

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

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ABSTRACT

Probiotics are live microbial feed supplements, which positively affect the host animal by improving its intestinal microbial balance. Studies have shown probiotic activities of *Lactococci* isolated from dairy foods, which include the ability to inhibit the growth of other bacteria and the reduction of cholesterol. However, there is limited documented work on the probiotic activity in *Lactococci* from plant materials. The present study isolated and tested cholesterol reduction ability (*in-vitro*) of *Lactococcus lactis* isolates from fermented smooth pigweed (*Amaranthus hybridus*) leaves. The specific objectives were to: isolate *L. lactis* bacteria from *A. hybridus* leaves harvested at maturity (30 days), determine cholesterol reduction ability of *L. lactis* isolated from the *A. hybridus* leaves and establish whether there were any differences in the amounts of cholesterol reduced from the growth media by *L. lactis* and *Lactobacillus acidophilus* ATCC 43121 (the positive control). It further aimed at determining whether fermentation affected 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 in Njoro. The leaves were harvested at maturity and fermented for five days. After fermentation, *L. lactis* strains were isolated and their ability to remove cholesterol from the growth medium tested. This ability was compared with that of *Lb. acidophilus* ATCC 43121. Consumer acceptability of the fermented leaves was also compared to freshly boiled leaves. The *Lactococcal* strains isolated reduced cholesterol level by 52 µg/ml and *Lb. acidophilus* ATCC 43121 reduced by 56 µg/ml indicating a similar reduction capability ($p < 0.05$) to that of the standard *Lb. acidophilus* ATCC 43121 probiotic. 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 ($p < 0.05$) in consumer acceptability whereby, the consumers scored 6.90 points for fermented leaves compared to av6.83 points scored by consumers for fresh boiled leaves. This study concluded that fermented amaranthus leaves dish is a potential source of probiotics as the level of cholesterol reduction by the isolated *Lactococcal* strains compares favourably with the reduction levels of the control *Lb. acidophilus* ATCC 43121 which is a known probiotic.

Key words: Probiotic, Cholesterol, *Lactococcus lactis*, *Amaranthus hybridus*, *Lactobacillus acidophilus* ATCC 43121

INTRODUCTION

Green leafy vegetables have for long been recognized as an affordable and abundant potential source of vitamins, minerals and protein [1]. This is because of their ability to utilize a wide range of virtually unlimited and readily available primary materials: water, CO₂, atmospheric nitrogen and sunlight [2]. For example, cassava leaves, depending on the variety, are rich in protein (14–40% of dry matter), minerals, vitamins B1, B2, C and carotenoids [1]. However, with the advent of modern farming, these plants are facing extinction and as a result food insecurity may worsen. Currently, the majority of Kenyans (especially town dwellers) are concentrating on 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 for example *Solanum nigrum* (black nightshade), *A. hybridus* (Smooth Pigweed) and *Cucurbita maxima* (pumpkin) leaves [3, 4]. Some of these vegetables are fermented and consumed in meals which make them a potential source of probiotics.

Probiotics are live microorganisms, which when administered in adequate amounts can confer a health effect on the host [5, 6]. For a microorganism to achieve probiotic status, it must have a demonstrable benefit for the host, be alive when administered, and be non-pathogenic. Fermented dairy products are the most widely used food vehicles for probiotic bacteria, but other forms of delivery include lyophilized form, tablets, capsules, sprays, chewing gums, lozenges. The ability to reduce serum cholesterol levels, antimicrobial substrate production and immune modulation are considered as effective properties [6]. Lactic acid bacteria (LAB) consist of twenty genera such as *Lactobacilli*, *Leuconostoc*, *streptococci* and *Lactococci* [2, 7]. The most widely used probiotic bacteria are *Lactobacilli* and *Bifidobacteria* and extensive studies on their beneficial effects on human health have been reported [8]. 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 (GIT) [9]. Several studies have, however, shown the possibility of the presence of *Lactococci* in human or animal GIT [2].

Lactococci can also be found in milk and milk products, plant materials (fermented vegetables and fruits) and intestines of fish. Probiotic activities of *Lactococci* isolated from dairy foods include the ability to inhibit the growth of other bacteria and cholesterol removal from growth media [9]. However, few studies have been published concerning the probiotic activity of *Lactococci* from plant materials [10]. One of the beneficial health effects related to probiotics is their ability to reduce serum cholesterol levels. A culture of *Lb. acidophilus* actively taking up cholesterol from growth media would function *in vivo* to exert a hypocholesterolemic effect [11, 12]. For example, *Lb. acidophilus* ATCC 43121 can incorporate some of the cholesterol removed from media into the cellular membrane during growth [13]. This property has beneficially influenced serum cholesterol levels in pigs [14]. 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* [15]. However, few such studies have been published concerning this ability by *Lactococci* from plant materials [16].

