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University of Mainz, Heidelberg,
55128 Mainz,
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African Journal of Biochemistry Research

Full Length Research Paper

Effect of kidney bean consumption on some lipid and haematological parameters of albino rats

Olanipekun, O. T.[1,3]*, Balogun, E. A.[2], Akinloye, O. A.[3], and Omotainse, S. O.[4]

[1]Product Development Program, Institute of Agricultural Research and Training; Obafemi Awolowo University, Nigeria.
[2]Department of Biochemistry, Faculty of Life Sciences, University of Ilorin, Ilorin, Kwara State, Nigeria.
[3]Department of Biochemistry, College of Biological Sciences, Federal University of Agriculture, Abeokuta, Nigeria.
[4]Department of Veterinary pathology, College of Veterinary Medicine, Federal University of Agriculture, Abeokuta, Ogun Stae, Nigeria.

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This study investigated changes in cholesterol levels and haematological parameters of rats subjected to diets supplemented with kidney bean seeds. The seeds were processed in three parts; the first part was milled raw; the second part was boiled, dried and milled; third part was germinated, dried and milled. The kidney bean seed flour was incorporated into the diet of different groups of rats at 10% inclusion. Thirty male rats were assigned into 5 groups (n=6); and fed for 30 days: A-normal diet, no cholesterol; B, normal diet + cholesterol; C, cholesterol diet+ raw kidney bean; D, cholesterol diet+ boiled kidney bean; E, cholesterol diet+ germinated kidney bean. At the end of the feeding trial, the rats were sacrificed; blood was taken from the jugular vein to determine some lipid and haematological parameters. The values for the plasma lipid profile for rats in groups D and E were significantly lower ($p<0.05$) than that of the control; TC (122.26 ± 0.30 and 120.20 ± 0.12 vs 136.82±0.37 mg/dl); TAG (102.72 ± 0.22 and 100.37 ± 0.37 vs 113.98 ± 0.16 mg/dl); LDLC (56.89 ± 0.24 and 44.00 ± 0.12 vs 74.45 ± 0.31 mg/dl); phospholipids (143.66 ± 0.81 and 145.11 ± 0.55 vs 154.39 ± 0.22 mg/dl), the same groups had significantly higher values ($p<0.05$) of HDLC (46.03±0.34 and 57.04±0.34 vs 39.11±0.46 mg/dl). Similar trend was found in the liver tissue. Haematological values were significantly higher ($p<0.05$) in groups D and E rats than in control, PCV (43.60±0.25 and 44.00±0.32 vs 37.80±0.20) %; Hb (14.14±0.05 and14.58±0.04 vs12.20±0.06) mg/dl; RBC (5.82±0.07 and 6.01±0.04 vs 5.02±0.01) $x10^9$/L; MCV (75.17±0.62 and 78.78±0.36 vs 67.37±0.55) fl. Processed kidney bean based diet could reduce cholesterol concentrations in plasma and liver of rats and improve their haematological parameters.

Key words: Kidney bean, cholesterol, lipid parameters, haematology, plasma, liver.

INTRODUCTION

Legumes are the edible seeds of pod bearing plants belonging to the Order *Leguminosae* and are widely grown throughout the world, being second only to cereals as a source of human and animal food (Singh et al.,

*Corresponding author. E-mail: yoyint@yahoo.com. Tel: 08060330088.

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2004). Legumes not only add variety to the human diet, but also serve as an economical source of supplementary proteins for the large human populations of the developing countries. In general, legumes as sources of complex carbohydrate, proteins and dietary fibre, have a significant amount of vitamins, and minerals as well as high energy values (Apata and Ologhobo, 1994). Legumes have been considered as the most significant food source for people of low incomes (Apata, 2008). They are important in alleviating protein malnutrition (Mbah et al., 2012). Despite the large number of existing legumes in Nigeria; their consumption as staple food has been centred on few ones like cowpea, groundnut, and just recently, soybean. There are other lesser known legumes, including kidney bean, bambara nut, pigeon pea, and lima bean, which can equally be of nutritional and health benefit to man especially in developing countries like Nigeria where the per capita income is very low.

Kidney bean (*Phaseolus vulgaris*) is an herbaceous annual plant, the red variety is the most common; the white and black varieties are also available but are less widely used (Katherine, 2002). The cooked beans are widely used in savoury cuisine throughout the world, for example, in casseroles, salads, curries, soups, and pasta and meat dishes, the straws are used as fodders (Katherine, 2002). Kidney bean (*Phaseolus vulgaris*) is currently underutilised in Nigeria, because it has not gained widespread industrial, economic and nutritional importance, because its acceptability and utilization have been limited (Audu and Aremu, 2011). The few works done on it is limited to its use in animal feed (Apata and Ologhobo, 1994). Other works (Okoye et al., 2008; Audu and Aremu, 2011) have been done on the red variety of the bean. In the authors' opinion, kidney bean has received little attention due to lack of adequate information on its nutritional and health benefits.

The role of dietary fibre and proteins from different sources, in reducing cholesterol levels have been established (Sirtoni et al., 2004; Martins et al., 2005, Oboh and Omofoma, 2008). Legumes could be used in solving problems of cardiovascular diseases arising from changes in dietary patterns and urbanization trends (Doss et al., 2011). This work investigated changes in lipid profile levels and haematological parameters of rats subjected to diets supplemented with kidney beans seeds, with the aim that the result will encourage kidney bean utilization at both household and industrial levels.

MATERIALS AND METHODS

Collection of kidney beans

Required quantity of kidney beans was purchased at Bode Market, Ibadan. The kidney beans were processed into flour before incorporating into the rat diet.

