PJN
ISSN 1680-5194

PAKISTAN JOURNAL OF NUTRITION

ANSInet
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan
Mob: +92 300 3008585, Fax: +92 41 8815544
E-mail: editorpjn@gmail.com

Pakistan Journal of Nutrition 4 (3): 126-134, 2005
ISSN 1680-5194

Humic Acid Substances in Animal Agriculture

K.M.S. Islam, A. Schuhmacher and J.M. Gropp
Institute of Animal Nutrition, Nutrition Diseases and Dietetics, Gustav-Kühn-Straße 8,
D-04159 Leipzig, University of Leipzig, Germany

Abstract: Humic acids (HA), a class of compounds resulting from decomposition of organic matter, particularly plants are natural constituents of drinking water, soil and lignite. It inhibit bacterial and fungal growth, thus decrease levels of mycotoxins in feed. Stress management, immune system, anti-inflamatory activity, antiviral properties as well as prevention of intestinal diseases, mainly diarrhoea in humans and animals are described as its beneficial effect. The use of HA and related products in feed improved gut health for better nutrient utilization as well as improved the health status by working against pathogens by developing immunity. Routine use of HA in feed improved growth of broilers by increasing digestion of protein and trace element utilization but a few researches has been conducted in this area. However, also contradictory findings in piglets are described. Most of the literature found is from companies but scientific articles are rather limited. There are also strong limitations in the knowledge of HA uses as feed additive for growth of other species of animal. It is really difficult to compare the actual effects of HA preparations due to different sources and nature as well as because rearing of animal in different region of the world varies as climatic conditions and aspects. So, bio-effect of HA product depends on specification.

Key words: Humic acid, animal and agriculture, immune system

Introduction

Antimicrobial feed additives are world wide used so far in animal husbandry to improve the economy and ecology of animal production by increasing growth rate, decreasing feed expenditure per gain and diminishing the risk of disease (Hays, 1981; Gropp *et al*., 1992). But the unavoidable spread of bacterial resistance and cross-resistance to antibiotics used in veterinary and human therapy (Barton, 1998; Khachatourians, 1998) increasingly considered as a hazard, therefore the approval of antimicrobial growth promoters will be phased out by EU legislation by the end of 2005. Among many alternatives Humic acids (HA) are described. There are literature states that it has growth related effect as well as health protection capacity by changing some physiology and developing immunity in different species of animal. However, also contradictory findings in piglets are described (Schuhmacher and Gropp, 2000). Most of the cases different companies claims the HA substances as beneficial for the production of farm animal. So, it is important review the literature to draw an actual picture in relation to animal agriculture.

Organic matter in the soil exists in 3 different forms: (1) Living plant and animal matter, (2) Dead plant and animal matter and (3) decomposed plant and animal matter (humic substances). So, humic substances are the most common forms of organic carbon in the natural environment.

Concept of humic acid

Most humic substances are chemically attached to inorganic components (clay and oxides), and a smaller part gets dissolved in the solutions of the soil, particularly under alkaline conditions. An important feature of humic substances is that they can combine with metal ions, oxides and clay minerals to form water soluble or insoluble complexes and can interact with organic compounds such as alkenes, fatty acids, capillary-active substances and pesticides.

Farmers use humates to accelerate seed germination and improve rhizome growth (Humet Product Documentation and Technical Information, 1999). These materials are able to stimulate oxygen transport, accelerate respiration and promote efficient utilization of nutrient by plants (Visser, 1987; Österberg and Mortensen, 1994). These observations prompted scientists to study the specific properties of humates and their possible benefits in improving health and well being of humans and animals. Several humic substances have been identified.

Humus: This is the fraction of humic substances that is not soluble in water at any pH value. These substances have the greatest molecular sizes, as their molecular weights can be around 300,000 Dalton. The oxygen content in this substance is the lowest and falls in the

Correspondence author: Khan Md. Shaiful Islam, Department of Animal Nutrition, Bangladesh Agricultural University, Mymensingh-2202, Bangladesh

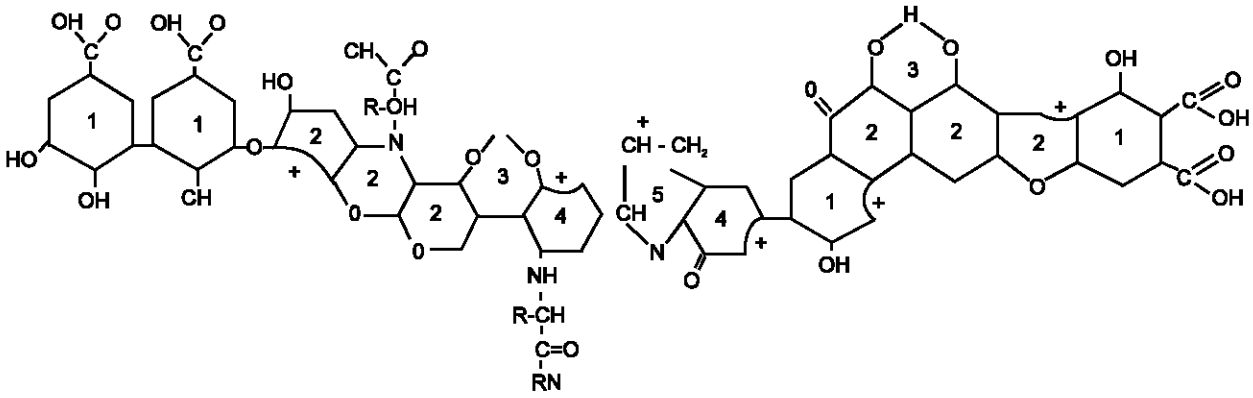


Fig. 1: Oxidized HA molecule

range of 32-34 %, while the nitrogen content is the highest, being around 4 %. Because of the high molecular weight, the negative surplus charge on their surfaces is insufficient for peptising the macromolecules even at strongly alkaline pH, and so their mobility in the soil is insignificant when in a coagulated state.

Humic acids: Humic acids are humic substances not soluble in water under acid conditions (below pH 2), but become soluble at a greater pH (HuminTech, 2004). Humic acids are soluble in dilute alkaline solutions and precipitate as soon as the solution becomes slightly acidic. These substances have medium molecular size and their molecular weight is around 5,000 to 100,000 Dalton. Oxygen represents 33-36 %, while nitrogen represents 4 % in this substance. Because of their medium molecular size, sufficient negative surplus charge on their surfaces for peptising the macromolecules will occur only in a more alkaline medium with a pH over 8 and thus their mobility in the soil is limited in neutral acidic-alkaline conditions.

Fulvic acids: Fulvic acids are soluble under all pH conditions. Those dissolve in dilute alkaline solution and will not precipitate even if the solution turns slightly acidic. These substances have the lowest molecular size, as their molecular weight is around 2,000 Dalton. This is the material with the highest oxygen content (around 45-48 %) and the lowest nitrogen content (less than 4 %). Because of their low molecular weight their surface negative surplus charge is sufficient to peptise the macromolecules even at neutral or slightly alkaline conditions resulting in significant mobility in the soil.

Phenolic acids: These substances are not defined based on solubility but identified as a component of humic substances.

Chemical structure of humic acid

Bio-Liquid Complex (Bio Ag Technologies International) derived from a type of leonardite (highly oxidized form of organic matter) differs from their theoretical formula because a part of its chemical structure has been oxidized. These broken bonds create places on the molecules where micronutrient ions can be absorbed. The oxidized sites give the entire molecule a negative charge enabling it to absorb micronutrients (Fig. 1). In the past these compounds have been largely overlooked because of the unavailability of adequate and sensitive analytical methods. A chemical structure for the basic skeleton of HA is based on alkylbenzenes,-napthhalenes and -phenanthrenes.

Soluble HA is available as either potassium humates or sodium humates (ie HA is only soluble in an alkaline base). Potassium humates are the product of choice for the soil because extra sodium is rarely required. Sodium humates are preferable for animals as sodium is an important inorganic electrolyte for animal.

Bioavailability and Composition of Humic Acid Products

A sample of Humisolve-R (Faust Bio-Agricultural Services) containing 73 % HA shows the following bioactive organic groups (Faust, 1998).

a) 3.32 % carbonyl, carboxyl and quinone groups
b) 28.1 % of phenol hydroxyls and nitrogen-containing aromatic groups
c) 7.78 % of aromatic and heterocyclic compounds
d) 44.7 % of protonated aromatic carbons
e) 16.06 % of methyl and methylene groups

Total bioactive organic group (a-d) in that sample of humate was 83-94 %. The activity had been confirmed by bioassay that had connected those bioactive organic chemicals to the humates on plants and animal cells (Faust, 1998).

Certified composition of HA/HuminFeed produced by Humintech GmbH, Heerdter Landstr. 189/D, D-40549 Düsseldorf, Germany, is (1) Water 14,50 %; (2) Ash (DM basis) 26,00 %; (3) Humic acids (DM basis) 74,00 %; (4) Sodium 8,9 %;(5) pH (in 10 % solution) 9 to 10. In a case study by Enviromate TM, 2002, the HA material contained

Table 1: Effect of dietary Farmagülatör DRY™ Humate on performance of male broilers (Kocabagli *et al.*, 2002)

Dietary regimes	Hatch weight	Body weight (g) (21 d)	Body weight (g) (42 d)	Feed:gain (g:g) (0-21d)	Feed:gain (g:g) (22-42)	Feed:gain (g:g) (0-42)	Overall mortality (%)
NAFH[1]	45.6	758	2346[b]	1.68	2.14[a]	1.99	8.0
$FH_{(0-21)}$[2]	45.5	735	2394[ab]	1.74	2.03[ab]	1.95	1.0
$FH_{(22-42)}$[3]	44.9	749	2451[a]	1.67	1.99[b]	1.89	5.0
$FH_{(0-42)}$[4]	44.6	737	2428[ab]	1.71	1.99[b]	1.92	7.0
SEM	0.31	7.9	25.9	0.039	0.037	0.032	2.03
P- value	0.144	0.140	0.029	0.545	0.0416	0.2522	0.114

[1]No added Farmagulator DRY™ Humate; [2]Farmagulator DRY™ Humate provided from 0 to 21 d (2.5 kg/ton feed); [3]Farmagulator DRY™ Humate provided from 22 to 42 d (2.5 kg/ton feed); [4]Farmagulator DRY™ Humate provided from 0 to 42 d (2.5 kg/ton feed)

Table 2: Effect of HA containing diets on live weight, feed conversion efficiency (FCE; gLWG/1000 g feed intake) and mortality of broiler[1]

Code	(1)	(2)	(3)	(4)	(5)	(6)
Humic acid (g kg^{-1} feed)	0	0.3	0.6	1.2	2.4	4.8
Composition of feed						
CP (g/100g air dry sample)	26.20	26,16	26.00	25.70	25.06	23.47
ME (MJ kg^{-1} feed)[2]	12,73	12,69	12,65	12,54	13,09	12,52
Performance of broiler						
Weight /bird on day 7	193.4[a]	193.5[a]	192.0[a]	192.3[a]	185.0[a]	174.2[b]
Weight /bird on day 14	547[a]	541[a]	543[a]	536a	521a	483[b]
Weight /bird on day 35	2408	2369	2335	2355	2310	2301
FCE from 0 to7 days	1011[a]	988[ab]	1020[a]	992[ab]	921[c]	930[bc]
FCE from 0 to 35 days	723[a]	712[ab]	715[ab]	709[ab]	701[ab]	689[b]
Mortality (%)	8.75	5.00	1.25	2.50	1.25	5.00

CP-Crude protein; [1]Research conducted at the Institute of Animal Nutrition, Nutrition Diseases and Diatetics, University of Leipzig, Germany. [2]Calculated from FMV (ME in MJ/kg = g Crude protein x 0.01551 + g Crude Fat x 0.03431 + g Starch x 0.01669 + g Sugar x 0.01301) according to German legal regulations (see Weinreich *et al.*, 2002). [abc]Different superscripts in the same row differ significantly ($p<0.05$)

(1) Crude protein 7.10 %; (2) Ash 8.33 %; (3) Crude fibre 12.50 %; (4) Carbohydrates 51.20 %; (5) Nitrogen 1.14 %; (6) Moisture 8.60 %; (7) Humic acids 42-48 %; (8) Fulvic Acids 12 % of HA. Many other literatures (not mentioned here) showed that the HA composition varies between the HA preparations of different companies but also between the different sources (soil), they were extracted from.

Humic acids as feed additive

Humic acids are not approved as feed additive, but as veterinary drug at EU level although many literatures indicated those as feed additives claiming growth promotion effect.

Better performance by improving nutritive value of feed: In recent years, it has been observed that humates included in feed and water of poultry promotes growth (Bailey *et al.*, 1996; Parks, 1998; Shermer *et al.*, 1998; Eren *et al.*, 2000). Kocabagli *et al.* (2002) studied to use (2.5 g kg^{-1}) of Farmagülatör DRY™ Humate (FH) (Farmavet International) on live performance, carcass weight, and the abdominal fat pad of broilers during different feeding periods (control-without FH), FH from 0-21 day (starter period), FH from 22-42 days (grower period). Feeding FH during the grower period had the most beneficial effect in terms of growth and feed conversion (Table 1). An other study (Eren *et al.*, 2000), compared the effects of dietary humate (Farmagülatör DRY™) supplementation at 1.5 and 2.5 g kg^{-1} feed on broiler performance from 0 to 42 d. Although there was no performance difference at 21 d, the authors found that dietary supplementation of humate at 2.5 g kg^{-1} significantly improved the live weights of broilers at 42d. They also showed that serum Na+ concentration and tibia bone ash of male broilers were significantly elevated when humate was fed at 2.5 g kg^{-1} but not 1.5 g kg^{-1}. Also a recent study conducted by the author at the Institute of Animal Nutrition, Nutritional Diseases and Diatetics, University of Leipzig, Germany indicated that the inclusion of HA during later stage in broiler diet could more beneficial in respect of performance (Table 2).

In 1998, a test was carried out on a wide scale at the Severny pedigree poultry breeding state farm near the town of Bratsk. The poultry losses decreased by 50%, while the live weight in five weeks increased by 30% (Teravita, 2004). The results, shown in the Fig. 2, strongly supported the recent findings of the author

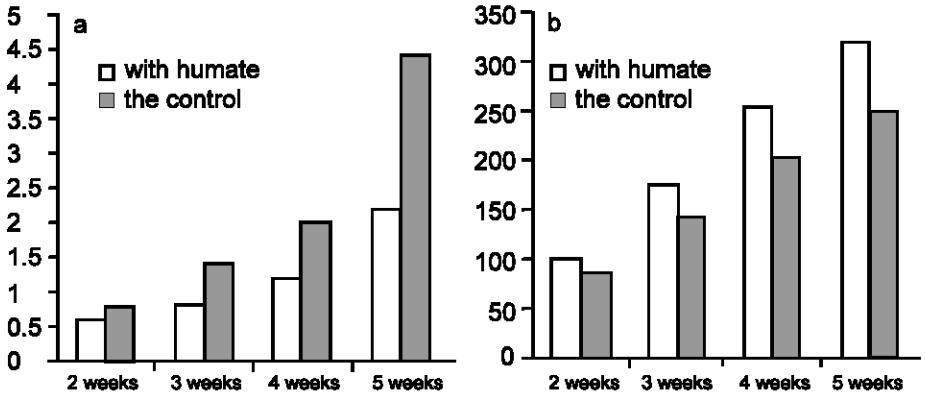


Fig. 2: The effect of the humate supplements on poultry loss (a) and live weight gain in poultry (b).

(Table 2) in respect of mortality but not strong for growth. Conversely Bailey *et al.* (1996) found that feeding 5 g Menefee® humate (MH) kg^{-1} feed (Sundine Enterprises, Inc.) to male broilers did not affect body weight, but improved feed conversion at 35 d. They also reported that dietary MH supplementation increased mortality significantly but also body weight by day 42 in female broilers. In another report with turkeys feeding MH improved weight gain and feed conversions from 8 to 12 wk of age ($P<0.05$), but this response did not persist until 20 wk of age. But, incorporation of humates in the feed of chickens has reduced unspecific deaths by 3 to 5 % (Stepchenko *et al.*, 1991) also supported by Kocabagli *et al.* (2002) and the recent work of the author (Table 2).

Humic acids stabilize the intestinal flora and thus ensure an improved utilization of nutrients in animal feed. This leads to an increase in live weight of the animal without increasing the amount of feed given to the animal (HuminTech, 2004). In the same brochure it is mentioned that diet digestibility as a result of maintaining optimum pH in the gut increases, resulting in lower levels of nitrogen excretion and less odour. Moreover, HA is said to improve protein digestion as well as calcium and trace element utilization.

The HA also leads to increased milk production and increased butterfat percentage in dairy cows, improved feed efficiency, decreased feed costs, reduced fly population and reduced costs for insect control. Furthermore, the weaning weights increased and faster weight gains were observed, while problems with scours greatly decreased (Livestock R. Us, 2003). On the whole, HA should increase the animal's resistance against heat stress.

Calves born from cows that have been fed humates, had a 13.4 % more weight within four months compared to control. The bull-calves that had been fed with humates, had an increase of 21.2 % body weight than control. The haematological data of animals in both humate-fed groups showed increased levels of haemoglobin by 11.5 %, phosphorus by 6.7 %, albumen by 24.3 %, and the β-globulin level increased by 32 %. In broilers feed activated the synthetic phase of albumineous exchange. As a result, there was a 10 % increase in body weight, and an immunity rise by 5-7 % (TeraVita, 2004).

Yasar *et al.*, 2002, concluded that HA caused increased weight gain in rats. The improved weight gain was associated with increased ileal epithelial mass, increased feed intake, improved feed:gain ratio and increased nitrogen retention in rats.

Replacing antibiotic growth promoter: Replacing antibiotic with HA as growth promoter in animal feed does not cause any loss in the performance of animals. On the contrary, performance factors (LWG, FI, FCR and faeces consistency - scour assessment) of animals are considerably improved. The use of HA in animal feed excludes of course the possibility of antibiotic residue or microbial resistance (Humin Tech, 2004). Simultaneously, as a result of a higher food conversion rate and enhanced absorption of nitrogen by the animal, nitrogenous wastes and odour are reduced. It was also concluded by Ceylan and Ciftci, (2002), that HA would be an alternative to antibiotic growth promoters in broiler diets.

Health value of humic acid substances

Scientists at the Drepropetrovish Agricultural Institute in Moscow revealed humate as harmless with respect to blood, cardio-vascular system, endocrine system and other vitally important organs using patho-histological and histo-chemical methods. The toxicity of naturally occurring HA is remarkably low (Thiel *et al.*, 1981). An LD_{50} of 0.536g kg^{-1} bw can be considered as confirmation of the harmlessness of humate (Lotosh, 1991). Current repeat toxicity studies in rodents indicated total safety at levels up to 50 mg kg^{-1} body weight (Laub, 1998b).

Humic acids should inhibit pathogenic bacterial growth and growth of moulds, thus decreasing levels of

Table 3: Relative organ weight (liver, spleen and Bursa fabricii) in percent of body weight of humic acid fed broiler

Code	(1)	(2)	(3)	(4)	(5)	(6)
Humic acid (g kg^{-1} feed)	0	0.3	0.6	1.2	2.4	4.8
Liver	2.21	2.34	2.20	2.31	2.22	2.19
Spleen	0.08	0.11	0.10	0.10	0.08	0.09
Bursa fabricii	4.22	4.46	3.23	4.15	2.92	3.26
Thyroid gland	0.009	0.012	0.011	0.011	0.011	0.010

NB: Experiment conducted for a period of 35 days at the Institute of Animal Nutrition, Nutrition Diseases and Dietetics, University of Leipzig, Germany. No significant differences observed among the treatments for different parameters ($p>0.05$)

Table 4: Morphometric parameters of thyroid gland of chicks offered different levels of humic acid in feed

Code	(1)	(2)	(3)	(4)	(5)	(6)
Humic acid (g kg^{-1} feed)	0	0.3	0.6	1.2	2.4	4.8
No. of follicles/location*	19.64^{a}	17.34^{ab}	18.38^{ab}	12.68^{b}	17.91^{ab}	18.04^{ab}
Diameter of follicles (µm)	64.41^{a}	70.58^{ab}	71.1^{ab}	84.47^{b}	70.30^{ab}	69.96^{ab}
[1]Height of epithelia (µm)	4.23	4.35	4.23	4.59	4.11	4.00
[1]Length of nucleus (µm)	5.31^{a}	5.11^{ab}	4.96^{ab}	4.72^{b}	4.74^{b}	4.74^{b}
[1]Width of nucleus (µm)	2.80	3.00	3.00	2.95	2.76	2.65
No. of hyperplasia	1.05^{a}	0.79^{ab}	0.67^{ab}	0.59^{ab}	0.51^{b}	0.75^{ab}
FEI	15.38	16.33	16.98	18.66	17.36	17.80

*(367.53X285.75 µm); NB. Ten birds per group and 11 location of each slide from right lobe were considered; [1]Ten replicates were considered per location of the slide from a bird and 110 replications per bird/slide. Follicle epithelium index (FEI) = Follicle diameter (µm)/Epithelial height (µm). [abc]Different superscripts in the same row differ significantly ($p<0.05$)

mycotoxin, which should lead to improved gut health (Humin Tech, 2004). Dermal, oral or subcutaneous application of HA leads to inhibitory effects on inflammation. The ability to inhibit inflammation is believes to be related with the flavonoid groups contained in HA.

Humic acids are able to form a protective film on the mucous epithelia of the gastro-intestinal tract against infections and toxins (Kühnert *et al.,* 1991). The macro-colloidal structure of HA ensures a good shielding on the mucous membrane of the stomach and gut, the peripheral capillaries and damaged mucous cells. As a result of this process, the resorption of toxic metabolites is reduced or fully prevented, especially after infections, in case of residues of harmful substances in animal feed or when it is switched to new feeds. Furthermore, HAs also help to prevent excessive loss of water via the intestine (HuminTech, 2004). Those are used in horses, ruminants, swine and poultry at an oral doses level of 500 to 2000 mg kg^{-1} bw for the treatment of diarrhoea, dyspepsia and acute intoxications.

There are some parameters indicating physiological benefits due to HA and related products, they are given below.

Blood parameters: For humans 100-300 mg kg^{-1} bw has no effect on bleeding time, clotting time, thrombin time, plate count, or induced platelet aggregation (Malinowska *et al.,* 1993). Red blood cells (RBC) and haemoglobin level remained on normal levels under the influence of humate in comparison with control (Lotosh, 1991). Literature has indicated that the RBC was capable to carry more oxygen in presence of humate. This additional oxygen causes feelings of euphoria, similar to hyperventilating, during the first few days of taking humate. Healing of injuries, as a result of additional oxygen, is much quicker. Cutting horses have ankle inflammations frequently from their rigorous training programs. Healing times for these injuries have been reduced by the usage of humates. According to Dabovich *et al.,* 2003, a HA product Promax has nutriceutical properties in that it stimulates neutrophil activity which may protect against bacterial pathogens and reduce mortality during acute bacterial infection.

Mineral transfer: Humic acids act as dilator increasing the cell wall permeability. This increased permeability allows easier transfer of minerals from the blood to the bone and cells. Calcification of a bovine implant was improved by 16% (Kreutz and Schlikekewey, 1992). There are also changes in intracellular divalent calcium levels (Yang *et al.,* 1996). Hoewever, literature also reports binding of iodine from foods (Summers *et al.*, 1989) so that antithyroideal effects could be supposed (Seffner *et al.,* 1995). But reverse concluded by (Huang *et al.,* 1994) that the HA do not induce goiter, but they may enhance the goitrogenic effect of low iodine. From a recent study by the author at the Institute of Animal Nutrition, Nutrition Diseases and Diatetics, University of Leipzig, Germany indicated the absence of goiter genic effect in broiler showing lack of dose related effects on visceral organs and histomorphometric parameters of thyroid gland (Table 3 and 4). Just as fulvic acid carries life-sustaining minerals to the body also captures and

removes toxic metals from the body. Fuchs *et al.,* 1982, indicated that the HA had differentiated effects upon trace elements in laboratory rat. Plasma iron levels were hardly affected, while copper and zinc levels were initially suppressed with a tendency for recovery after 60 days.
Seffner *et al.*, 1995, could show that small amounts of HA given to the rat for 8-14 weeks result in histological signs of goitre and trace it back to a reduced iodine availability in the intestine. So, it is contradictory of the recent findings of the author because (Table 3 and 4) Seffner *et al.*, 1995, applied to drinking water, were comparable to our 2.4 g kg^{-1} feed. The rat diet contained (by certificate of the producer) 0.9 mg I kg^{-1}. Authors experimental diet contained 2.5 mg added I kg^{-1} feed. Both dosages were quite above the requirement or the allowance data. Probably, (i) high iodine of diet inhibited the potential occurrence of goitre, (ii) the method was too rough to detect the initial stage of iodine deficiency, and (iii) length of the feeding trial was to short.

Stress management: Literature reports that humates reduce the production of stress causing hormones. This has been cumulated from animal behaviour, in particular from calves first entering the arena. Animals on humate are less affected by the outside stimulus of the crowds or confining areas of the arena. This effect has also been noted on sheep, horses, cattle and hogs. In dairy operations, those animals not on humate aggressively eat their rations while humate animals leisurely graze (Enviromate, 2002).

Microbial interaction: In soil tested for microbial activity, levels increased 400 to 5000 times with the addition of 300 ppm humate into the soil. Humates added to feed stimulate the microbial growth and the extent can be quite large depending upon the species, the culture medium, and the environment (Huck *et al.,* 1991). Species for which natural humic substances have been shown to be inhibitory include *C. albicans, Ent. cloacae, Prot. vulgaris, Ps. aeruginosa, S. typhimurium, St. aureus, St. epidermidis, and St. pyogenes* (Riede *et al.,* 1991). It seems that within the body, humates stimulate the "good" microbes while suppressing the "bad" microbes.
Testing of milk during field trials often indicates an increase of microbes in the milk, an indication to the dairyman of impending mastitis. As a result of feeding humates, mastitis cases within the milking herd dropped from an average of 3 to 4 cases daily to 4 cases in a month (Mosley, 1996). Additional confirmation of reduction of mastitis was observed in lactating female goats.

Immune system: By improving immune functions in the animal, HA are able to reduce the incidence of diarrhoea and other digestive upsets to a considerable extent as well as to improve the animal's defences against pathogens such as *E. coli* (HuminTech, 2004). According to CVMP, 1999, the intramuscular injection of the HA sodium salt (1 mg kg^{-1} bw) to rabbits had no effects on haematological parameters and the glucose concentration in blood, but affected the albumine/globuline ratio in plasma (marked increase of the β-globulin fraction). Pukhova *et al.* (1987) found that sodium humate increases the lifespan of mongrel rats exposed to lethal doses of cobalt radiation.

Anti-inflammatory properties: Humic acids isolated from peat exhibited significant efficacy for adhesions when tested on female rats that had standardized lesions placed on both uterine horns and the peritoneum of the anterior abdominal wall (Yang *et al.,* 1996). According to Kühnert *et al.,* 1982 the humic substances, including peat and sodium humates, are known to exhibit anti-inflammatory properties. Not only does the humate relieve from inflammation, it has been shown to bond to the collagen fibers to aid in repair of damaged tendons and bone. Tendon strength has been shown to increase by as much as 75% (Iubitskaia and Ivanov, 1999; Kreutz and Schlikekewey, 1992).