The use of probiotics as a biological procedure of cholesterol reduction is increasing rapidly although most probiotic products in the market are derived from animal products, mainly milk. These products are expensive, thus, probiotics from plant materials such as amaranthus will be a promising remedy in cholesterol reduction. This is because plants probiotics are better priced and readily available in most parts of Kenya. The study had the overall aim of isolating and testing the cholesterol reduction ability (*in vitro*) of *L. lactis* from fermented smooth pigweed (*A. hybridus*) leaves. The specific objectives of this study were to: (a) isolate *L. lactis* bacteria from *A. hybridus* leaves harvested at maturity (30 days), and (b) determine cholesterol reduction ability of *L. lactis* isolated from *A. hybridus* leaves harvested at maturity (30 days) and whether there were any differences in the amounts of cholesterol removed from the growth media of *L. lactis* and *Lb. acidophilus* 43121 (the positive control). It further sought to establish whether fermentation has any influence on the protein, mineral and moisture content of amaranthus leaves and also the consumer acceptability of the fermented amaranthus leaves as compared to fresh boiled leaves.

MATERIALS AND METHODS

Study Area

Amaranthus hybridus was planted in a field within the Kenya Agricultural Research Institute in Njoro Kenya, whose location lies at 0° 22 S, 35° 56 E and at 2267M above sea level. The field was used because of the available infrastructure and closeness to the university research laboratory. The daily temperatures ranged from 22° C to 28° C. The rain is erratic with an average of 600 to 900 mm annually. The seeds used in the study were obtained from the Kenya Seed Company, Nakuru and no fertilization was done. The experimental design used was a completely randomized design at $\alpha = 0.05$ as the experiment was conducted at two different locations within the KARI farms in Njoro. The location was considered a random effect to enable make inferences toward a larger area of what might be expected to occur in the Njoro region. Treatments were considered to be a sample of the harvested leaf population to which inferences could be made. The leaves were harvested fresh after 30 days of growth from germination. Only the tender young leaves were harvested during the study. The samples were packed loosely in high-density polyethylene (HDPE) bottles which were leak proof and chemical resistant. HDPE bottles feature heavy duty walls that resist splitting and puncturing and also minimised heat build up.

Fermentation

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

meter [17]. A time interval of 5 days was selected to simulate the time frame used in traditional fermentation.

Isolation of *L. lactis* Strain

After a time interval of 5 days based on the traditional processing time, the fermented vegetables were used as a source to isolate Lactic acid bacteria (LAB). The isolation of the lactic acid strains was done as described by Kimoto [16]. This entailed obtaining one g of each sample, which was then homogenized with 9 ml of 0.85% (w/v) sterilized NaCl solution using a Waring Blender 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, Himedia Laboratories Mumbai, India) to make the second dilution (10^{-2} concentration). This was repeated up to dilution 7 (10^{-7} concentration). From the appropriate dilutions, 0.1 mls were drawn and pour plated using de Man, Rogosa and Sharpe (MRS) and plate count agar (Himedia, Himedia Laboratories Mumbai, India). The MRS medium is designed to favour the luxuriant growth of *Lactobacilli* for laboratory study. The media was sterilized in an autoclave (Ato Provert, Czech Republic) at 121 ± 1 °C for 15 minutes. For total viable count, Plate count agar (Himedia, Himedia Laboratories Mumbai, India) was used where incubation was done in an aerobic incubator (Carbolite pin30 (201)) for 24 hrs at 30 °C. For the LAB, the plates were incubated in an anaerobic incubator (Leec, Czech Republic) at 30°C for 48 hours 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 MRS broth (Sigma, UK). The broths which were inoculated with each colony were cultivated at 30°C and tested for catalase reaction. The cultures were centrifuged for 10 min at $5,400 \times g$ and 4 °C, and 2 mls of 3% (v/v) H_2O_2 solution added to the pellets. Isolates were identified through gram staining, KOH reaction, catalase test and gas production from glucose, trehalose, sucrose, mannitol, fructose, maltotriose, arabinose, lactose and raffinose. Isolated strains were maintained in nutrient agar (NA). *Lactobacillus (Lb.) acidophilus* ATCC 43121 was used as a positive control for reduction of cholesterol. Isolated *lactococcal* strains (using M17 agar) were maintained by subculturing into M17 broth (Sigma, UK) supplemented with 0.5% (w/v) glucose (GM17) for *Lactococci* or MRS broth for *Lb. acidophilus* and incubating them at 30 °C for *Lactococci* or at 37 °C for *Lb. acidophilus* for 18 hrs. The cultures were stored at 4 °C between transfers in a fridge (Kelvator, UK) and subcultured before being used in the cholesterol reduction trials.

Cholesterol Removal Ability of *L. lactis* Strains

Isolates identified as Gram-positive, catalase-negative cocci were tested for their cholesterol removal ability. M17-THIO broth (M17 broth with 0.2% sodium thioglycollate-oxygen scavenger) was prepared as per the manufacturer and used to resuscitate *Lb. acidophilus*. The broth was then supplemented with 0.3% ox gall (as a bile salt). A filter-sterilized cholesterol solution (10 mg/ml in ethanol) 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 a GasPak anaerobic

System for 24 hrs at 37 °C. Although the optimum growth temperature of *Lactococci* is 30°C, these experiments were carried out at 37 °C to simulate the conditions in the intestine. After 24 hrs, cells were removed by centrifugation for 7 min at 5,400 × g and 4 °C. The remaining cholesterol concentration in each spent broth was determined colorimetrically as described below:

The broth samples (0.5 ml) were placed into clean test tubes (duplicates for each sample), 3 ml of 95% ethanol added to each tube, followed by 2 ml of 50% potassium hydroxide. The contents of all tubes were mixed thoroughly after the 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 20s, 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 the 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 and the cholesterol amount was then determined from the standard curve. Uninoculated sterile broth was also analyzed [12].