Processing of kidney bean seed flour

The beans were sorted and treated as follows. 100g portion was milled raw, another 100g portion was boiled, followed by drying in the oven at 40°C until weight was constant, the last 100g portion was soaked for 12 hours and then allowed to sprout) at room temperature for 48 hours; the sprouted seeds were then dried at 40°C. The processed kidney bean seeds were milled into flour and packed in air tight containers. The kidney bean seed flour was incorporated into the rat diets at 10% inclusion level.

Experimental animals

Adult male Wistar albino rats of weight range (120-150 g) were bought from Covenant Farm Nigeria Enterprises, Ibadan. They were housed in the rat house of Institute of Agricultural Research and Training (IAR&T) and grouped in five standard cages of six rats each, under clean environmental conditions: temperature (27°C); humidity (55%); and 12 h/ 12 h light/darkness cycles. The rats were allowed to acclimatise for one week while being given free access to commercial pelleted diet and water *ad libitum*. The rats later received different diets for 30 days as presented in Table 1.

Estimation of plasma total cholesterol (TC) concentration

The total cholesterol concentration was determined using commercially available diagnostic kit (RX MONZA, Randox laboratories, United Kingdom). This involved mixing 5 µL of sample with 500 µL of cholesterol reagent, the mixture was incubated at 37°C for 5 min. Also, 5 µL of calibrator standard was mixed with 500 µL of cholesterol reagent, and incubated at 37°C for 5 min. Absorbance of calibrator (standard) and samples was then read at 500 nm in a spectrophotometer (Biorad) against reagent blank. The absorbance values of samples and calibrator standard was then used to calculate the cholesterol level in all samples.

$$Total\ Cholesterol\ concentration = \frac{Absorbance\ \ of\ sample}{Absorbance\ \ of\ standard} \text{ x } Concentration\ of\ standard$$

Estimation of plasma high density lipo-protein (HDL) cholesterol concentration

HDL cholesterol was determined using commercially available diagnostic kit (RX MONZA, Randox laboratories, United Kingdom). Test tubes were labelled as reagent blank, standard, and samples A- E. 10 µL of distilled water was added into reagent blank, 10 µL of standard into standard tube, and 10 µL of each sample into sample tubes.1000 µL of the HDL reagent was added into all the tubes. The reaction mixtures were incubated separately for 5 min at 37°C. The absorbance of each was read at 500 nm in a spectrophotometer (Biorad) against reagent blank. Concentration of HDL cholesterol was calculated using the expression:

$$Conc.of\ HDLC = \frac{Absorbance\ \ of\ sample\ \ (supernatant\ \ )}{Absorbance\ \ of\ supernatant\ \ \ standard} \text{ X } Conc.\ of\ supernatant\ standard$$

Table 1. Composition of Experimental Diets given to the different rat groups.

Ingredients	Non-cholesterol feed A (%)	Cholesterol Feed B (%)	Cholesterol + raw KB Feed C (%)	Cholesterol + boiled KB Feed D (%)	Cholesterol + germinated KB Feed E (%)
Casein	22.0	22.0	22.0	22.0	22.0
Vitamin/mineral mixture	4.0	4.0	4.0	4.0	4.0
Corn starch	54.5	53.5	43.5	43.5	43.5
Sugar	10.0	10.0	10.0	10.0	10.0
Cholesterol	-	1.0	1.0	1.0	1.0
NaCl	0.5	0.5	0.5	0.5	0.5
Dried KB flour	-	-	10.0	-	-
Soaked and Boiled KB flour	-	-	-	10.0	-
Germinated KB flour	-	-	-	-	10.0
Hydrogenated fat	9.0	9.0	9.0	9.0	9.0

Salt/mineral mix contains the following (in g/100 g): calcium phosphate, 49.50; sodium powder, 11.80; potassium sulfate, 5.20; sodium chloride, 7.40;magnesium oxide, 2.40; potassium citrate, 22.40; ferric citrate, 0.60; mangnesium carbonate, 0.35; cupric carbonate, 0.03; zinc carbonate, 0.16; chromium potassiumsulfate, 0.055; potassium iodate, 0.001; sodium selenate, 0.001; choline chloride, 0.50; thiamine HCl, 0.06; riboflavin, 0.06; niacin, 0.30; calcium pantothenate, 0.16;biotin, 0.01; vitamin B12, 0.10; vitamin D3, 0.025; vitamin E acetate, 1.00; pyridoxine, 0.07; folic acid, 0.02; vitamin A acetate, 0.08.; KB—Kidney bean. Source: (Vadde et al., 2007).

Estimation of plasma triacylglycerol (TAG) concentrations

Plasma triglycerides were determined using commercially available colorimetric kit RX MONZA, Randox laboratories, United Kingdom). Test tubes were labelled as reagent blank, standard and samples A- E. 10 µL of distilled water was added into reagent blank, 10 µL of standard into standard tube, and 10 µL of each sample into sample tubes. 1000 µL of the triacylglycerol reagent was added into all the tubes. The reaction mixtures were incubated separately for 5 min at 37°C. The absorbance of each was read at 500 nm in a spectrophotometer (Biorad) against reagent blank. Concentration of plasma triacylglycerol was calculated using the expression:

$$Triacylglycerol\ concentration = \frac{Absorbance\ \ of\ sample}{Absorbance\ \ of\ standard}\ \text{X Concentration of standard}$$

Estimation of plasma low density lipo-protein (LDL) cholesterol concentrations

LDL Cholesterol was determined by calculation using the formula:

$$LDL\ Cholesterol = TC - \frac{TAG}{5} - HDLC$$

Where, TC= Total cholesterol, TAG= triacylglycerol, HDLC= high density lipo-protein cholesterol.

Estimation of plasma phospholipid concentrations

Phospholipids was determined by using commercially available colorimetric kit (Abnova phospholipids assay kit; KA1635). Standard solution was prepared by mixing 24 µL of phospholipids with 216 µL distilled water. 20 µL of the prepared standard was mixed with 80 µL of working reagent, also 20 µL of each sample was separately mixed with 80 µL of working reagent, each mixture was incubated for 30 min at 37°C, absorbance of each mixture (sample and standard) was read at 570 nm in a spectrophotometer 21D against the reagent blank.

