the study (day 5).

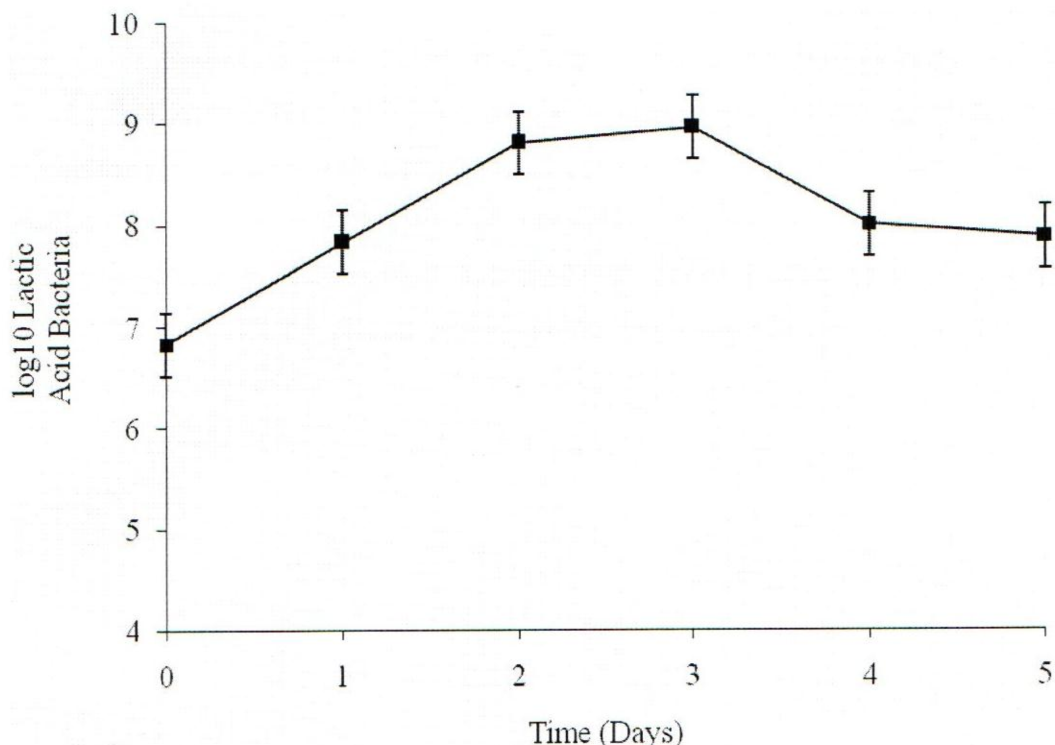


Figure 4.2. The growth of lactic acid bacteria during fermentation of *Amaranthus hybridus* leaves.

To isolate lactic acid bacteria, de Mann Rogosa Sharpe agar (MRS) was used. The colonies identified as lactic acid bacteria were then isolated and *L. lactis* identified from the isolates using, M17 broth. For this study the maximum growth of 8.9 x 10^8 cfu/ml was obtained on day three after which there was a decline up to the fifth day. From figures 4.1, 4.2 and Appendix 1, the maximum microbial population for both the total viable count and LABs was reached on the third day. This could be as a result of presence of various nutrients (soluble sugars) leaching out of exposed cut cells due to high osmotic pressure of the brine favouring their growth. This agrees with the findings by Sanchez *et al.* (2000) in which he found out that the highest microbial count in cabbage was reached on the third day, after which there was a decrease in the microbial population. After day 3 the decrease in microbial population could be

attributed to the decrease in fermentable substrates (glucose, fructose and sucrose) which serve as substrate for the microorganisms. The majority of organisms are dependent on nutrients for both energy and growth (Dora and Glenn, 2002). Organisms vary in their specificity towards different substrates and usually only colonize foods which contain the substrates they require (Peter, 2002). Sources of energy vary from simple sugars to complex carbohydrates and proteins (Parvathy and Puthuvallil, 2005). The energy requirements of micro-organisms are very high. Limiting the amount of substrate available thus checks their growth. Besides nutrients depletion, a change of growth environment that includes accumulation of waste products of metabolism could also be the cause of decrease in microbial populations (Parvathy and Puthuvallil, 2005).
Table 4.1 below shows the physiological and biochemical profile of lactic acid bacteria isolates from the *A. hybridus* leaves.

Table 4.1 Physiological and biochemical profile of lactic acid bacteria isolates from the *A. hybridus* leaves

Characteristic	A1	A2	A3	A4	A5	A6	A7
Growth at							
10°C	+ve	+ve	+ve	+ve	+ve	+ve	+ve
45°C	-ve	-ve	-ve	-ve	-ve	-ve	-ve
pH 9.6	+ve	+ve	+ve	-ve	+ve	-ve	-ve
Growth in							
4% NaCl	+ve	+ve	+ve	+ve	+ve	+ve	+ve
6.5% NaCl	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Gas Production							
Glucose	+ve	+ve	+ve	+ve	+ve	+ve	+ve
Sucrose	+ve	+ve	+ve	+ve	+ve	+ve	+ve
Trehalose	+ve	+ve	+ve	+ve	+ve	+ve	+ve
Mannitol	+ve	+ve	+ve	+ve	+ve	+ve	+ve
Maltotriose	+ve	+ve	+ve	+ve	+ve	+ve	+ve
Fructose	+ve	+ve	+ve	+ve	+ve	+ve	+ve
Raffinose	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Lactose	+ve	+ve	+ve	+ve	-ve	+ve	-ve
Arabinose	-ve	+ve	+ve	-ve	+ve	-ve	+ve
Gram stain test	+ve	+ve	+ve	+ve	+ve	+ve	+ve
Catalase test	-ve	-ve	-ve	-ve	-ve	-ve	-ve
KOH test	-ve	-ve	-ve	-ve	-ve	-ve	-ve

+ve: positive, -ve: negative, A1, A2, A3, A4, A5, A6 and A7: strain from *A. hybridus* leaves.