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. This was used for the determination of the cholesterol amounts in the spent broths as indicated above [18].

Determination of crude protein, moisture and ash content of *A. hybridus* Leaves

The crude protein, moisture and ash content 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 over of the vegetable leaves to avert fungal growth.

Crude Proteins: Micro-Kjeldhal Method

A dried sample (0.2g) was weighed, placed into micro-kjeldhal digestion tubes and 10 mL of concentrated nitrogen free sulphuric acid were added into each tube with one selenium tablet used as catalyst for each tube. The samples were then digested in a digester (Gallenhamp digester) at 445 °C for 3 hours. The digested samples were cooled to room temperature, then distilled using kjeldhal distillation unit (Velp scientifica, Italy). The distillate was collected in 15 mL of 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:

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

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.

Moisture Content: Oven Method

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 as follows:

% Moisture content = $\{(\text{weight of original sample}) - (\text{weight of dry sample}) / (\text{weight of original sample})\}$

Ash Content: Gravimetric Method

A 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 & Bertsens) at 550°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 = $\{(\text{weight of crucible} + \text{ash}) - (\text{weight of crucible}) / (\text{weight of original sample})\} \times 100$

Sensory Evaluation

The fresh *A. hybridus* leaves were coded as XYZ while fermented leaves were coded as ABC. A total of 63 consumer panelists were served with the samples and then asked to rank the samples depending on general acceptability. A nine-point hedonic scale was provided on which panelists were to score depending on their preference. Where 1 represented *dislike extremely* and 9 represented *like extremely* [19].

Analysis of Data

A two way analysis of variance (ANOVA) was used to analyse the data at significance level $P < 0.05$. Completely randomized design (CRD) replicated twice at $\alpha = 0.05$ was used. The statistical package for social sciences (SPSS) computer package for analysis was used.

RESULTS

The maximum total viable count for the study was 1.1×10^9 cfu/ml (table 1, figure 1) which was reached on the third day after which a reduction was witnessed up to the last day of the study (day 5).

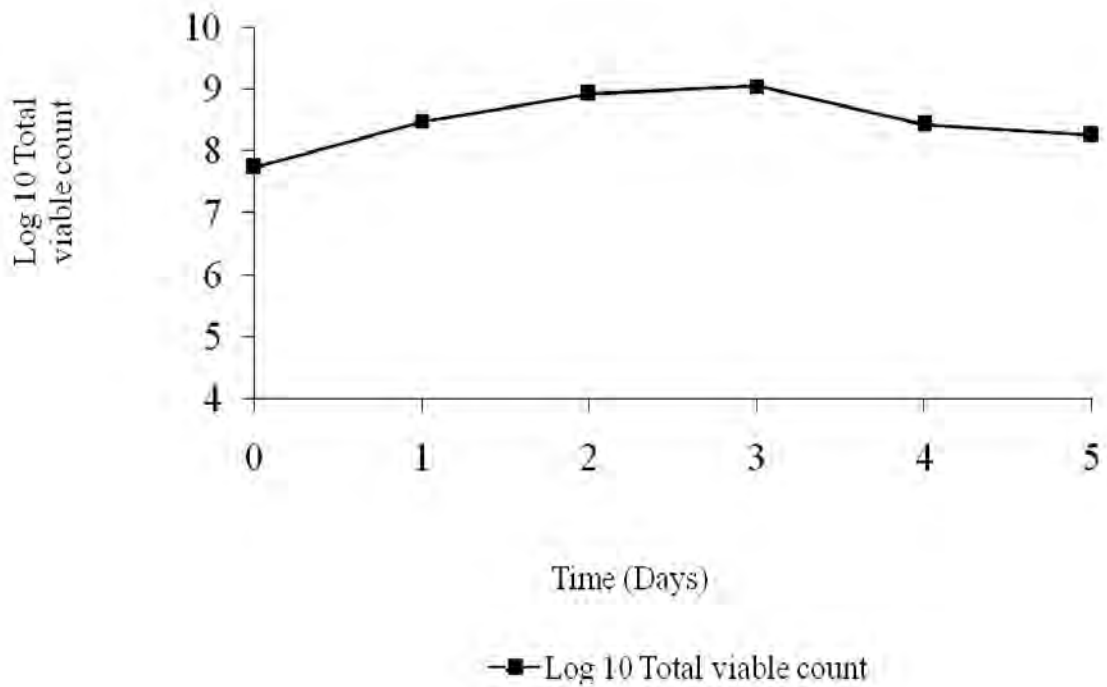


Figure 1: The growth of microorganisms during fermentation of *A. hybridus* leaves

To isolate LABs, de Mann Rogosa Sharpe agar (MRS) was used. The colonies identified as LAB were then isolated and *L. lactis* identified from the isolates using, M17 agar. For this study the maximum growth of 8.9×10^8 cfu/ml (table 1) was obtained on day three after which there was a decline up to the fifth day as shown in figure 2.