Concentration of phospholipids was calculated by the formula:

$$Conc. of\ phospholipids = \frac{Absorbance\ \ of\ sample}{Absorbance\ \ of\ standard}\ \text{x Conc. of standard}$$

Preparation of the liver samples

Composite paste of the liver tissue was prepared by homogenizing 0.3 g of the liver with 2.7 ml of chloroform/methanol mixture (1:1 v/v). The resulting suspension mixture was then filtered and rinsed with additional 10 ml of the chloroform/methanol mixture to give a homogenate. The filtrate was centrifuged for 10 min at 1,500 rpm and the resultant supernatant was used for the determination of liver lipid profile in the rats.

Estimation of liver lipid profile

The liver lipid profile (TC, HDLC, TAG and Phospholipids) was

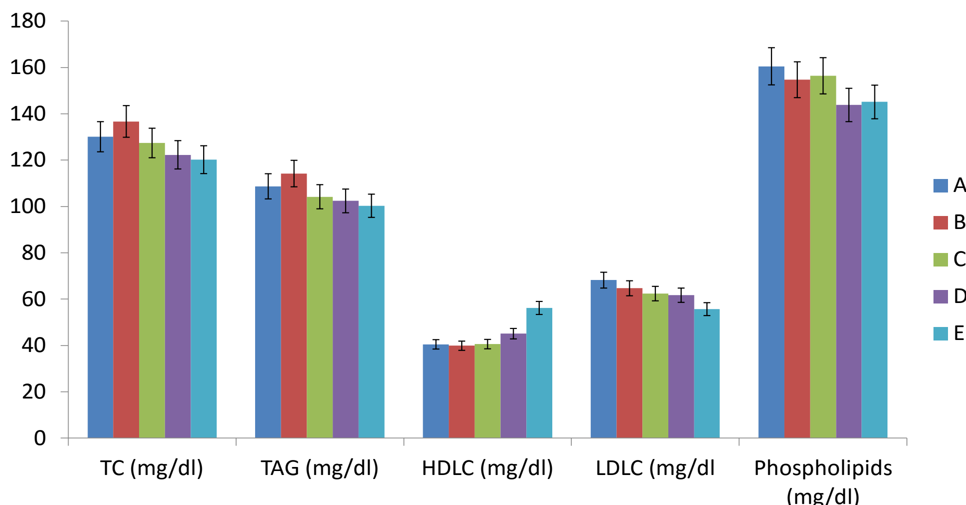

Figure 1. Plasma lipid profile of rats fed differently processed Kidney bean seeds. Each bar represents the means ± SEM of 5 determinations.TC= total cholesterol. TAG= triacylglycerol; HDLC= high density lipo-protein cholesterol; LDLC= low density lipo-protein cholesterol.

estimated as outlined for plasma estimations.

Estimation of red blood cell (RBC)

This was determined by using Neabeauer counting chamber as described (Lamb, 1981). Blood sample was collected and diluted at a ratio of 1:200 (blood: red cell diluting fluid). The cells were counted with a hand tally under a compound microscope. Red blood cell counts were obtained using the relationship:

RBC/µl= Number of red blood cell counted x 5 x10 x 200

Estimation of white blood cell (WBC)

This was determined by using Neubauer counting chamber as described by (Lamb, 1981). Blood sample was collected and diluted at a ratio of 1:20 (blood: white cell diluting fluid). The cells were counted with a hand tally under a compound microscope. White Blood Cell counts were obtained using the relationship:

WBC/µl= Number of white blood cell counted x 0.25 x 10 x 20.

Estimation of packed cell volume (PCV)

This was estimated by spinning 75 µl of each blood sample in a haematocrit micro-centrifuge at1200 revolution per minute (rpm) for five minutes. The value was read on the haematocrit reader as a percentage of the total blood volume according to the equation:

$$PCV = \frac{\text{Height of packed cell column}}{\text{Height of whole blood column}} \times 100$$

Estimation of haemoglobin (Hb) concentrations

Haemoglobin was measured using haemoglobinometer which compares the colour of light passing through a haemolysed blood sample with a standard blood colour. A drop of blood was placed on one side of the chamber glass and stirred with the end of a haemolysis applicator (which has been dipped in saponin solution) until the blood became transparent. The cover glass was positioned on top of the blood plate and introduced into the haemoglobinometer where reading took place.

Estimation of mean cell volume (MCV)

This was calculated using the expression:

$$\frac{PCV X 10}{RBC}$$

Where PCV = Packed cell volume; RBC = red blood cell

Statistical analyses

Results were reported as mean ± standard error of mean (SEM). The levels of homogeneity among the groups were assessed using Analysis of Variance (ANOVA). Where heterogeneity occurred, the groups were separated using Duncan Multiple Range Test (SAS, 1995).