All isolated strains fermented glucose, trehalose, sucrose, mannitol, fructose, and maltotriose; but did not ferment raffinose. Most strains fermented lactose. Some strains fermented arabinose, but some strains did not. On the basis of these results, they were tentatively identified as *L. lactis*. The seven isolates (Table 4.1) were preliminary identified using Gram staining, KOH reaction,

catalase test and gas production from glucose. All the seven strains were Gram positive, catalase negative and KOH negative. Figure 4.3 below shows the pH profile of the amaranthus leaves.

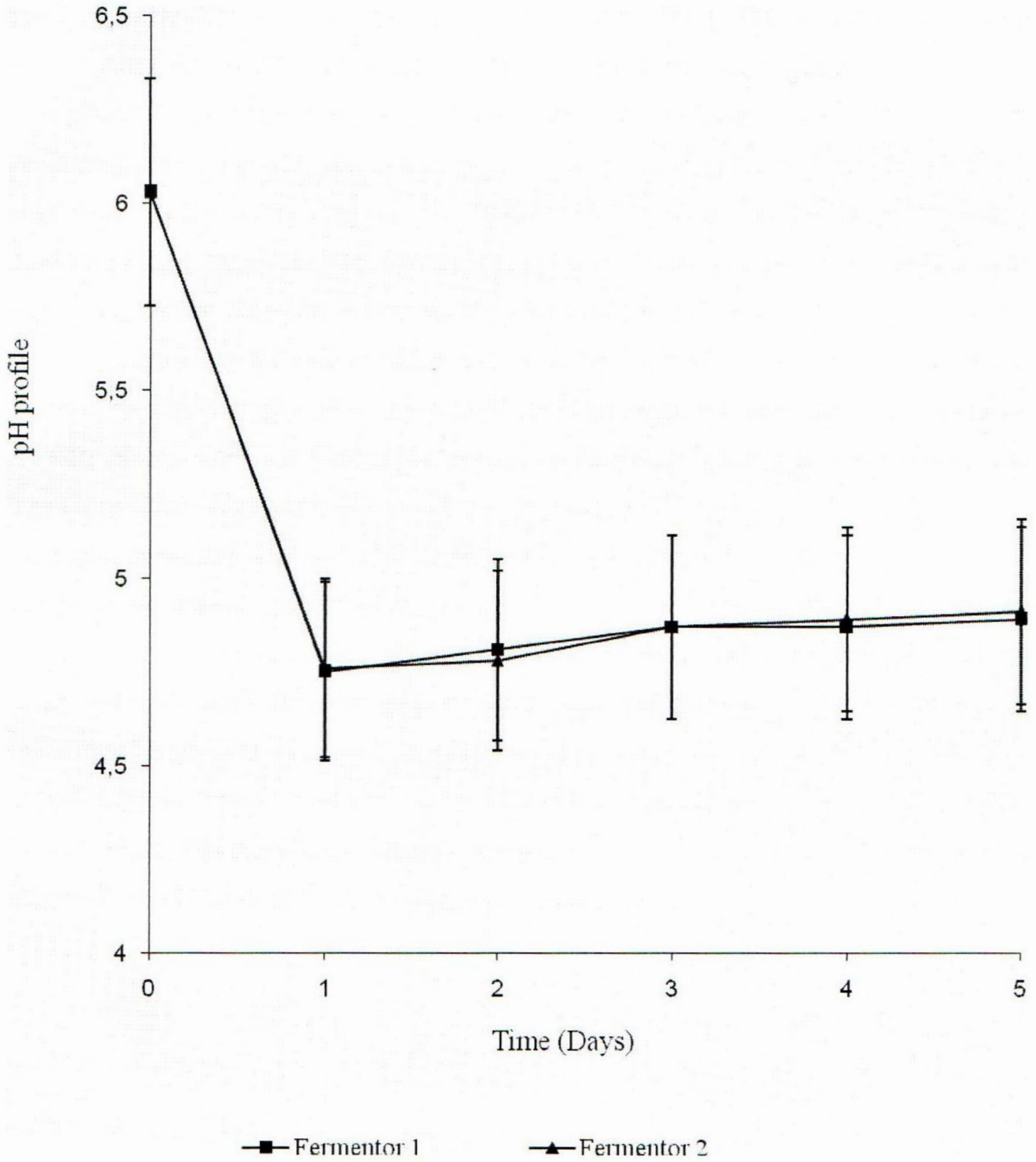


Figure 4.3. The pH profile during fermentation of *Amaranthus hybridus* leaves.

The fermentation progress of this study was monitored using the pH profile. The pH decreased rapidly from day 0 (6.03) to 4.75 on day one. After day one, the pH was stable to the last day of the study (Figure 4.3).