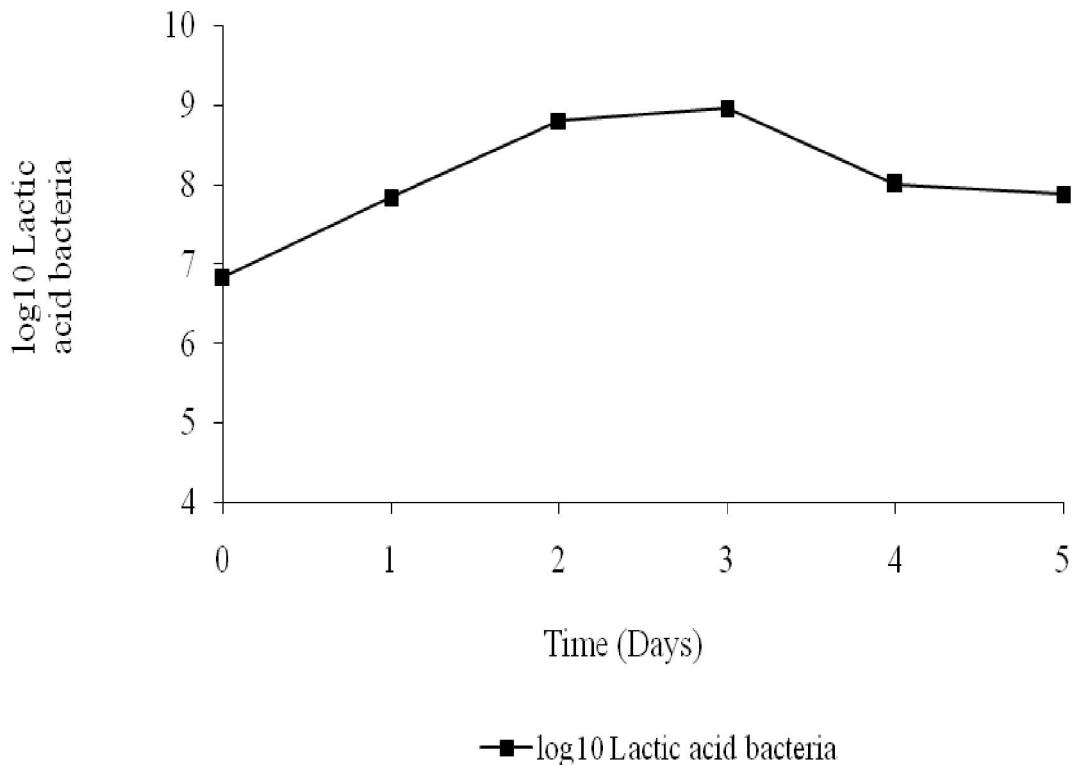


Figure 2: The growth of LAB during fermentation of *A. hybridus* leaves

All isolated strains fermented glucose, trehalose, sucrose, mannitol, fructose, and maltotriose, but did not ferment raffinose. Most strains fermented lactose. From table 2 it is evident that some strains fermented arabinose, but other strains did not.

The fermentation progress was monitored using the pH profile, which decreased rapidly from 6.03 (day 0) to 4.75 (day 1). After day one, the pH was stable to the last day of the study as in figure 3.