RESULTS

Figures 1 and 2, respectively gives the plasma lipid profile and Liver lipid profile of rats fed diets with processed kidney bean seeds. The total cholesterol and triacylglycerol, were found to be significantly lower ($p<0.05$) in both plasma and liver of the rat groups fed processed kidney bean seeds than the control. The values of HDL-cholesterol was significantly higher ($p<0.05$) in the rat groups fed processed kidney bean diet

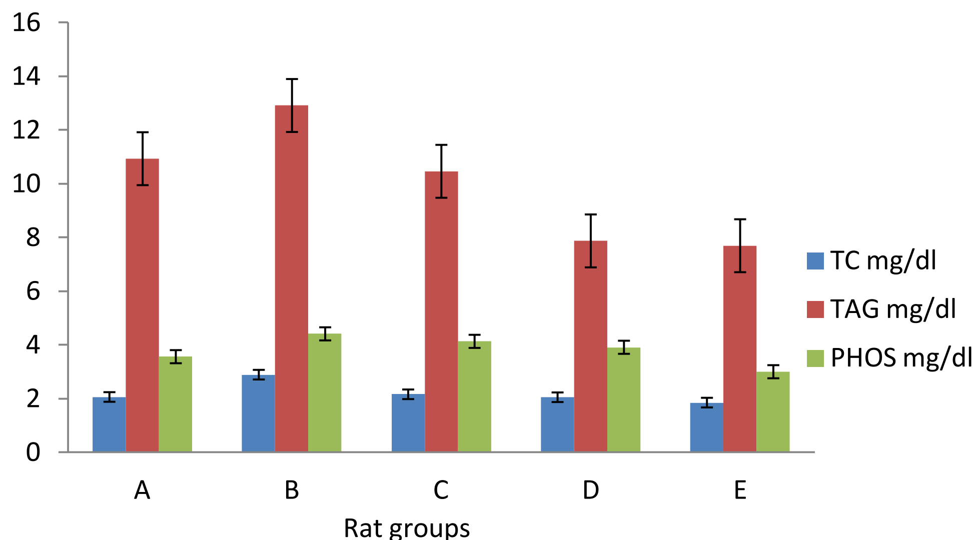

Figure 2. Liver lipid profile of rats. Each bar represents the means ± SEM of 5 determinations**.** TC= Total cholesterol. TAG= triacylglycerol; PHOS= phospholipids; Group A = rats on non-cholesterol diet only; group B = rats on cholesterol diet only; Group C = rats on cholesterol diet +raw kidney bean; group D = rats on cholesterol diet + boiled kidney bean; group E = rats on cholesterol diet + germinated kidney bean.

Table 2. Mean haematology result of rats fed different diets of kidney bean seeds.

Rat group	PCV	Hb (g/dL)	RBC ($x10^9$/L)	WBC ($x10^{12}$/L)	MCV(fl)
A	41.00±0.45[b]	14.05±0.07[b]	6.15±0.02[a]	7.34±0.01[d]	72.88±0.57[c]
B	37.80±0.20[c]	12.20±0.06[d]	5.02±0.01[b]	6.19±0.01[e]	67.37±0.33[d]
C	40.00±0.40[b]	13.26±0.09[c]	5.24±0.14[b]	8.10±0.01[c]	74.33±0.41[bc]
D	43.60±0.25[a]	14.14±0.05[b]	5.82±0.07[a]	11.41±0.01[b]	75.17±0.62[b]
E	44.00±0.32[a]	14.58±0.04[a]	6.07±0.04[a]	12.18±0.04[a]	78.78±0.36[a]

Values are means of 5 determinations ± SEM. Means within the same column with different superscripts are significantly different at p<0.05. ; Group A = rats on non-cholesterol diet only; group B = rats on cholesterol diet only; Group C = rats on cholesterol diet +raw kidney bean; group D = rats on cholesterol diet + boiled kidney bean; group E = rats on cholesterol diet + germinated kidney bean.

than the control for both plasma and liver lipid profile. The plasma LDL-cholesterol values of the rats on processed kidney bean diets were significantly lower ($p<0.05$) than that of the control. Also, phospholipids value was significantly lower ($p<0.05$) in rats fed processed kidney bean diets than in the control group.

The rats fed processed kidney bean diets (germinated and boiled) demonstrated significantly higher PCV level than the control; and those that had germinated kidney bean diet demonstrated significantly higher Hb level ($p<0.05$); whereas boiled kidney bean diet did not increase Hb level significantly ($p>0.05$) in the rats (Table 2). There was no significant difference ($p>0.05$) in RBC level of rats on processed kidney bean diet and control (rats on non-cholesterol diet). The red blood cell (RBC) counts of the control group were highest but not significantly different ($p>0.05$) from the values obtained for the group fed on germinated kidney beans (Group E), and boiled kidney bean (Group D); the rats on cholesterol diet (Group B) and those on the raw kidney bean (Group C) also had similar values which were significantly lower than the other groups. The value of white blood cell (WBC) counts was significantly higher ($p<0.05$) in the rat group on processed kidney bean diets than in the control. The values of mean corpuscular volume (MCV) of rats fed processed kidney bean diet was significantly higher ($p<0.05$) than that of the control group (Table 2).

DISCUSSION

The result of the plasma and liver lipid profile of the rats revealed that consumption of processed kidney bean seeds (boiled and germinated) could serve as a means of reducing the cholesterol level of consumers. In the human body, high levels of triacylglycerol in the blood stream have been linked to atherosclerosis, and by extension, the risk of heart disease and stroke (Akinkugbe,

2000). HDL-Cholesterol is termed good cholesterol because of their ability to remove cholesterol from atheroma within arteries, and transport them to the liver for excretion (Wingerd, 1994). HDL-Cholesterol is protective against atherosclerosis. A high level of HDL is an indication of a healthy metabolic system. The result suggests that the intake of germinated kidney beans will reduce the level of LDL-Cholesterol circulating in the blood. Studies have shown that higher levels of LDL cholesterol promote health problems and cardiovascular disease. LDL-Cholesterol is often called “bad” cholesterol because high levels of it in the plasma are linked with increased deposition of cholesterol in the arterial walls and higher incidences of heart attacks (Vander et al., 1998). Phospholipids play an important role in the cell membranes and nervous tissues; as well as during all stages of atherosclerosis (Berliner, 2009). The low phospholipids value of rat groups fed boiled kidney bean and germinated kidney bean diets shows that the consumption of processed kidney bean could reduce cholesterol concentration in the blood. Some studies (Martins et al., 2005; Viveros et al., 2007) have reported the cholesterol reducing effect of some legumes on different animals like chicken, pigs and rats.