The pH decreased rapidly from 6.03 in day 0 to 4.7 on day 1 (Fig. 4.3). This was the lowest pH reached in this research. This could be due to a high concentration of fermentable sugars and a high initial microbial population as indicated by the total viable count 5.4×10^7 cfu/ml and the lactic acid bacteria populations of 6.7×10^6 cfu/ml. The sudden drop in the pH provides pH shock which grossly affects the survival of both the pathogenic and the spoilage organisms present in the vegetables. This makes the vegetable safe for consumption by even the immune challenged groups in the community given other documented health benefits of fermented food products (Sanchez *et al.,* 2000). This drop in the pH is as a result of multiplication of the LABs. After the first day the pH was almost constant. This could probably be due to a decrease in the fermentable sugars. However the pH values attained in this study were high as compared to the case of sauerkraut that attains a final pH of 3.4 to 4. This pH has been reported to suppress growth of spoilage microorganisms (Mutegi, 2002). According to Ayras (1980) cabbages have a high sugar content of the range 1.2 to 2.2% and this provides enough of fermentable sugars and thus a high percent lactic acid.

4.2 Cholesterol reduction abilities of *L. lactis* and *Lb. acidophilus*

Information recently published by the Lipid Research Clinics (Carmen, 2007) indicates that the higher the total serum cholesterol level is in humans, the greater the risk for development of coronary heart disease. Some studies have reported that ingestion of probiotics e.g. *Lb. acidophilus* has resulted in decreased serum cholesterol levels in humans (Dilmi, 2006) and animals (Gilliland and Buck, 1994). None of these studies has suggested that *L. lactis* isolated from plants products has this ability. Figure 4.4 below shows the manner of cholesterol removal from the growth media by both the *L. lactis* and *Lb. acidophilus.*

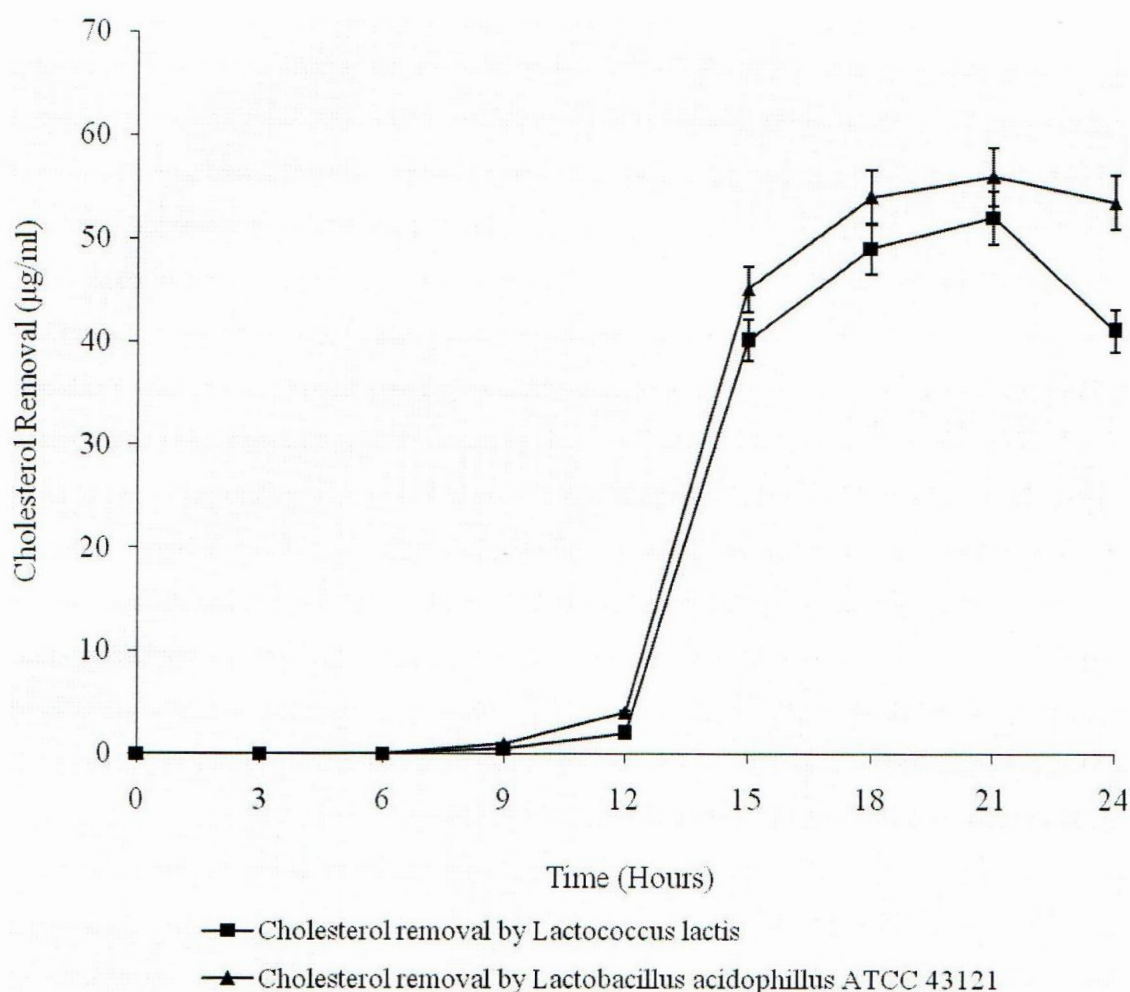


Figure 4.4. Cholesterol removal ability of *L. lactis* (■) and *Lb. acidophilus* (▲).