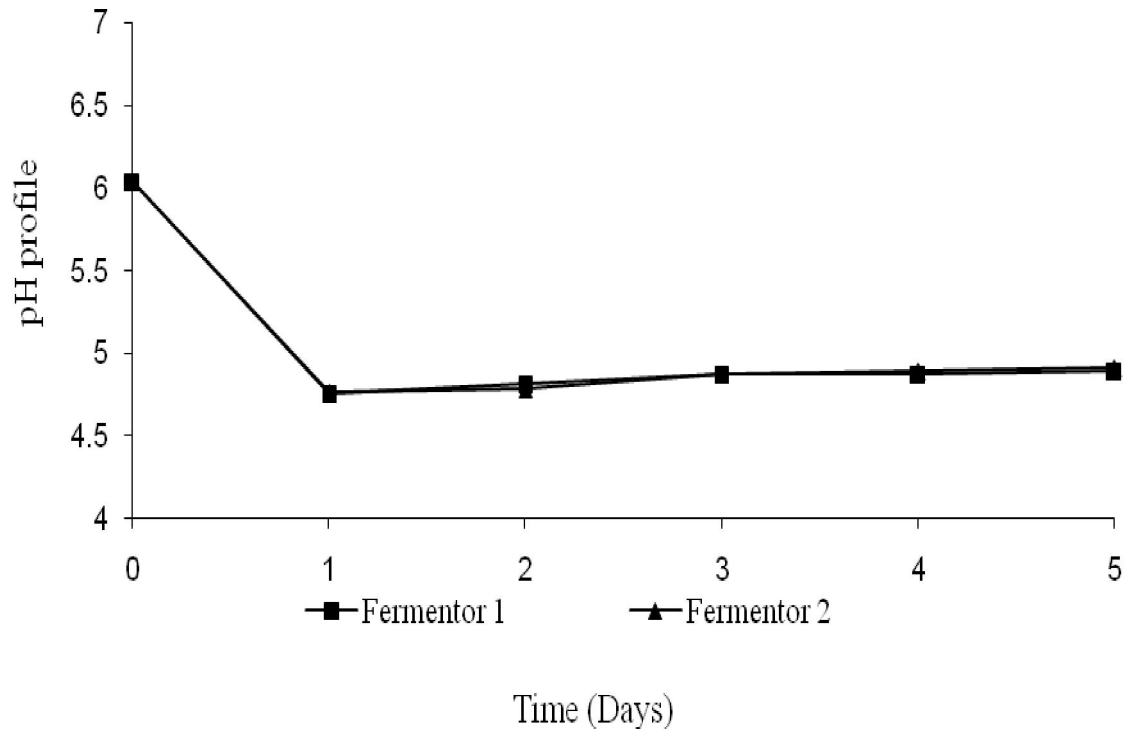
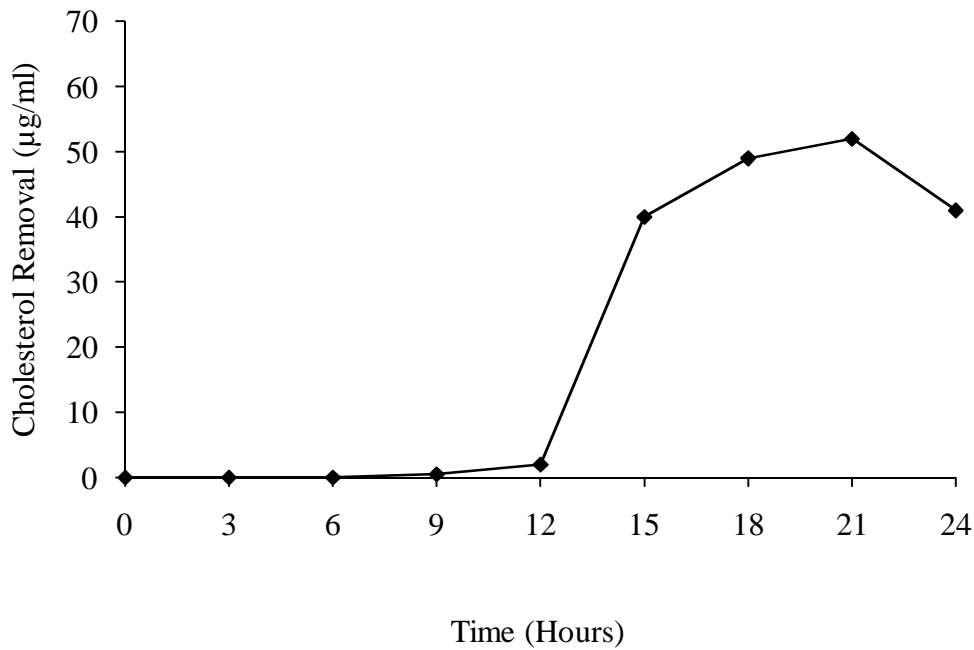


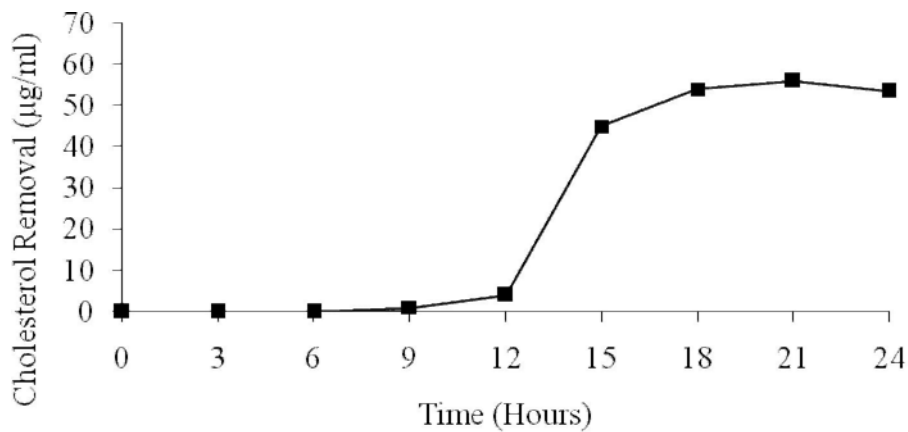
Figure 3: The pH profile during fermentation of *A. hybridus* leaves

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, 5 and 6).