Anti-viral properties: Humates are effective media additives for the production of antibiotics in the soil (Huck *et al.,* 1991). Humic substances have long been known to exhibit antiviral properties in particular against rhinoviruses (Enviromate, 2002). Viral pathogens for which soil-extract materials have been shown to be effective include in particular Coxsackie virus A9, herpes simplex virus type 1 and 2 (Schiller *et al.,* 1979; Thiel *et al.,* 1981; Thiel *et al.,* 1977; Laub 1998a; Laub 1998b, and Knocking, 1991), human immunodeficiency virus (HIV) (Laub 2000; Laub, 1995), influenza type A and B (Laub 2000 and Enviromate, 2002), as well as other respiratory tract infections (Schultz, 1965; Knocking, 1991 and Jankowski *et al.,* 1993).
In earlier times, HAs have also been employed as veterinary medicine therapy successfully employing peat mull (extracted HA) to prevent the transmission of foot and mouth disease in pigs (Schultz, 1965). Lotosh, 1991, mentioned the humate as a pharmacy that raises resistance against non-specific diseases. This fact was confirmed by using such models as atoxic anemia, toxic hepatitis, peptic ulcer and hypercholesterolemia.

Liver effects: In an experimental model with partially hepatectomised rats, long-term application of HA resulted in the stimulation of omithine decarboxylase, an increase in spermidine and histamine as well as DNA and RNA levels, and in overall liver mass (Maslinski *et al.,* 1993). It is also clear that the humate plays a role in the liver function and protects somewhat from disease and/or disturbances (Lotosh, 1991).

Odour reduction: Texas A and M University System researchers have discovered that using humate decreases volatile ammonia in animal waste by 64%, reduces odour, and improves the nitrogen to phosphorus ratio in the waste (Parker *et al.*, 2001). Scientists are developing rations formulated to enhance manure characteristics while maintaining the performance of animal (Greene and Cole, 2000; Mosley, 1996).

Residue in food material

In residue studies swine orally received a mixture of Humocarb and concentrated HA (ratio 16:1) at a dose level of 500 and 2000 mg kg^{-1} bw day^{-1} for 30 days and sheep orally received 1000 to 2000 mg kg^{-1} bw day^{-1}. At the end of the treatment periods no HA could be detected by a photometric method (limit of detection: 10 to 50 µg ml^{-1}) in blood plasma and muscle, liver and kidney (CVMP, 1999). The results obtained by different researchers (Lange *et al.*, 1996; Kühnert *et al.*, 1989) indicated that HA 1500 is toxicologically not harmful after oral administration. Taking into account the pharmacokinetic data, residues of the substance in animal tissues can be ruled out with high significance. They also found that after oral administration of 500 mg HA 1500 kg^{-1} bw, the half life period was 1.5 hours and maximum plasma concentration was 3 µg ml^{-1}.

Conclusion: There is no doubt that HA has many beneficial effect like antibacterial, antiviral and anti inflammatory in animals, improves immune system, stress management and reduce odour in faeces. It also has positive effect on liver functioning. Ultimately reduces mortality and increases growth in poultry. But the level of benefits is now questionable for ruminants because due to its antimicrobial affect may cause depression of protein synthesis by reducing rumen microorganism. Supported literature those indicates the HA as growth promoter in ruminants seems weak in this aspects and facing question. It's affect as goitrogenic substance in rat is rejected in case of poultry by recent findings. So, in relation to growth promoter, using routinely is not so positive but where health risk is higher might be reflect beneficial due to protection of diseases. It is also difficult to compare the actual effects of HA preparations due to different sources and preparations as well as because rearing of animal in various region of the world differing the climate.

References

Barton, M.D., 1998. Does the use of antibiotics in animals affect human health. Aust. Vet. J., 763: 177-180.

Bailey, C.A., K.E. White and S.L. Donke, 1996. Evaluation of Menefee Humate™ on the performance of broilers. Poult. Sci., 75(Suppl. 1): 84. (Abstr.).

Ceylan, N. and I. Ciftci, 2002. The effects of some alternative feed additives for antibiotic growth promoters on the performance and gut microflora of broiler chicks. Turk. J. Vet. Anim. Sci., 27: 727-733.

CVMP (Committee for Veterinary Medicinal Products), 1999. Humic acid and their sodium salts (summary report). The European Agency for the Evaluation of Medicinal Products. EMEA/MRL/554/99-FINAL February 1999.

Dabovich, L.A., L. Hulbert, A. Rudine, S. Kim F. Ji and J.J. McGlone, 2003. Evaluation of nutriceutical effects on pig immunity: Effects of Promox. 2003 Southern Section ASAS meeting. Pork Industry Institute, Department of Animal and Food Science, Texas Tech University, Lubbock, TX 79409.

Enviromate, T.M., 2002. Effects of humic acid on animals and humans (literature review and current research). Effects of humic acid. Enviromate Inc. 8571 Boat Club Road, Forth Worth, Texas 76179. http://www. enviromateinc.com /effect sha.asp

Eren, M., G. Deniz, S.S. Gezen and I.İ. Türkmen, 2000. Broyler yemlerine katilan humatlarin besi performansi, serum mineral konsantrasyonu ve kemik külüüzerine etkileri. Ankra Üniv. Vet. Fak. Derg., 47: 255-263.

Faust, R.H., 1998. What is the formula for humic and fulvic acids? New information!! Untitled article. http://www.bioag.com/whats-in-humates.html

Fuchs, V., S. Golbs, M. Kühnert, W. Schopeck and B. Stier, 1982. Studies into action of humic acids on selected trace elements in laboratory rats. Arch. Exper. Vet. Med., Leipzig 36. March, 2: 187-191.

Gropp, J., D. Birzer and A. Schuhmacher, 1992. Vom Gesamtnutzen der Futterzusatz-stoffe; ein Beitrag zur Auflösung des Widerstreits von Ökonomie und Ökologie. pp.168-204. Schriftenreihe der Akademie für Tiergesundheit, Bonn, Band 3. Verlag der Ferber'schen Universitätsbuchha-ndlung Gießen.

Greene, L.W. and A. Cole, 2000. Efficient waste and odor management for feedlots.-USDA/ARS. The Agriculture Program, Texas A & M University System, AGCOM 5-1-00. http://agprogram.tamu.edu/; press release, May, 2000.

Hays, V.W., 1981. The Hays Report: Effectiveness of feed additive usage of antibacterial agents in swine and poultry production. Long Beach, CA: Rachelle Laboratories, Inc.; Report, 12476-01,5/pp81.91.

Huck, T.A., N. Porter and M.E. Bushell, 1991. Effect of humates on microbial activity. Gen. Microbiol. 137: 2321-2329.

Huang, T.S., F.J. Lu, C.W. Tsai and I.J. Chopra, 1994. Effect of humic acids on thyroidal function. J. Endocrinol. Invest., 17: 787-791.

HuminTech., 2004. Huminfeed-Tierfutterzusätze and Veterinär Medizin and Huminsäure Basierende Produkte. Humintech®Humintech GmbH, Heerdter Landstr. 189/D, D-40549 Düsseldorf, Germany, http://www.fulvic.de/049/ani malfeeds/products/ huminfeed.html http://www.fulvic.de/049/ animalfeeds/ products/huminfeed.html

Humet Product Documentation and Technical Information, 1999. Horizon Multiplan LTD.: Budapest, 1999.

Iubitskaia, N.S. and E.M. Ivanov, 1999. Sodium humate in the treatment of osteoarthrosis patients. Vopr Kurortol Fizioter Lech Fiz Kult., 5: 22-24.

Jankowski, A., B. Nienartowicz, B. Polañska and A. Lewandowicz-Uszyñska, 1993. A randomised, double-blind study on the efficacy of Totpa Torf Preparation (TTP) in the treatment of recurrent respiratory tract infections. Arch. Immunol. Ther. Expt., 41: 95-97.

Kocabagli, N., M. Alp, N. Acar and R. Kahraman, 2002. The effects of dietary humate supplementation on broiler growth and carcass yield. Poult. Sci., 81: 227-230.

Kühnert, V.M., V. Fuchs and S. Golbs, 1982. Chemical characterisation and pharmacologico-toxicological peculiarities of humic acid. Arch. Exp. Veterinarmed. Leipzig, 36: 169-177.

Kühnert, V.M., K.P. Bartels, S. Kröll and N. Lange, 1991. Huminsäurehaltige Tierarzneimittel in Therapie and Prophylaxe bei gastrointestinalen Erkrankungen von Hund und Katze. Monatshefte Vet. Med., 46: 4-8.

Kühnert, V.M., V. Fuchs and S. Golbs, 1989. Pharmakologish-toxikologische Eigenshaften von Huminsäuren und ihre Wirkungsprofile für eine veterinarmedizinische Therapie. Deutsche Tierärztl. Wschr, 96: 3-10.

Knocking, R., 1991. Interaction of humic acids and humic acid like polymers with herpes simplex virus type 1. Humic Substances in the Aquatic and Terrestrial Environment, Berlin, pp: 408-412.

Kreutz, and W. Schlikekewey, 1992. Effects of Implanted bovine calcium hydroxyapatite with humate. Arch. Orthop. Trauma Surg, 111: 259-264.

Khachatourians, G.G., 1998. Agricultural use of antibiotics and the evolution and transfer of antibiotic-resistant bacteria. Can. Med. Assoc. J., 159: 1129-1136.

Lange, N., M. Kühnert, A. Haase, H. Höke and B. Seubert, 1996. Studies concerning the resorption properties of a low molecular humic substance after single oral application to rats. Dtsch. Tierärztl. Wschr, 103: 134-135.

Laub, R., 1995. The chemically induced inhibition of HIV-1 replication. Laub BioChem Corp., January 1995. www.laubiochem.com http://www.laubiochem.com/.

Laub, R., 1998a. Acute systemic toxicity studies of natural product and synthetic humates. Laub BioChem Corp, August 1998. www. laubbiochem. com http:// www. laubbiochem. com/.

Laub, R., 1998b. The chemically induced inhibition of HSV infection. Laub BioChem Corp., August 1998. www.laubiochem.com http://www.laubiochem.com/.

Laub, R., 2000. Laub developing humate with anti-HIV, HSV, HPV and other antiviral activity. Biotechnology Information Institute, February 2000. Antiviral Drug and Vaccine Development Information, Vol. 12, No. 2. ISBN 0897-9871.

Livestock R. US, 2003. Field trials on Dairy Cattle. ENVIROMATE Inc. 8571 Boat Club Road, Fort Worth, Texas 76178. www.livestockrus.com/ consignments/envi romate/enviromate.htm http://www.livestockrus.com/consignments/envi romate/enviromate.htm

Lotosh, T.D., 1991. Experimental bases and prospects for the use of humic acid preparations from peat in medicine and agricultural production. Nauchnye Doki Vyss Shkoly Biol. Nauki., 10: 99-103.

Maslinski, C., W. A. Fogel. and W. Andrzejewski, 1993. An examination of humate stimulated liver functions. *Acta Pol. Pharm.* 50(4-5):413-416.

Malinowska, M.H., D. Pietraszek and E. Chabielska, 1993. Acta Pol. Pharm., 50: 507-511.

Mosley, R., 1996. Field trials of dairy cattle. Non-published research. Enviromate, Inc. August 1996.

Österberg, R. and K. Mortensen, 1994. The growth of fractal humic acids: cluster correlation and gel formation. Radiat. Environ. Biophys., 33: 269-276.

Parks, C.W., 1998. The use of Menefee HumateTM in typical and low-crude protein diets for turkey toms and in the bioremediation of petroleum-contaminated soil amended with poultry litter as a co-substrate and nutrient source. Master's Thesis. North Carolina State University, Raleigh, NC.

Parker, D., B. Auvermann and W. Greene, 2001. Effect of chemical treatments, ration composition and feeding strategies on gaseous emissions and odor potential of cattle feedyards. Pre-publication Texas A&M Extension Service, December 2001. TAMU Ag Research and Extension Ctr. Amarillo,Tx.

Pukhova, G.G., N.A. Druzhina, L.M. Stepchenko and E.E. Chebotarev, 1987. The influence of natrium humate on animals irradiated with lethal doses. Radiobiologiia. 27: 650-653.

Riede, U.N., G. Zeck-Kapp, N. Freudenberg, H.U. Keller and B. Seubert, 1991. Humate induced activation of human granulocytes. Virchows Arch B Cell Pathol Incl Mol. Pathol., 60: 27-34.

Schiller, F., R. Klöcking, P. Wutzler and I. Färber, 1979. Tropical ammoniumhumat treatment of human HSV infections-Preliminary clinical results. Dermatol Monatsschr, 165: 505-509.

Seffner, W., F. Schiller, R. Heinze and R. Breng, 1995. Subchronic application of humic acids and associated compounds provokes histological changes of goitre in the rat. Exp. Toxic. Pathol., 47: 63-70.

Schultz, H., 1965. Investigations on the viricidal effects of humine-acids in peat-mull. Deutsche Tierärztl Vochenschr., 72: 294-297.

Shermer, C.L., K.G. Maciorowski, C.A. Bailey, F.M. Byers, and S. Ricke, 1998. Caecal metabolites and microbial populations in chickens consuming diets containing a mined humate compound. J. Sci. Food Agri. 77: 479-486.

Summers, R.S., F. Fuchs and H. Sontheimer, 1989. The fate and removal of radioactive iodine in the aquatic envrionemnt. In: Advances in Chem. Aquatic Humic Substances (ed. J.H. Suffet and P. McCarthy). 219: 623-636.

Schuhmacher, A. and J.M. Gropp, 2000. Effect of humic acids on health state and performance of weaners. Proc. Soc. Nutr. Physiol., 9: 77.

Stepchenko, L.M., L.V. Zhorina and L.V. Kravtsova, 1991. The effect of sodium humate on metabolism and resistance in highly productive poultry. Nauchnye Doki Vyss Shkoly Biol. Mauki., 10: 90-95.

TeraVita TM, 2004. Humates in Poultry and Stock Farming. http://www.teravita.com/Humates/ Chapter 9.htm

Thiel, K.D., R. Klocking, H. Schweizer and M. Sprossig, 1977. In vitro studies of the antiviral activity of ammonium humate against herpes simplex virus type 1 and type 2. Zentralbl Bakteriol., 239: 304-321.

Thiel, K.D., B. Helbig, R. Klöcking, P. Wutzler, M. Sprössig and H. Schweizer, 1981. Comparison of the in vitro activities of ammonium humate and of enzymically oxidized chlorogenic and caffeic acids against type 1 and type 2 human herpes virus. Pharmazie, 36: 50-53.

Visser, S.A., 1987. Effect of humic substances on mitochondiral respiration and oxidative phosphorylation. The Science of the Total Environment. 62: 347-354.

Weinreich, O., P. Radewahn and B. Krüsken, 2002. Schätzgleichungen zur Berechnung des Energiegehaltes von Mischfuttermitteln. *In:* Futtermittelrechtliche Vorschriften. Agrimedia GmbH, Spithal 4. D-29468 Bergen/Dumme, pp: 179-180.

Yasar, S., A. Gokcimen, I. Altunas, Z. Yonden and E. Petekkaya, 2002. Performance and ileal histomorphology of rats treated with humic acid preparations. J. Anim. Physi. Anim. Nutr., 86: 257-264.

Yang, H.L., H.C. Chiu and F. Lu, 1996. Effects of humic acid on the viability and coagulant properties of human umbilical vein endothelial cells. Am. J. Hematol., 51: 200-206.

Pakistan Journal of Nutrition 4 (3): 135-141, 2005
ISSN 1680-5194

Antioxidant Actions of Dried Flower Extracts of *Hibiscus sabdariffa* L. On Sodium Arsenite - Induced Oxidative Stress in Rats

I.F. Usoh[1], E.J. Akpan[1], E.O. Etim[1] and E.O. Farombi[2]
[1]Department of Biochemistry, Faculty of Basic Medical Sciences, University of Uyo, Nigeria
[2]Department of Boichemistry, Faculty of Basic Medical Sciences, University of Ibadan, Nigeria

Abstract: The antioxidant actions of 80% ethanolic extract of dried flowers of *Hibiscus sabdariffa* L. (HSE) on lipid peroxidation (LPO), reduced glutathione (GSH), glutathione-s-transferase (GST), catalase (CAT), superoxide dismutase (SOD) and vitamin C (VITC), were examined using a model of sodium arsenite (SA) - induced oxidative stress in rats. The oral administration of the extracts (200 and 300mg/kg body weight) significantly ($P<0.05$) decreased by 37% SA - induced malondialdehyde (MDA) formation in liver, suggesting the role of the extract in protection against pro-oxidant induced membrane damage. Pretreatment with the extracts prior to the intra-peritoneal administration of 10mg/kg body weight of SA reduced significantly (by 86%) and induced non-significantly (by 37%) the level of GSH depletion and GST activity respectively in a dose - dependent manner. The extract also attenuated SA - induced reduction in the serum level of VITC as evidenced by a significant ($P<0.05$) dose - dependent increase (by 60%) in serum VITC level. Pretreatment with the extracts showed a significant ($P<0.05$) increase in liver and decrease in whole blood activities of SOD (by 369% and 85%) and CAT (by 829% and 58%) respectively, hence revealing the hepatoprotective and antioxidant effectiveness of the extracts. Furthermore, the extracts, evaluated (*in vitro*) by their capacity of quenching 1, 1-diphenyl - 2-picrylhydraxyl (DPPH) free radical, showed strong scavenging effects on DPPH free radical at concentration of 0.20mg/ml (1C50 = 0.20mg/ml). The extracts at low and high concentrations showed no inhibitory effect on nitric oxide radical. These findings are suggestive of the possible chemopreventive and antioxidative role played by dried flower extract of *Hibiscus sabdariffa* L.

Key words: Sodium arsenite, oxidative stress, *Hibiscus sabdariffa* L., antioxidant activity

Introduction

Reactive oxygen species (ROS) or free radicals are generated as byproducts or intermediates of aerobic metabolism and through reactions with drugs and environmental toxins. The elevated cellular levels of free radicals cause damage to nucleic acid, proteins, and membrane lipids and have associated with many aging-related problems including carcinogenesis and heart diseases (Halliwell *et al*., 1992; Halliwell, 1996; Wang and Jiao, 2000). The balance between the production and scavenging of ROS can therefore determine the susceptibility of the body to oxidative damage. Although almost all organisms possess antioxidant defense and repair systems, which quench or minimize the production of oxygen-derived species, thus protecting them against oxidative damage, these protective systems are insufficient to entirely prevent the damage (Simic, 1988) caused by endogenous or exogenous oxidants (Sun, 1990). In view of this, much attention has been focused on the protective biochemical function of naturally occurring antioxidants in biological systems, and on the mechanisms of their actions. The phenolic compounds, which are widely distributed in plants, were considered to play an important role as dietary antioxidants for the prevention of oxidative damage in living systems (Block, 1992; Hertog and Feskens, 1993).

The study of numerous compounds that could be useful antioxidants has generated increasing interest in the field of food or medicine. The dried flowers of *Hibiscus sabdariffa* L. (Malvaceae) commonly called “zobo” by Nigerians have gained importance as local soft drink and medical herb in local regions. Studies revealed that the dried flowers of *Hibiscus sabdariffa* L., a Chinese herbal medicine, have been used effectively in folk medicine against hypertension, pyrexia, and liver disorders (Tseng *et al*., 1997). The present study was aimed at evaluating the antioxidant effects of ethanolic extracts of dried flowers of *Hibiscus sabdariffa* L., on some biomarkers of oxidative stress in rats treated with sodium arsenite.

Materials and Methods

Plant material: The fresh flowers of *Hibiscus sabdariffa* L. were purchased at Bodija Market, Ibadan. They were dried at room temperature and sohxlet extracted with 80% ethanol (three changes), lyophilized, weighed and preserved at 4^{0}C and used as and when required (Rana *et al*., 2000).

Experimental animals: Twenty female Wistar rats (160-200g) obtained from the Department of Physiology, University of Ibadan were used. They were maintained

and housed in cages in the departmental animal house and fed on commercial rat pellets obtained from Mokola, Ibadan.

Experimental design: Animals were randomized into five groups with four in each group and put on normal diet. The extract and sodium arsenite, dissolved in distilled water were administered orally and intraperitoneally (ip) respectively.

Group A (control): Only normal diet: Group B: Animals were treated with only 10mg/kg body weight of SA.
Group C Animals were treated with 200mg/kg body weight of HSE for 7 days.
Group D Animals were treated for 6 days with 200mg/kg body weight of HSE and 10mg/kg body weight SA on the 7th day.
Group E Animals were treated for 6 days with 300mg/kg body weight of HSE and 10mg/kg body weight of SA on the 7th day.

Preparation of serum and microsomal fractions of liver homogenate: The animals were sacrificed 24 hours after the sodium arsenite administration by cervical dislocation. The blood was collected by heart puncture and serum was separated by centrifugation (3000rpm at 4°C for 10 mins). The serum was used for the determination of vitamin C. The whole blood was also collected for the determination of catalase and SOD activities. The liver was immediately removed, washed in ice cold 1.15%kcl solution, blotted, weighed and homogenized in 4 volumes of the homogenizing buffer (pH7.4) using a potterelvegin homogenizer. The resulting liver homogenate was centrifuged at 10,000g for 30mins, in a Beckman L5 - 50B ultracentrifuge with a 220.78VD2 rotor at 4°C. The solution (supernatant) was decanted and part of it used for GST assay. The other portion was further centrifuged at 105,000g for 1 hour with a type 35 fixed angle rotors in the same ultracentrifuge. Pellet microsomes were suspended in 0.25M sucrose solutions and this was stored in a frozen condition. These procedures were carried out at temperature between 0°C and 4°C so as to retain enzyme activity. The liver microsome was used to determine lipid peroxidation, reduced glutathione, SOD, catalase and free radical scavenging capacity.

Assessment of lipid peroxidation: Lipid peroxidation in microsomes prepared from liver was estimated spectrophotometrically by Thiobarbituric acid - reacting substances (TBARS) method as described by Varshney and Kale (1990). The reaction mixture contained 0.4ml of microsomal sample mixed with 1.6ml 0.15M Tris KCl buffer, 0.5ml of 30% TCA and 0.5ml of 52mMTBA. The mixture was placed in a water bath for 45min at 80°C, cooled in ice and centrifuged at room temperature for 10min at 3,000rpm. The absorbance of the clear supernatant was measured against reference blank of distilled water at 532nm in a spectrophotometer.

Determination of catalase: Whole blood and liver catalase was estimated in a UV recording spectrophotomer at 240nm by monitoring the decomposition of H_2O_2 as described by Aebi (1984). The reaction mixture (1ml, vol.) contained 0.02ml of suitably diluted cytosol in phosphate buffer (50mM, pH7.0) and 0.1ml of 30mM H_2O_2 in phosphate buffer. The specific activity of catalase has been expressed as moles of H_2O_2 reduced per minute per mg protein.

Superoxide dismutase (SOD) assay: Whole blood and liver superoxide dismutase was assayed utilizing the technique of Fridovich (1989). 1ml of whole blood was diluted in 9ml of distilled water to make a one in ten dilution of whole blood. An aliquot of 2.0ml of the diluted blood was added to 2.5ml of 0.05M carbonate buffer (pH 10.2) to equilibrate in the spectrophotometer and the reaction started by the addition of 0.3ml freshly prepared 0.3mM adrenaline to the mixture which was quickly mixed by inversion. The reference cuvette contained 2.5ml buffer, 0.3ml of substrate (adrenaline) and 0.2ml of water. The increase in absorbance at 480nm was monitored every 30 seconds for 150sec. A single unit of enzyme is defined as the quantity of SOD required to produce 50% inhibition of autoxidation.

Hepatic reduced glutathione (GSH) determination: Hepatic GSH was estimated by a colorimetric method using Ellman's reagent as described by Sedlak and Lindsay (1968). Liver of rats was removed, blotted and homogenized at 4 volume of ice-cold homogenizing buffer (pH 7.4). An aliquot of the homogenate was deproteinized by addition of an equal volume of 4% sulfosalicylic acid and after centrifugation at 17,000g for 15min at 2°C. 0.5ml of the diluted supernatant was added to 4.5ml of Ellman's reagent. A blank was prepared with 0.5ml of the diluted precipitate solution (diluted twice with 0.1ml phosphate buffer and 4.5ml Ellman's reagent). GSH was proportional to the absorbance at 412nm. The GSH level was quantified using a standard curve prepared by plotting with different concentration of GSH.

Vitamin C assay: Vitamin C in serum was assayed according to the method of Wilson and Guillan (1969). 0.5ml of serum was added to 2ml of freshly prepared TCA (6g/100ml) in 13x100mm test tubes and mixed well on a vortex mixer. This mixture was centrifuged for 10min at 2500rpm. 1.2ml of the clear supernatant was pipetted into 12x100mm screw cap test tubes. The standards were prepared in duplicate. 1.2ml of TCA (6g/100ml)

were added to two test tubes to use as blank. 0.4ml of dinitrophenyl hydrazine-thiourea-copper sulphate (DTCS) reagent was added to all tubes, which were capped, mixed and incubated in a water bath at 37°C for 3 hours. The tubes were removed from water bath and chilled for 10min in ice bath, while mixing slowly. 2ml of cold 12M H_2SO_4 was mixed and the mixture checked to make sure it did not exceed room temperature. The spectrophotometer was adjusted with the blank to read zero absorbance at 520nm and then absorbance of standards and unknown read. The concentration of each working standard was plotted against absorbance. The vitamin C level was quantified using the standard curve.

Glutathione-s-transferase (GST) assay: The cytosolic GST activity was determined spectrophotometrically at 37°C according to the method of Habig *et al.* (1974). The total reaction mixture contained 2.79ml 0.1M phosphate buffer (pH 6.5), 0.15ml 20mM 1-chloro-2, 4-dinitrobenzene (CDNB), 0.03ml 0.1M GSH. The reaction mixture was preincubated at 37°C which after the reaction was started by the addition of 0.03ml diluted cytosol and the absorbance was read at 30 sec, 1min, 2min, 3min, interval at 340nm. The reaction mixture without the enzyme was used as blank. The specific activity of GST is expressed as µmol of GSH-CDBN conjugate formed/min/mg protein using an extinction coefficient of 9.6mM-1CM-1

Determination of nitric oxide radical inhibition activity: Nitric oxide, generated from sodium nitroprusside in aqueous solution at physiological pH, interacts with oxygen to produce nitrite ions, which were measured by Griess reaction. The method of Green *et al.* (1982) and Macocci *et al.* (1994) was used. The reaction mixture (3ml) containing sodium nitroprusside in phosphate buffered saline and the extract (from 10 to 1000 µg/ml) was incubated at 25°C for 150min. After incubation 0.5ml of Griess reagent was added. The absorbance of the chromophore formed was evaluated at 546nm.

Determination of free radical-scavenging capacity: This involves testing the free radical-scavenging capacity of the extract by bleaching (at 517nm) the staple 1,1-diphenyl-2-picrylhydrazine (DPPH) according to the method of Ursin *et al.* (1994). A reaction mixture containing 3ml of methanol, 30µl of DPPH and the extract of *Hibiscus sabdariffa* (at a final concentration of 0.01, 0.05, 0.20, 0.30, 0.40 or 0.50 mg/ml) was left to stand at room temperature for 30min before being mixed with redistilled water (1ml) and toluene (3ml). The solution was then centrifuged, and the absorbance of the upper phase was read at 517nm against a blank without crude extract. The percentage of DPPH bleaching was calculated thus:

% of DPPH bleaching = $[Ab_C - Ab_T / Ab_C] \times 100\%$

Where: Ab_C = Absorbance of control group
Ab_T = Absorbance of the extract added group

Protein determination: Protein was determined by following the method of Lowry *et al.* (1951) using bovine serum albumin (BSA), at 660nm.

Statistical analysis: The results were reported as means±SD from four repeated determinations and evaluated with the analysis of student's t-test. Differences were considered to be statistically significant at $P<0.05$.

Results and Discussion

An explosion of interest in studying the involvement of free radicals in carcinogenesis (Troll and Weisner, 1985), has led to the use of dietary antioxidant treatment to terminate free radical attacks, hence promoting general human health (Neff, 1997; Chung *et al.*, 1999; Baubles *et al.*, 2000). The antioxidants may act as free radical scavengers, reducing agents, chelating agents for transition metals, quenchers of singlet oxygen molecules, and activators of antioxidative defense enzyme systems to suppress radical damage in biological systems (Zielinski and Kozlowska, 2000).