The amounts of cholesterol in the spent broth were plotted against time for a 24 h growth period (each value is the average from three trials). As time increased, the amount of cholesterol detected in the spent broth decreased (Fig. 4.4). In the 21st hour, both organisms assimilated maximum amounts of cholesterol with *L. lactis* and *Lb. acidophilus* ATCC 43121 assimilating 52 and 56 µg/ml respectively after which there was a decline in its reduction. Cholesterol removal from the growth media by both the test and the positive control were significant at $P < 0.05$. However, there was no significant difference at $P < 0.05$ in the amounts of cholesterol removed by *L. lactis* and *Lb. acidophilus* (positive control).

The manner of cholesterol removal by the two organisms corresponded to the manner of their growth as evident in Figure 4.4. The rapid cholesterol removal during 12 to 15 h of

incubation corresponded to exponential growth phase of the organisms thus, the fast cholesterol reduction rate witnessed during the same time. Kimoto *et al.* (2002) studying cholesterol removal from media by *Lactococci* concluded the same. This uptake of cholesterol occurs only when the culture is growing anaerobically in the presence of bile. The amount of bile required to enable the cultures to remove cholesterol from the growth medium is not in excess of the levels normally encountered in the intestines (Gilliland and Buck, 1994). Thus, the conditions required in the *in vitro* system for cholesterol uptake by these cultures would also be expected to occur in the intestinal tract. This should enable the organism to assimilate at least part of the cholesterol ingested in the diet, thus making it unavailable for absorption into the blood. A similar action could be exerted on endogenous cholesterol in the intestines. Thus, the reduction of cholesterol from growth media by microorganisms can be extrapolated to humans (Nicole and Martin, 2006). Studies on animal models (rat) have shown the ability of *L. lactis* isolated from animal products to reduce cholesterol and to survive in the rat GIT (Kimoto *et al.,* 2002). Further, the study also showed the need for ingestion of the organisms in order to keep them in adequate numbers so as to effect health benefits (e.g. cholesterol reduction) to the host. Likewise, for most probiotics, ingestion of the organisms continously is reguired. Therefore, amaranth leaves (fermented) should be consumed regularly.

4.3 Proximate composition of *A. hybridus* leaves

Proximate composition of any food is a reflection of its nutritive value. The higher the values of protein, soluble carbohydrates, crude fibre, ash and vitamins, the higher the nutritive value of that particular food. The proximate composition of amaranth leaves was determined and tabulated in Table 4.2 below.

Table 4.2. The proximate composition of amaranth leaves

Sample	Moisture (%)	Ash	Protein
Fresh dry amaranthus leaves	5.44 ± 1.5^{a}	19.76 ± 2.8^{a}	36.07 ± 1.2^{b}
Fermented dry amaranthus leaves	6.22 ± 1.7^{b}	36.21 ± 3.1^{b}	16.65 ± 1.4^{a}

Means in the same column followed by the same letter are not significantly different ($P < 0.05$)

Crude protein was significantly higher for fresh solar dried leaves than fermented solar dried leaves at $P < 0.05$. The moisture content was significantly higher for the fermented leaves at $P < 0.05$. Ash content for fermented amaranth leaves increased significantly at $P < 0.05$. This low protein value could be attributed to protein utilization by the fermentative bacteria, leaching of nutrients and draining away of brine that preceeds drying (Kasangi *et al.*, 2010). Moisture content was significantly higher for fermented leaves at $P < 0.05$ compared to fresh solar dried leaves (Table 4.2). The higher moisture content of fermented leaves could be attributed to the high content of single cell protein due to high microbial count. Ash content increased significantly ($P < 0.05$) from 19.76% for fresh solar dried leaves to 36.21% for the fermented leaves (Table 4.2). This could be attributed to addition of 2.5% sodium chloride at the start of fermentation (Sanchez *et al.*, 2000). Green leafy vegetables e.g. *A. hybridus* leaves are normally consindered to be a very good source of minerals and with fermentation, the mineral content tends to rise. This impilies that fermented *A. hybridus* leaves are a superior source of minerals as compared to fresh leaves. Thus, fermentation should be encouraged since, apart from the fermented vegetables being a source of probiotics they are also a good source of minerals.

4.4 Ranking tests for *A. hybridus* leaves based on general acceptability

In any processing of food product, the ultimate goal is to satisfy the consumer who is the most important in deciding the direction the processor will take. The acceptability of a food product is dependent on the ability of the processing method to preserve the natural quality of the food material as is acceptable to the consumer. Table 4.3 below gives the scores given by consumer panelists on the listed products.

Table 4.3 Ranking scores of amaranth leaves as given by panelists based on general acceptability.

Sample	Mean Score
Fresh, boiled amaranthus leaves	6.83 ± 1.9^{a}
Fermented amaranthus leaves	6.90 ± 2.0^{a}

N = 63

From this study there was no significant difference in the mean ranking scores for boiled and the fermented amaranth leaves at $P < 0.05$. However, most panelists associated the fermented leaves

with fermented sour flavour which the majority are not acquainted with. The sour flavour of the fermented leaves could be as a result of coexistence of high counts of LABs and yeasts that positively contribute to flavour (Sanchez *et al.,* 2000). Trail *et al.* (1996) observed that a high ratio of volatile to non-volatile acids has a positive effect on flavour of sauerkraut, which could be the case here. In some part of kenya, this vegetable is fermented after addition of cream from cow's milk to make it less sour. However, sourness due to fermentation is not a major problem in Kenya since majority of Kenyans are used to fermented food products. These fermented products include, porridge (made from cereals), *mursik* (traditional fermented milk), *mala*, yoghurt and cheese hence fermentation will not affect the consumption of *A. hybridus* leaves.