◆ Cholesterol removal by *Lactococcus lactis*

Figure 4: Amount of cholesterol removed from MRS Broth by *L. Lactis*



■ Cholesterol removal by *Lactobacillus acidophilus* ATCC 43121

Figure 5: Amount of cholesterol reduced by *Lb. acidophilus* ATCC 43121 (positive control) in MRS Broth

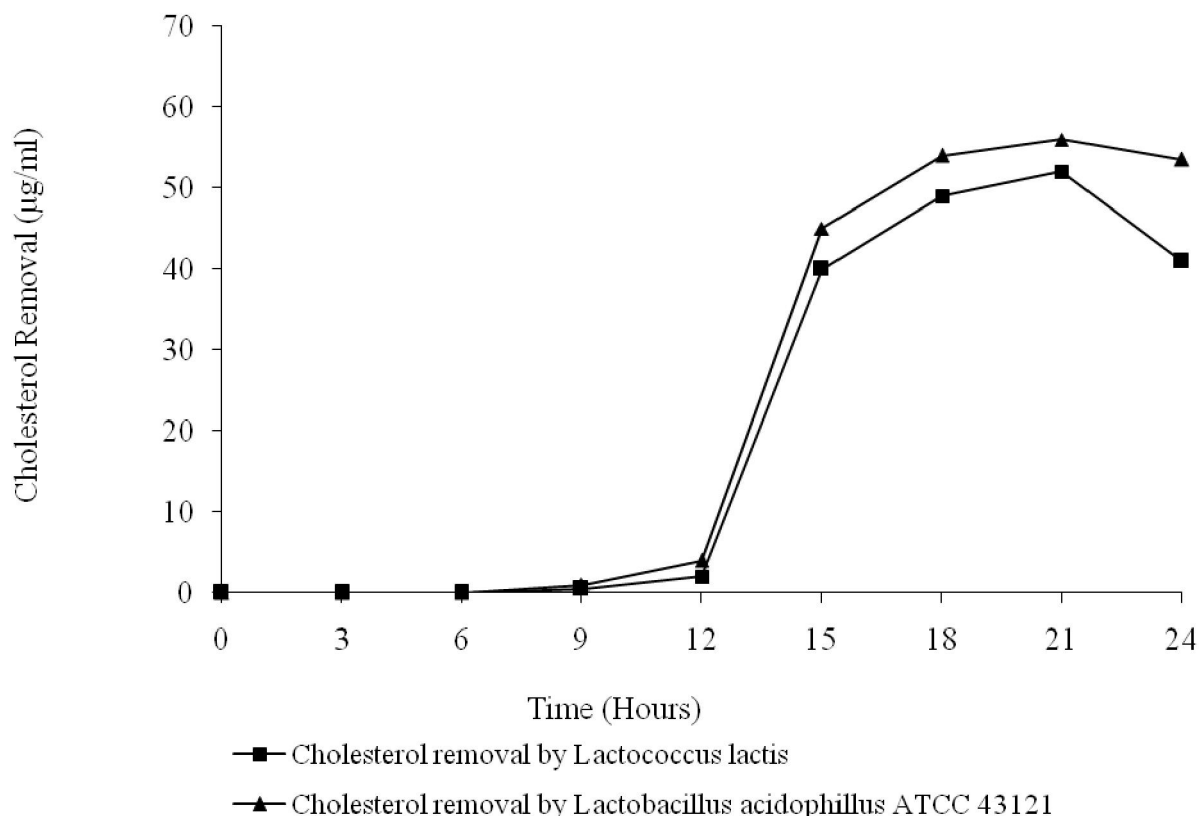


Figure 6: Cholesterol reduction ability of *L. lactis* () and *Lb. acidophilus* ()

On 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 the reduction. Cholesterol removal from the growth media by both the test and the positive control were significant ($P < 0.05$). However, there was no significant difference ($P = 0.05$) in the amounts of cholesterol removed by *L. lactis* and *Lb. acidophilus* (positive control).

The crude protein, moisture content and ash values are summarized in Table 3. 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 ($P < 0.05$).

Table 4 gives the scores by consumer panelists on the listed products. From this study there was no significant ($P = 0.05$) difference in the mean ranking scores for boiled and the fermented amaranth leaves.

DISCUSSION

Lactic acid fermentation involves utilisation of fermentable sugars by LABs with subsequent production of lactic acid, carbon dioxide among other by-products. This phenomenon is a result of the multiplication of the LAB facilitated by the availability of fermentable substrate. From table 1, figures 1 and 2, 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.* [20]. After day 3, there was a decrease in the microbial population. This decrease could be attributed to the decrease in fermentable substrates (glucose, fructose and sucrose), which serve as food for the microorganisms. The majority of organisms are dependent on nutrients for both energy and growth [21]. Organisms vary in their specificity towards different substrates and usually only colonize foods which contain the substrates they require [22]. Sources of energy vary from simple sugars to complex carbohydrates and proteins [23]. 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 the decrease in microbial populations [23]. 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 did not. On the basis of these results, they were tentatively identified as *L. lactis*. The seven isolates were Gram positive, catalase negative and KOH negative (Table 2). The pH decreased rapidly from 6.03 on day 0 to 4.7 on day 1 (Fig. 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 LAB 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 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 [10]. This drop in pH is as a result of multiplication of the LAB. After the first day the pH was almost constant. This could be probably due to a decrease in the fermentable sugars. However, the pH values attained in this study were high as compared to sauerkraut that attains a final pH of 3.4 to 4.0. This pH has been reported to suppress growth of spoilage microorganisms [23]. Cabbages have a high sugar content (1.2 to 2.2%) and this provides enough fermentable sugars and thus a high percent lactic acid. Information recently published by the Lipid Research Clinics [24], indicates that the higher the total serum cholesterol level is in humans, the greater the risk of developing coronary heart disease. Some studies have reported that ingestion of probiotics such as *Lb. acidophilus* decreases serum cholesterol levels in humans and animals [25, 26]. None of these studies has suggested that *L. lactis* isolated from plant products had this ability. The manner of cholesterol removal by the two organisms corresponded to the manner of their growth evident in figures 4, 5 and 6. The rapid cholesterol removal