It is evident from our results (Table 1) that pretreatment with HSE significantly ($P<0.05$) decreased (by 37%) SA-induced hepatic lipid peroxidation when compared with the group that received only 10mg/kg of SA, hence exhibiting a dose dependent effect. Also increasing the conc. of HSE (from 200 to 300mg/kg) non-significantly delayed the lipid oxidation (by 2%) when compared with the control. The concentration of malondiaaldehyde (MDA), an index of lipid peroxidation was increased in rats treated with 10mg/kg of SA alone, but pretreatment with HSE decreased its formation.

The free radical chain reaction is widely accepted as a common mechanism of lipid peroxidation. Radical scavengers may directly react with and quench peroxide radicals to terminate the peroxidation chain reaction and improve the quality and stability of food products (Bran – Williams *et al.*, 1995; Chen and Ho, 1997). HSE may suppress lipid peroxidation through different chemical mechanisms, including free radical quenching, electron transfer, radical addition, or radical recombination (Liangli Yu *et al.*, 2002).

It is well established that reduced glutathione (GSH), the most important biomolecule protecting against chemically induced cytoloxicity, can participate in the elimination of reactive intermediates by conjugation and hydro peroxide reduction, or of free radicals by direct quenching. It plays a key role in the detoxification of its reactive toxic metabolites, and liver necrosis begins when GSH stores are markedly depleted (Davies *et al.*, 1974; Mitchell *et al.*, 1973). Our results (Table 1) demonstrated a significant ($P<0.05$) decrease (by 43%)

Table 1: Effect of *Hibiscus sabdariffa* Extract (HSE) on Hepatic lipid peroxidation (LPO), Reduced Glutathione (GSH), Glutathione-s-transferase (GST) activity and protein concentration in rats treated with Sodium Arsenite (SA)

Group/ treatment	Mean ± SD			
	LPO(MDA mM/cm)	GSH (mg/ml)	GST (µmol/ min/mg protein)	Protein (mg/ml)
A: Control (only normal feed)	102.60±9.051	5.80±0.46	0.11±0.07	38.11±19.28
B: 10mg/kg HSE only	160.30±23.13 (56%)	3.29±0.67 (43%)*	0.051±0.02 (116%)*	22.49±10.10
C:200mg/kg HSE only	86.53±37.18 (16%)* (46%)**	6.87±0.34 (18%)* (108%)**	0.13±0.07 (18%)* (61%)**	37.02±16.40
D:200mg/kg HSE and 10 mg/kg SA	111.5.40±33.92 (12%)* (28%)**	4.789±0.69 (17%)* (46%)**	0.053±0.03 (52%)* (3%)**	27.77±11.09
E: 300mg/kg HSE and 10 mg/kg SA	100.95±25.32 (29%)* (37%)**	6.13±0.79 (6%)* (86%)**	0.07±0.06 (36%)*(37%)**	25.16±6.36

and increase (by 18%) in the conc. of GSH in rats treated with only 10mg/kg of sodium arsenite and 200mg/kg of the extract respectively as compared to the control. The depletion of GSH promotes generation of ROS and oxidative stress with cascade of effects thereby affecting functional as well as structural integrity of cell and organelle membrane (De Leve *et al.*, 1996). However, pretreatment with HSE significantly reduced sodium arsenite - induced hepatic GSH depletion; the result which is probably due to the decreased bioactivation of sodium arsenite to reactive species by the extracts. The elevated level of GSH protects cellular proteins against oxidation through glutathione redox cycle and also directly detoxifies ROS and/or neutralize reactive intermediate species generated from exposure to xenobiotics including chemical carcinogens (Ketterer, 1998). Our in vivo study showed that SA reduced GSH levels in the rat liver and a high dose of HSE blocked the phenomenon effectively.

GSH is largely mediated through the activity of GST, and form abducts with the toxic metabolites of SA. Results in Table 1 showed that HSE also induced (by 37%) the activity of hepatic GST (though not significantly), by preventing the SA - induced reduction (by 116%) of this enzyme. GSTs catalyze the conjugation between electrophiles and nucleophiles GSH. Their primary function is to detoxify electrophiles capable of DNA binding, a typical reaction of phase II metabolism (Pickett *et al.*, 1995). They play a critical role in protecting tissues against product of oxidative stress and electrophiles. Persuasive evidences abound which support the induction of GST and protection against a wide spectrum of cytotoxic, mutagenic and carcinogenic chemicals (De Flora and Ramel, 1989; Ketterer, 1988; Reed, 1990). The protective effect of many naturally occurring chemopreventive agents against carcinogenesis have been ascribed to decreased bioavailability of potential DNA damaging entities and their destruction into excretable metabolites facilitated through induction of GST (Coles and Ketterer, 1990). In this study the specific activity of the enzyme measured was the sum of all its isoforms. The results in our study of HSE against SA-induced hepatotoxicity may be related to increased levels of GSH content or increased GST activity and the possibility exists that HSE is involved in the direct quenching of the reactive metabolites of SA deactivation such as conjugation with glucuronide and sulphate.

The results in Table 2 revealed significant ($P<0.05$) reduction (by 50%) and elevation by (10%) in levels of vitamin C in animals that received only the toxicant and the extract respectively as compared to the control. HSE pretreatment also prevented significantly SA-induced reduction of serum vitamin C levels, hence exhibiting a dose dependent effect. Vitamin C (ascorbic acid) is an outstanding powerful antioxidant that reacts rapidly with a variety of oxidants, including the rather poorly reactive superoxide anion radical (Nishikimi, 1975). Vitamin C within the body is maintained in the reduced form by shuttling the dehydroascobate across the erythrocyte membrane for reconversion to ascorbate (Orroinger and Roear, 1979), a mechanism that may not cope with oxidative stress. The decrease in the levels of vitamin C could be attributed to increased production of ROS, and the increased levels is possibly due to the ability of HSE to mitigate ROS produced by the compound.

The activities of physiological antioxidants such as catalase and superoxide dismutase were studied. The results (Table 2) revealed a dose - dependent change in the activities of whole blood and liver catalase, and whole blood and liver SOD in HSE treated groups. Catalase is a tetrameric hemoprotein that undergoes reduction at its active site in the presence of its substrate, H_2O_2, and catalyses the dismutation of H_2O_2 to water and molecular oxygen. The result of our study showed an increase and a decrease in the activity of whole blood and liver catalase respectively due to administration of SA alone. This increase is thought to be due to an induction in response to increased production of H_2O_2 following increased oxidant stress

Table 2: Effect of Extract of *Hibiscus sabdariffa* (HSE) on Serum Vitamin C levels, Whole Blood and Hepatic Catalase (CAT) and Superoxide Dismutase (SOD) activities in rats treated with Sodium Arsenite

Group/ treatment	Mean ± SD				
	Vitamin C (mg/100ml)	Whole blood CAT (Kat f)	Liver CAT (Kat f)	Whole blood SOD Unit of activity	Liver SOD unit of activity
A: Control (only normal feed)	0.186±0.0087	0.89±0.09	2.63±0.07	0.58±0.23	1.56±0.46
B: 10mg/kg HSE only	0.081±0.022 (56%)*	1.32±0.29 (48%)*	0.28±0.02 (89%)	1.93±0.038 (232%)*	0.35±0.129 (75%)*
C:200mg/kg HSE only	0.206±0.012 (10%)* (56%)**	0.46±0.06 (48%)* (65%)**	2.99±0.13 (14%)* (968%)**	0.48±0.02 (17%)* (75%)**	1.97±0.15 (28%)*(462%)**
D:200mg/kg HSE and 10 mg/kg SA	0.126±0.030 (32%)* (56%)**	0.63±0.066 (29%)* (52%)**	2.36±0.061 (10%)* (742%)**	0.56±0.04 (3%)* (71%)**	1.42±0.46 (9%)* (31%)**
E:300mg/kg HSE and 10 mg/kg SA	0.130±0.030 (30%)*(60%)**	0.55±0.064 (38%)* (58%)**	2.60±0.005 (10%)* (829%)**	0.28±0.17 (52%)* (85%)**	1.64±0.31 (5%)* (369%)**

Table3: Effect of Extract of *Hibiscus sabdariffa* on Nitric Oxide radical and DPPH bleaching

Dose (mg/ml)	IC_{50}	Dose (mg/ml)	% of DPPH Bleaching
10-1000	N.D	0.01	42.4±3.60
		0.05	44.8±5.70
		0.20	49.7±2.80
		0.30	57.7±7.10
		0.40	66.7±4.60
		0.50	75.6±8.10

* % change with respect to control group, A
** % Change with respect to toxicant treated group, B
Data are calculated from four experiments in each case
N.D = not detected

which injured the liver and caused a leakage of the enzyme into the blood, hence increasing and decreasing its activities in the blood and liver respectively. Moreso, the activities of whole blood catalase were shown to be decreased and liver catalase increased following HSE pretreatment which inhibited SA-induced liver injury in rats. The hepatoprotective capacity of the extract is evident in the significant ($P<0.05$) increase in the activity of liver catalase when compared with the toxicant treated group alone. It has been proposed that glutathione peroxidase is responsible for the detoxification of H_2O_2 in low concentration whereas catalase comes into play when glutathione peroxidase pathway is saturated with substrate (Gaetani *et al.*, 1989).

Superoxide dismutase (SOD) has an antitoxic effect against the superoxide anion. The augmented activity of metalloenzyme, SOD accelerates dismutation of superoxide radicals to H_2O_2, which is removed by catalase (Aebi, 1984). H_2O_2, a reaction product of the SOD reaction, inactivates SOD, and in the presence of H_2O_2, SOD acts as a pro-oxidant (Bast *et al.*, 1991; Yim *et al.*, 1990). It is observed from the results (Table 2) of this experiment that administration of SA alone increased and decreased respectively whole blood and liver SOD activities. This observation may not be unconnected with a response to an increased production of ROS induced by the toxicant. The pharmacokinetics of SA may play a significant role in the difference observed on the activities of SOD in the blood and liver. The decrease in SOD activity observed in the liver might be attributed to the high distribution of the toxicant in the liver, a major target organ for most xenobiotics. The high concentration therefore in the liver and the resultant damage should be responsible for the decrease in SOD activity observed in this study. The affinity of xenobiotics for plasma protein determines the volume of distribution in this medium as well as the concentration of free (active) drug. The generation of H_2O_2 from metabolism of SA and perhaps elsewhere may be responsible for the induction of SOD activity in the blood coupled with relatively low distribution in plasma compared with the liver. The experiment also revealed a decrease and an increase respectively in the activity of whole blood and liver SOD following administration of the extract of *Hibiscus sabdariffa*. It is obvious from the result that HSE inhibits hepatotoxicity induced by 10mg/kg body weight of SA, evidently shown by a reduction and elevation in the whole blood and SOD activities. The elevated level of liver SOD suggests that HSE is hepatoprotective and contains antioxidant property.

Nitric oxide radical generation at physiological pH from sodium Nitroprusside was not found to be inhibited by the extract of *Hibiscus sabdariffa* as its $1C_{50}$ (mg/ml) was not detected, even at low and high concentrations of the extract (Table 3). This suggests that the extract may not scavenge nitric oxide radicals. The bleaching of DPPH by the extract of *Hibiscus sabdariffa* was measured in order to determine the free radical quenching capacity of the extract. DPPH was used to provide stable free radicals, which were scavenged by the extract in a dose-dependent manner (Table 3). Although a decrease in absorbance, in other words, loss of DPPH free radicals indicates the capacity of the extracts to capture free radicals; it is not a clear-cut definition of antioxidant effect (Tseng *et al.*, 1997). However, the present study revealed that the extract has the capacity to quench

about 50% DPPH free radicals at the concentration of 0.2mg/ml. This result might not be unconnected with the contribution of the phenolic constituents of the crude extract.

The present study demonstrates that the extracts of *Hibiscus sabdariffa* L. have dose-dependent protective effect against sodium arsenite-induced oxidative damage. It is not unlikely that this protective effect is probably mediated by its inhibitory effect on sodium arsenite bioactivation. More would have been comprehended if this study was extended to correlate the exact mechanisms and constituents of the extract on the levels of other parameters such as glutathione peroxidase, glutothione reductase, â-carotene, vitamin A, and phase I and II drug metabolizing enzymes in different tissues. Moreover, since the data reported in this study were generated for short-term treatment with HSE; it is recommended that long-term animal studies to evaluate the effects of these extracts on biomarkers of oxidative stress and biochemical mechanisms involving xenobiotic enzymes be carried out. This will increase our understanding of the role of *Hibiscus sabdariffa* L. in chemical carcinogenesis.

References

Aebi, H., 1984. Catalase *In vitro* In: S.P. Colowick, N. O., Kaplane(eds) Methods in Enzymology, 105: 121-126.

Bast, A., G.R. Haesen and C.J.A. Doelman, 1991. Oxidants and antioxidants State of the art of Review. A. M. J. Med., 91(Suppl. 3c), 25435.

Baubles, A., E.A. Decker and F.M. Clydes Dale, 2000. An antioxidant effects of aqueous extracts from wheat-based ready-to-eat breakfast cereals. Food Chem., 68: 1-6.

Block, G., 1992. The data support a role for antioxidants in reducing cancer risk. Nutr. Rev., 50: 207-213.

Bran - Williams, W., M.E. Cuvelier and C. Berset, 1995. Use of a free radical method to evaluate antioxidant activity. Lebensmittelwissens-chaft and technologic, 28: 25-30.

Chen, J.H. and C.T. Ho, 1997. Antioxidant activities of caffeic acid and its related hydroxycinnamic acid compounds. J. Agri. Food Chem., 45: 2374-2378.

Chung, H.S., L.C. Chang, S.K. Lee, L.A. Shamon, R.B.V. Breemen, R.G. Mehta, N.R. Farnsworth, J.M. Pezzuto and A.D. Kinghorn, 1999. Flavonoids constituents of Chorinzan the diffuse with potential cancer chemopreventive activity. J. Agri. Food Chem., 47: 36-41.

Coles, P., B. Ketterer, 1990. The Role of Glutathione and Glutathione-s-transferases in Chemical Carcinogenesis. Crit. Rev. Biochem. Mol. Biol., 25: 47-50.

Davies, D.C., W.Z. Potter, D.J. Jollow and J.R. Mitchell, 1974. Species differences in hepatic glutathione depletion, covalent binding and hepatic necrosis after acetaminophen. Life Sci., 14: 2099-2109.

De Flora, S. and C. Ramel, 1989. Mechanism of Inhibition of Mutagenesis and Carcinogenesis: Classification and Overview. Mutat. Res., 202: 285-306.

De Leve, L.D., X. Wang, J.F. Kuhlenkamp and N. Kaplowitz, 1996. Toxicity of azathioprine and monocrotaline in murine sinusoidal endothelial cells and hepatocytes. The role of glutathione and relevance to hepatic venocclusive disease. Hepathology, 23: 589-599.

Fridovich, I., 1989. Superoxide dismutase. An adaptation to paramagnetic gas. J. Biol. Chem., 254: 7761-7764.

Gaetani, G.F., S. Galiano, L. Canepa, A.M. Ferraris and H.N. Kirkman, 1989. Catalase and glutathione Peroxidase are equally effective in detoxification of hydrogen peroxide in human erythrocytes. Blood, 73: 344-339.

Green, L.C. D.A. Wagner, J. Glogowski, P.L. Skipper, J.S. Wishnok and S.R. Tannenbaum, 1982. Anal Biochem., 1216: 131.

Habig, W.H., M.J. Pabst and W.B. Jakoby, 1974. Glutathione - S-transferase-The first step in mercapturic acid formation. J. Biol. Chem., 249: 7130-7139.

Halliwell, B., 1996. Antioxidant in human health and disease. Ann. Rev. Nutr., 16: 33-50.

Halliwell, B., J.M.C. Gutteridge and C.E. Cross, 1992. Free radicals, antioxidants, and human disease. Where are we now? J. Lab. Clin. Med., 119: 598-620.

Hertog, M.G.L. and E.J.M. Feskens 1993. Dietary antioxidant flavonoids and risk of coronary heart disease the Zutphen Elderly Study. Lancet., 342: 1007-1011.

Ketterer, B., 1988. Protective Role of Glutathione and Glutathione S-Transferases in Mutagenesis and Carcinogenesis: Mutat. Res., 202: 343-361.

Ketterer, B., 1998. Glutathione-s-transferase and Prevention of Cellular Free Radical Damage. Free Rad., 28: 647-658.

Liangli, Yu, Scott Haley, Jonathan Perret and Mary Harris, 2002. Antioxidant properties of hard winter wheat extracts. Food Chem., 78: 457-461.

Lowry, O.H., N.J. Rosenbrough, A. Farr and R.J. Randall, 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem., 193: 265-275.

Marcocci, L., J.J. Maguire, M.T. Droy-Lefaix and L. Parker, 1994. Biochem. Biophys. Res. Commun., 201: 748.

Mitchell, J.R., D.J. Jollow, D.C. Potter, J.R. Gillete and B.B. Brodie, 1973. Acetaminnophen-induced hepatic necrosis IV: Protective role of glutathione. J. Pharmacol. Exp. Ther., 187: 211-213.

Neff, J., 1997. Big Companies take nutraceuticals to heart. Food Processing, 58: 37-42.

Nishikimi, M., 1975. Oxidation of ascorbic acid with superoxide anion generated by the xanthine - xanthine oxdase system. Biochem. Biophys. Res. Commum., 1-63, 463-8.

Orroinger, E.P. and M.E.S. Roear, 1979. An ascorbate-Mediated trans membrane reducing system of the human erythrocyte. J. Clin. Invest., 63: 53-58.

Pickett, C.B. and A.Y.H. Lu, 1995. Glutathione S-Transferases: Gene Structure, Regulation, and Biological Function, Annu Rev. Biochem. 58: 743-764.

Rana, P.S. P. Bandhuvula and R.R. Araga, 2000. Modulatory influence of Adhatoda vesica (Justicia adhatoda) leaf extract on the enzymes of xenobiotic metaolism, antioxidant status and lipid peroxidation. Mol. and Cell. Biochem., 213: 99-109.

Reed, D.J., 1990. Glutathione: Toxicological Implications. Ann. Rev. Pharmacol. Toxicol., 30: 603-631.

Sedlak, J. and R.H. Lindsay, 1968. Estimation of total protein bound and non-protein sulfhydryl groups in tissues with Ellman's reagent. Anal. Biochem., 25: 192-205.

Simic, M.G., 1988. Mechanisms of Inhibition of Free-Radical. Processed In Mutagenesis and Carcinogenesis. Mutation Res., 202: 377-386.

Sun, Y., 1990. Free radicals, antioxidant enzymes and carcinogenesis. Free Radical. Biol. Med., 8: 583-599.

Troll, W. and R. Weisner, 1985. The role of oxygen radical as possible mechanism of tumor promotion. Ann. Rev. Pharmacol. Toxicol., 25: 509-528.

Tseng, T.H., E.S. Kao, C.Y. Chu, F.P. Chou, H.W. Linwu, and C.J. Wang, 1997. Protective effects of dried flower extracts of *Hibiscus sabdariffa* L against Oxidative stress in rat primary hepatocytes. Food and Chemical Toxicol., 35: 1159-1164.

Ursin, F., M. Maiorino, P. Morazzoni, A. Roveri and G. Pifferi, 1994. A novel antioxidant flavonoid affecting molecular mechanisms of cellular activation. Free Rad. Biol. Med., 16: 547-553.

Varshney, R. and R.K. Kale, 1990. Effects of Calmodulin antagonist on radiation induced lipid peroxidation in microsomes. Int. J. Rad. Biol., 58:733-74.

Wang, S.Y. and H. Jiao, 2000. Scavenging capacity of berry crops on superoxide radicals, hydrogen peroxide, hydroxyl radicals, and singlet oxygen. J. Agri. Food Chem.,48: 677-684.

Wilson, S.S. and R.A. Guillan, 1969. Clin. Chem., 17: 282.

Yim, M.B., P.B. Chock and E.R. Stardtman, 1990. Copper-zinc superoxide dismutase catalyzes hydroxyl radical production from hydrogen peroxide. Proc; Nat l. Acad. Sc. USA., 87: 5006-10.

Zielinski, H. and H. Kozlowska, 2000. Antioxidant activity and total phenolics in selected cereal grains, their different morphological fractions. J. Agri. Food Chem., 48: 2008-2016.

Pakistan Journal of Nutrition 4 (3): 142-147, 2005
ISSN 1680-5194

Effect of Dried Dates Extract on the Growth and Viability of *Bifidobacteria* in Different Milk Types

Meshref A. Al-Rwaily[1], Saqer, M. Herzallah[2], Mohammed Ali Humeid[3] and Mohammad I. Yamani[3]
[1]Quality Control Lab, Ministry of Commerce and Industry, Haditha, Kingdom of Saudi Arabia
[2]Department of Nutrition and Food Technology, Faculty of Agriculture, Mu'tah University, Jordan
[3]Department of Nutrition and Food Technology, Faculty of Agriculture, Jordan University, Jordan

Abstract: The objectives of the study were to screen the ability of two of dates (hilwa and sukary) extracts to stimulate the growth of two selected *strains of Bifdobacteria* (*B. infantis, B. angulatum*) inoculated in three milk types (cow, goats and camel), to investigate their effects on the stability of the bacteria during cooled storage and to study the feasibility of production of acceptable healthy drinks based on milk. Dates extract were prepared by hot soaking of dried fruits at 70°C, then sterilized at 120°C and finally mixed with milk types at three concentration levels (10, 15, 20% v/v). The growth study was conducted by inoculation of milk types and their pasteurized products (90°C / 20 min) with *Bifidobacterium infantis* or *B. angulatum* to contain 10^6 cfu/ml and incubated at 37°C for 16 h. The bacterial counts were conducted at the beginning and every four hours for a period of 16 hr. The inoculated milk products were also stored at 4°C for 15 days during which the viability of the bacteria, hydrogen ion concentration was measured and the coagulation was monitored every three days for the same periods. No significant differences were found in the growth of the two starter cultures in most treatments, regardless of the type of milk and dates extract as well as concentration and incubation time. The pH of the control milk samples as well as that of the different milk preparations decreased gradually during incubation and refrigerated storage at 4°C for 15 days. This indicated activity of the bacteria and/or their enzyme systems. The sensory evaluation of six selected bifidus milk prepared from cow's, goat's and camel's milk and hilwa dates extracts at 10% (v/v) level, revealed a moderate acceptability with no significant differences from cow's milk with hilwa dates extract and camel's milk with hilwa dates extract preparations, the least significant acceptance at ($P< 0.05$) was observed for goat's milk preparations.

Key words: *Bifdobacteria*, hilwa, sukary, camel, goat

Introduction

Bifodobacteria were isolated from various animals (Scardovi, 1986). All species derived from human are non-spore forming, non-motile, anaerobic, Gram-positive bacteria. *Bifidobacteria* had long been recognized as bacteria with probiotic, nutritive and therapeutic properties (Bezkoravainy, 2001; Holzapfel *et al*., 2001). Since Bifidobacteria do not grow well in milk, the manufacturing of fermented milk products with *Bifidobacteria* often requires the use of an inoculum containing the final number of cells of *Bifidobacteriam* required for the products (Crittenden, 1999). In recent years, there has been an increasing interest in the incorporation of the intestinal species *Lactobacillus acidophilus* and *Bifidobacterium* species into fermented milk products. These species are frequently associated with health promoting effects in human and animal intestinal tract.

These probiotic effects are generally related to inhibition of pathogenic species, reducing the risk of colon cancer, increasing the immune response and decreasing concentration of cholesterol in blood plasma (Gilliland, 1990; Gurr, 1987).

In selecting strains to produce these products, criteria include not only properties related to the intestinal effects, but also multiplication in milk and survival during storage in acidic milk. Growth of *Bifidobacterium* in the intestine is often related to the presence of specific growth factors (Modler *et al*., 1990). *Bifidobacteria* have a number of beneficial effects on host health in both infants and adults. The most important of these include inhibition or displacement of undesirable microorganisms, elimination of procarcinogens, immunomodulation and vitamin production (Gibson and Wang, 1994; Modler *et al*., 1990).

Bifidobacteria are now big business in Japan and Europe where bifid-amended foods and beverages are one of the fastest-growing segments of culture-products. *Bifidobacteria* are not true lactic acid bacteria in the sense of a *Lactococcus* or *pediococcus* (Hughes and Hoover, 1991). *Bifidobacteria* produce both acetic and lactic acids as primary metabolites in the molar ratio of 3:2. Glucose is degraded characteristically by the fructose 6-phosphate shunt metabolic pathway (Bezkoravainy, 2001; Holzapfel *et al*., 1998).

Dates are widely consumed and produced in the Kingdom of Saudi Arabia and are considered to be one of the most rich sources in minerals and sugars.

Furthermore, milk is one of the best sources of nutrients for child growth. Milk and dates are major food sources of Arabs in all desert region and they are often eaten simultaneously or in different combinations. The addition of fruit extracts may enhance the growth of *Bifidobacteria* by providing essential nutrients, enhancing the sensory quality of the products since the flavor of bifido culture in milk is not favorable, and providing consumers with certain nutrients especially minerals and energy. The objectives of the study were to screen the ability of adding extracts of date to cow's, goat's, and camel's milk in stimulating the growth and stability of selected *Bifidobacteria* and to investigate feasibility of production of acceptable health drinks based on milk and date extracts fermented by *Bifidobacteria*, and to evaluate sensory acceptability of products as health drinks.

Materials and Methods

Raw milk: Raw Cow's milk was obtained from Almarai Company Ltd., Saudi Arabia. Camel's milk was obtained from Alwatania and Goat's milk was obtained from local farm in Gurayat province in Saudi Arabia.

Heat treatment of milk: Whole milk samples (2000 ml each) were heat treated at 90°C for 20 min in water bath, then cooled (in a water bath mixed with ice).

Sources and maintenance of cultures (enumeration of *Bifidobacteria* strains)

Lyophilized *Bifidobacterium infantis* ATCC 15697 was obtained from Deutsche Sammlung von Microorganismen und Zellkulturen GmbH, Braunschweig (DSMZ), Germany.

Forty-eight hours prior to the start of each experiment, cultures were revived by transfer twice into 10 ml of MRSL (MRS broth with 5% lactose), (Hughes and Hoover, 1995) and incubation at 37°C for 24 hours in an anaerobic chamber (Gaspak system; BBL, Cockeysville, MD, U.S.A).

Determination of viability during refrigerated storage: *Bifidobacterium* strains *B. infantis* 15697 was cultured anaerobically at 37°C for 48 h with 0, 10, 15, and 20% (v/v) extracts of suckary dates and hilwa dates product of Saudi Arabia and samples containing no fruits (0%) were used as controls. All inoculated samples after fermentation were stored at 4.0 ± 1°C for 15 d. One ml of each milk samples was diluted with 9 ml of sterile 0. 1% (w/v) peptone water (Difco) and mixed uniformly with a vortex mixer (Waring Blendor, model 32BL80). Subsequent ten fold serial dilutions were prepared and viable numbers enumerated using pour plate technique. *Bifidobacteria* were enumerated in duplicate using MRSL agar (Difco, Laboratories. Detroit, MI, U.S.A). The inoculated plates were incubated anaerobically at 37°C for 48 h using Gas Paks. Cell counts were carried out on day 0, 3 6, 9 12 and 15. The colonies were counted using a colony counter (Model, EC1-video colony counter AES, Laboratoire).

Growth studies: Growth characteristics of cultures of *Bifidobacteria* in cow, goat and camel milks were evaluated. Each culture was inoculated at 1% (v/v) into 100 ml of milk and was incubated at 37°C for 16 hours in the anaerobic chamber (BBL). Initial viable counts for each culture were standardized by the use of standard curve so that they were approximately the same for all cultures (= $1x10^8$ cfu/ml).