CHAPTER FIVE

CONCLUSIONS AND RECOMMENDATIONS

5.1 CONCLUSIONS

In this study, *L. lactis* strain was isolated successfully from fermented *A. hybridus* leaves harvested at maturity (30 days). Their potential in reduction of cholesterol from the growth media was tested and it was established that *L. lactis* has the same potential in cholesterol reduction as that of the control (*Lb. acidophilus* ATCC 43121). Thus, from this study, it can be concluded that *L. lactis* isolated from fermented *A. hybridus* can reduce cholesterol *in vitro*. The fermentation trends of amaranth leaves have been found to be similar to other studied fermented vegetables. However, the level of pH attained was higher (4.7) compared to other vegetables like cabbages with a pH level of 3.4, attained in sauerkraut fermentation. There was a decrease in the protein content after fermentation from 36.07 to 16.65%. There was an increase in the mineral content from 19.76 to 36.21% while moisture content in this research increased from 5.44 to 6.22%. For acceptability test, fresh amaranth scored 6.03 points while fermented scored 6.3. Thus, there was no significant difference in acceptability of fermented and fresh boiled amaranthus leaves at $P < 0.05$. This means that fermentation did not impart any undesirable changes except a sour taste, an attribute of fermented products.

5.2 RECOMMENDATION

1. Trials should be carried out in experimental animal models and N.O.A.L (No Observable Adverse Levels) determined for *L. lactis* with a view of adaptation to human nutraceutical foods.
2. Increase acceptability of the fermented amaranthus through coming up with various products.

REFERENCES

Aletor, O. R., Oshodi, A. A. and Ipinmoroti, K. 2002. Chemical composition of common leafy vegetables and functional properties of their leaf protein concentrates. Journal of Food Chemistry. **78**: 63–68.

Alexander, B., Patrick, W., Stéphane, M., Olivier, J., Karine, M., Jean, W., S. Dusko, E. and Alexei, S. 2001. The complete genome sequence of the lactic acid bacterium *Lactococcus lactis ssp. lactis* IL1403. Cold Spring Harbor Laboratory Press. **11(5)**: 731-753.

Amy, C. B. and Ana, V. M. 2004. Probiotics and medical nutrition therapy. Journal of Nutritional Clinical Care. **7(2)**: 56–68.

Ayras, J. C. 1980. Microbiology of foods. W.H. Freeman and Co. San Francisco.

Brooijmans, R. J., Poolman, B., Schuurman-Wolters, G. K., de Vos, W.M. and Hugenholtz, J. 2007. Generation of membrane potential by *Lactococcus lactis* through aerobic electron transport. Journal of Bacteriology. **(15)**: 123-125.

Carmen, C. M., Jussi M. and Seppo, S. 2007. *In vitro* analysis of probiotics strains inhibit pathogen adhesion to human intestinal mucus. Journal of International Food Research. **79**: 125-136.

Catherine, S., Paul, R. R., Gerald, F. F. and Douwe, S. 2005. Fermented functional foods based on probiotics and their biogenic metabolites. Current Opinion in Biotechnology. **16**: 198–203.

Corzo, G. and Gilliland, S. E. 1999. Measurement of bile salt hydrolase activity from *Lactobacillus acidophilus* based on disappearance of conjugated of bile salts. Journal of Dairy Science. **82**: 466–471.

De Rodas, B. Z., Gilliland, S. E. and Maxwell, C. V. 1996. Hypocholesterolemic action of *Lactobacillus acidophilus* ATCC 43121 and calcium in swine with hypercholesterolemia induced by diet. Journal of Dairy Science. **79**: 2121-2128.

Dhellot, J. R., Matouba E., Maloumbi M.G., Nzikou J.M., Ngoma D.G., Linder M., Desobry S. and Parmentier, M. 2006. Extraction, chemical composition and nutrional characterization of vegetable oils: Case of *Amaranthus hybridus* (var 1 and 2) of Congo Brazzaville. African Journal of Biotechnology, **5(11)**: 1095-1101

Dilmi, B. A. 2006. Assimilation (*in vitro*) of cholesterol by yoghurt bacteria. Annual Agriculture, Environment and Medical Journal, **13(1)**: 49-53.

Dora, A. P. and Glenn, R. G. 2002. Cholesterol assimilation by lactic acid bacteria and Bifidobacteria isolated from the human gut. International Journal of Applied and Environmental Microbiology, **68(9)**: 4689-4693.

Fabian, E. and Elmadfa, I. 2006. Influence of daily consumption of probiotics and conventional yoghurt on the plasma lipid profile in young healthy women. Journal of Nutrition, **50(4)**: 387-393.

Furgus, G. P. and Michael, G. 2000. Lactic Acid Bacteria. Applied Microbial Systems. Kluwer Academic Publishers. Dordrecht, The Netherlands. 367-388.

Gerber, S.D. and Solioz, M. 2007. Efficient transformation of *Lactococcus lactis* IL1403 and generation of knock-out mutants by homologous recombination. Journal of Basic Microbiology, **(47)**: 281-286.