during 12 to 15 h of incubation corresponded to exponential growth phase of the organisms. Kimoto *et al.*, 2002; Liong and Shah, 2005 [11, 16] studying cholesterol removal from media by *Lactococci* concluded the same. It is assumed that the reduction of cholesterol from growth media by microorganism can be extrapolated to humans. In animal models (rat), the ability of *L. lactis* isolated from animal products to reduce cholesterol and to survive in the rat GIT was demonstrated. However, 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 to the host. Therefore, amaranth leaves (fermented) could be able to influence health in a positive way.

The nutritive value of any food is a reflection of its health benefits. The higher the protein, soluble carbohydrates, crude fibre, ash and vitamins the higher the nutritive value of that particular food. The crude protein, moisture and ash content of amaranth leaves was determined and tabulated in table 3; crude protein was significantly higher for fresh solar dried amaranth leaves than the fermented leaves at $P < 0.05$. This low protein value could be attributed to protein utilization by the fermentative bacteria, leaching out of nutrients and draining away of brine that preceds drying [27]. Moisture content was significantly ($P < 0.05$) higher for fermented leaves compared to fresh solar dried leaves (table 3). 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 (table 3). This could be attributed to addition of 2.5% sodium chloride at the start of fermentation. In any processing of food product, the ultimate goal is to satisfy the consumer who has the upper hand 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 or alter the natural quality of the food material as is acceptable to the consumer. From this study, there was no significant difference in the mean ranking scores for boiled and the fermented amaranth leaves ($P > 0.05$). However, most panellists associated the fermented leaves with fermented flavour which the majority were not acquainted with. The sour flavour in the fermented solar leaves could be as a result of co-existence of high counts of *Lactobacilli* and yeasts that positively contribute to flavour [20]. The high ratio of volatile to non-volatile acids has a positive effect on flavour of sauerkraut [28].

CONCLUSIONS

This study established that fermented amaranthus leaves is a potential source of probiotics as the level of cholesterol reduction by the isolated *Lactococcal* strains compared favourably with that of cholesterol reduction by the control *Lb. acidophilus* ATCC 43121, which is a known probiotic. Thus, from this study, it can be concluded that *L. lactis* isolated from fermented *A. hybridus* can reduce cholesterol *in vitro*.

Since *L. lactis* isolated from fermented amaranthus leaves have the potential to reduce cholesterol, the leaves could be consumed regularly to help manage serum cholesterol levels. African traditional fermented foods remain the main source of nutrition for many rural communities in Africa. Although lactic acid bacteria are integral to many

of these foods, more needs to be known about the specific health benefits they confer or the properties of their strains. Further research should thus be done to;

- a. Determine the survival ability of *L. lactis* isolated in this study in the GIT.
- b. Find how the extract from the fermented amaranth leaves could be put into further use since water soluble nutrients are lost by draining away of the extract.
- c. Determine whether cholesterol removal ability can benefit humans (for example do a study on *in-vivo* cholesterol reduction).

Table 1: Total Viable Count and LABs Growth on PCA and MRS, respectively

Colony Forming Units (C.F.U)		
Day	Total Viable Count	Lactic Acid Bacteria
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

Table 2: Physiological and Biochemical Profile of LAB 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
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: strains from *A. hybridus* leaves.

Table 3: The crude protein, moisture and ash content (%) of amaranth leaves

	Moisture	Ash	Protein
Fresh dry amaranthus leaves	5.44 ^a	19.76 ^a	36.07 ^a
Fermented dry amaranthus leaves	6.22 ^b	36.21 ^b	16.65 ^b

N = 5

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

Table 4: Ranking scores of amaranth leaves as given by panelists based on general acceptability

Sample	Mean Score
Fresh, boiled amaranthus leaves	6.83 ^a
Fermented amaranthus leaves	6.90 ^a