Samples were drawn from each flask at, 0, 4, 8, 12 and 16 h. and gas generating envelope was replaced at each interval.

Viable counts were done by serial dilution with 0.1% peptone-water and pour plating in duplicate using MRSL agar. The pH of samples and coagulation were determined at each interval.

Preparation of fermented and unfermented milk: The procedure of Hughes and Hoover (1995) was used to prepare fermented and unfermented whole milks from cows, goats and camels, respectively.

Fermented milks were made by a 1% (v/v) inoculation of 100 ml of each milk. Flask openings (Corring Brand Milk Dilution Bottles, Screw Cap, Cat. No. 1372) were sealed with a single layer of parafilm (Parafilm, Laboratory Film, American National Can. Chicago, IL. 60631). Fermented milk was incubated for 16 hrs at 37°C in the anaerobic chamber for growth studies. Unfermented samples were prepared by inoculation of milks that had been prechilled to 4°C for 15 days. After that the sample flasks were sealed and capped as described for fermented samples. Samples were stored at 4°C immediately after inoculation. Bacterial counts, coagulation and pH were evaluated in the fermented and unfermented milks on days 0, 3, 6, 9, 12 and 15. Viable counts were determined as mentioned in the growth studies. The pH of samples were recorded prior to dilution.

Dried date samples: Two varieties of commercial dates product of Saudi Arabia (hilwa and suckary dates) were used. The extracts were prepared with ca. 50% total solids.

Dried dates extraction method: Samples of dates without stones (250 g each) were soaked in 500 ml distilled water at 70°C for 1 hour, blended using Waring Blender, (model 32BL80), then strained in cheese cloth. The extracts were filled in 500ml glass bottles and sterilized at 121°C for 15 minutes in an autoclave.

Measurement of pH: The pH of the fermented and unfermented samples was measured using a pH meter (Model, Jenway 3410 Electrochemistry analyzer),

calibrated with fresh pH 4.0 and 7.0 standard buffers.

Statistical analysis of experimental data: Analyses of variance (ANOVA) procedure of SAS institute, Inc. (2000) software, general linear model (GLM) and Fisher's least significant difference (LSD) were used to differentiate between means within and among the treatments. All analysis were preformed using procedures for the general linear model(PROC GLM) of SAS ™ (Version 8 e SAS institute. (All the experiments were repeated in duplicates on different days. The data obtained were analyzed at 5% level of significance.

Sensory evaluation: A hedonic (5 points) scale test as described by Linda *et al.* (1991), was used to evaluate the acceptance of milks from cow, goat, and camel containing 10% hilwa dates extract. Twenty panelists from Quality Control Lab of ministry of commerce and industry in Haditha - Kingdom of Saudi Arabia conducted the sensory evaluation. Panelists were asked to evaluate aroma, taste, color and overall acceptability of the samples.

Results

Growth of *Bifidobacterium infantis* in flavored fruit milks containing Date extract and incubated 37°C for 16 hours

Hilwa dates extract: Results in Table 1 show changes in *Bifidobacterium* counts (log cfu/ml) in milks from cow, goat and camel containing different concentration of hilwa dates extract and inoculated with 1% culture of *B. infantis*. The changes in the *Bifidobacterium* counts (log cfu/ml) in milks were not significantly different at (P< 0.05) between 0, 10 ,15 and 20% concentrations, respectively of hilwa dates extract after incubation for 16 h at 37°C.

The bacterial counts (log cfu/ml) in cow's milk increased from 5.63 to 7.25, 6.53 to 7.26 , 6.82 to 7.14 and 6.79 to 7.10 at 0% ,10%, 15% and 20% concentrations, respectively of hilwa dates extract after incubation for 16 h at 37°C.

The bacterial counts (log cfu/ml) in goat's milk increased from 6.80 to 6.86, 6.81 to 6.86, 6.81 to 6.86, 6.82 to 6.86 at all concentrations of hilwa dates extract throughout the incubation for 16 h at 37°C.

In camel's milk containing different concentration of hilwa dates extract and inoculated with 1% culture of *B. infantis* the changes in *Bifidobacterium* counts (log cfu/ml) were also not significantly different at (P< 0.05) throughout the period of the incubation for 16 h at 37°C. The counts increased from 6.76 to 6.78 and 6.72 to 6.84, 6.79 to 6.84, 6.79 to 6.82 at 0%, 10% , 15% and 20% concentrations, respectively at the end of incubation for 16 h at 37°C.

The changes in pH of cow's, goat's and camel's milk containing different concentrations of hilwa dates extract and inoculated with 1% culture of *B. infantis* are shown in Table 2. The pH at all time intervals tested, were not significantly different at (P< 0.05) between 0, 10 ,15 and 20% concentrations, respectively of hilwa dates extract after incubation for 16 h at 37°C.

The pH of cow's milk decreased and ranged from 6.69 to 6.46, 6.68 to 6.59, 6.697 to 6.57 and 6.67 to 6.56 at 0, 10 ,15 and 20% concentrations, respectively, of hilwa dates extract after incubation for 16 h at 37°C.

The pH in goat's milk containing different concentrations of hilwa dates extract and inoculated with 1% culture of *B. infantis* decreased from 6.45 to 6.38, 6.43 to 5.52 , 6.42 to 5.95, and from 6.41 to 5.55 at 0, 10, 15 and 20% concentrations, respectively, of hilwa dates extract after incubation for 16 h at 37°C.

The bacterial growth (log cfu/ml) in goat's milk increased from 6.77 to 6.86, 6.76 to 6.84, 6.74 to 6.83 and from 6.79 to 6.83 at all concentrations of suckary dates extract throughout the incubation for 16 h at 37°C.

In camel's milk containing different concentration of suckary dates a extract and inoculated with 1% culture of *B. infantis* the changes in *Bifidobacterium* counts (log cfu/ml), the counts increased from 6.53 to 6.48 , 6.55 to 6. 73, 6.49 to 6.84 and from 6.53 to 6.69 after 12 hr and then decreased to 6.48 at 0%, 10% , 15% and 20% concentrations, respectively, at the end of incubation for 16 h at 37°C.

In camel's milk containing different concentrations of hilwa dates extract inoculated with 1% culture of *B. infantis*, the pH of each concentration decreased from 6.61 to 6.05, 6.61 to 5.54, 6.62 to 5.48 and from 6.60 to 5.63 during incubation for 16 h at 37°C.

Suckary dates extract: Results in Table 3 show changes in *Bifidobacterium* counts (log cfu/ml) in milks from cow, goat and camel containing different concentration of hilwa dates extract and inoculated with 1% culture of *B. infantis*. The changes in the *Bifidobacterium* counts (log cfu/ml) in milks did not significantly differ at (P<0.05) between 0, 10 ,15 and 20% concentrations, respectively of suckary dates extract after incubation for 16 h at 37°C.

The bacterial counts (log cfu/ml) in milk from cow increased from 7.13 to 7.37, 7.18 to 7.29, 7.12 to 7.18 and 7.24 to 7.48 at 0, 10,15 and 20% concentrations, respectively of suckary dates extract after incubation for 16 hr at 37°C.

The bacterial growth (log cfu/ml) in goat's milk increased from 6.77 to 6.82, 6.76 to 6.84, 6.74 to 6.83 and from 6.79 to 6.83 at all concentrations of suckary dates extract through the incubation for 16hr at 37°C.

In camel's milk containing different concentrations of suckary dates extract and inoculated with 1% culture of *B. infantis*, the changes in *Bifidobacterium* counts (log cfu/ ml) increased from 6.53 to 6.48, 6.55 to 6.73, 6.49 to 6.84 and from 6.53 to 6.48 at 12 hr, at 0, 10, 15 and 20% concentrations, respectively, at the end of incubation for 16 hr at 37°C.

Table 1: Changes in the *Bifidobacterium* counts (log cfu/ml) in cow's , goat's and camel's milk containing different concentrations of hilwa dates extract and inoculated with 1% culture of *B. infantis* when incubated at 37°C

Milk	Time (h)	Hilwa dates concentration %			
		0	10	15	20
Cow	0	6.63[a1]	6.53[a]	6.82[a]	6.79[a]
	4	6.51[a]	6.79[a]	6.84[a]	6.81[a]
	8	6.68[a]	6.61[a]	7.26[a]	6.71[a]
	12	7.04[a]	6.91[a]	6.76[a]	7.05[a]
	16	7.26[a]	7.26[a]	7.14[a]	7.10[a]
Goat	0	6.80[a]	6.81[a]	6.81[a]	6.82[a]
	4	6.80[a]	6.83[a]	6.86[a]	6.83[a]
	8	6.84[a]	6.86[a]	6.86[a]	6.86[a]
	12	6.86[a]	6.86[a]	6.86[a]	6.86[a]
	16	6.86[a]	6.86[a]	6.85[a]	6.86[a]
Camel	0	6.76[a]	6.72[a]	6.79[a]	6.79[a]
	4	6.81[a]	6.84[a]	6.81[a]	6.85[a]
	8	6.84[a]	6.84[a]	6.85[a]	6.88[a]
	12	6.85[a]	6.85[a]	6.93[a]	6.86[a]
	16	6.78[a]	6.84[a]	6.84[a]	6.82[a]

[1]means in the same rows and columns with the same letter are not significantly different at (P<0.05)

Table 2: Changes in the pH of cow's, goat's and camel's milk containing different concentrations of hilwa dates extract and inoculated with 1% culture of *Bifidobacterium infantis* when incubated at 37°C

Milk	Time (h)	Hilwa dates concentration %			
		0	10	15	20
Cow	0	6.69[a1]	6.68[a]	6.69[a]	6.67[a]
	4	6.64[a]	6.64[a]	6.65[a]	6.66[a]
	8	6.6[a]	6.62[a]	6.6[a]	6.62[a]
	12	6.6[a]	6.57[a]	6.54[a]	6.55[a]
	16	6.46[a]	6.59[a]	6.57[a]	6.56[a]
Goat	0	6.45[a]	6.43[a]	6.42[a]	6.41[a]
	4	6.38[a]	6.33[a]	6.32[a]	6.31[a]
	8	6.37[a]	6.33[a]	6.31[a]	6.31[a]
	12	6.42[a]	6.15[a]	6.19[a]	6.26[a]
	16	6.38[a]	5.52[a]	5.95[a]	5.55[a]
Camel	0	6.61[a]	6.61[a]	6.62[a]	6.60[a]
	4	6.29[a]	6.25[a]	6.27[a]	6.31[a]
	8	6.15[a]	6.27[a]	6.28[a]	6.29[a]
	12	6.09[a]	5.79[a]	5.89[a]	6.13[a]
	16	6.05[a]	5.54[a]	5.48[a]	5.63[a]

[1]Means in the same rows and columns with the same letter are not significantly different at (P < 0.05).

The changes in pH of cow's, goat's, and camel's milk containing different concentrations of suckary dates extract inoculated with 1% culture of *B. infantis* are shown in Table 4. The pH at all time intervals tested, were not significantly different at (P<0.05) between 0, 10,15 and 20% concentrations, respectively of suckary dates extract after incubation for 16 h at 37°C.

The pH of cow's milk decreased from 6.92 to 5.24, 6.90 to 5.41, 6.91 to 5.92 and from 6. 86 to 5.98 at 0, 10, 15 and 20% concentrations, respectively, of hilwa dates extract after incubation for 16 h at 37°C.

The pH of goat's milk containing different concentrations of hilwa dates extract and inoculated with 1% culture of *B. infantis* decreased from 6.79 to 6.16, 6.75 to 5.86, and 6.72 to 5.81, and from 6.70 to 5.80 at 0, 10, 15 and 20% concentrations, respectively, of hilwa dates extract after incubation for 16 h at 37°C.

In camel's milk containing different concentrations of suckary dates extract inoculated with 1% culture of *B. infantis*, the pH decreased from 6.73 to 6.17, 6.73 to 6.02, 6.75 to 6.05 and from 6.76 to 6.29 at 0, 10, 15, and 20% concentrations, respectively, of hilwa dates extract after incubation for 16 h at 37°C.

Sensory evaluation: The results in Tables 5 show that the cow's milk drinks with hilwa dates extract obtained the highest overall acceptability of 3.5 and 3.4 respectively; this means that their acceptance is between "neither dislike nor like" and "like" in other words they were of moderate acceptance.

Discussion

Dates was selected in this study for their high carbohydrates, dietary fibers and mineral contents. Furthermore, they are available all over the year and are stable and relatively of low cost per unit extractable dry matter. In addition, dates are the most popular and favorable food items during the holly month of Ramadan. The results of the study of growth, pH and viability and activity of *Bifidobacterium* during refrigerated storage, presented in the tables 1 to 4 generally revealed no significant growth of bacterial numbers, but considerable and sometimes significant decrease of the pH of the milks. The decrease of the pH with incubation time indicates the activity of the bacteria. A gradual and variable pH drop was recorded almost in all treatments due to incubation time. Generally, the drop was higher in camel's and goat's milk preparations than in cow's milk preparations. On the other hand, the drop was higher in enriched milk than in the pure milk (control). One can also observe a general trend of a greater decrease in milk preparations with 10 and 15% extracts additions than those with 20% extract additions, that indicates the existence of a concentration optimum for the bacterial activity. However, no clear relationship was observed between pH drop and coagulation of the milk preparation. Most coagulations occurred after 12 or 16 h of incubation or 12 days of refrigerated storage at pH values higher than the isoelectric point of casein. This

Table 3: Changes in the *Bifidobacterium* counts (log cfu/ml) in cow's, goat's and camel's milk containing different concentrations of suckary dates extract and inoculated with 1% culture of *B. infantis* when incubated at 37°C

Milk	Time (h)	suckary dates concentration %			
		0	10	15	20
Cow	0	7.13[1a]	7.18[a]	7.12[a]	7.24[a]
	4	7.73[a]	6.85[a]	6.68[a]	7.36[a]
	8	7.41[a]	7.47[a]	6.80[a]	6.68[a]
	12	6.97[a]	7.07[a]	7.31[a]	7.13[a]
	16	7.37[a]	7.29[a]	7.18[a]	7.48[a]
Goat	0	6.77[a]	6.76[a]	6.74[a]	6.79[a]
	4	6.73[a]	6.74[a]	6.78[a]	6.77[a]
	8	6.84[a]	6.83[a]	6.34[a]	6.82[a]
	12	6.83[a]	6.84[a]	6.83[a]	6.84[a]
	16	6.82[a]	6.83[a]	6.83[a]	6.83[a]
Camel	0	6.53[a]	6.55[a]	6.49[a]	6.53[a]
	4	6.60[a]	6.60[a]	6.81[a]	6.60[a]
	8	6.51[a]	6.87[a]	6.77[a]	6.51[a]
	12	6.69[a]	6.67[a]	6.72[a]	6.69[a]
	16	6.48[a]	6.73[a]	6.84[a]	6.48[a]

[1]Means in the same rows and columns with the same letter are not significantly different at (P < 0.05).

Table 4 : Changes in the pH of cow's, goat's and camel's milk containing different concentrations of suckary dates extract and inoculated with 1% culture of *Bifidobacterium infantis* when incubated at 37°C

Milk	Time (h)	Suckary dates concentration %			
		0	10	15	20
Cow	0	6.92[1a]	6.91[a]	6.91[a]	6.86[a]
	4	6.71[a]	6.76[a]	6.81[a]	6.84[a]
	8	6.69[a]	6.71[a]	6.77[a]	6.80[a]
	12	6.42[a]	6.42[a]	6.55[a]	6.77[a]
	16	5.24[a]	5.41[a]	5.92[a]	5.98[a]
Goat	0	6.79[a]	6.76[a]	6.72[a]	6.70[a]
	4	6.48[a]	6.41[a]	6.42[a]	6.43[a]
	8	6.44[a]	6.48[a]	6.49[a]	6.52[a]
	12	6.43[a]	6.36[a]	6.37[a]	6.27[a]
	16	6.16[a]	5.86[a]	5.81[a]	5.8[a]
Camel	0	6.73[a]	6.73[a]	6.75[a]	6.76[a]
	4	6.62[a]	6.6[a]	6.6[a]	6.61[a]
	8	6.57[a]	6.53[a]	6.58[a]	6.60[a]
	12	6.52[a]	6.47[a]	6.57[a]	6.56[a]
	16	6.17[a]	6.03[a]	6.05[a]	6.29[a]

[1]Means in the same rows and columns with the same letter are not significantly different at (P < 0.05).

indicates that the coagulation is not due to acidity but probably due to microbial enzymes. Goat's milk showed the highest occurrence of coagulation followed by camel's milk, whereas cow's milk showed low coagulation occurrence.

Table 5: Sensory evaluation[1] of cow's, goat's and camel's milk containing 10% of hilwa dates extract

Milk	Aroma	Taste	Color	Overall acceptability
Cow	3.6[2ab]	3.3[a]	3.3[bc]	3.4[a]
Goat	3.5[ab]	3.2[a]	3.1[c]	3.2[ab]
Camel	3.5[ab]	3.3[a]	3.6[bc]	3.2[ab]

[1]A 5 points-hedonic scale whereby 1 means dislike very much, 2 dislike, 3 neither dislike nor like, 4 like and 5 like very much.
[2]Values represented means (n=20). Means with different litters within a column are significantly different at (p<0.05).

The loss in viability of *Bifidobacteria* occurs in fermented milks due to several factors including acid and presence of oxygen (Shah, 2000).

Bifidobactria could grow well in milk inoculated with cultures prepared in a synthetic medium.

Retention of viability of *Bifidobacteria* was greatest with high amylose corn starch (hi-maize). The average pH of skim milk at the end of 4 weeks storage averaged between 4.34 (for *B. animals* with raftilose) to 4.07 (for *B. longum* with inulin). The highest levels of acetic acid and lactic acid were produced, respectively, (Bruno *et al.*, 2002).

Loss of viability of *Bifidobacteria* is typically more pronounced in fermented milk than in unfermented milk due to acid injury to the organism (Dave and Shah, 1997). Lankaputhra *et al.* (1996) observed that viability of *Bifidobacteria* strains such as *Bifidobacterium infantis* in 12% skim milk at pH 4.3 was decreased by 30% after 12 d of storage at 4°C. After 24 d the same temperature, the counts decreased by more than 82%. Medina and Jordan (1994) observed a 93% reduction in bifidobacterial counts of fermented milk produced in Spain at 7°C .

Possible reasons for limited growth: The starter cultures selected for this study do not seems to be suitable for the growth in the different pure milks nor in the enriched milk preparations. Al-Saleh (2001) found similar results in camels milk, where he used *B. angulatum*, *B. infanti*, and four other species. A minor substantial growth was observed only in *B. Longum* whereas a substantial decrease in number was recorded in all other species. In contrast to that, Ustunol and Gandi (2001) observed doubling of the bacterial number of *B. Bifidum* cultured in skim milk enriched with sucrose, honey, fructose or glucose within at least 222 minutes in honey- enriched milk.

The non-encouraging results of the growth study of the two Bifidus species used, raise the question how the growth can be promoted? The use of other more suitable commercial pure or mixed culture may be most promising (Medina and Jordan, 1994). The use of additives such as cysteine (Biavati, 1992) amylose (Hughes and Hoover, 1995) or oligosaccharide (Roberfroid, 1998) may be practical. Another aspect to be tested is to ensure the prevalence of strict anaerobic

conditions. This could be accomplished by exposing the milk to high vacuum to expel soluble oxygen just before incubation.

In the light of the restricted growth of the *Bifidobacterium* species, the judgment regarding the suitability of the produced milk preparation, as a healthy drink, should be based on two aspects: the first one is the efficacy of the surviving bacterial number (> 10^6 cfu/ml) to reside in the intestinal estral and causing a positive health impact and the second is the sensory acceptance of the drinks. Since most of the cfu numbers in all preparations are 10^6 cfu/ml or higher, it is concluded, according to the Adhikari *et al.* (2003), that all preparations are adequate, provided a minimum of 100 ml is consumed daily.

Regarding the sensory quality, the results of the sensory evaluation (Table 5) indicate a moderate acceptance for cow's milk preparations with hilwa dates extracts and less than moderate for camel's milk and the least acceptance for goat's milk preparation.

These results confirm reports which mention a low sensory acceptability of Bifidus milk (Al-Saleh, 2001). The sensory acceptability of Bifidus milk preparation could be improved by combining Bifidus with acidophillus fermentations (Gomes and Malcata, 1999) or by two steps fermentation (Adhikari *et al*., 2003), since this will result in increasing the sourness of the product.

Conclusions: The cold storage of inoculated milks with Bifidobacteria revealed high rate of bacterial survival that encourage the production of Bifidus milk just by inoculation. On the other hand, the pH in both incubated and refrigerated treatments decreased gradually and sometimes with significant differences at ($p<0.05$ level), that indicating activity of the starter cultures used.

References

Adhikari, K., A. Mustapha and I.U. Grun, 2003. Survival and metabolic activity of microencapsulated *Bifidobacterium longum* in stirred yogurt. J. Food Sci., 68: 275-280.

Al-Saleh, A. A., 2001. Bfidus camel milk powder: Sensory evaluation and viability of *Bifidobacteria*. J. King Saud Univ. Agri. Sci., 13: 45-53.

Bezkoravainy, A., 2001.propioticcs determinants of survival and growth in the gut Am. J. Clin. Nutr., 73 (suppl): 3995-4055.

Biavati, B., T. Sozzi, P. Mattarelli and L.D. Trovatelli, 1992. Survival of *Bifidobacteria* from human habitat in acidified milk. Microbiologica, 15: 197- 200.

Bruno, F.A., W.E.V. Lankaputhta and N.P. Shah, 2002. Growth, viability and activity of *Bifidobacterium* spp. In skim milk containing prebiotics. J. Food Sci. 67: 740-2744.

Crittenden, R., 1999. In; Probiotics; A Critical Review, Tonnock GW edit. Norfolk, England: Horizon Scientific Press, pp: 141-156.

Dave, R.I. and N.P. Shah, 1997. In Viability of probiotic bacteria in youghrt made from commercial starter cultures. Int. Dairy J., 7: 707- 715.

Gibson, G.R. and X. Wang, 1994. Regulatory effects of *Bifidobacteria* on the growth of other colonic bacteria. J. Appl. Bacteriol., 77: 413-420.

Gilliland, S.E., 1990. Health and nutritional benefits from lactic acid bacteria. FEMS Microb. Rev., 87: 175-188.

Gomes, A.M. and F.X. Malcata, 1999. Bifidobacterium spp. and *Lactobacillus acidophilus*: biological, biochemical, techonological and therapeutical properties relevant for use as probiotics. Trends in Food Sci. Tec., 10: 139-157.

Gurr, M.I., 1987. Nutritional aspects of fermented milk products. FEMS Microbial. Rev., 46: 337-342.

Holzapfel, W.H., P. Habere, J. Snel, U. Schillinger and J.H. Huis, 1998. Overview of gut flora probiotics. Int. J. Food Microbiol., 41: 85-101.

Holzapfel, W.H., P. Habere, R. Gisen, J. Bjorkroth and U. Schillinger, 2001.Taxonomy and important features of probiotic microorganisms in food and nutrition. Am. J. Clin. Nutr., 73 (suppl): 365s-373s.

Hughes, D.B. and D.G. Hoover, 1991.*Bifidobacteria*: Their potential for use in American dairy products. Food Tec., 45: 4 74-83.

Hughes, D.H. and D.G. Hoover, 1995. Viability and enzymatic activity of *Bifidobacteria* in milk. J. Dairy Sci., 78: 268-276.

Lankaputhra, W.E.V., N. Shah and M. Britz, 1996. Survival of *Bifidobacteria* during refrigerated storage in presence of acid and hydrogen peroxide. Milchwissenschft., 17. 2: 65-70.

Linda, P.M., M.A. Deborah, B. Gail and E. Larmond, 1991. Laboratory Methods for Sensory Evaluation of Food. 2ed ED. Canadian Dept. of Agri. Puplication, Ottawa. Canda.

Medina, I.M. and R. Jordan, 1994. Survival of constitutive microflora in commercially fermented milk containing *Bifidobacteria* during refrigerated storage. J. Food Protec., 56: 731-733.

Modler, H.W., R.C. McKellar, H.D. Goff and D.A. Mackie, 1990. Using ice-cream as a mechanism to incorporate *Bifidobacteria* and fructooligo-saccharides into the human diet. Cultured Dairy Prod. J., 25: 4-9.

Roberfroid, M.B., 1998. Prebiotics: preferential substrates for specific germs. Am. J. Clin. Nutr., 73: 406S-409S.

Scardovi, V., 1986. The genus *Bifidobacterium*. In: Bergey's Manual of System Bacteriology. J. G. Holt, ed. Williams and Wilkins Co., Baltimore, MD., P: 1418.

Shah, N.P., 2000. Probiotics, prebiotic bacteria: selective enumeration and survival in dairy foods. J. Dairy Sci., 83: 894-907.

Ustunol, Z. and H. Gandi 2001. Growth and viability of commercial *Bifidobacterium* spp. in honey-sweetened skim milk. J. Food Protec., 64: 1775-1779.

Pakistan Journal of Nutrition 4 (3): 148-153, 2005
ISSN 1680-5194

The Effect of Phytase and Zinc Supplementation on Palm Kernel Cake Toxicity in Sheep

H.D. Akpan[1], E.O. Udosen[2], A.A. Udofia[3], E.J. Akpan[1] and A.A. Joshua[1]
[1]Department of Biochemistry, Faculty of Basic Medical Sciences,
University of Uyo, Uyo, Akwa Ibom State, Nigeria
[2]Department of Biochemistry, College of Medical Sciences,
University of Calabar, Calabar, Cross River State, Nigeria
[3]Department of Chemical Pathology, University of Calabar Teaching Hospital,
Calabar, Cross River State, Nigeria
E-mail: henrydan4u@yahoo.com

Abstract: The toxicity effect of excess palm kernel cake and the effect of phytase and zinc supplementation on palm kernel cake (PKC) toxicity in sheep were investigated. Three experimental diets A, B, and C were prepared using grass and PKC at 10% and 90% respectively. Diet A contained grass and PKC only (PKC group). Diet B was same as A but in addition was supplemented with Ronozyme™ P at 750 FYT/Kg (PKC + Phytase (P) group). Diet C was also as A, but in addition, was supplemented with zinc sulphate at 500µg/g (PKC + Zn group). Another diet D, containing corn and fish meal at 20% and grass at 80% served as control. These diets were fed to twelve male West African Dwarf sheep (average weight 20.0±0.8kg) divided into 3 groups and the control. Clinical signs and body weights were monitored for 20 weeks. The animals were slaughtered and the right lobes and renal cortex were isolated for copper and Zinc estimation and histopathological evaluation. The results obtained showed that animals in the PKC + phytase group, PKC + Zinc group and the control performed alike in terms of clinical signs, gross and histopathological lesions. In terms of body weight gain, animals in PKC + phytase group performed better than animals in PKC + Zinc group and the control, although the differences were not statistically significant ($p>0.05$). Animals in the PKC group all died of PKC toxicity. Supplementation with zinc and phytase prevented chronic PKC toxicity and can be useful in the therapy for PKC toxicity in sheep. Supplementation with zinc however, led to significant elevation in zinc concentration in the liver and kidney. It is interesting to discover that the use of phytase did not elevate the concentration of zinc in these organs.

Key words: Phytase, zinc, supplementation, palm kernel cake, toxicity, sheep

Introduction

Palm kernel cake (PKC) is a by-product obtained after extraction of palm kernel of the fruits of the oil palm tree *Elaeis guineensis*. Palm kernel cake is produced by grinding the palm kernel followed by pressing which could be done with or without an intermediary flaking and cooking stages (Tang and Toeh, 1985). Raw palm kernel oil is obtained during the pressing and is diverted for classification while the residual cake (Palm kernel cake) is cooled and stored for use (Tang and Toeh, 1985).