Gilliland, S. E. and Buck, L. M. 1994. Comparisons of freshly isolated strains of *Lactobacillus acidophilus* of human intestinal origin for ability to assimilate cholesterol during growth. Journal of Dairy Science, **77**: 2925-2933.

Gilliland, S. E., Buck, L. M. and Brashear, M. M. 1998. Bile salt deconjugation and cholesterol removal from media by *Lactobacillus casei*. Journal of Dairy Science, **81**: 2103-2110.

Grahn, E., Holm, S. E., Lilja, H. and Sellgren, K. 1994. Interference of a *Lactococcus lactis* strain on the human gut flora and its capacity to pass the stomach and intestine. Scandinavian Journal of Nutrition, **38**: 2–4.

Gruzza, M., Duval, I. Y. and Ducluzeau, R. 1992. Colonization of the digestive tract of germ-free mice by genetically engineered strains of *Lactococcus lactis*: Study of recombinant DNA stability. Microbial Releases. **1**: 165–171.

Hols, P., Kleerebezem, M., Schanck, A., Ferain, T., Hugenholtz, J., Delcour, J. and de Vos, W.M. 1999. Conversion of *Lactococcus lactis* from homolactic to homoalanine fermentation through metabolic engineering. Nature Biotechnology. **(17)**: 588-592.

Hurst, A., Branen, A. L. and Davidson, P. M. 1983. Antimicrobials in Foods. Nisin and other Inhibitory Substances from Lactic Acid Bacteria.. Marcel Dekker, Inc., New York. Pp 327-351.

Iheanacho, M. E. and Udebuani, A. C. 2009. Nutritional Composition of Some Leafy Vegetables Consumed in Imo State, Nigeria J. Appl. Sci. Environ. Manage. **13**(3) 35 – 38.

Ingrid, W., Gerhard, R. and Beatrice, L. 2001. Protective role of probiotics and prebiotics in colon cancer. American Journal of Clinical Nutrition. **73**: 451–455.

James, G., Julie, M., Glory, M. and Terese, F. M. 2003. African traditional leafy vegetables and the urban and peri-urban poor. Food Policy. **28**: 221–235.

Jeanne, D., Bette, B., Matthew H. and Richard, M. 2005. Treating irritable bowel syndrome with a food elimination diet followed by food challenge and probiotics. Journal of Nutrition. **135**: 1752-1756.

Joseph, R. 2004. The effects of probiotics on colon cancer development. Nutrition Research Reviews. **17**: 277–284.

Kasangi, D. M. 2010. Fermentation of cowpea leaves as a method of food preservation and improvement of food security. International Food Research Journal. **7**: 721-732.

Katuriina, M. W. 2000. Wild vegetables used by the Sambaa in the Usambara Mountains N.E. Tanzania. Fenninci Annuall Botanical Journal. **37**: 57-67.

Kelly, W. J., Davey, G. P. and Ward, L. J. H. 1998. Characterization of *Lactococci* isolated from minimally processed fresh fruit and vegetables. International Journal of Food Microbiology. **45**: 85-92.

Kim, K. P., Rhee, C. H. and Park, H. D. 2002. Degradation of cholesterol by *Bacillus subtilis* SFF34 isolated from Korean traditional fermented flatfish. Letters in Applied Microbiology. **35**: 468–472.

Kimoto, H., Kurisaki, J., Tsuji, N. M. Ohmomo, S. and Okamoto, T. 1999. *Lactococci* as probiotic strains: Adhesion to human enterocyte-like Caco-2 cells and tolerance to low pH and bile. Letters in Applied Microbiology. **29**: 313–316.

Kimoto, H., Nomura, M., Kobayashi, M., Okamoto, T. and Ohmomo, S. 2004. Identification of probiotic characteristics of *Lactococcus* strains from plant materials. Letters in Applied Microbiology. **38 (2)**: 111-117.

Kimoto, H., Ohmomo, S. and Okamoto, T. 2002. Cholesterol removal from media by *Lactococci*. Journal of Dairy Science. **85**: 3182–3188.

Kimoto, H., Ohmomo, S., Nomura, M., Kobayashi, M. and Okamoto, T. 2000. *In vitro* studies on probiotic properties of *Lactococci*. Milchwissenschaft. **55(5)**: 245-249.

Klaenhammer, T. R. and Kullen, M. J. 1999. Selection and design of probiotics. International Journal of Food Microbiology. **50**: 45– 57.

Klijn, N., Weerkamp, A. H. and de Vos, W. M. 1995. Genetic marking of *Lactococcus lactis* shows its survival in the human gastrointestinal tract. Applied and Environmental Microbiology. **61**: 2771-2774.

Larsen, L. A., Raben, A., Haulrik, N., Hansen, A. S., Manders, M. and Astrop, A. 2000. Effect of 8-week intake of probiotic milk products on risk factors for cardiovascular diseases. European Journal of Clinical Nutrition. **54**: 288–297.

Laura, J. F. and Glenn, R. G. 2002. *In vitro* investigations of the effect of probiotics and prebiotics on selected human intestinal pathogens. Journal of Microbiology Ecology. **39**: 67-75.

Linda, J. H., Henry, P. F. and Todd, R. K. 1992. Characterization of two nisin-producing *Lactococcus lactis subsp. lactis* strains isolated from a commercial sauerkraut fermentation. Journal of Applied and Environmental Microbiology. **58(5)**: 1477-1483.

Liong, M. T. and Shah, N. P. 2005a. Acid and bile tolerance and cholesterol removal ability of *Lactobacilli* strains. Journal of Dairy Science. **88**: 55–66.