Iron (Fe) is a major constituent of the Hb, the relative reduction in the Hb level of rats fed boiled kidney bean diet is an indication that boiling is not as effective as germination in making iron available in legume based foods; this corroborates earlier report by Tizazu et al. (2011). Processing improved the nutrient availability of the bean, with germination being the most effective. Kidney beans have been reported to be an excellent source of molybdenum, which is involved in protein synthesis, and is vital for the utilization of iron (Katherine, 2002). The low WBC values found in the rats fed cholesterol diet only, and raw kidney bean diet could compromise immunity and predispose the animals to opportunistic infections. WBC contributes to the defense mechanism of animals (Adepoju and Adebanjo, 2011). The normal MCV range for rats is 55.1- 62.2 fl (Bruce et al., 2005). A low MCV indicates iron deficiency. None of the rats was anaemic, it seemed that high intake of cholesterol reduced the availability of nutrients in the rats fed cholesterol diet only, and that consumption of processed kidney bean diet ameliorated the deleterious effect, and also improved the nutritional wellbeing of the rats.

Conclusions

From the study, it was observed that the inclusion of boiled and germinated kidney bean in the diet reduced the concentration of cholesterol in the plasma and liver of rats, and also improved haematology parameters. Therefore, there is a distinct beneficial effect of the consumption of heat treated kidney bean based foods, especially as it relates to reducing the risk of atherogenic dyslipidemia.

Conflict of interests

The author(s) did not declare any conflict of interest.

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Full Length Research Paper

Modulation in membrane lipids of *Escherichia coli* and *Staphylococcus aureus* in response to varying NaCl concentrations

Omotoyinbo Oluwasegun Victor, Sanni David Morakinyo* and Olawoye Theophilus

Department of Biochemistry, Federal University of Technology, Akure, Ondo State, 340252, Nigeria.

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The effect of varying NaCl concentrations on membrane lipids of *Escherichia coli* and *Staphylococcus aureus* cultivated at 37°C was studied. Lipid profiles of the membrane extracts were resolved by thin layer chromatography (TLC) using polar and neutral lipids solvent systems, with their fatty acid constituents determined via gas chromatography-mass spectrometry (GC-MS). Polar lipids identified were cardiolipin, phosphatidic acid, phosphatidylglycerol, phosphatidylethanolamine and lysophosphatidylglycerol, while neutral lipids for both microorganisms were steryl-esters, triacylglycerols, diacylglycerols and monoacylglycerols at the different NaCl concentrations of growth however triacylglycerol was not detected in *S. aureus* at 3% NaCl. Fatty acids for *E. coli* were C15:0, C16:0 and C18:1 at 0% NaCl but C16:2 was also detected at 3% NaCl. *S. aureus* fatty acids were C13:0, C14:0, C16:0 and C18:1 for 0% NaCl while at 3% NaCl, additional fatty acids observed include C12:0, C15:0 and C16:1. The lipids expressed were relatively similar; however, their fatty acids differed in constituents and abundance.

Key words: NaCl concentration, lipid profiles, fatty acids, membranes, *Escherichia coli*, *Staphylococcus aureus.*

INTRODUCTION

Every organism must find in its environment all of the substances required for energy generation and cellular biosynthesis. The chemicals and elements of this environment that are utilized for bacterial growth are referred to as nutrients. Therefore, changes in composition of surrounding environment such as varying saline concentrations, pH and other conditions may result in membranes with altered physical or thermal characteristics. It has been established that most microorganisms alter their membrane lipid composition when grown in the presence of high NaCl concentrations (Kogut and Russel, 1984; Komaratat and Kates, 1975). Also, it has been suggested that the phospholipid head group alterations of Gram-positive halotolerant bacteria differ from those of Gram-negative halophilic bacteria when grown in media containing high NaCl concentrations (Miller, 1985).

The plasma membrane of microorganisms enables the cell to sense changes in the surrounding osmotic conditions

*Corresponding author. E-mail: moraksanni@yahoo.co.uk. Tel: +234-806-925-6694.

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so that it can adjust its internal glycerol concentration to achieve osmotic equilibrium (Peeler et al., 1989). In fact, it has been suggested that the NaCl-induced alterations of membrane lipid composition may be important in controlling the ionic permeability of halotolerant or halophilic bacteria (Komaratat and Kates, 1975). For example, bacteria belonging to the Enterobacteriaceae family, such as *Escherichia coli* and *Salmonella*, do not tolerate high salt levels, but some species, such as *Serratia rubidea*, are very salt-tolerant (up to 10% NaCl) (Zambonelli et al., 1992). Also, halotolerant strains of *E. coli* are able to survive and grow even at very high salt concentrations; this high osmotic strength is due to the production of proline in the cells (Brewer, 2000; Tiecco, 2000). However, tolerance of *Staphylococcus aureus* to high concentrations of NaCl in liquid medium has been reported by Hurst et al. (1973). This study is aimed at identifying the phospholipids and fatty acids expressed by *E. coli* and *S. aureus* at different NaCl concentrations.