The most important usage of PKC is as ingredient for the formulation of animal feeds (Collingwood, 1958). It has a high nutritive value and is considered to be excellent for ruminants but also suitable for use in feed formulations for swine, poultry and horses (Hutagulung *et al.*, 1982; Yeong *et al.*, 1983; Collingwood, 1958). It usage in excess in sheep are known to cause chronic toxicity and death due to hepatic necrosis (Abdul Rahman *et al.*, 1989; Wan Mohammed *et al.*, 1989). Jaundice and haemoglobinuria are the most remarkable clinical signs demonstrated (Hair-Bejo and Alimon, 1995).

Changes occurring after ingestion of excess PKC in sheep are consistent to those of chronic copper toxicity (Sandstead *et al.*, 1970; McCall *et al.*, 1971; Ishmeal *et al.*, 1971; Underwood, 1977; Soli, 1980). Supplementation of diets with sodium molybdate and ferrous sulphate reduces the incidence of the disease (Abdul Rahman *et al.*, 1989; Wan Mohammed *et al.*, 1989). The toxicity can be prevented by dietary zinc supplementation either with or without ammonium molybdate (Hair-Bejo and Alimon, 1995) and is possible due to antagonistic interaction between copper and zinc, which make zinc to be preferentially absorbed instead of copper (Hair-Bejo and Alimon, 1995). However, the use of supplementary zinc in the prevention of PKC toxicity in sheep led to an elevated blood, hepatic and renal zinc concentration (Hair-Bejo and Alimon, 1995). The long-term effect of this alteration is not known. Furthermore, little information is available on the use of supplementary zinc in the prevention of PKC toxicity in

sheep (Hair-Bejo and Alimon, 1995). Few approaches are made to prevent PKC toxicity in sheep (Abdul Rahman *et al.*, 1989; Wan Mohammed *et al.*, 1989; Hair-Bejo and Alimon, 1995). In view of the abundance of PKC in many West African Countries, the potential of PKC as cheap material for feeding sheep and other animals and livestock and the economic importance of sheep product such as cheese, wool, and meat etc, it is desirable to investigate widely on PKC toxicity and ways of prevention in sheep.

It is known that copper bioavailability depends on the relative proportion of zinc. The zinc and copper contents of PKC have been reported to fall between the ranges of 43.8-77.0mg/kg and 11-28.5mg/kg respectively (Yeong *et al.*, 1983; Abdul Rahma *et al.*, 1989; Jalaludin *et al.*, 1991; Mustaffa *et al.*, 1991; Hair-Bejo and Alimon, 1995). It is obtained naturally in the ratio of 1:4 of copper to zinc. It is suggested that if most of the natural zinc in PKC were bioavailable, it may make zinc to be preferentially absorbed instead of copper and reduce the burden of having to source for inorganic zinc.

Factors that aggravate zinc deficiency include high copper, phosphorus, calcium and phytate (Maga, 1982; Davis and Olpin, 1979; Bingham, 1978; Davis and Reid, 1979; Sandberg *et al.*, 1982). Siew (1989) gave the composition of zinc, copper, phosphorus and calcium in PKC (out of the seventy eight samples from 23 palm kernel crushers analyzed) to be 77.0, 28.5, 47.4 and 17.4 mg/kg respectively. Therefore, calculated molar proportion of zinc to copper; zinc to phosphorus and zinc to calcium is 3:1; 2:1 and 4:1 respectively. At this ratio, copper, phosphorus and calcium appear not to exhibit significant effect to interfere with zinc absorption in PKC. Only phytate may play significant role, as phytate level as high as 1309±20.8mg/kg was obtained for Nigerian PKC (Akpan and Joshua, 2003, unpublished). Davis and Olpin (1979) demonstrated zinc deficiency symptoms in rat fed a diet with phytate: zinc ratio of 10:1.

The objective of the experiment was to investigate the effect of supplementary phytase and zinc on palm kernel cake toxicity in sheep and to assess the toxicity effect of excess palm kernel cake in sheep.

Ronozyme™ P was used to represent the universe of phytase and is a highly active phytase derived from *Peniophora lycil.* It is formulated as a multi coated granulate (CT) with superior heat stability and function well above pelleting temperature of 80°C. With proven efficacy in pigs and poultry, Ronozyme ™P makes better use of phosphorus and other nutrients such as calcium, zinc, magnesium, and phytate-bound proteins, which are present naturally in the diet, thus reducing the need for inorganic supplements and saving unnecessary cost. It is recommended at 750 FYT/kg diet.

Materials and Methods

Three experimental diets A, B, and C similar to the experimental feed of Hair-Bejo and Alimon, (1995), were prepared using grass and PKC at the level of 10 and 90% respectively. The PKC used in the study was purchased from Basu Palm Kernel crushing industry located at Itam in Itu Local Government Area in the South-Southern part of Nigeria. Grass was only elephant grass. Diet A contained grass and PKC only (PKC group). Diet B was same as A but in addition was supplemented with Ronozymes™ P (phytase) at 750FYT/Kg (PKC + Phytase (P) group). Diet C was also as A, but in addition was supplemented with Zinc sulfate ($ZnSO_4.7H_2O$) at 500µg/g (PKC + zinc (Zn) group). Another diet D containing corn and fishmeal at 20% and grass at 80% was prepared to serve as control.

Twelve male West African Dwarf sheep weighing 20.0 ± 0.8kg were divided into 4 groups of 3 animals each and were randomized to the three experimental diets and the control. They were stall-fed for 20 weeks. All animals were monitored for clinical signs. Body weights were recorded at two weeks interval. Blood samples were also collected every two weeks from the jugular vein and analyzed same day for copper and zinc content or stored at 0 - 4°C until required for analysis. At the end of the 20 weeks, the animals were slaughtered and carcasses were analyzed for gross lesions. The right liver lobes and renal cortex were isolated for histopathological evaluation and estimation of copper and zinc content.

Histopathological studies: 10% formalin was freshly prepared and both the right lobe and the renal cortex were fixed in the formalin for 48 hours and subsequently dehydrated in alcohol, cleared with xylene and embedded in paraffin wax. Sections of the lobe and cortex at about 5µm were mounted on glass slides and stained with haematoxylin and eosin (Lillie, 1965).

Analysis of zinc and copper: Copper and zinc were estimated in blood, liver lobe, renal cortex and the PKC used for this study. The liver and kidney from each of the animals in each group were prepared for copper and zinc analysis by oven drying at 70°C until they reached a constant weight. The dry weights were recorded and the material ground to powder, sieved with 0.5 mesh and samples of the sieved were digested. The PKC used for this study was also oven dried to constant weight, ground to powder and sieved with 0.5 mesh and samples of the sieved were also digested. Also, 2.0ml of the blood from each animal collected fortnightly over the 20 weeks were also digested.

Digestion was carried out in a pyrex glass tube (150mm x 18mm) using 70% aristar grade Nitric acid (BDH Chemicals Ltd) and 60% spectrosol grade perchloric acid (BDH Chemical Ltd) in 2 to 1 (v/v) respectively. Added into each recovery tube was fifty µl (1mg/ml) of spectrosol grade cupric nitrate and zinc nitrate solutions (BDH Chemical Ltd). The tubes were then covered with

Table 1: Effect of different dietary treatments of PKC on the average daily weight gain in sheep (0-20 weeks)

Treatment	Average weight gain (g)
PKC	40.1 ± 0.05[b]
PKC + Zn	50.3 ± 0.10[a]
PKC + P	55.3 ± 0.02[a]
Control	54.0 ± 0.11

[a]Mean with different superscript are significantly different (p>0.05)

Table 2: Effect of different dietary treatments of PKC in sheep on blood, hepatic and renal copper concentration

Treatment	Blood (µg/ml)	Right liver (µg/g)	Renal cortex (µg/g)
PKC	3.02±0.82[b]	1058.3±0.20[b]	430.5±0.50[b]
PKC+Zn	1.25±0.11[a]	458.8±20.20[a]	40.7±10.50[a]
PKC+P	1.05±0.20[a]	430.3±30.20[a]	34.2±5.80[a]
Control	1.05±0.11[a]	430.0±0.50[a]	30.5±7.00

[a]Mean with different superscript are significantly different (p>0.05)

glass marbles, left overnight and on the next morning heated until they were completely digested and turned from dark brown to colourless.

The digest were further diluted in distilled water to 10ml in volumetric flask and analyzed for copper and zinc using Atomic Absorption Spectrophotometer (Virian Spectra 400) at wavelengths of 324.7nm and 213.9nm respectively. The spectrophotometer was standardized using a solution containing 2.00, 4.00, 6.00, 8.00 and 10.00µg/ml of copper and 0.20, 0.40. 0.60, 0.08 and 0.10µg/ml of zinc prepared from spectrosol grade cupric nitrate and zinc nitrate (1mg/ml) respectively, in 0.1M Nitric acid. The spectrophotometer was closely monitored and restandardised if necessary to ensure better performance. Triplicate determinations were carried out per sample and the result of copper and zinc concentration were expressed as the mean of the three determination ± standard error of mean of the groups in P-g/g dry weight or µg/ml. Student's t-test was employed to test the significance of difference between treatment means.

Results

There was no mortality except in the PKC group. All animals died in the PKC group. The clinical signs were jaundice observed in the first animal on day 54, second and third on day 56 and followed by haemoglobinuria at day 57 for that first animal and at day 58 by the other two animals. The animals severely flake off their hairs at day 58 and were generally weak, depressed and anorexic and all died on day 130, the first one in the morning followed by the other two in the evening. The animals in the PKC + Zn group, PKC + phytase group and the control group did not die and did not show any clinical abnormalities throughout the trial.

The average daily gain of the animals in the various groups is shown in Table 1. The average daily weight gain was similar in all groups over the 20 weeks except at two weeks prior to death where it began to reduce in the PKC group and at one week to death was significantly lowered ($P < 0.05$) The numerical values of the daily gain were 35.6g for the PKC group, 50.5g for the PKC + Zn group, 55.3g for the PKC + P group and 54.0g for the control. The daily gain was numerically higher in the PKC + P group than the PKC + Zn group and the control, though the difference was not statistically significant ($P>0.05$).

Analysis of carcasses for gross lesions revealed that animals that died in the course of the experiment died of copper toxicity of PKC. All animals that died had severe generalized jaundice. All animals in the PKC group had liver that was moderately yellowish with multi focal pale area of necrosis. Kidneys were firmed, enlarged and dark black in appearance at both the cortex and medulla. Carcasses of animals of the other groups analyzed did not show significant gross lesions.

The hepatocytes of animals that died in PKC group were highly swollen, vacuolated and necrotized at the periacinal zone. Moderate fibrosis occurred at the periportal zone. Tubular epithelial cells of the liver lobes and renal cortex of animals in the other groups did not show remarkable histological lesions.

The copper concentration in the PKC used in this study was 20.5 ± 0.09µg/g. The copper concentration in the blood, renal cortex and liver for each treatment is shown in Table 2. The blood copper concentration in all the groups remained alike for the first 4 weeks of the trial (PKC group, PKC + Zn, PKC + P and the control had blood copper concentration of 1.85 ± 0.02µg/ml, 1.02 ± 0.20µg/ml and 1.00 ± 0.35µg/ml respectively). It was slightly elevated in the PKC group from 4th to 8th week ranging from 2.45 ± 0.20µg/ml but highly increased to 7.50 ± 0.10µg/ml at the 10th week and thereafter (which was about 2 weeks after the appearance of haemoglobinuria). The blood copper concentration in the PKC + Zn group, PKC + P group and the control increased only slightly to 1.30 ± 0.20µg/ml, 1.08 ± 0.02µg/ml and 1.2 ± 0.85µg/ml respectively, throughout the duration of the experiment. The mean blood copper concentration of the PKC (3.02 ± 0.82µg/ml) was significantly higher ($P>0.05$) than those of the PKC + Zn group, PKC + P group and the control. The blood copper concentration of PKC + Zn, PKC + P and the control were similar.

The copper concentration in the right liver lobe and renal cortex in the PKC group (1058.3 ± 0.20 and 430.5 ± 0.5µg/g respectively) were significantly higher ($P > 0.05$) when compared with those of the control (460.3 ± 0.50 and 32.5 ± 7.0µg/g respectively) and those of the PKC + Zn group (458 ± 0.20 and 40.7 ± 10.5µg/g respectively) and those of PKC + P group (430.0 ± 30.2 and 34.2 ± 5.8

Table 3: Effect of different dietary treatments of PKC in sheep on blood, hepatic and renal Zinc concentration

Treatment	Blood (µg/ml)	Right liver (µg/g)	Renal cortex (µg/g)
PKC	3.50±0.10[c]	90.50±15.20[c]	102.30±12.50[c]
PKC+Zn	6.09±1.80[a]	219.20±30.20[a]	212.50±35.30[a]
PKC+P	4.90±0.50[b]	110.00±8.20[b]	118.20±8.50[b]
Control	4.90±1.50[b]	108.50±18.20[b]	108.20±12.10

[b]Mean with different superscript are significantly different (p>0.05)

µg/g respectively). But those of PKC + Zn group (430.0 ± 30.2 and 34.2 ± 58µg/g respectively) and the PKC + P group (430.0 ± 30.20 and 34.2 ± 38µg/g respectively) were not significantly different when compared with those of the control.

The Zinc content in the PKC used in this study was 75.0 ± 0.2µg/g. The result of the Zinc concentration of the blood, renal cortex and the right liver lobe for each treatment is shown in Table 3. The blood Zinc concentration in all the groups remained alike for the first 4 weeks of the trial (PKC group 3.2 ± 0.21µg/ml, PKC + Zn group 3.91 ± 0.5µg/ml, PKC + P group 3.5 ± 0.5µg/ml and the control 3.4 ± 1.5µg/ml). It was slightly elevated in the PKC + Zn group from 4th to 8th week ranging from 3.9 ± 0.8µg/ml but highly increased to 7.02 ± 0.10µg/ml at the 10 week and thereafter (which was 2 weeks after haemaglobinuria). The blood Zinc concentration in the PKC group, PKC + P group and the control increased only slightly throughout the duration of the trial. The mean blood Zinc concentration in the PKC + Zn group (6.09 ± 1.80µg/ml) was significantly higher (P >0.05) than those of the other groups. The mean blood Zinc concentration of the PKC + P group (4.90 ± 0.50µg/ml) was significantly higher (P>0.05) than those of the PKC group (3.50 ± 0.10µg/ml) but was consistent with the control (4.90 ± 1.5µg/ml).

The mean Zinc concentration in the right liver lobe and renal cortex in the PKC + Zn group (219.2 ± 30.2µg/g and 212.5 ± 35.3µg/g respectively) were significantly higher (P > 0.05) when compared with those of the PKC group (90.50 ± 15.2 and 102.3 ± 12.5µg/g respectively), PKC + P group (110.0 ± 8.2 and 118.2 ± 8.5µg/g respectively) and the control group (108.5 ± 18.2 and 108.2 ± 12.1µg/g respectively). The mean Zinc concentration in the right liver lobe and renal cortex in the PKC + P group, and the control were similar.

Discussion

The objective of the study was to assess the effect of phytase and zinc supplementation on palm kernel cake toxicity in sheep. It was to further assess the toxicity effect of excess palm kernel cake in sheep. The results obtained show that animals with phytase supplementation (PKC + P group) performed as those of the zinc supplementation (PKC + Zn group) and the control in terms of clinical signs, gross and histological lesions. In terms of body weight gain, animals with phytase supplementation (PKC + P group) even performed better than animals with the zinc supplementation (PKC + Zn group) and the control, though the differences were not statistically significant (P>0.05).

Animals in the PKC group all died of PKC toxicity. Supplementation with zinc and phytase prevented chronic PKC toxicity and can be useful in the therapy for PKC toxicity in sheep. Supplementation with zinc led to a significant elevation of zinc concentration in the blood, right liver lobe and renal cortex. These findings on the elevated zinc concentration in the blood and these organs support previous findings by Hair-Bejo and Alimon (1995). We obtained a significant elevation in our study. Hair-Bejo and Alimon (1995), in their study did not obtain a significant elevation. The variation in the results might be due to the differences in the composition of PKC used which might be caused by differences in soil type, species of palm tree and processing instruments etc. Our PKC material contained 75.0 ± 0.2µg/g of zinc and 20.5 ± 0.09µg/g of copper while those of Hair-Bejo and Alimon (1995) contained 43.8 ± 0.1µg/g of zinc and 21.6 ± 0.2µg/g of copper. It was interesting to observe that the use of supplementary phytase did not at all elevate the concentration of zinc in those organs.

Phytases are enzymes specialized in breaking down phytates (myo-inositol hexakisphosphates). In addition to phosphorus, phytate also binds tightly to other nutrients such as proteins and minerals and render them unavailable. Phytase breaks down phytate and release bound nutrients, thus improves the digestibility of those nutrients. In our work, we observed that the addition of phytase increased blood zinc concentration significantly (P>0.05) in the PKC + P group compared with other groups. The mean blood zinc concentration was 4.9 ± 0.5µg/ml in the PKC + P group and was significantly higher (P>.0.05) than the mean blood zinc concentration in the PKC group 3.50 ± 0.10µg/ml. This result suggests that the added phytase increased the availability of natural zinc of the PKC. Contrarily, we observed that while the blood zinc concentration was increased with the added phytase, the blood copper concentration was decreased. The enzyme appears to improve the utilization of zinc of PKC, which then make zinc to be preferentially absorbed instead of copper. Similar observations of the role of phytase in copper and zinc utilization have been reported by a number of researchers. Aoyagi and Baker (1995) reported decreased utilization of copper in soya bean meal by 50% when microbial phytase was added. There is also report showing increased calcium availability and decreased copper and sulphur availability in barley grains when phytate level was reduced. In growing and finishing pigs, Murray (1998) reported increased availability of zinc, magnesium, phosphorus and iron,

but decreased availability of calcium and copper utilization when a low level of phosphorus corn-soya bean meal was fed with phytase enzyme. Perhaps the increased zinc utilisation in PKC when phytase was added overcame the copper toxicity in the PKC + P group.

But why should sheep as a ruminant requires an extrinsic phytase to utilize phytate when ruminant readily utilize phytate because of the phytase produced by the rumen microorganism? More recent findings shows that the efficacy of phytase depend not only on the present of the enzyme but also on the type of feed (phytate), the dose and the source of phytase (Dekker *et al.,* 1992; Eeckhout and De Paepe, 1992; Dungelhoef *et al.,* 1994). The toxicity effect of excess PKC in sheep without an added phytase may be caused by the inability of the endogenous phytase to handle the increased phytate level in the PKC. The supplemental phytase may have had an additive effect on the ruminal phytase activity. An increased phytase activity was demonstrated with an increased phytase level (Simons *et al.,* 1990).

This study agrees with previous findings that feeding PKC in excess (90%) in sheep caused chronic copper toxicity (Hair-Bejo and Alimon, 1995; Abdul Rahman *et al.,* 1989; Wan Mohammed *et al.,* 1989). The clinical signs, gross lesion and histopathological changes were consistent with those reported by Hair-Bejo and Alimon (1995) except that in our study, the kidney, were completely darkened. Our work also agrees with the work of Hair-Bejo and Alimon (1995), who also observed an elevated hepatic, renal and blood copper in the PKC group. Our work, supports other works that the PKC toxicity in sheep is consistent to those of acquired chronic copper poisoning (Ishmael *et al.,* 1971; Soli 1980). Our works also agree with Hair-Bejo and Alimon (1995) that dietary zinc supplementation can prevent chronic copper toxicity of excess PKC in sheep.

It is concluded that feeding PKC in excess in sheep can cause chronic copper toxicity in sheep but supplementing the diet with zinc and phytase can prevent this effect. Supplementation with phytase does not elevate zinc concentration in the liver and kidney, as it is the case with zinc supplementation.

References

Abdul Rahman, M.V, H.K. Wong, H. Zaini and H. Sharif, 1989. Preliminary observation on the alleviation of copper in sheep fed with palm kernel meal based diet. Proc of 12 Conf. MSAP, pp: 75-78.

Akpan, H.D. and A.A. Joshua, 2003. Nutritional Evaluation of Nigerian Palm Kernel cake as feedstuff for poultry. Undergraduate Project work, University of Uyo, Nigeria (unpublished).

Aoyagi, Segi and D.H. Baker, 1995. Effect of microbial phytase and 1,25 Dihydroxycholecalciferol on Dietary copper utilization in chicks. Poult. Sci., 74: 121-126.

Bingham, S., 1978. Nutritional: A Consumers guide to good eating. Transworld publishers London.

Collingwood, J.G., 1958. Palm Kernel meal. Processed plant protein foodstuffs. Academic press Inc., New York. P: 677-701.

Davis, N.T. and S.E. Olpin, 1979. Studies on the phytate: zinc molar contents in diets as a determinant of zinc availability to young rats. Br. J. Nutr., 41: 591-603.

Davis, N.T. and H. Reid, 1979. An Evaluation of the phytate, zinc, copper, iron and manganese contents of, and zinc availability from soybased textured vegetable protein meat substitutes or meat extenders. Br. J. Nutr., 41: 579-589.

Dekker, R.A., P.A. Kemme and A.W. Jongblood, 1992. Methodological comparison of the assessment of P digestibility of tapioca and maize, and the influence of amount and origin of phytic acid on the efficacy of microbial phytase from Aspergillus niger. Report IWO - DLO, elystad, - nr. 244.

Düngelhoef, M., M. Rodehutscord, H. Spiekers and E. Pfeffer, 1994. Effect of supplemental microbial phytase on availability of P contained in maize, wheat and triticale to pigs. Anim. Feed Sci. Tech., 49: 1-10.

Eeckhout, W. and M. De Paepe, 1992. Betere benutting van de voeders I.I. synthetische fytase.

Hair-Bejo, M. and A.R. Alimon, 1995. The protective role of zinc in palm kernel cake (PKC) toxicity in sheep. Mal. J. Nutr., 1: 75-82.

Hutagulung, R.I., M.D. Mahyuddin and S. Jalaludin, 1982. Feeds for farm animals from the oil palm. Proc. Of the oil palm in the eighties (push parajah, P. and Chew, P. S. eds). Vol. II. P: 609-622.

Ishmael, J., C. Gopinath and J.M.C.C. Howel, 1971. Experimental chronic toxicity in sheep. Histological and histochemical changes during development of the lesions in the liver. Res. Vet. Sci., 12: 358-366.

Jalaludin, S., Z.A. Jelan, N. Abdullah and Y.W. How, 1991. Recent developments in the oil palm by-product based ruminant feeding system. In recent advances on the nutrition of Herbivores (eds. Y. W. How *et al.,* pp: 35 - 44, MSAP).

Lillie, R.D., 1965. Nuclei, nuclei acids, general oversight stains. In histopathotogic Technique and practical histochemistry, 3rd edition pp: 142-179, McGraw Book Company.

Maga, J.A., 1982. Phytate: its chemistry, occurrence, food interactions, nutritional significance and methods of analysis J. Agri, Food Chem., 30, 1-9.

McCall, J.T., N.P. Goldstein and L.H. Smith, 1971. Implication of trace metals in human diseases. Fed. Proc., 30: 1011-1015.

Murray, A.C., 1998. Effect of microbial phytase on calcium and phosphorus digestibility and serum mineral concentrations in growing and finishing pigs. UGA Animal and Diary Science. Annual Report, pp: 301-304.

Mustaffa, B., S. Mizairi and H. Hawari, 1991. Palm Kernel cake in cattle feedlotting. Asean Food J., 6: 102-103.

Sandberg, A., L. Hasselblad and K. Hasselblad, 1982. The effect of wheat bran on the absorption of minerals in the small intestine. Br. J. Nutr. , 48: 185-191.

Sandstead, H.H., G.H. Burk, G.H. Booth and W.J. Darby, 1970. Current concepts on trace minerals. Clinical considerations. Med. Clin. North Am., 54: 1509-1531.

Siew, W.L., 1989. Characteristics and uses of Malaysian palm kernel cake. PORIM Technology No., 14: 2-3.

Simons, P.C.M., H.A.J. Versteegh, A.W. Jongloed, P.A. Kemme, Slump; K.D. Bos, M.G.E. Wolters, R.F. Beu Deker and G.J. Verschoor, 1990. Improvement of phosphorus availability by microbial phytase in broilers and pigs. Br. J. Nutr., 64: 525-54.

Soli, N.E., 1980. Chronic copper poisoning in sheep. A review of the literature. Nord. Vet. Med., 32: 75-80.

Tang, T.S. and P.K. Toeh, 1985. Palm kernel cake oil extraction – the Malaysian experience. J. Am. Oil Chem. Soc., 62: 254-258.

Underwood, E.J., 1977. Copper. In trace elements in human and animal nutrition. 4th eds. pp: 56-108, New York, San Francisco, London Academic Press.

Wan Mohammed, W.E., A. Abdul Rahman, N. Mobamad, and H.F. Koh, 1989. Oil palm by-products in prime lamb production. Proc of 12th Conf. MSAP, pp: 69-74.

Yeong, S.W., T.K. Mukhergee and R.I. Hutagulung, 1983. The nutritive value of palm kernel cake as a foodstuff for poultry. Proc. of the national workshop on oil palm by products utilization, p: 100-107.

Pakistan Journal of Nutrition 4 (3): 154-157, 2005
ISSN 1680-5194

Feeding Practices, Growth Rate and Management of Ostrich Chicks in Sudan

H.H. Musa[1], A.H. Suleiman[1], T.P. Lanyasunya[2], O. Olowofeso[3] and D.M. Mekki[4]
[1]Department of Animal Production, Faculty of Veterinary Science, University of Nyala, Sudan
[2]Kenya Agricultural Research Institute (KARI), Kenya
[3]Department of Agricultural Science, Adeyemi College of Education, P.M.B., 520, Ondo, Nigeria
[4]Department of Animal Science, Faulty of Natural Resource and Environmental Studies,
University of Kordofan, Sudan

Abstract: Seventeen male Ostrich chicks (*Ostruiothio, Camelus camelus*) aged one month old were brought from Radom National Park to the research farm of University of Nyala, both in Sudan, and reared indoor, concentrate rations were formulated and provided *ad-libitum* and water equally supplied. During the study period, chick behaviour were observed, body weight gained (g/day), feed intake (g/day), water consumption (liter/day) and feed conversion ratio (FCR) were found to be 178.64, 447.91, 4.18 and 2.39, respectively. Rapid growth rate during the age of 2 to 4 months resulted to leg deformation which eventually leads to the death of some chicks with mortality rate 41.20% at the end of the experiment. The body measurements at different ages of chicks were highly significant ($P< 0.05$). There were high positive correlations between live weight and other parameters estimated (shank diameter and length, nick diameter and length, wing length, body length, heart girth and bird high).

Key words: Ostrich chicks, feeding practices, Sudan, nutritional requirements

Introduction

Wildlife has changeable dynamic importance according to human situation and his needs. Previous studies carried out by Babiker and Mohamed (1988) indicated that wildlife can survive and produce under adverse climatic conditions, yet accurate statistics of total population of species inhabiting Sudan are lacking. Sudan has a large number of Ostrich populations in its natural environment. Studies by Ciliers and Huchzermeyer (1998) and Tully and Shane (1996) indicated that Ostrich farming has spread worldwide and fully established in South Africa. Studies by Ciliers and Huchzermeyer (1998) characterized Ostrich by low-fat and low-cholesterol meat to be the primary product. There is also a market for leather, feather and oil by-products derived from Ostrich production.

Experiments conducted on Ostrich so far have been very scanty in literature and available evidences mainly focused on nutritional requirements of Ostrich and it was suggested that the turkey is the best avian model from which to predict Ostrich nutrient needs (Ullrey and Allen, 1996).

Also, knowledge on various nutritional aspects is not well defined Ciliers and Huchzermeyer (1998). Ostrich in different regions showed different development of body weight and high concentrate feed intake resulted to rapid growth during age of four months (More, 1996). The purpose of this study was to evaluate some characteristics of Ostrich chicks in Southern Darfur, Sudan, particularly the husbandry and the feeding practices.