Liong, M. T. and Shah, N. P. 2005b. Optimization of cholesterol removal by probiotics in the presence of prebiotics by using a response surface method. Journal of Applied and Environmental Microbiology. **71(4)**: 1745–1753.

Ljungh, A. and Wadstrom, T. 2006. Lactic acid bacteria as probiotics. Journal of Current Issues on Intestinal Microbiology. **7(2)**: 73-89.

Mainville, I., Arcand, Y. and Farnworth, E. R. 2005. A dynamic model that simulates the human upper gastrointestinal tract for the study of probiotics. International Journal of Food Microbiology. **99**: 287– 296.

Mann, G. V. and Spoerry, A. 1974. Studies of a surfactant and cholesterolemia in the Maasai. American Journal of Clinical Nutrition. **27**: 464–469.

Marteau, M., Christophe, C. J., Philippe, R. and Jürgen S. 2007. Protection from gastrointestinal diseases with the use of probiotics. American Journal of Clinical Nutrition. **73**: 430–436.

Marteau, P., Seksik, P. and Jian, R. 2002. Probiotics and health: New facts and ideas. Current Opinion in Biotechnology. **13(5)**: 486-489.

McNaught, C. E. and MacFie, J. 2001. Probiotics in clinical practice: a critical review.

Mutegi C. K. 2002. Preservation of kale by lactic fermentation and solar drying. Msc. Thesis, University of Nairobi, Nairobi.

Mwangi, D. 2003. Introduction to Grain Amaranth. Joypet Services and Printers, Nairobi. 80-102.

Myers, R. L. 1998. Nitrogen fertilizer effect on grain amaranth. Agronomy Journal. **90(5)**: 597-602.

Nakarai, T., Morita, K., Nojiri, Y., Nei, J. and Kawamori, Y. 2000. Liver abscess due to *Lactococcus lactis* subspecies cremoris. Pediatrics International. **42(6)**: 699-701.

Ndegwa, O. C. and Aagaard, H. J. 2003. Traditional gathering of wild vegetables among the Luo of Western Kenya-a nutritional anthropology project 1. Ecology of Food and Nutrition. **42**: 78-89.

Nicole, M. and Martin, B. K. 2006. Effects of probiotic bacteria on diarrhoea, lipid metabolism, and carcinogenesis: A review of papers published between 1988 and 1998. American Society for Microbiology. **4(1)**: 425-434.

Noh, D. O., Kim, S. H. and Gilliland, S. E. 1997. Incorporation of cholesterol into the cellular membrane of *Lactobacillus acidophilus* ATCC 43121. Journal of Dairy Science. **80**: 3107-3113.

Ole, H., Torben, L., Lars, P. C., Ulla, K., Nazmul, H. and Shakuntala, H. T. 2004. Contents of iron, calcium, zinc and β-carotene in commonly consumed vegetables in Bangladesh. Journal of Food Composition and Analysis. **17**: 587–595.

Oliveira, A.P., Nielsen, J. and Förster, J. 2005. Modeling *Lactococcus lactis* using a genome-scale flux model. BMC Microbiology. **(5)**: 39.

O'May, G. A. and MacFarlane, G. T. 2005. Probiotic efficacy: Are the claims justified? In Probiotic Dairy Products. Blackwell Publishing, London. Pp 138–166.
of the evidence. Journal of Nutrition Research. **21**: 343–353.

Parvathy, S. N. and Puthuvallil, K. S. 2005. Biochemical characterization of lactic acid bacteria isolated from fish and prawn. Journal of Culture Collections. **4**: 48-52.

Perdigon, G., Alvarez, S. and de Macias, M. E. 1990. The oral administration of lactic acid bacteria increases the mucosal intestinal immunity in response to enteropathogens. Journal of Food Protection, **53**: 404–410.

Peter, J. J. 2002. Clinical nutrition: 7. Functional foods-more than just nutrition. Canadian Medical Association Journal. **166 (12)**: 55-57

Peter, K., Mary, C. and Nathan, W. L. 2006. Intestinal bacterial microflora: A potential source of chronic inflammation in patients with chronic kidney disease. Journal of Nephrology Dialysis Transplantation. **21(8)**: 2057-2060.

Pigeon, R. M., Cuesta, E. P. and Gilliland, S. E. 2002. Binding of free bile acids by cells of yogurt starter culture bacteria. Journal of Dairy Science. **85**: 2705–2710.

Psomas, E. I., Fletouris, D. J. Litopoulou, T. E. and Tzanetakis N. 2003. Assimilation of cholesterol by yeast strains isolated from infant feces and feta cheese. Journal of Dairy Science. **86**: 3416–3422.

Sanchez, A.H., Montano, A., Castro, A. and Rejano, L. 2000. Comparative study on chemical changes in olive juice and brine during green olive fermentation. Journal of Agriculture & Food Chemistry. **48**: 5975-5980. Scandinavian Journal of Nutrition. **48 (1)**: 32-36.

Sean, B. H, Andrew, T. C., Hitchin, E. and Jerry, M. W. 2006. Mucosal delivery of a Pneumococcal vaccine using *Lactococcus lactis* affords protection against respiratory infection. The Journal of Infectious Diseases. **195**: 185-193.

Shanahan, F. and O'Sullivan, G. C. 2000. Genes, bacteria and T cells: ingredients for inflammatory bowel disease. Gastroenterology. **115**: 1595–1600.