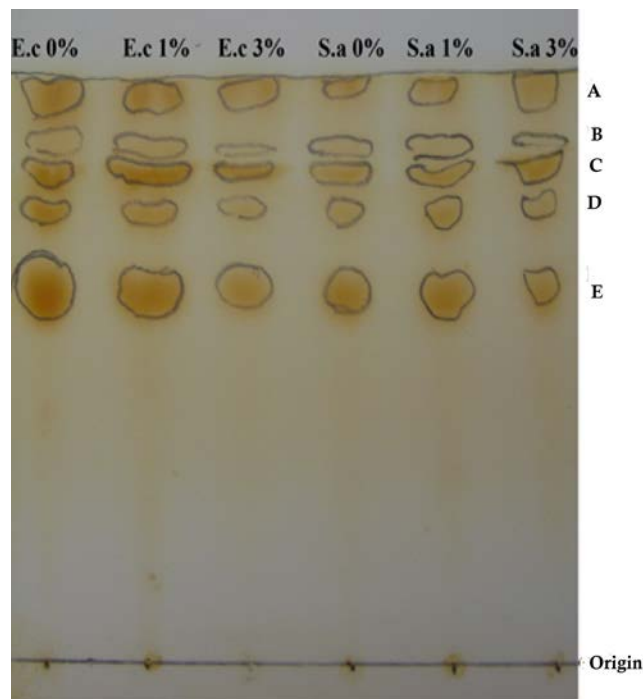

Plate 1. Chromatogram of polar lipids from *E. coli* and *S. aureus.* Adsorbent: Coated alumina plates. Solvent: chloroform: methanol: diisobutyl ketone: glacial acetic acid: water, 45:15:30:20:4 (v/v/v/v/v). A = cardiolipin, B = phosphatidic acid, C = phosphatidylglycerol; D = Phosphatidylethanolamine; E = Lysophosphatidylglycerol. R_f values of spots were calculated and compared with those of Wuthier (1966) and Hunter et al. (1981).

MATERIALS AND METHODS

Bacterial culture conditions

Clinical isolates of *E. coli* and *S. aureus* were grown for 18 h in 150 ml nutrient broth containing additional NaCl concentrations to give a salt inclusion level of 0, 1 and 3% (w/v). Each culture medium was prepared in triplicate.

Culture harvesting

The cultures were harvested by centrifugation (1000 x *g*, 10 min). In addition, wet cell paste obtained after decanting the clear supernatant, was then re-suspended in 100 ml; 1.0% NaCl (w/v), and centrifuged. Second supernatant was discarded and cell pellet frozen overnight and stored in sealed McCartney bottles.

Lipid extractions

Lipids were extracted from bacterial cells following the procedure of Bligh and Dyer (1959). 3.75 ml (1:2 v/v) chloroform ($CHCl_3$): methanol (MeOH) were added to each 1 ml of sample (wet cell paste), and then vortex well in a test-tube. 1.25 ml $CHCl_3$ was later added to the mixture in test-tube and then vortex well. Finally, another 1.25 ml distilled H_2O was added and vortex well before centrifuging at 6000 rpm in an automated cold centrifuge for 15 min at 4°C to give a two-phase system (aqueous top and organic bottom). The bottom phase which is the organic phase containing extracted lipids was then recovered through a Pasteur pipette, making sure to avoid the interface or upper face. Extracted lipids from the bottom phase were then placed in an evaporator and the residue obtained was later re-dissolved in 1 ml of $CHCl_3$: MeOH (2:1 v/v) ready for further lipid analysis.

Lipid analysis

The first analysis was carried out using a preparative thin layer chromatography (TLC). The total lipids extract in chloroform: methanol, 2:1 (v/v) were run on the outside lanes of the same TLC plate to enable identification of the sample lipid classes; polar lipids were resolved on activated 0.25 mm layers of silica gel H (Merck)and, developed using chloroform: methanol: diisobutyl ketone: glacial acetic acid: water, 45:15:30:20:4 (v/v/v/v/v) (Hunter et al., 1981; Wuthier, 1966). Resolution of neutral lipids were carried out on activated 0.25 mm layers of silica gel G (Merck), with solvent system hexane: diethyl ether: acetic acid, 80:20:1 (v/v/v) (Hunter et al. 1981; Kupke and Zeugner, 1966). Lipids were detected by inserting the TLC plates in chamber containing few crystals of iodine which has been already saturated with iodine vapour, after which the TLC plates are then carefully removed and the spots are gently circled with a dull pencil. Lipid compositions were further analyzed by gas chromatography-mass spectrometry (GC-MS). The GC-MS analysis of lipid extracts was performed using a GC-MS QP2010 PLUS Shimadzu, Japan. Interpretation on mass spectrum of GC-MS was done using the database of National Institute Standard and Technology (NIST), having more than 62,000 patterns. The mass spectrum of the unknown component was compared with the spectrum of the known components stored in the NIST library. The name, molecular weight and structure of the components of the test materials were ascertained.

RESULTS AND DISCUSSION

TLC of the resolved polar lipids of *E. coli* and *S. aureus* is presented in Plate 1. Five spots containing cardiolipin (CL), phosphatidic acid (PA), phosphatidylglycerol (PG),

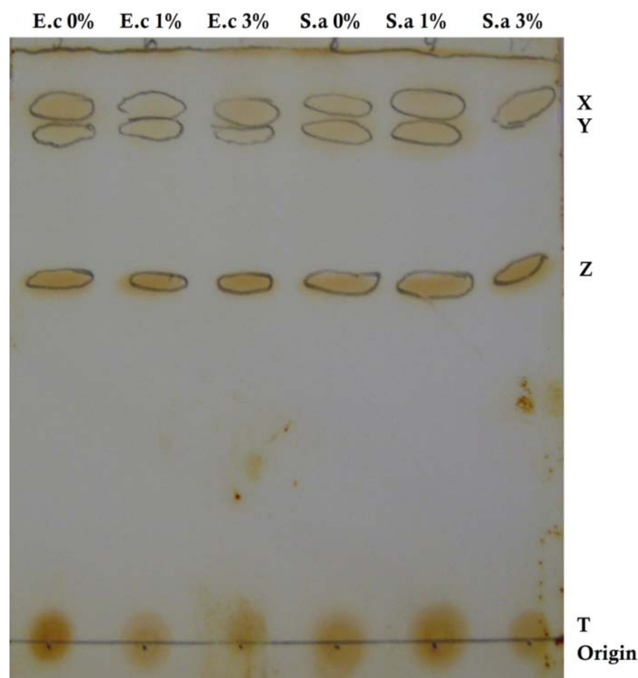

Plate 2. Thin-Layer chromatogram of neutral lipids from *E. coli* and *S. aureus.* Adsorbent: Coated alumina plates. Solvent: hexane: diethyl ether: glacial acetic acid, 70:30:1 (v/v/v). X = Steryl esters, Y = Triacylglycerols, Z = Diacylglycerols, T = Monoacylglycerols / Phospholipids. R_f values of spots were calculated and compared with those of Kupke and Zeugner (1966) and Hunter et al. (1981).