Table 1: Concentrate rations (%DM) and composition for Ostrich chicks in Western Sudan

Stuffs	(%DM)
Ingredients	
Sorghum	61.00
Ground nut cake	18.00
Ground nut husk	14.00
Fish meal	5.00
Oyster shell	1.80
Salt	0.20
Total	100.00
Chemical composition	
Dry matter (DM)	92.40
Ash	14.50
Crude protein (CP)	20.20
Ether extract (EE)	3.60
Crude fibre (CF)	7.40

Materials and Methods

Housing: This experiment was conducted at University of Nyala research farm, Sudan, between February to August, 1999. Two pens each with dimensions of 4x4x2.4-m shaded with a door of 200x90-cm and three windows 125x180-cm made up of fine wire net were used. The floor of the pen was covered with ravel and sawdust. Each pen was supplied with two feeding and three water troughs made up of zinc and plastic, respectively.

Source of animals: Seventeen Ostrich chicks aged one month were brought from Radom National Park, Sudan,

Table 2: Means and standard deviation for different parameters of Ostrich chicks

Parameters	Age (weeks)				
	1	3	6	9	12
Live weight (kg)	2.71±0.50	5.52±1.59	9.72±2.12	14.50±3.05	20.08±4.49
Shank diameter (cm)	4.91±0.31	6.13±0.10	7.58±0.61	8.61±0.25	10.28±0.34
Shank length (cm)	12.53±0.59	16.83±1.13	25.20±1.38	30.50±1.11	35.22±1.22
Neck diameter (cm)	10.28±0.79	14.85±0.82	18.85±1.30	20.78±1.12	25.15±1.42
Neck length (cm)	22.10±2.03	31.78±3.25	49.75±4.17	62.58±3.04	71.53±1.72
Wing length (cm)	15.15±0.86	22.72±2.56	34.90±2.87	44.67±3.00	49.93±2.91
Body length (cm)	16.50±2.10	22.66±1.30	31.53±2.76	38.02±2.43	46.58±3.92
Heart girth (cm)	10.60±1.21	15.69±1.89	19.54±1.49	23.90±2.60	29.60±1.31
Bird high (cm)	28.40±3.05	48.20±3.94	67.70±3.02	83.95±3.78	107.59±3.87

Table 3: Correlation coefficient of body weight and body measurements of Ostrich chicks

Parameters	Live weight	Shank diameter	Shank length	Neck diameter	Neck length	Wing length	Body length	Heart girth	Bird high
Live weight	1.000								
Shank diameter	0.993**	1.000							
Shank length	0.982**	0.992**	1.000						
Neck diameter	0.975**	0.995**	0.987**	1.000					
Neck length	0.980**	0.990**	1.000**	0.984**	1.000				
Wing length	0.976**	0.987**	0.998**	0.982**	0.999**	1.000			
Body length	0.992**	0.999**	0.996**	0.992**	0.994**	0.991**	1.000		
Heart girth	0.991**	0.998**	0.987**	0.993**	0.985**	0.983**	0.996**	1.000	
Bird high	0.991**	1.000**	0.991**	0.995**	0.989**	0.987**	0.999**	0.999**	1.000**

Correlation is significant at the 0.01 level (2-tailed).

divided into two groups, identified by shank tag. The initial weight of each Ostrich was taken; daily feed and water intake were recorded. Chopped alfa-alfa, gravel, vitamins and minerals were provided three times a week. The chicks were not vaccinated throughout the experimental periods.

Feeding: Vitamin B complex and Vitamin C were injected intramuscularly into the Ostrich chicks and the concentrate mash ration formulated, supplied to the chicks and its chemical composition is presented in (Table 1).

Experimental measurements: Daily records of total amount of feed and volume of water consumed were kept and other parameters recorded fortnightly. Weight of chicks were carried out using spring balance and shank diameter taken from the mid, shank length taken from hook joint to meta-carpopharngeal joint, neck diameter taken from around its bas, neck length taken from last cervical vertebrate to poster end of the atlas, wing length taken from carcoid to great trochanter of femur, heart girth behind wings and bird height taken from claws to dorsal of the thorax, Data collected were analyzed using one-way analysis of variance (ANOVA) and correlation coefficient using SPSS 11.5, respectively.

Results

The study revealed that pens with these dimensions are suitable for Ostrich chicks at two months old, after that age dimensions need to be added and spaces to run round are necessary. Feed and water troughs should be adequate and evenly distributed to avoid injury with sharp edges of metallic one. Chicks behaviour like body temperature regulation, scratching the ground and backing feed trough were observed. At third month of age, chicks clustered to flapping their feathers, wings moved forward and lateral, there were pecking and social vices among the birds.

Average feed intake, water consumption per liter, daily weight gained and feed conversion ratio (FCR) were estimated and were as depicted in Fig. 1, 2, 3 and 4, respectively. High concentrate feed intake increase growth rate during the age of 2 to 4 months causing leg deformities in the hook joint resulting in death among the birds with mortality rate up to 41.20% at 45 days old, fowl-pox like lesions was also observed, but disappeared a week later, there were no other diseases observed.

Table 2, showed the mean and standard deviation for body measurements at different ages of chicks, highly significant difference ($P<0.05$) was found among body measurements and age of chicks. Similarly, there was

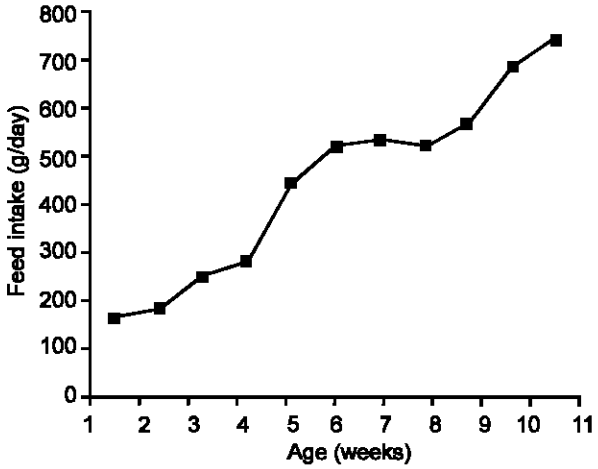


Fig. 1: Feed intake of Ostrich Chicks (g/day)

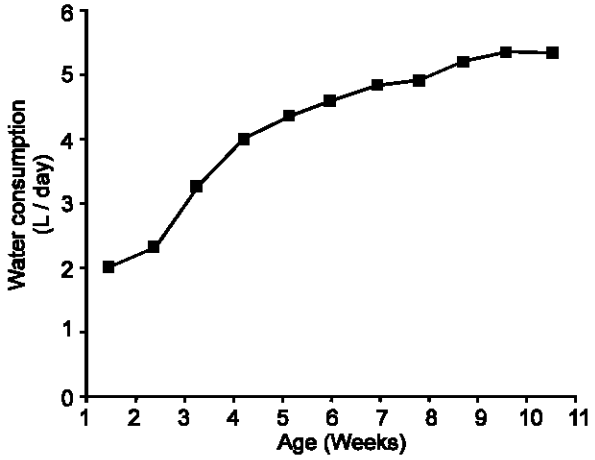


Fig. 2: Water consumption of Ostrich chicks (L/day)

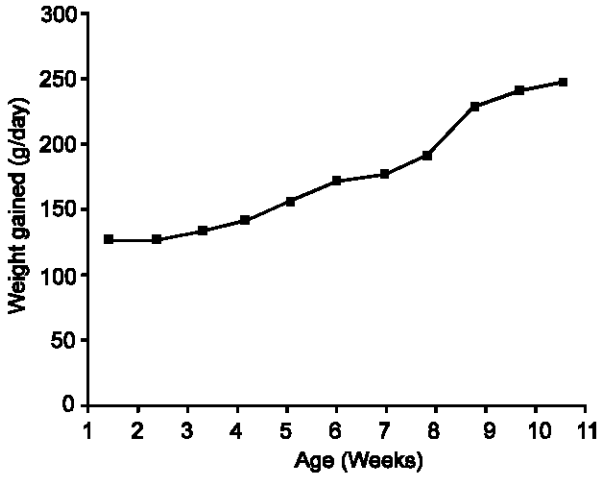


Fig. 3: Weight gained of ostrich chicks (gram/day)

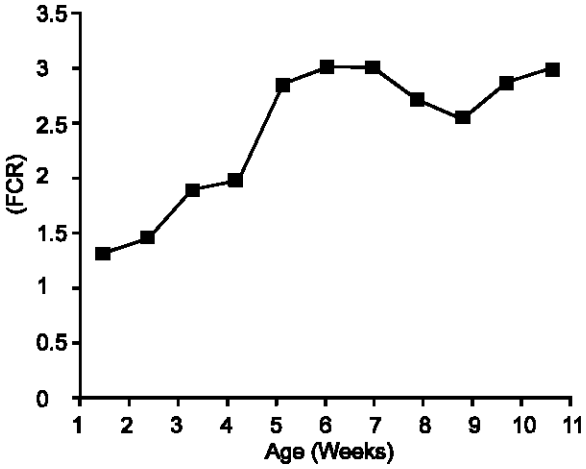


Fig. 4: Feed conversion ratio of Ostrich chicks (FCR)

positive correlation between live weight and (shank diameter and length, nick diameter and length, wing length, body length, heart girth and bird high) (Table 3).

Discussion

The dimensions of pen in this experiment were convenient for chicks at 2 months old. Thereafter extra space can be added as previously reported by (Kreibich and Sommer, 1995). Metal trough can change to plastic to avoid injury and bleeding which resulted in pecking and cannibalism. Chicks behaviour observed in this study were same as observed by (Kreibich and Sommer, 1995).

Average feed intake per day, water consumption per day, feed conversion ratio (FCR) and weight gained as shown in the accompanied Fig. 1, 2, 3 and 4 were not different from that estimated by (Mushi *et al.*, 1998). Also, Kreibich and Sommer (1995) explained that feed conversion ranged from 1.4:1 to 1.6:1 for younger birds 4 to 6 months old, while for older birds ranging from 4:1 to 6:1.

The high concentrate feed intake increased weight gained during 2 to 4 months and absence of space to run around caused 41.20% mortality, 35.30% dead by leg deformation and 5.90 % were injury by sharp edges of metal trough. This information was in agreements with (Kreibich and Sommer, 1995 and More, 1996). Body measurements were found highly significant among chicks age. The observations of the present study for body measurements (shank diameter and length, nick diameter and length, wing length, body length, heart girth and bird high) is similar to previous studies carried out by Mushi *et al.* (1998) indicated that metatarsal length increased rapidly at weekly rate of 2.50-cm and that body weight was highly correlated with metatarsal length 0.90 and the mean body length reached 134-cm in 4 months.

References

Babiker, S.A. and A.S. Mohamed, 1988. A note on yield and carcass characteristics of Red-buck. (*Redunca redunca*) in Sudan. Sudan J. Anim. Prod., Vol. 1, 2: 65-70.

Ciliers, S.C. and F.W. Huchzermeyer, 1998. Foodstuff evaluation, metabolizable energy and amino acid requirements for maintenance and growth in Ostrich in Ostriches ratite congress, Oudtshoorn, South Africa, 21st -25th Sept., 12-23.

Kreibich, A. and M. Sommer, 1995. Ostrich farm management. German, pp: 450.

More, S.J., 1996. The performance of farmed Ostrich chicks in Eastern Australia. Preventive Veterinary Medicine, 29: 91-106.

Mushi, E.Z., M.G. Binta, R.G. Chaba, J.F. Lsa and L. Modisa, 1998. Serum biochemical values of farmed Ostrich (*Struthio camelus*) in Botswana, onderstepoort. J. Vet. Res., 65, 3:189-193.

Tully, T.N. and S.M. Shane, 1996. Husbandry practices as related to infectious parasitic disease of farmed ratites, Revue. Scientifgue-et- technique-office-international- des- Epizootic, 15: 73-89.

Ullrey, D.E. and M.E. Allen, 1996. Nutrition and feeding of Ostriches. American Nutrition Conference III. Anim. Feed Sci. Tec., 59: 27-36.

Corresponding Author: H. H. Musa, College of Animal Science and Technology, Yangzhou University, Yangzhou, 225009, China

Pakistan Journal of Nutrition 4 (3): 158-161, 2005
ISSN 1680-5194

A Seven Month Survey for the Detection of *E. coli* O157:H7 from Ground Beef Samples in the Markets of Turkey

Mehmet Elmali[1], Zeynep Ulukanli[2], Hilmi Yaman[1], Mehmet Tuzcu[3], Kenan Genctav[2] and Perihan Cavli[2]
[1]Department of Food Hygiene and Technology, Faculty of Veterinary,
University of Kafkas, 36100 Kars, Turkey
[2]Department of Biology, Faculty of Science and Arts, University of Kafkas, 36100 Kars, Turkey
[3]Department of Veterinary Division, High School of Vocational, University of Kafkas, 36100 Kars, Turkey

Abstract: A seven month period from the beginning October to the end of April 2004, a total of 126 ground beef samples were analyzed to determine the incidence of *Escherichia coli* (*E. coli*) O157:H7. Among the sampling months, the incidence of EHEC serotypes were only observed in April. Of the 126 ground beef samples, only one ground beef sample was positive for *E. coli* O157:H7, having a prevalence of 0.79 %. Five samples were found positive for *E. coli* O157 serotype, having a prevalence of 3.96 %. The results of this study reveal that the occurrence of EHEC serotypes in ground beef in the Kars (Eastern Turkey) seem to be seasonal and the level of *E. coli* O157 was significantly higher as compared to other studies in Turkey, but it is similar to studies performed in many countries around the world. Of the tested eighteen antibiotics, resistance towards three or more antibiotics were observed among the all isolates.

Key words: *E. coli* O157:H7, ground beef, antibiotic resistance

Introduction

The need for hygienic meat production has gained importance due to awareness among consumers about health risks associated with contaminated meat (Yashoda *et al*., 2000). The microbiological quality of either spoilage or food poisoning microorganisms depends on the meat used for mincing, sanitary conditions, practices in preparation time and temperature of storage (Duitschaever *et al*., 1973; Khalafalla *et al*., 1993). These factors may cause a major risk for subsequent foodborne infection in human (Huffman, 2002; Reid *et al*., 2002). The thread posed by EHEC diseases spread via contaminated and improperly cooked meat has been well recognized and the epidemiological impact of such diseases is considerable (Huffman, 2002; Reid *et al*., 2002). Shiga toxin-producing *Escherichia coli* (*E. coli*) (STEC) produce cytotoxins identical at the genetic and protein level to the shiga toxins produced by *Shigella dysenteria* I. Enterohemorrhagic *E. coli* (EHEC) are a subset of STEC. *E. coli* O157:H7 can be considered as a significant protype of the EHEC (Tutenel *et al*., 2002). *E. coli* O157:H7 has been well recognized as cause of human diseases including diarrhea, haemorrhagic colitis (HC) and the haemolytic uremic syndrome (HUS), or thrombotic thrombocytopenic purpura (TTP) (Nataro and Kaper, 1998). Healthy cattle have been regarded as a natural reservoir of VTEC organisms for infections (Hancock *et al*., 1994; Armstrong *et al*., 1996). The natural reservoirs of this pathogen are also many kinds of animals especially sheep, goats, wild animals. Consumption of contaminated and improperly cooked ground beef is often implicated in foodborne outbreaks of *E. coli* O157:H7, though a variety foods including unpasteurized milk, apple cider, fermented sausage, mayonnaise, and water, have also been linked as routes of transmission for human illness. The contamination of this potentially pathogenic strain may be secondarily transmitted from infected animals or person to person (Mead and Griffin, 1998).

With the exception of studies that describe the presence of EHEC in raw meat and raw meat based products Turkey, there is no information available in the literatures about the true incidence of this bacterium in ground beef. The objectives of this study were (I) to determine the incidence of this bacterium in ground beef through seven months and (II) to determine the resistance towards the antibiotics used in veterinary and human therapy (III) to compare the results with other studies performed in many countries in the world.

Materials and Methods

Samples: A total of 126 ground beef samples were collected eighteen times per month from the beginning October to the end of April, 2004. For each sampling, 100 g was collected from the markets and butchers, placed in a sterile bag, and stored in a cool box for transportation to the laboratory and analyzed within 1 h.

Analyses: To detect the presence of E. coli O157:H7 in ground beef samples, a 25 g of sample was pre-enriched with modified novobiocin EC broth (mEC+n, Merck 14582, Berlin, Germany) at 37°C for 24 h. A swap of the enrichment broth was then spread onto selective

CT-SMAC (Cefixime-Tellurite Supplement and Sorbitol MacConkey Agar, Oxoid CM 813 and SR 172 E, Basingstoke, UK) and incubated at 42°C for 24-48 h. End of the incubation, colourless, sorbitol negative (-), suspected colonies were streaked onto Fluorocult Violet Red Bile (VRB) (Merck 1.04030, GERMANY) and these plates were incubated at 42°C for 24-48 h. aerobically. Colonies grown on VRB were checked under UV light. The suspected colonies were Gram stained and IMVIC tests were performed. The colonies were then subjected to the agglutination test to determine the serotype of the bacteria using specific antisera to *E. coli* O157 (Oxoid, 200075, UK) and Dryspot *E. coli* O157 latex agglutination test (Oxoid, UK) for E. coli O157 carried out in parallel. Cultures identified as *E. coli* O157 were tested with antisera H7 (Oxoid, 211057, UK) as described by the manufacturer.

Antimicrobial testing: Antibiotic resistance patterns of *E. coli* O157 and *E. coli* H7:O157 were determined by the disk diffusion method using Mueller Hinton Agar (Bauer *et al.*, 1966). Zone interpretations were based on the recommendations of the National Committee on Clinical Laboratory Standards (NCCL). The antibiotic discs used were: ampicillin (10 µg), cefoperazone (30 µg), ceftriaxone (30 µg), cephazolin (30 µg), ceftazidime (30 µg), chloramphenicol (30 µg), ciprofloxacin (10 µg), gentamicin (10 µg), kanamycin (30 µg) nalidixic acid (30 µg), norfloxacin (10 µg), oxytetracycline (30 µg), ofloxacin (5 µg), enrofloxacin (5 µg), streptomycin (10 µg), tetracycline (30 µg), trimethoprim (5 µg), trimethoprim-sulfamethoxazole (25 µg). *E. coli* ATCC 25922 was used as control organism.

Results and Discussion

In the present investigation, we used mEC+n broth and CT-SMAC method. Of the 126 ground beef samples, only 1 (0.79%) was contaminated with *E. coli* O157:H7. Five (3.96%) of the 126 ground beef samples were positive for *E. coli* O157. A seven months of ground beef survey revealed that EHEC serotypes were not detected between October and March. It has been previously noted that the occurrence of *E. coli* O157:H7 in cattle feces is often seasonal, with the warmer and increased moist conditions of summer season contributing the highest incidence (Kudva *et al.*, 1997; Johnsen *et al.*, 2001). Fecal samples harboring highest incidence of the EHEC in the warmer season may directly contaminate the ground beef samples and concordantly increased the level of detection of the pathogens in the samples analyzed in this work. No recovery of the EHEC serotypes in cooler seasons in this study may be as consequences of low infectious dose or non homogenous distribution of the organism within sample or may be the background microflora in ground beef predominate the growth of *E. coli* O157:H7. In a study of Heuvelink *et al.* (1997), they evaluated the efficacy of selective enrichment and plating media for the isolation of O157 serogroup from minced meat and concluded that mEC+n and CT-SMAC were the most efficient media for selective enrichment and isolation, respectively. It appears from the present data, detecting level of bacteria in ground beef samples collected in the period of April seems to be augmented in warmer season rather than cooler season, using the novobiocin (mEC+n) and CT-SMAC. Hence, it can be said that more sensitive techniques such as immunomagnetic separation or else are required with the pre- enrichment procedure in cooler season.

Previously existing studies regarding the prevalence of *E. coli* O157:H7 in beef and ground beef samples were noted as a 3.7% of the 164 beef, 29.4% of the 17 beef samples in USA (Pai *et al.*, 1984; Doyle and Schoeni, 1987) and 6 % of the 50 ground beef samples in Egypt (Abdul-Raouf *et al.*, 1996). Although high prevalence was reported in these earlier studies, no recovery or false-positive *E. coli* O157:H7 serotypes resulted from the two recent studies of Silveira *et al.* (1999) and Uhitil *et al.* (2001). Silveira *et al.* (1999) investigated 886 hamburger samples from the southeast of Brazil. Uhitil *et al.* (2001) analyzed 114 beef and baby beef samples from 30 different grocery stores for the presence of *E. coli* O157:H7 in Croatia. The number of the samples examined in those studies was not low for detecting a microorganism but it is presumably due the differences and difficulties arising from the methodology.

The prevalence found in this investigation shows similarity to those reported by Vernozy-Rozand *et al.* (2002), who found the prevalence of *E. coli* O157:H7 in minced beef was 0.12% (four of 3450), but dissimilar to the more recent study of Chinen *et al.* (2001), who detected *E. coli* O157:H7 from the 6 (3.75%) of 160 ground beef samples between February and May in Argentina. In contrast, the prevalence of O157 was approximately equal to the number of the *E. coli* O157:H7 serotypes found in Chinen *et al.* (2001). Our result showed a higher prevalence when compared to the study of Heuvelink *et al.*, 1997, who detected positive *E. coli* O157 from the 1 % of the 571 minced beef in Netherlands.

Reports conducted in Turkey related to the incidence of *E. coli* O157 in raw meat and raw meat based products was found at 2% and 2.58% in hamburgers (Sarimehmetoglu *et al.*, 1998; Cebiroglu and Nazli, 1999; Noveir *et al.*, 2000), 5% in meatballs (Sarimehmetoglu *et al.*, 1998), 0.4% in minced meat, 0.99% in sodjouk (Noveir *et al.*, 2000). In a study of Gun *et al.* (2003), the presence of *E. coli* O157:H7 was investigated in the bovine carcasses and abattoir environment in Istanbul between January 2000 and April 2001. Eight (2.4%) of the 330 cattle carcasses and six positive isolates from the environments in abattoir were

shown to be positive for this serotype. Nonetheless, the incidence of this pathogen in the sampling months is not clearly mentioned. Therefore, results of the present study may be more representative of ground meat in the Kars, Eastern part of the Turkey than previous studies conducted in other parts of the country. If so, risk exposure to EHEC O157 from ground beef is higher than previous reports by Noveir *et al.* (2000). Unfortunately, the infection cases of *E. coli* O157:H7 have not been officially known in Turkey. Therefore further work is essentially needed to determine the genes encoding the virulence factors to prevent risk exposures to livings.

Food consumption is an important pathway for bacteria to enter humans, the presence of antimicrobial-resistant bacteria in foods warrants particular attention (Schroeder *et al.*, 2004). Many human *E. coli* O157:H7 infections are acquired from eating undercooked contaminated beef. Therefore, it is crucial to determine if resistant *E. coli* O157:H7 is a possible reservoir for spread of resistance factors to other microorganisms (Galland *et al.*, 2001). In the present work, both *E. coli* O157 and *E. coli* O157:H7 serotypes were resistant to three or more antibiotics. Chloramphenicol, streptomycin and trimethoprim type resistance was common among all isolates as shown in Table 1.

Table 1: Antibiotic resistance of the EHEC isolates

Isolates	Resistance pattern*
O157	C, S, W
O157	C, S, TE, W
O157	C, S, W
O157	C, S, OT, TE,W
O157	C, S, W
O157:H7	C, S, OT, TE,W

*Abbreviated symbols in the Table were C (Chloramphenicol, 30µg); S (Streptomycin, 10µg), OT (Oxytetracycline, 30µg); TE (Tetracycline, 30µg); W (Trimethoprim, 5µg).

Resistance towards at least one or more antibiotics such as ampicillin, chloramphenicol, and tetracycline has been reported for O157 and H7 serotypes by previous workers from several materials (Meng *et al.*, 1998; Radu *et al.*, 2001). In the present investigation, data concluded from antibiotic resistance pattern is in agreement as compared to the previous studies. This is possibly as a consequence of extensive usage of these antibiotics in the treatments of the cattle or in other sources e.g. a contact with feces of the animals, feeding, water sources or agriculture, which may be as a cause of the transmission of resistant genes from various vectors to food production animal. General conclusion can not be derived due to the low number of microorganism used in this preliminary test. However, three to five antibiotic resistance among the isolates reveal may be an alert for the consumption of improperly cooked meat. Molecular studies are needed to determine the genes encoding the resistance.

Transmission vehicles are not examined in this preliminary work, it is rather to determine the true incidence of this pathogen in ground beef during the three seasons (fall, winter and spring). This harmony of seasonal rates of *E. coli* O157:H7 in cattle, retail meats and human disease is evidence not only of causation but also of the potential for farm level interventions to be transmitted to the consumer in the farm of lower risk (Hancock *et al.*, 2001). Epidemiological and comprehensive studies are required to find out the incidence of this pathogen throughout the all year and major possible contamination sources including abattoir conditions, transportation of carcass, butcher's hygiene, utensils and tools used in butcher's shop and storage of ground beef should be checked routinely.

References

Abdul-Raouf, U.M., M.S. Ammar and L.R. Beuchat, 1996. Isolation of *Escherichia coli* O157:H7 from some Egyptian foods. Int. J. Food Microbiol., 29: 423-426.

Armstrong, G.L., J. Hollingsworth and J.R. Morris Jr., 1996. Emerging foodborne pathogens: *Escherichia coli* O157:H7 as a model of entry of a new pathogen into the food supply of the developed world. Epidemiol. Rev., 18: 29-51.

Bauer, A.W., W.M.M. Kirby, J.C. Sherris and M. Turck, 1966. Antibiotic susceptibility testing by a standardized single disc method. American J. Clin. Pathol., 45: 493-496.

Cebiroglu, H. and B. Nazli, 1999. Dondurulmus hamburger kofte ve diger kofte cesitlerinde enterohemarojik *Escherichia coli* O157:H7 susunun varligi uzerine arastirmalar. J. Fac. Vet. Uni. Istanbul, 25: 107-121.

Chinen, I., J.D. Tanaro, E. Miliwebsky, L. Haydee Lound, G. Chillemi, S. Ledri, A. Baschkier, M. Scarpin, E. Manfredi and M. Rivas, 2001. Isolation and characterization of *Escherichia coli* O157:H7 from retail meats in Argentina. J. Food Prot., 64: 1346-1351.

Doyle, M.P. and J.L. Schoeni, 1987. Isolation of *Escherichia coli* O157:H7 from retail fresh meats and poultry. Appl. Environ. Microbiol., 53: 2394-2396.

Duitschaever, C.L., D.R. Arnott and D.H. Bullock, 1973. Bacteriological quality of raw refrigerated ground beef. J. Milk and Food Tec., 36: 375-377.

Galland, J.C., D.R. Hyatt, S.S. Crupper and D.W. Acheson, 2001. Prevalence, antibiotic susceptibility, and diversity of *E. coli* O157:H7 isolates from a longitudinal study of beef cattle feedlots. Appl. Environ. Microbiol., 67: 1619-1627.

Gun, H., A. Yilmaz, S. Turker, A. Tanlasi and H. Yilmaz, 2003. Contamination of bovine carcasses and abattoir environment by *Escherichia coli* O157:H7 in Istanbul. Int. J. Food Microbiol., 84: 339-344.

Hancock, D.D., T.E. Besser, M.L. Kinsel, D.H. Tarr, D.H. Rice and M. Paros, 1994. The prevalence of *Escherichia coli* O157:H7 in dairy and beef cattle in Washington State. J. Epidemiol. Infect., 113: 199-207.

Hancock, D.D., T. Besser, J. Lejeune, M. Davis and D. Rice, 2001. The control of VTEC in the animal reservoir. Int. J. Food Microbiol., 66: 71-78.