Sirisansaneeyakul, S., Luangpipat, T., Vanichsriratana, W., Srinophakun, T., Chen, H.H. and Chisti, Y. 2007. Optimization of lactic acid production by immobilized *Lactococcus lactis*. Journal of Industrial Microbiology & Biotechnology. **(34)**: 381-391.

Tanous, C., Chambellon, E. and Yvon, M. 2007. Sequence analysis of the mobilizable *Lactococcal* plasmid pGdh442 encoding glutamate dehydrogenase activity. Microbiology. **(153)**: 1664-1675.

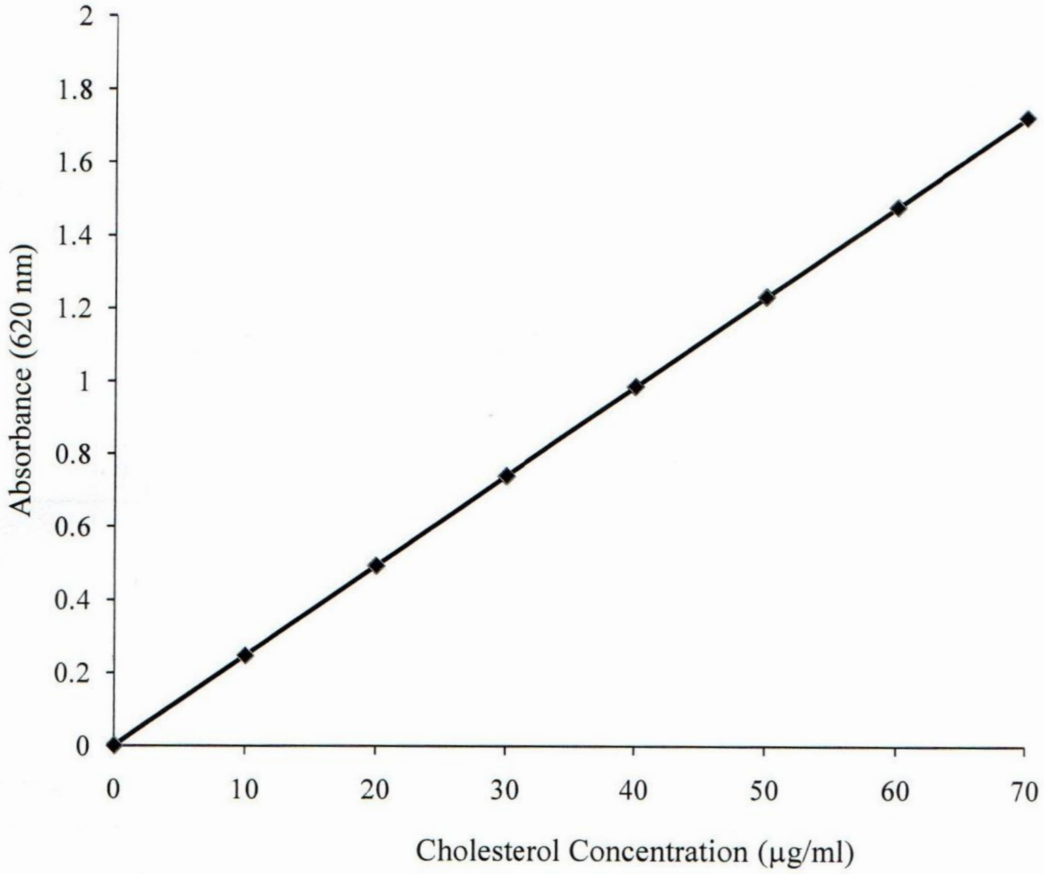
Trail, A. C., Fleming, H. P., Young, C. T. and Mcfeeters, R. F. 1996. Chemical and sensory characterization of commercial sauerkraut. Journal of Food Quality. **19**: 15-30.

APPENDICES

Appendix 1. Total viable count and lactic acid bacteria growth on PCA and MRS respectively.

	Colony Forming Units (C.F.U)	
Day	Total Viable Count (TVC)	Lactic Acid Bacteria (LAB)
0	5.5×10^7	6.8×10^6
1	3.0×10^8	6.9×10^7
2	8.4×10^8	6.4×10^8
3	1.1×10^9	9.0×10^8
4	2.7×10^8	1.0×10^8
5	1.8×10^8	7.6×10^7

Appendix 2. Cholesterol standard curve.



Appendix 3. Questionnaire for preference test ranking (hedonic scale - scoring analysis).

The following was the questionnaire used to score the samples based on general acceptability.

PANELIST NUMBER………………. DATE…………………………….

Taste these samples and check how much you like or dislike each one. Use the appropriate scale to show your attitude by checking at the point that best describes your feeling about the sample. Please give a reason for this attitude. Remember you are the only one who can tell what you like. An honest expression of your personal feeling will help.

CODE	XYZ			ABC					
Like extremely									
Like very much									
Like moderately									
Like slightly									
Neither like or dislike									
Dislike slightly									
Dislike moderately									
Dislike very much									
Dislike extremely									
Comment									

Where, XYZ is fermented *A. hybridus* while ABC is fresh boiled *A. hybridus*.

Appendix 4. *Amaranthus hybridus* plants growing at KARI-Njoro.

Appendix 5. *Amaranthus hybridus* leaves fermented in the food microbiology lab in the Dairy and Food Science and Technology Department of Egerton University, Egerton.

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