$\bar{N} = \hat{63}$

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

REFERENCES

1. **Aletor O, Oshodi R and K Ipinmoroti** Chemical composition of common leafy vegetables and functional properties of their leaf protein concentrates. *J. Food Chem.*, 2002; **78**: 63–68.
2. **Klijn N, Weerkamp AH and WM de Vos.** Genetic marking of *Lactococcus lactis* shows its survival in the human gastrointestinal tract. *Appl. Environ. Microbio.* 1995; **61**: 2771-2774.
3. **Katuriina MW** Wild vegetables used by the Sambia in the Usambara Mountains N.E. Tanzania. *Ann Bot Fenn.* 2000; **37**: 57-67.
4. **Ndegwa OC and HJ Aagaard** Traditional gathering of wild vegetables among the Luo of Western Kenya-a nutritional anthropology project 1. *Eco. Food Nutr.*. 2003; **42**: 78-89.
5. **Marteau M, Christophe CJ, Philippe R and S Jürgen** Protection from gastrointestinal diseases with the use of probiotics. *Ameri. J. Clin. Nutr.* 2007; **73**: 430–436.
6. **Marteau P, Seksik P and R Jian** Probiotics and health: New facts and ideas. *Curr. Opin Biotech.* 2002 **13**(5): 486-489.
7. **Furgus GP and G Michael** Lactic Acid Bacteria. Applied Microbial Systems. Kluwer Academic Publishers. Dordrecht, The Netherlands 2000: 367-388.
8. **Perdigon G, Alvarez S and MN de Macias** The oral administration of lactic acid bacteria increases the mucosal intestinal immunity in response to enteropathogens. *Food Prot. J.* 1990; **53**: 404–410.
9. **Grahn E, Holm SE, Lilja H and K Sellgren** Interference of a *Lactococcus lactis* strain on the human gut flora and its capacity to pass the stomach and intestine. *Scandinavian J. Nutr.* 1994; **38**: 2–4.
10. **Kelly WJ, Davey GP and LJ Ward** Characterization of *Lactococci* isolated from minimally processed fresh fruit and vegetables. *Int. J. Food Microbio.* 1998; **45**: 85-92.
11. **Liong MT and NP Shah** Acid and bile tolerance and cholesterol removal ability of *Lactobacilli* strains. *J. Dairy Sci.* 2005; **88**: 55–66.
12. **Liong MT and NP Shah** Optimization of cholesterol removal by probiotics in the presence of prebiotics by using a response surface method. *J. Applied Environ.l Microbio.* 2005; **71**(4): 1745–1753.
13. **Noh DO, Kim SH and SE Gilliland** Incorporation of cholesterol into the cellular membrane of *Lactobacillus acidophilus* ATCC 43121. *J. Dairy Sci.* 1997; **80**: 3107-3113.

14. **De Rodas BZ, Gilliland SE and CV Maxwell** Hypocholesterolemic action of *Lactobacillus acidophilus* ATCC 43121 and calcium in swine with hypercholesterolemia induced by diet. *J. Dairy Sci.* 1996; **79**: 2121-2128.
15. **Gilliland SE, Buck LM and MM Brashear** Bile salt deconjugation and cholesterol removal from media by *Lactobacillus casei*. *J. Dairy Sci.* 1998; **81**: 2103-2110.
16. **Kimoto H, Ohmomo S and T Okamoto** Cholesterol removal from media by *Lactococci*. *J. Dairy Sci.* 2002; **85**: 3182-3188.
17. **Gilliland SE** Bacterial Starter Cultures for Foods. CRC Press, Boca Raton, Florida. U.S.A. 97-117.
18. **Rudel LL and MD Morris** Determination of cholesterol using o-phthalaldehyde. *J Lipid Res.* 1973; **14**: 364-366.
19. **Morten CM, Gail VC and B Thomas** Sensory Evaluation Techniques. CRC Press 4th Edition Boca Raton, U.S.A. 2007; 26-58.
20. **Sanchez AH, Montano A, Castro A and L Rejano** Comparative study on chemical changes in olive juice and brine during green olive fermentation. *J. Agric. & Food Chem.* 2000; **48**: 5975-5980.
21. **Dora AP and RG Glenn** Cholesterol assimilation by lactic acid bacteria and *Bifidobacteria* isolated from the human gut. *Int. J. Applied and Env. Microbio.* 2002; **68(9)**: 4689-4693.
22. **Peter JJ** Clinical nutrition: 7. Functional foods-more than just nutrition. *Canadian Med. Assoc. J.* 2002; **166 (12)**: 55-57
23. **Parvathy SN and KS Puthuvallil** Biochemical characterization of lactic acid bacteria isolated from fish and prawn. *J.Culture Collections.* 2005; **4**: 48-52.
24. **Carmen CM, Jussi M and S Seppo** In vitro analysis of probiotics strains inhibit pathogen adhesion to human intestinal mucus. *J. Int. Food Res.* 2007; **79**: 125-136.
25. **Dilmi BA** Assimilation (in vitro) of cholesterol by yoghurt bacteria. *Annual Agri. Env. Med. J.* 2006; **13(1)**: 49-53.
26. **Gilliland SE and LM Buck** Comparisons of freshly isolated strains of *Lactobacillus acidophilus* of human intestinal origin for ability to assimilate cholesterol during growth. *J. Dairy Sci.* 1994; **77**: 2925-2933.
27. **Viander B, Maarit M and A Palva** Impact of low salt concentration, salt quality on natural large-scale sauerkraut fermentation. *Food Microbio.* 2003; **20(4)**: 391-395.
28. **Trail AC, Fleming HP, Young CT and RF Mcfeeters** Chemical and sensory characterization of commercial sauerkraut. *J. Food Qual.* 1996; **19**: 15-30.