Heuvelink, A.E., T.M. Zwartkruis-Nahuis and E.D. Boer, 1997. Evaluation of media and test kits for the detection and isolation of *Escherichia coli* O157 from minced beef. J. Food Prot., 60: 817-824.

Huffman, R.D., 2002. Current and future technologies for the decontamination of carcasses and fresh meat. Meat Sci., 62: 285-294.

Johnsen, G., Y. Wastegon, E. Heir, O.I. Berget and H. Herikstad, 2001. *Escherichia coli* O157:H7 in feces from cattle, sheep and pigs in the southwest part of Norway during 1998 and 1999. Int. J. Food Microbiol., 65: 193-200.

Khalafalla, F., A.F. Gergis and A. El-Sherif, 1993. Effect of freezing and mincing technique on micobial load of minced meat. Die Nahrung, 5: 422-427.

Kudva, I.T., P.G. Hatfield and C.J. Hovde, 1997. Characterization of *Escherichia coli* O157:H7 and other shiga toxin-producing of *E. coli* serotypes isolated from sheep. 1997. J. Clin. Microbiol., 35: 892-899.

Mead, P.S. and P. Griffin, 1998. *Escherichia coli* O157:H7. Lancet, 352: 1207-1212.

Meng, J., S. Zhao, M.P. Doyle and S.W. Joseph, 1998. Antibiotic resistance of *Escherichia coli* O157:H7 and O157:NM isolated from animals, food, and humans. J. Food Prot., 61: 1511-1514.

Nataro, J.P. and J.B. Kaper, 1998. Diarrheagenic *Escherichia coli*. Clin. Microbiol. Rev., 11: 142-201.

Noveir, M.R., H.B. Dogan and A.K. Halkman, 2000. A note on *Escherichia coli* O157:H7 serotype in Turkish meat products. Meat Sci., 56: 331-335.

Pai, C.H., R. Gordon, H.V. Sims and L.E. Bryan, 1984. Sporadic cases of haemorrhagic colitis associated with *Escherichia coli* O157:H7. Ann. Int. Med., 101: 738-742.

Reid, C.A., A. Small, S. Avery and S. Buncic, 2002. Presence of food borne pathogen in cattle hides. Food Control., 13: 411-415.

Radu, S., O.W. Ling G. Rusul, M.I. Karim and M. Nishibuchi, 2001. Detection of *E. coli* O157:H7 by multiplex PCR and their characterization by plasmid profiling, antimicrobial resistance, RAPD and PFGE analyses. J. Microbiol. Methods, 46: 131-139.

Sarimehmetoglu, B., O. Kuplulu and S. Kaymaz, 1998. Hamburger ve inegol koftelerinden *Escherichia coli* O157:H7 izolasyonu. Ankara Uni. Vet. Fak. Derg., 45: 221-227.

Schroeder, C.M., D.G. White, B. Ge, Y. Zhang, P.F. Mcdermott, S. Ayers, S. Zhao and J. Meng, 2004. Isolation and antimicrobial-resistant *Escherichia coli* from retail meats purchased in Greater Washington, DC, USA. Int. J. Food Microbiol., 85: 197-202.

Silveira, N.F.A., N. Silva, Contreras, L. Miyagusku, M. Lourdes, De., F. Baccin, E. Koono and N.J. Beraquet, 1999. Occurrence of *Escherichia coli* O157:H7 in hamburgers produced in Brazil. J. Food Prot., 62: 1333-1335.

Tutenel, A.V., D. Pierard, J. Uradzinski, E. Jozwik, M. Pastuszczak, J. Van Hende, M. Uyttendaele, J. Debevere, T. Cheasty, J. Van Hoof and D.L. Zutter, 2002. Isolation and characterization of enterohaemorrhagic *Escherichia coli* O157:H7 from cattle in Belgium and Poland. Epidemiol. Infect., 129: 41-47.

Uhitil, S., S. Jaksic, T. Petrak and K. Botka-Petrak, 2001. Presence of *Escherichia coli* O157:H7 in ground beef and ground baby beef meat. J. Food Prot., 64: 862-864.

Vernozy-Rozand, C., S. Ray-Gueniot, C. Ragot, C. Bavai, C. Mazuy, M.P. Montet, J. Bouvet and Y. Richard, 2002. Prevalence of *Escherichia coli* O157:H7 in industrial minced beef. Lett. Appl. Microbiol., 35: 7-11.

Yashoda, K.P., N.M. Sachinra, P.Z. Sakhare and R.D. Narasimha, 2000. Microbiological quality of hygienically processed buffalo carcasses. Food Control, 11: 217-224.

Pakistan Journal of Nutrition 4 (3): 162-169, 2005
ISSN 1680-5194

The Risk of Mycotoxins Contamination of Dairy Feed and Milk on Smallholder Dairy Farms in Kenya

T.P. Lanyasunya[1], L.W. Wamae[3], H.H. Musa[4], O. Olowofeso[2] and I.K. Lokwaleput[1]
[1]KARI-NAHRC, P.O. Box 25, Naivasha, Kenya
[2]Department of Agricultural Science, Adeyemi College of Education, Ondo, Nigeria
[3]KARI, Headquarter Nairobi, Kenya
[4]Department of Animal Production and Veterinary Science, University of Nyala, Sudan

Abstract: Mycotoxins are toxic secondary metabolites produced by fungi that thrive in warm humid environments. Because Kenyan climate is favourable for growth of mycotoxins causing moulds, the threat of mycotoxin related livestock and human poisoning is real and of major concern. This threat is made even more palpable by the fact that, staple diets in many Kenyan households are based on crops such as maize, which are highly susceptible to mycotoxins contamination. The objective of the current study was to highlight the existing but grossly ignored danger of mycotoxin contamination of dairy feeds possibly leading to animal and human poisoning. During the study, qualitative and quantitave information were obtained through extensive review of scientific articles, magazines and books touching on this subject. Consultations were also held with resource persons (Toxicologists) to help validate some of the assertions made by various authors. A very clear illustrated facts, as revealed by the current study are that, aflatoxin is one of the most widely occurring and dangerous of all mycotoxins known. The term aflatoxin refers to a closely related group of metabolites produced by toxigenic strains of *Aspergillus flavus* and *A. parasiticus*. Aflatoxins are potent carcinogenic, mutagenic, teratogenic, and immunosuppressive agents. Four different aflatoxins, B1, B2, G1 and G2, have been identified with B1 being the most toxic. Their contamination of agricultural feed grains poses a serious threat worldwide. Although occurrence and magnitude of mycotoxin contamination varies with geographical and seasonal factors and also with the conditions under which a food or feed crop is grown, harvested, and stored, those grown under tropical and subtropical conditions are more prone to contamination than those in temperate regions due to favourable humidity and temperature levels for mould growth (10 - 40°C, pH range of 4 - 8 and above 70% equilibrium relative humidity). Aflatoxin B1 is potent when it contaminates food grains. This potency was illustrated by an outbreak of aflatoxin poisoning in Kenya (January - July 2004). This outbreak resulted in 125 recognized deaths and hospitalization of over 300 others across various districts (Makueni: N = 148; Kitui: 101; Machakos: 19; Thika: 12 and Kenyatta National Hospital: 37). Of 342 samples tested, a total of 182 (53.2%) had >20 ppb of aflatoxin. In addition, a substantial percentage of samples from each district had aflatoxin levels >1,000 ppb: Makueni (12.1%), Kitui (9.6%), Thika (3.9%), and Machakos (2.9%). Livestock get poisoned when they consume contaminated feeds. Virtually all feeds are susceptible so long as conditions permit mould colonization. Mouldy protein supplements, poultry manure, cereal grains and their by-products are the primary sources of mycotoxins found in homemade dairy concentrates on smallholder farms. Aflatoxin M1 is metabolic breakdown product of aflatoxin B1 and can appear in the milk of lactating cows consuming significant quantities of aflatoxin B1 emanating from mouldy feedstuffs. When the level of M1 appearing in milk and other dairy products is more than 20 ppb (concentration accepted by Kenya authorities), then it becomes a food safety hazard. Control of mycotoxins in dairy diets on smallholder farms would reduce the likelihood of livestock poisoning and concentration of mycotoxin residues in milk and other animal products destined for human consumption.

Key words: Aflatoxins poisoning, ruminant livestock, mould colonization

Introduction

In most developing countries, livestock production is an important part of the national economy and more importantly, of the subsistence and semi-commercial smallholder farming systems, dominated by resource-poor farm households. In Kenya, dairy industry is increasingly becoming a smallholder farmers' domain. At preset, they own over 80% of the 3 million heads of dairy cattle, producing about 56% of the total milk production and contributing 80% of the marketed milk (Peeler and Omore, 1997; Staal *et al*., 1999; Conelly, 1998; Thorpe *et al*., 2000). This therefore means, focusing dairy development efforts to this category of farmers will not only provide a good potential to improve national milk self-sufficiency and family incomes, but also a greater potential in improving public health

through improvement of milk quality and hygiene. Currently, a major obstacle to increased milk production on smallholder farms is the chronic shortage of affordable feeds of adequate quality and quantity, particularly during the dry season. A closer look a the use of available feed resources on these farms, indicates that, the problem of feed shortage is further complicated by farmers inability to use them before they spoil, especially during the wet season when there is a production peak. During the peak production, one of the major agents of feed spoilage, are moulds. Though their prevalence and rate of colonization are highly dependent on the climate and farm practices, the growth of moulds in raw materials and finished feeds for livestock is a universal problem. Mould spores, of the so-called field fungi, can contaminate cereal grains and stover in the standing crop and in severe conditions (too wet or too dry season). Their spores can germinate, especially under high moisture conditions. During storage a different range of moulds (the storage fungi) become more important. These moulds can grow at moisture contents above 11%. Both the field and storage fungi can lead to a build up of mycotoxins. Mycotoxins are metabolites produced by these fungi (Moulds), which serve as a protective mechanism (Merck Veterinary Manual, 1986). Mycotoxins are among the most common contaminants in animal feeds, causing great economic loss in both the livestock industry and aquaculture (Sharlin *et al.*, 1981; Hafez *et al.*, 1982; Jantrarotai and Lovell, 1990). Problems associated with mycotoxins tend to be worse in the tropics where high humidity and temperature create optimal conditions for fungal growth. Mycotoxins include metabolic by-products produced by a number of different fungi that may or may not be toxic. Aflatoxin is one of the most widely occurring and dangerous mycotoxins (C.A.S.T., 1989). Aflatoxin, is produced by the fungi *Aspergillus flavus* and *Aspergillus parasiticus*. Four different aflatoxins, B1, B2, G1 and G2, have been identified with B1 being the most toxic, carcinogenic, hepatotoxic and potentially mutagenic, while also being the most prevalent (Stoloff, 1980). Mycotoxins are not only toxic to animals but also to exposed humans. Usually, exposure is through consumption of contaminated foods (Nelson *et al.*, 1993). This paper examines some of the potential factors and pathways that could expose both livestock, particularly dairy cattle and human beings to mycotoxins poisoning. Special reference is hereby made to the smallholder resource-poor farmers in Kenya where lack of awareness and limited feeds are believed to aggravate chronic mycotoxins (i.e aflatoxin) poisoning.

Materials and Methods

The current study was conducted over 2-month period (May - June, 2005) in Kenya. During the study, relevant scientific publications touching on mycotoxicosis and aflatoxicosis in both livestock and humans formed the primary source of qualitative and quantitative information discussed herein. Secondary sources included books, newsletter, newspapers and magazines. Consultations were also held with technical persons specialized in toxicology and institutions that have registered cases of aflatoxin poisoning (hospitals). The purpose of consultations was to help validate some of the obtained baseline data and assertions made by various authors of documents reviewed. The objective of the study was to highlight the present risks of feed and therefore milk contamination on smallholder dairy farms in Kenya. By highlighting this highly ignored subject, the authors of this paper hope to create awareness on the need to take appropriate health precautionary measures during feed handling and feeding of dairy animals on these farms so as to minimize chances of feed and food contamination. Thus, minimizing the risk of mycotoxin related poisoning on both livestock and humans. Relevant data and graphic presentations from different sources were adapted to illustrate various aspects of the discussion and to provide the basis for drawing conclusive inferences and recommendations.

Results and Discussion

Mycotoxin causing moulds: Mycotoxins are secondary metabolites of fungal origin that are toxic. The term Mycotoxin literally means poison from a fungi. Among the thousands of species of fungi, only about 100 are known to produce mycotoxins. There are three major genera of fungi that produce mycotoxins: *Aspergilius*, *Fusarium* and *Penicilium*. And, although between 300 and 400 mycotoxins are known, those mycotoxins of most concern, based on their toxicity and occurrence, are aflatoxin, deoxynivalenol (DON or vomitoxin), zearalenone, fumonisin, T-2 toxin, and T-2-like toxins (trichothecenes). Deoxynivalenol (DON), zearalenone, T-2 toxin and fumonisin are all produced by mould of the genus *Fusarium*. Moulds in this genus are found in virtually every cereal crop, especially maize (in the field or in store) and collectively are capable of producing 70 different mycotoxins. Some strains of *Fusarium* may produce as many as 17 mycotoxins simultaneously. Thus *Fusarium* mycotoxins are the most frequently identified group of mycotoxins in grains and feeds. Aflatoxins are one of the most potent toxic substances that occur naturally. These are a group of closely related mycotoxins produced by fungi *Aspergillus flavus, A. parasiticus* and *A. nomis*. The genus *Aspergillus* includes over 185 species. Around 20 species have so far been reported as causative agents of opportunistic infections in man. Among these, *Aspergillus fumigatus* is the most commonly isolated species, followed by *Aspergillus flavus* and *Aspergillus niger*. *Aspergillus clavatus*, *A. nomis, Aspergillus parasiticus*, *Aspergillus glaucus* group, *Aspergillus nidulans, Aspergillus oryzae, Aspergillus terreus, Aspergillus ustus*, and *Aspergillus versicolor* are among the other species less commonly

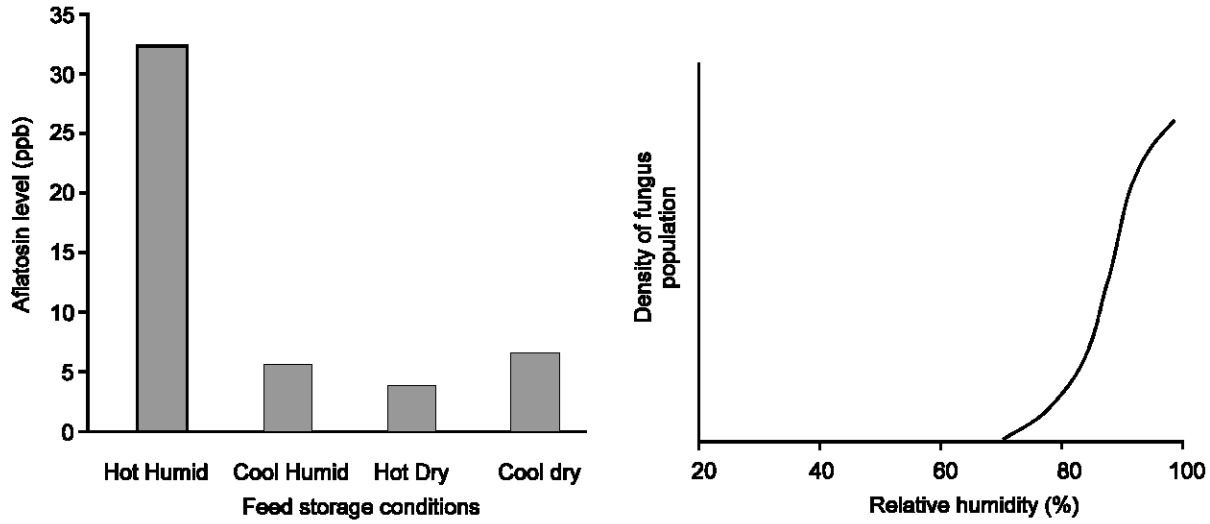
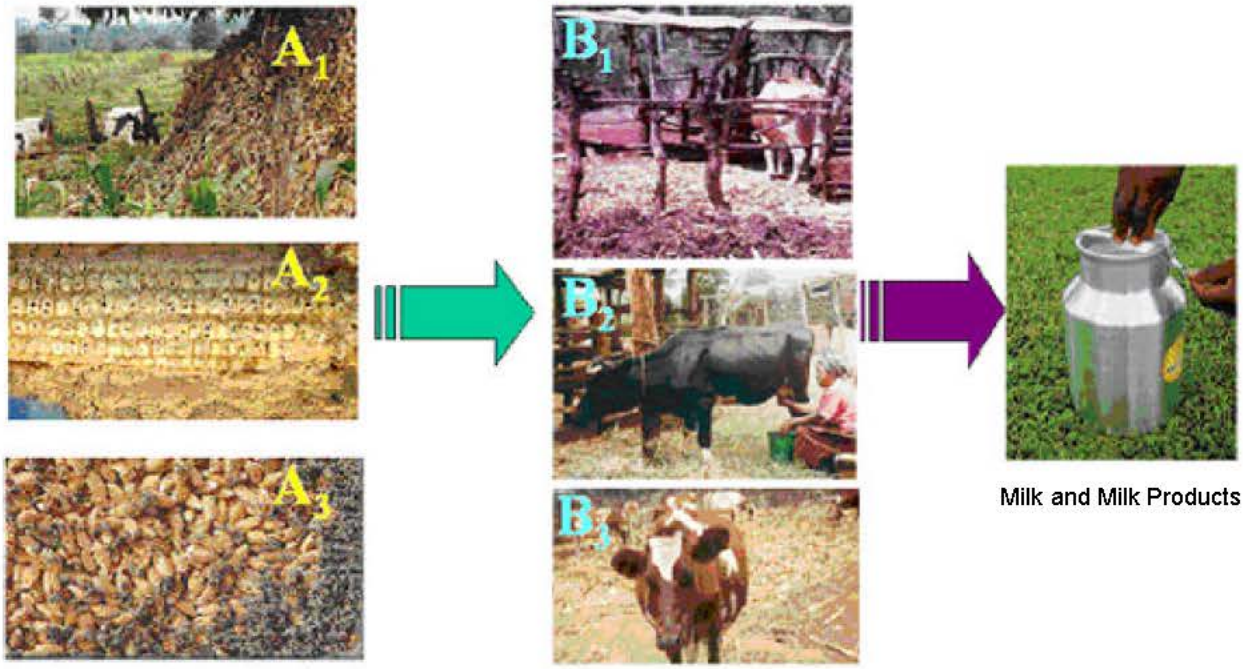


Fig. 1: Influence of temperature and relative humidity on fungal colonization and aflatoxin production in animal feeds (Thomson and Henke, 2000)

isolated as opportunistic pathogens. *Aspergillus* is a group of moulds, which is found worldwide. These fungi are ubiquitous and their potential for contamination of foodstuffs and animal feeds is widespread. Under favourable environmental conditions, some of these toxigenic moulds can produce mycotoxins on a wide range of agricultural commodities. The occurrence and magnitude of any mycotoxin contamination varies with geographical and seasonal factors, and also with the conditions under which a food or feed crop is grown, harvested, and stored. Crops in tropical and subtropical areas are more susceptible to contamination than those in temperate regions, since the high humidity and temperature (Fig. 1) in these areas provide optimal conditions for toxin formation.

Feed and food contamination: Many different mycotoxins have been found to occur on forages and grains either in the field or in storage (Lacey, 1991). Main factors contributing to mould colonization and therefore mycotoxins production include: the feedstuff (quality), moisture level of feed, temperature, relative humidity, pH, plant stresses such as drought or insect infestation, and damaged or broken grain kernels (Jacques, 1988; Ashworth *et al.*, 1969). Weather condition is the primary factor influencing mould colonization (Coulumbe, 1993) (Fig. 1). Moulds grow over a temperature range of 10 - 40°C, pH range of 4-8 and above 70% equilibrium relative humidity. Unlike yeast, which requires free water, moulds are also capable of growing on a dry surface (Lacey, 1991) and on feeds containing 12-13% moisture (Christensen *et al.*, 1977). In wet feeds such as silage, higher moisture levels allow rapid mould growth if oxygen is available. Because most moulds are aerobic, high moisture concentrations that exclude adequate oxygen can prevent mould growth. *Aspergillus flavus* colonization and aflatoxin production in maize and oil seeds are encouraged by high humidity (86-87%) and heat (Davis and Deiner, 1983; Smith, 1997). Drought stress has also been found to increase the number of *Aspergillus* spores in the air (Sorenson *et al.*, 1984). Thus, when drought stress occurs during pollination, the increased population of *Aspergillus* spores in the air greatly increases the chance of infection. Also, nitrogen stress (low soil fertility) and other stresses that affect plant growth during pollination can increase the level of aflatoxin produced by the *Aspergillus* fungi. Mature maize that remains in the field (as dry heaps - a common practice in Kenya) or maize that is stored without properly drying can be susceptible to *Aspergillus* growth and aflatoxin production. Though poorly stored feeds and grains can and indeed does become contaminated with aflatoxin (Lillehoj and Fennell, 1975; Shotwell *et al.*, 1975), past studies have clearly demonstrated that most aflatoxins contamination originates from the field (Smith, 1997). Time of harvest has also been shown to have an influence on aflatoxin levels because *Aspergillus* does not compete well with other molds when maize is above the 20 percent moisture. Thus, harvesting maize with a moisture content of above 20 percent and then drying it down to at least a moisture content of 15 percent, within 24 to 48 hours of harvest, will keep *Aspergillus* growth and toxin production at a minimum. This requirement is hard to achieve on smallholder resource-poor farms in Kenya. Insect (i.e Weevil) and physical damage (running trucks on maize grain as often seen in maize growing belts such as Kitale) can greatly increase *Aspergillus* infection and the levels of aflatoxin.

Protein rich supplements (cotton seed cake, sun flower



A1 - Poorly conserved maize stover (susceptible to moulding); A2 - Heavily pest infested un-shelled maize cob; A3 - Mould colonized wheat grain; B1 - Dump (dairy cow) feeding environment; B2 - Smallholder farmer milking and grass hay on dirty ground; B3 - Dairy cows scavenging on maize stover on dirty yard

Fig. 2: Potential sources of aflatoxin poisoning of dairy cows and pathway for contamination of milk on smallholder farms in Kenya

cake, fish meals and other oil seed by-products), cereal grains and their by-products (maize bran, maize germ, wheat bran and other grain milling by-products) which are often poorly stored, are the primary source of the moulds found in homemade dairy concentrates on smallholder farms. Majority of smallholder farmers in Kenya are not keen on controlling moisture through appropriate storage, which is the single most important factor in determining if and how rapidly moulds will grow in feeds. This is further compounded by the farmers' wide spread practice of using spoilt (physically, pest or mould damaged) grains and other feed resources (mouldy crop residues) to formulate dairy rations (Fig. 2). Today, crop residues constitute the major part of ruminant livestock rations on smallholder farms. However, their efficient utilization is highly militated against by rapid mould colonization, exacerbated by poor handling and storage. Majority of farmers do not protect maize stover against scorching sunshine, termite and other pest damage and more importantly mould colonization. This leads to both chronic aflatoxin poisoning of dairy animals and heavy wastage of this very important feed resource. To control mould growth, obvious sources of moisture in the feed handling and storage must therefore be eliminated. In sound feed management systems the first important step in controlling mould and therefore feed contamination, is to reduce moisture level in hay, cereal and protein supplements used for ration formulation. It is important to note that, although drying of feed has been shown to reduce mould counts many mould spores remain in the feed or feed material after it has been dried. After drying, the remaining spores can grow if conditions are right. In Kenya majority of smallholder dairy herds are kept in either total (zero-grazing) or semi-confinement (semi-zero-grazing) in highly moist environments. Their respiration, defecation, water spillages and rainfall make the environments where these animals are kept highly laden with moisture. This therefore means that, feed that was initially very low in moisture content will rapidly gain moisture when placed into this kind of environment, hence spurring the growth of mould and production of mycotoxin. Under such environment provision of feed troughs will greatly reduce growth of mould and production of mycotoxin on the feed being offered to the animals.

Livestock poisoning: Mycotoxin related poisoning (i.e. Aflatoxicosis) is reported from all parts of world in almost all domestic animals (poultry, cattle, horses, rabbits, and dogs). Aflatoxicosis is poisoning that result from ingestion of aflatoxins in contaminated feed. The

aflatoxin group consists of B1, B2, G1, and G2 (Cotty *et al.*, 1994; Smela and Curier, 2001). Aflatoxin B1 is the most potent of the group and has been shown to be a potent carcinogen (Allcroft and Carnagham, 1963). Like other mycotoxins, aflatoxins occur in a variety of feedstuffs, which are routinely used by farmers to feed dairy animals. In agreement with this assertion, past research studies have confirmed that, most cases of animal poisoning by aflatoxins can be traced to the growth of fungi in poorly handled feeds (Jacques, 1988; Smith, 1997). When ingested through contaminated feed mycotoxins produce a wide range of harmful effects in animals. Though it depends on the affected animal species, the general effects of aflatoxicosis include decreased feed utilization and efficiency (reduced appetite) leading to low weight gain, liver and kidney damage, gastrointestinal dysfunction (hemorrhage, and necrosis throughout the digestive tract), embryonic and early death of the newborn (interferes with conception, ovulation, implantation, fetal development, and the viability of newborn animals), teratogenicity (birth defects), tumors and suppressed immune system function (even when low levels are consumed), reduced productivity (reduced milk production and reproductive efficiency), anaemia, jaundice, carcinogenesis, and death, (Pier, 1992). Depending on interaction with other factors, aflatoxin concentration as low as 100 ppb may be toxic to cattle (Garrett *et al.*, 1968). However, the toxic level is generally considered to be between 300 and 700 ppb. Garrett *et al.* (1968) reported a significant negative effect on weight gain and dry matter intake of cattle offered diets containing 700 ppb aflatoxins. A significant increase in liver weight was also observed during the same study and this was used as an indicator for toxicity. Guthrie (1979) reported a decline in reproductive efficiency when lactation dairy cattle fed on diets containing 120 ppb aflatoxin. When the same cows were offered aflatoxin-free diets, milk production increased by more than 25%. The general indication, as pointed out by many past research reports is that, milk production loss appears to occur when diets contain more than 300 ppb (DON). However, Patterson and Anderson (1982) and Masri *et al.* (1969) suggested that depending on many other factors, aflatoxin concentration of as low as 100 ppb may be detrimental to the dairy stock. Young animals in all species are more sensitive to the effects of aflatoxin than mature animals. Calves may be affected by exposure to aflatoxin metabolites secreted in the milk. Pregnant and growing animals are less susceptible than young animals, but are more sensitive to aflatoxin than mature non-pregnant animals. Even when fed at non-lethal levels, aflatoxin will reduce productive efficiency and may increase the disease susceptibility of the animals. Because of partial degradation in rumen, mycotoxins are less toxic to cattle than most other farm animals. However, mycotoxins are not completely degraded and some of the degradation products remain toxic (Kiessling *et al.*, 1984). The extent of ruminal degradation appears to be variable. It is speculated that feeds and/or feeding situations resulting in a faster rate of ruminal feed passage or a low population of protozoa in the rumen may reduce mycotoxin degradation in the rumen (Kiessling *et al.*, 1984). Ruminal degradation of mycotoxins appears to be more dependent on protozoan than bacterial activity (Kiessling *et al.*, 1984). Acute toxicity is caused when large doses of aflatoxins are ingested through heavily contaminated feed. Though it has been reported that mouldy feed is unpalatable, totally confined dairy animals on smallholder farms in Kenya have little chance of selecting feed. Farmers offer what is available and the question of whether the feed is mouldy or not, is less considered. This is particularly critical during dry season when feeds are scarce. Rather than acute type aflatoxin toxicity, chronic type posses more serious challenge on these farms. Chronic toxicity occurs through long-term exposure of dairy (and other farm) animals to low - moderate aflatoxin concentrations which impacts negatively on herd productivity and posses serious risks to humans that depend on it for milk (Robens and Richard, 1992; Reeds and Kasali, 1987). The economic impact of chronically reduced animal productivity, increased incidences of morbidity due to immunosuppression, damage to vital organs, interferences with reproductive capacity and the ever present source of milk contaminant, is many times greater than the economic impact caused by an animal death due to acute poisoning. For this reason therefore, preventive detection in the feed is essential.