phosphatidylethanolamine (PE) and lysophosphatidylglycerol (LPG) were identified in both organisms at the different NaCl concentration investigated by comparing their R_f values with those of Hunter et al. (1981) who used the same solvent system. Also, Oku et al. (2004) reported the presence of phosphatidylethanolamine in *S. aureus* and *E. coli* at different salt stress condition. This further establishes the suggestion, that halophiles, including halococci exhibit highly similar lipid profiles (Kates, 1978).

The resolved neutral lipids from these bacteria showed four components on TLC for both organisms at the different NaCl concentration as shown in Plate 2; however, the triacylglycerol spot was not detected for *S. aureus* at 3% NaCl inclusion. The lipids identified include: steryl esters (SE), triacylglycerols (TAG), diacylglycerols (DAG) and monoacylglycerols (MAG)/phospholipids (PL).

Gram-negative bacteria are surrounded by two membranes, and the major lipids of such enteric bacteria cells are phosphatidylethanolamine, phosphatidylglycerol and cardiolipin (diphosphatidylglycerol) in varying ratios (Goldfine, 1982), while in the Gram-positive cocci and the lactobacilli the major polar lipids are phosphatidylglycerol and cardiolipin (Goldfine, 1982). The presence of phosphatidylethanolamine and phosphatidylglycerol in the bacteria considered is not unusual as most groups of bacteria, with the exception of the *Lactobacillaceae* and *Micrococcaceae*, contain phosphatidylethanolamine (Shaw, 1970). Phosphatidylglycerol, its O-amino acid esters, and cardiolipin are even more widely distributed among bacterial lipids (Shaw, 1970). To survive in this salt stressed environment, microorganisms considered would have tried to modify their membrane structures to modulate the chemical composition of lipids (Morrow et al., 1995).

Nagamachi et al. (1992) in their study reported that cardiolipin can stabilize liposomes during osmotic stress. It is also required for the growth of *Escherichia coli* and *Bacillus subtilis* under high-salt conditions (Lopez et al., 2006; Romantsov et al., 2007) hence, some strains of *E. coli* and coliforms are able to tolerate quite high NaCl levels (Colavita et al., 2003). Therefore, it can be inferred that membrane lipid like cardiolipin plays a major role in the adaptive mechanism of the bacteria subjected to the condition of high salt stress. Also, other mechanisms, including species-specific systems such as variations in cell wall proteins (Kuroda et al., 2008), enhanced *Staphylococci* ability to cope with high-salt stress (Wilkinson, 1996). In addition, the free glycerol, a well-known osmoprotectant, could contribute to the resistance of the cell to osmotic stress. Miller (1985) however observes a significant change in the amount of Phosphatidylethanolamine upon increase in the NaCl concentration of the medium where organism grows.

From the GC-MS analysis of *E. coli* at 0 and 3% salt inclusion as seen in Figure 1, four compounds; three of which are fatty acids namely: pentadecylic acid, oleic acid and palmitic acid where resolved for control condition, while for salt stressed condition, five compounds where resolved with four of them being fatty acids: pentadecylic acid, palmitinoleic acid, oleic acid and palmitic acid. Also, GC-MS analysis of *S. aureus* at 0 and 3% salt inclusion (Figure 2), reveals six compounds with four of them being fatty acids, namely: malonic acid, myristic acid, oleic acid and palmitic acid, while for salt stressed conditions, eight compounds were resolved with seven, being fatty acids: lauric acid, malonic acid, myristic acid, pentadecylic acid, palmitoleic acid, oleic acid and palmitic acid, respectively.

The level of unsaturation of fatty acids was generally observed to increase in the salt stressed *S. aureus* condition (3% salt inclusion) as compared to the control, just as the crucial role of unsaturated fatty acids in the microbial stress response mechanisms has been previously reported by Russel et al. (1995). There was a decrease in relative abundance of C 18:1 (oleic acid) observed in both Gram negative *E. coli* and Gram positive *S. aureus* at salt stressed condition than in control conditions. Gale and Llewellin (1971) suggested that oleate of the membrane may be linked with K^+ accumulation; therefore, they inferred that oleate is also necessary for the stability of *S. aureus* membrane. The inner concentration of NaCl depends on the medium in

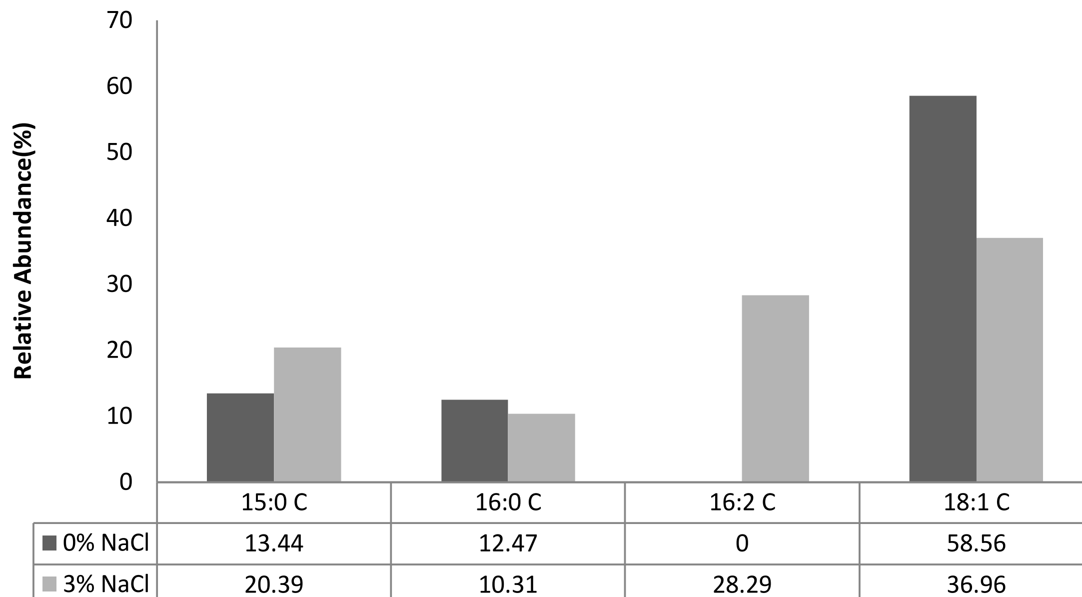

Figure 1. Relative abundance of fatty acids expressed from GC-MS of *E. coli* at 0 and 3% salt enhanced growth medium.

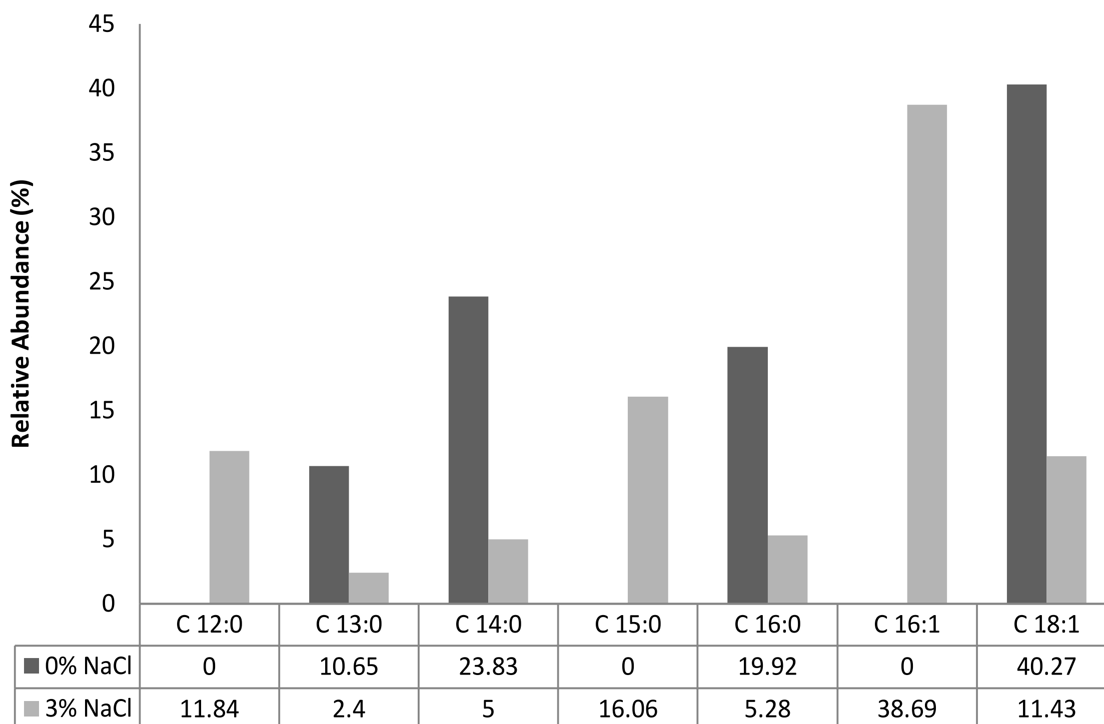

Figure 2. Relative abundance of fatty acids expressed from GC-MS of *S. aureus* at 0 and 3% salt enhanced growth medium.

which *S. aureus* is grown (Heller et al., 1998) and appears to be strictly related to internal K^+ concentration, indicating membrane activity (Hurst et al., 1973).

The presence of C16:2 in the cell extracts of *E. coli* (3% NaCl conc.) contradict the generally accepted observation that polyunsaturated fatty acids do occur among

members of the order *Eubacteriales* (Shaw and Stead, 1971). However, many authors have reported polyunsaturated fatty acids in bacteria. Katalinic and Fischer (1998), reported a C18:2 among fatty acids of *Bacillus megaterium* about 5% of the total acids. Yano et al. (1971), studied the fatty acids of *Arthrobacter simplex* grown in a variety of hydrocarbon-based media and reported the presence of C18:2 to be about 20% of the total fatty-acid composition, and confirmed their results by using mass spectroscopy. Therefore, that bacteria are capable of *de novo* synthesis of polyunsaturated fatty acids is evidenced by the work of Yano et al. (1971), who showed conversion of hydrocarbons, used as sole carbon sources, to polyunsaturated fatty acids.

Medium chain fatty acids with chain length between C8 to C14 were observed to be expressed in *S. aureus* at both control and salt -stressed condition which was not seen in *E. coli* at any of its salt conditions. This may further suggest one of the mechanisms *S. aureus* uses naturally to survive in salt stressed environment confirming it been halotolerant.

Conclusions

The lipid and fatty acid compositions of both bacteria considered were altered when the NaCl concentration of the growth medium were varied and in response to changes in growth salt conditions, the alteration of lipids obtained by on-probe GC-MS sample pre-treatment showed increased expression of unsaturated fatty acids at 3% NaCl concentration than 0% NaCl condition. Findings of this work also contribute to the comprehension of the membrane fatty acids modulation as it occur in bacteria in relation to their exposure to salt environment as previously suggested.

Conflict of interests

The author(s) did not declare any conflict of interest.

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