Human poisoning: Outbreaks of mycotoxin related human poisoning is a worldwide phenomenon. Though the problem of mycotoxin contamination of animal products destined for humans is not as frequent as observed with grains, the risk is still real and of major concern. While remaining hopeful that many of our healthy ruminant livestock are capable of "filtering out" or detoxifying many of the mycotoxins to which they are exposed during feeding, the issue of mycotoxin residues in milk, milk products and meat should not be ignored. Of the many reported mycotoxins, aflatoxins are the most potent toxic fungal substabstance that occurs naturally. These toxins have continued to wreck havoc across the tropical world, especially where cereal grains form the basal human diet. Human exposure to levels of aflatoxins from nanograms to micrograms per day occurs through consumption of maize, peanuts and other contaminated agricultural foodstuffs. Maize is the staple food in Kenya. It is milled into flour to make delicacies referred to locally as “Ugali” and “Uji” (a porridge). During periods of food scarcity the government of Kenya imports maize grains to bridge the shortfall. It is the contamination of these foodstuffs that

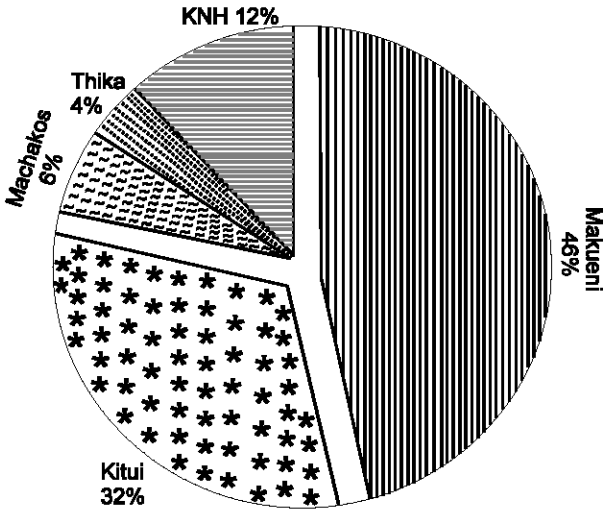


Fig. 3: Distribution of aflatoxcosis cases in five recording site and according to date of reporting in Eastern and Central provinces in Kenya (January - July, KMOH, 2004)

has been incriminated for both acute (Fig. 3) and chronic aflatoxin poisoning in Kenya. The chronic incidence of aflatoxin in diets is evident from the presence of aflatoxin M1 in human breast milk in Ghana, Kenya, Nigeria, Sierra Leone, Sudan, Thailand, and the United Arab Emirates, and in umbilical cord blood samples in Ghana, Kenya, Nigeria, and Sierra Leone (Bhat and Vasanthi, 2003; Maxwell *et al.*, 1998). Frequent consumption of low levels of aflatoxin has also been associated with chronic diseases like cancer. If animals consume contaminated feeds (sometimes containing high levels of aflatoxin up to 8,000 ppb) and then these toxins find their way from feed into milk or meat, they become a food safety hazard in these products too (Bhat and Vasanthi, 2003). This often happens, especially where animals exposed to aflatoxin contaminated feeds such as observed on many smallholder resource-poor farms in Kenya (Fig. 2). One structural type of aflatoxins - aflatoxin B1 is considered the most toxic and the type commonly found type in Kenya.

Aflatoxin M1 is metabolic breakdown product of aflatoxin B1 and can appear in the milk of lactating cows consuming significant quantities of aflatoxin B1 (Fig. 2). Aflatoxin M1 is not as carcinogenic as B1 but can be as toxic. The conversion of aflatoxin B1 in feed to aflatoxin M1 in milk is about 1% to 2%. Aflatoxin M1 residues in milk is reportedly approximately equal to 1 to 2 percent (1.7 percent average) of the dietary level. This ratio is not influenced greatly by milk production level since higher producing cows consume more feed and have a slightly higher transmission rate. Due to risks of milk residues, dietary aflatoxin should be kept below 25 ppb.

In May 2004, the Kenya Ministry of Health (KMOH, 2004) and key stakeholders undertook an investigation to establish the cause of an outbreak of jaundice with a high case-fatality rate (CFR) in the districts of Makueni and Kitui, Eastern Province. Laboratory testing of food collected from the affected area revealed high levels of aflatoxin, suggesting that, the outbreak was due to aflatoxin poisoning. Evidence that this outbreak resulted from aflatoxin poisoning included 1) high levels of aflatoxin (up to 8,000 ppb) in maize samples collected from patient households, 2) a clinical illness consistent with acute aflatoxin poisoning, 3) clustering of cases among residents of the same household, and 4) reports of deaths among animals known to have eaten the same maize as the patients during the same period. Food samples collected from household visits during May 10-19 included maize flour, maize grains, dry maize cobs, muthokoi (i.e., maize in which the outer hulls have been removed), millet, sorghum, and beans. During this investigation, a total of 342 samples were submitted for testing at the Kenya National Public Health Laboratory Services. The results revealed varied aflatoxin B1 levels (range: 20 to 8,000 ppb). Out of the 342 samples tested, a total of 182 (53.2%) had >20 ppb of aflatoxin. In addition, a substantial percentage of samples from each district had aflatoxin levels >1,000 ppb: Makueni (12.1%), Kitui (9.6%), Thika (3.9%), and Machakos (2.9%).

An outbreak of acute aflatoxicosis (20 cases; CFR = 60%) was reported previously in Makueni district, Eastern Province, Kenya, in 1981 (Ngindu, 1982). During the Kenya ministry of health study in Eastern province in 2004, patients were clustered in family groups that shared meals consisting of aflatoxin-contaminated maize (1,600-12,000 ppb). Acute hepatitis associated with consumption of moldy grains also has been reported in other areas in Africa, Western India, and Malaysia (Krishnamachari *et al.*, 1975a, b; Lye *et al.*, 1995), where most affected persons came from areas prone to drought and malnutrition. Unpredictable change in rainfall pattern sometimes causes forced harvest of grains before adequate drying, leading to heavy mould colonization and mycotoxin production. Typically, increased reports of jaundice and hepatitis followed within weeks of such harvests (Krishnamachari *et al.*, 1975a; b; Lye *et al.*, 1995). Ingestion of 2-6 mg/day of aflatoxin for a month can cause acute hepatitis and death (Patten, 1981; Krishnamachari *et al.*, 1975a, b). Locally produced maize associated with this outbreak was harvested in February during peak rains, and the first illnesses were reported in Makueni district in late March and early April. As of 20th July, a total of 317 cases had been reported, with 125 deaths (CFR = 39%). An increase in case reports began in the third week of April, with new cases continuing to occur through mid-July. It is worth pointing out that, for every symptomatic case of aflatoxicosis identified in affected areas in Kenya, several other additional persons were likely to

have been exposed to unsafe levels of aflatoxin through consumption of contaminated grains or animal products and thus, might face future adverse health consequences. It is not only by consumption of contaminated food that humans get poisoned. It has also been reported that, handling mouldy feeds (hay, stover, silage and others) may be harmful due to the presence of mycotoxins and actinomycetes which are responsible not only for poisoning but also for the allergic disease affecting man known as 'farmer's lung'. This is particularly serious in asthmatics and patients suffering from cystic fibrosis. There is no cure for aflatoxin B1 poisoning and it damages vital organs in the body including the liver, kidneys and lungs. Symptoms include yellow eyes, swollen legs, vomiting and bleeding.

Conclusion: It is uncontestable fact that mycotoxins pose a serious health risk to both livestock and human beings world-over, particularly in the tropics where climatic conditions (high moisture and temperature) spur the growth of moulds. Other than the direct health risk, economic losses arising from mycotoxicoses are equally enormous. Controlling mould growth and mycotoxin production is therefore very important. Solutions to mycotoxin menace can be based only on application of recommended preventive measures and research focusing on detoxification/decontamination of mycotoxin-contaminated foods and feeds. The corner stone to a successful mycotoxin reduction in feeds is control of moisture and temperature, which in turn has a direct bearing on the extent of mould colonization. Thus, control of mould growth in feeds can be accomplished by keeping moisture low, temperature moderately low, keeping feed fresh, keeping equipments used on-farm clean, and where possible using mould inhibitors. First step in mould control is to ensuring that the food (grains) or feed (crop residues, hay and agro-industrial processing by-products) is dried adequately. The dried grains and feeds (including poultry manure) should then be stored at a well-aerated barn and at low moisture level (14 percent or less) to discourage mould growth. In silages (forage, brewers waste) mycotoxins can be prevented by following accepted ensiling practices aimed at inhibiting quality deterioration primarily through elimination of oxygen. Some silage additives (such as ammonia, propionic acid, microbial cultures, or enzymatic silage) may be beneficial in preventing mycotoxins because they are effective at reducing mould growth. Silage silo size should be matched to herd size to ensure daily removal of silage at a rate faster than deterioration. Feed troughs and barns should be cleaned regularly to prevent contamination of fresh feed. At research level, deliberate efforts should be made to prevent mycotoxin contamination before harvest through crop and forage breeding research. Longer-term solutions would include strengthening nationwide surveillance, increased food and feed inspections to ensure food safety, and local education and assistance to ensure that food grains and animal feeds are harvested correctly, dried completely, and stored properly. Agricultural and public health frontline extension staffs need to demonstrate improved methods of feed utilization to resource-poor farm households and educate them against poor practices that encourage mycotoxin contamination of food and feeds on-farm.

References

Allcroft, R. and R.B.A. Carnagham, 1963. Groundnut toxicity: an examination for toxin in human products from animals fed toxic groundnut meal. Vet. Rec., 75: 29-263.

Ashworth, L.J. Jr., J.L. McMeans and C.M. Brown, 1969. Infection of cotton by *Aspergillus flavus* and epidemiology of the disease. J. Stored Prod. Res., 5: 193-202.

Bhat, R.V. and S. Vasanthi, 2003. Mycotoxin food safety risks in developing countries. In: Food safety in food security and food trade. Food, Agriculture and Environment. Focus 10 Vision 2020, pp: 1-2.

Christensen, C.M., C.J. Mirocha and R.A. Meronuck, 1977. Molds, mycotoxins and mycotoxicoses. Agricultural Experiment Station Miscellaneous Report 142. University of Minnesota, St. Paul.

Cotty, P.J., P. Bayman, D.S. Egel and D.S. Elias, 1994. Agriculture, aflatoxins and *Aspergillus*. In: K.A. Powell, A. Fenwick and J.F. Peberdy (Eds.). The Genus *Aspergillus*" Plenum Press. New York, N.Y., p: 1-27.

Coulumbe, R.A., 1993. Symposium: Biological action of mycotoxins. J. Dairy Sci., 76: 880-891.

Conelly, W.T., 1998. Colonial era livestock development policy: Introduction of improved dairy cattle in high-potential farming areas of Kenya. World Development 26: 1733 -1748.

Council for Agricultural Science and Technology (C. A. S. T), 1989. Mycotoxins: Economic and health risks. Council on Agricultural Science and Technology, Report, 116: 1-91.

Davis, N.D. and U.L. Deiner, 1983. Some characteristics of toxigenic and nontoxigenic isolates of *Aspergillus flavus* and *Aspergillus parasiticus*. In: U.L.

Garrett, W.N., H. Heitman, Jr. and A.N. Booth, 1968. Aflatoxin toxicity in beef cattle. Proc. Soc. Exp. Biol. Med. 127:188-190.

Guthrie, L.D., 1979. Effects of Aflatoxin in corn on production and reproduction in dairy cattle. J. Dairy Sci., 62: 134 (abstr.).

Hafez, A.M., S.E. Megalla, H.M. Abdel-Fattah and Y.Y. Kamel, 1982. Aflatoxin and Aflatoxicosis. II. Effects of Aflatoxin on Ovaries and Testicles in Mature Domestic Fowls. Mycopathologia, 77: 137-139.

Jantrarotai, W. and R.T. Lovell, 1990. "Subchronic Toxicity of Dietary Aflatoxin B1 to Channel Catfish". J. Aquatic Anim. Health, 2: 248-254.

Jacques, K., 1988. Molds: The hidden killer in feeds. Large Animal Veterinarian, 52: 96-105.

Kiessling, K.H., H. Patterson, K. Sandholm and M. Olsen, 1984. Metabolism of aflatoxin, ochratoxin, zearalenone and three trichothecenes by intact rumen fluid, rumen protozoa and rumen bacteria. Appl. Environ. Microbiol., 47: 1070-1073.

Krishnamachari, K.A., V. Nagaarajan, R.V. Bhat and T.B. Tilak, 1975a. Hepatitis due to aflatoxicosis: An outbreak in Western India. Lancet, 305: 1061-1063

Krishnamachari, K.A., R.V. Bhat, V. Nagarajan and T.B. Tilak, 1975b. Investigations into an outbreak of hepatitis in parts of Western India. Ind. J. Med. Res., 63: 1036-1049.

Kenya Ministry of Health (KMOH), 2004. Outbreak of aflatoxin poisoning in Eastern and Central Provinces, Kenya (January-July, 2004). Nairobi, pp: 1-5.

Lacey, J., 1991. Natural occurrence of mycotoxins in growing and conserved forage crops. In: J.E. Smith and R.E. Henderson (Eds.). Mycotoxins and Animal Foods. CRC Press, Boca Raton, Fla., p: 363-397.

Lillehoj, E.B. and D.I. Fennell, 1975. Fungi and aflatoxin in a bin of stored white maize. J. Stored Prod. Res., 11: 47-51.

Lye, M.S., A.A. Ghazali, J. Mohan, N. Alwin and R.C. Nair, 1995. An outbreak of acute hepatic encephalopathy due to severe aflatoxicosis in Malaysia. Am. J. Trop. Med. Hyg., 53: 68-72.

Masri, M.S., V.C. Garcia and J.R. Page, 1969. The aflatoxin M1 content of milk from cows fed known amounts of aflatoxin. Vet. Rec., 84: 146 -147.

Merck Veterinary Manual, 1986. 6th Edition. Merck Company, Ames, Iowa, 1, pp: 365.

Maxwell, S.M., F. Apeagyei. H.R. de Vries, D.D. Mwanmut and R.G. Hendrickse, 1998. Aflatoxins in breast milk, neonatal cord blood and sera of pregnant women. J. Toxicol. Toxin. Rev., 1989, 8: 19-29.

Nelson, P.E., A.E. Desjardins and R.D. Plattner, 1993. Fumonisins, mycotoxins produced by *Fusarium* species: Biology, chemistry and significance. Ann. Rev. Phytopathol., 31: 233-249.

Ngindu, A., 1982. Outbreak of acute hepatitis by aflatoxins poisoning in Kenya. Lancet, 319: 754-1348.

Patten, R.C., 1981. Aflatoxins and disease. Am. J. Trop. Med. Hyg., 30: 422-425.

Peeler, E.J. and A. Omore, 1997. Manual of livestock Production Systems in Kenya. 2nd Edition. KARI (Kenya Agricultural Research Institute), Nairobi, Kenya, pp: 138.

Pier, A.C., 1992. Major biological consequences of aflatoxicosis in animal production. J. Anim. Sci., 70: 3964-3967.

Patterson, D.S.P. and P.H. Anderson, 1982. Recent aflatoxin feeding experiments in cattle. Vet. Rec., 110: 60-61.

Robens, J.F. and Richard, J.L., 1992. Aflatoxins in animal and human health. Rev. Environ. Contam. Toxicol., 127: 69-94.

Reed, J.D. and O.B. Kasali, 1987. Hazards to live stock of consuming Aflatoxin contaminated meal in Africa ICRISAT proceedings of international workshop on Aflatoxin contamination in groundnuts 6-9 oct 1987.

Smela, M.E. and S.S. Curier, 2001. Elisabeth A Bailey and John M Essingmann: The chemistry and biology of aflatoxin B1, Carcinogenesis., 22: 535-545.

Sharlin, J.S., B. Howarth, F.N. Thompson and R.D. Wyatt, 1981. Decreased Reproductive Potential and Reduced Feed Consumption in Mature White Leghorn Males Fed Aflatoxin. Poult. Sci., 60: 2701-2708.

Smith, J.E., 1997. Aflatoxins. *In* Handbook of plant and fungal toxicants, J. P. Felix D'Mello (ed.). CRC Press, Boca Raton, Florida, pp: 269-285.

Stoloff, L., 1980. Aflatoxin control: Past and present. J. Assoc. Off. Ana. Chem., 63: 1,067-1,073.

Staal, S.J., R. Kruska, I. Balteweck, M. Kenyanjui, A. Wokabi, Njubi., P. Thornton and W. Thorpe, 1999. Combined household and GIS analysis of smallholder production systems: An application to intensifying smallholder dairy systems in central Kenya. Paper presented at the 3rd international symposium of Systems Approaches for Agricultural Development (SAAD-III) held in Lima, Peru, 8-10th November 1999, National Agrarian University, La Molina, Lima, Peru.

Shotwell, O.L., M.L. Gaulden, R.J. Battrast and C.W. Hesseltine, 1975. Mycotoxins in hot spots in grains. 1. Aflatoxins and zearalenone occurrence in stored corn. Cereal Chem., 49: 458-465.

Sorenson, W.G., W. Jones, J. Simpson and J.I. Davidson, 1984. Aflatoxin In Respirable Airborne Peanut Dust, J. Toxicol. and Environ. Health, 14: 525-533.

Thorpe, W., H.G. Muriuki, A. Omore, M.O. Owango and S. Staal, 2000. Dairy development in Kenya: The past, the present and the future. Paper presented at the annual symposium of the Animal Production Society of Kenya, Nairobi, Kenya, 22-23, March, 2000.

Thomson, C. and S.E. Henke, 2000. Effects of climate and type of storage container on aflatoxin production in corn and its associated risks t wildlife species. J. Wildlife Dis., 36: 172-179.

Pakistan Journal of Nutrition 4 (3): 170-174, 2005
ISSN 1680-5194

Effect of Dried Raisins and Apricots Extract on the Growth of *Bifidobacteria* in Cows and Goats Milk

Saqer M. Herzallah
Department of Nutrition and Food Technology, Faculty of Agriculture, University of Mu'tah, Karak, Jordan

Abstract: The study confirms the growth of *Bifidobacteria* (*B. infantis*) in milk mixed with dried fruits (apricots and raisins) extract with no significant difference at ($p<0.05$) regardless of the milk type and the fruit extract concentration. The sensory evaluation of *bifidus milk* extracts fermented by *B. infantis* prepared from cow's and goat's milk with dried raisin and apricot extract each at 10% (v/v) concentration, showed a significant acceptability at ($P< 0.05$) for both cow's and goat's milk with apricot extract against a lower acceptability obtained for milks with raisin extract with a significant differences at ($P< 0.05$).

Key words: *B. infantis*, goat's milk, raisin, apricot

Introduction

Bifidobacteria are considered to be one of the most important genera of bacteria in terms of human health. They account for 85 to 99% of the intestinal flora in infants (Mitsuoka, 1990). All species derived from human are non-spore forming, non-motile, anaerobic, Gram-positive bacteria. In a healthy adult person *Bifidobacteria* constitute third to fourth largest group of microflora in the lower gastrointestinal tract, while coliforms, *clostridia* and *lactobacilli* normally account for less than 15% of the intestinal flora (Mitsuoka, 1984). At birth, *Bifidobacterium infantis, B. breve* and *B. longum* are dominant but are gradually replaced with *B. adolescentis*. *Bifidobacterium longum* persists from birth throughout life in most healthy individuals. *Bifldobacterial* counts of 10^9 to 10^{10} per gram of stool are common in adults (Mitsuoka, 1982).

Bifidobacteria had long been recognized as bacteria with probiotic, nutritive and therapeutic properties (Bezkoravainy, 2001). Since Bifidobacteria do not grow well in milk, the manufacturing of fermented milk products with *Bifidobacteria* often requires the use of an inoculum containing the final number of cells of *Bifidobacteriam* required for the products (Crittenden, 1999).

Recently there has been an increasing interest in the incorporation of the intestinal species *Lactobacillus acidophilus* and *Bifidobacterium* species into fermented milk products. These species are frequently associated with health promoting effects in human and animal intestinal tract. These probiotic effects are generally related to inhibition of pathogenic species, reducing the risk of colon cancer, increasing the immune response and decreasing concentration of cholesterol in blood plasma (Gilliland, 1990; Gurr, 1987).

Bifidobacteria are not true lactic acid bacteria in the sense of a *Lactococcus* or *pediococcus* (Hughes and Hoover, 1991). *Bifidobacteria* produce both acetic and lactic acids as primary metabolites in the molar ratio of 3:2. Glucose is degraded characteristically by the fructose 6- phosphate shunt metabolic pathway (Bezkoravainy, 2001; Holzapfel *et al.,* 1998).

Dried apricots and raisins are widely consumed and produced in Jordan and Syria and are considered to be one of the most rich sources in minerals and sugars. Furthermore, milk is one of the best sources of nutrients for child growth. The addition of fruit extracts may enhance the growth of *Bifidobacteria* by providing essential nutrients, enhancing the sensory quality of the products since the flavor of bifido culture in milk is not favorable, and providing consumers with certain nutrients especially minerals and energy.

Therefore, the objectives of the study were first to screen the ability of adding extracts of dried apricots and raisins to cow's, goat', and sheep's milk to stimulate the growth and stability of selected *Bifidobacteria* and second to investigate the acceptability of health drinks made from Milk and dried fruit (apricots and raisins) extracts fermented by *Bifidobacteria*.

Materials and Methods

Milk source and heat treatment: Whole raw Cow's milk was obtained from Alsanabel Dairy Company Ltd., and Goat's milk was from the local market (Jordan). Whole milk samples (1000 ml each) were heat treated at 93 ± 1°C for 20 min in water bath, then cooled (in fridge).

Bifidobacteria cultures: Lyophilized *Bifidobacterium infantis* ATCC 15697 was obtained from Deutsche Sammlung von Microorganismen und Zellkulturen GmbH, Braunschweig (DSMZ), Germany.

Forty-eight hours prior to the start of each experiments, cultures were revived by transfer twice into 10 ml of MRSL (MRS broth with 5% lactose), (Hughes and Hoover, 1991) and incubation at 37°C for 24 hours in an anaerobic chamber (Gaspak system; BBL, Cockeysville, MD, U.S.A).

Viability determination: *Bifidobacterium* strains *B. infantis* 15697 was cultured anaerobically at 37°C for 48 h with 0, 10, 15, and 20% (v/v) extracts of dried apricots and dried red raisin (product of Jordan). Samples containing no fruits (0%) were used as controls. All inoculated samples after fermentation were stored at 4.0 ± 1°C for 15 d. One ml of each milk samples was diluted with 9 ml of sterile 0. 1% (w/v) peptone water (Difco) and mixed uniformly with a vortex mixer (Waring Blendor, model 32BL80). Subsequent ten fold serial dilutions were prepared and viable numbers enumerated using pour plate technique. *Bifidobacteria* were enumerated in duplicate using MRSL agar (Difco, Laboratories. Detroit, MI, U.S.A). The inoculated plates were incubated anaerobically at 37°C for 48 h using N_2 gas. Cell counts were carried out on day 0, 3 6, 9 12, and 15. The colonies were counted using a colony counter (Model, EC1-video colony counter AES, Laboratoire).

Growth studies: Growth characteristics of cultures of *Bifidobacteria* in cow and goat were evaluated. Each culture was inoculated at 1% (v/v) into 100 ml of milk and was incubated at 37°C for 16 hours in the anaerobic chamber (BBL). Initial viable counts for each culture were standardized by the use of standard curve so that they were approximately the same for all cultures (=1x10^8 cfu/ml). Viable counts were done by serial dilution with 0.1% peptone -water and pour plating in duplicate using MRSL agar. Then samples were drawn at 0, 4, 8, 12 and 16 h from each flask and flushed with inert gas (N_2) after closing every sampling time.

Preparation of fermented and unfermented milk: Milk from goats and cows were used for preparation of fermented and unfermented according to Hughes and Hoover (1991) method.
Fermented milks were made by a 1% (v/v) inoculation of 100 ml of each milk. Flask openings (Corring Brand Milk Dilution Bottles, Screw Cap, Cat. No. 1372) were sealed with a single layer of parafilm (Parafilm, Laboratory Film, American National Can. Chicago, IL. 60631). Fermented milk was incubated for 16 hrs at 37°C in the anaerobic chamber for growth studies. Unfermented samples were prepared by inoculation of milks that had been prechilled to 4°C for 15 days. After that the sample flasks were sealed and capped as described for fermented samples. Samples were stored at 4°C immediately after inoculation. Bacterial counts was evaluated in the fermented and unfermented milks on days 0, 3, 6, 9, 12 and 15. Viable counts were determined as mentioned in the growth studies.

Dried raisins and apricots samples: One variety of dried apricots of and dried raisin product of Jordan were used(free of preservatives). The extracts were prepared with ca. 35% total solids.

Dried fruit extraction method: Dried apricots and raisin (250 g each) were soaked in 500 ml distilled water at 70° C for 1 hour, blended using Waring Blender, (model 32BL80), then strained in cheese cloth. The extracts were filled in 500ml glass bottles and sterilized at 121°C for 15 minutes in an autoclave.

Statistical analysis of experimental data: General linear model (GLM) and Fisher's least significant difference (LSD) were used to differentiate between means within and among the treatments using SAS™ (Version 8 , SAS institute (The data obtained were reduced at a significance level of 5%($\propto$ = 0.05).

Sensory evaluation: A hedonic (5 points) scale test as described by Munoz *et al*. (1992), was used to evaluate the acceptance of milks from cow and goat containing 10% raisin or apricot extract. Fourty panelists from Mu'tah University, Faculty of Agriculture conducted the sensory evaluation. Panelists were asked to evaluate aroma, taste, color and overall acceptability of the samples.

Results

***Bifidobacterium infantis* Growth in milks containing dried Apricots extract:** Dried apricot and raisin are added to different types of milk for flavoring and sweetening purposes, that may supply the added culture with suitable nutrients.
Preliminary investigations were carried out to compare growth behaviors of one species of *Bifidobacteria*. Initial viable counts for each culture were standardized by the use of standard curve so that they were approximately the same count for all (= 1x10^8 cfu/ml). Results in Table 1 show changes in *Bifidobacterium* counts (log cfu/ml) in milks from cow and goat containing different concentrations of dried apricots extract and inoculated with 1% culture of *B. infantis*. The changes in the *Bifidobacterium* counts (log cfu/ml) in milks were not significantly different at (P< 0.05) between 0%, 10% and 20% concentrations respectively of dried apricots extract after incubation for 16 h at 37±1.0°C.
The bacterial counts (log cfu/ml) in cow's milk at all time intervals tested were ranged from 6.8 to 7.6 after incubation for 16 h at 37±1.0°C regardless of the dried apricots extract concentration against 6.3 to 6.8 of the bacterial counts (log cfu/ml) in goat's milk.

Growth in milks containing raisin extract: Results in Table 2 show changes in *Bifidobacterium* counts (log cuf/ml) in milks from cow and goat containing different concentration of raisin extract and inoculated with 1% culture of *B. infantis*. The changes in the *Bifidobacterium* counts (log cfu/ml) in milks were also not significantly different at (P<0.05) between 0, 10 and 20% concentrations.

Table 1: *Bifidobacterium* counts (log cfu/ml) in cow's and goat's milk containing different concentrations of dried apricots extract and inoculated with 1% culture of *B. infantis* when incubated at 37 ±1.0°C

Milk	Time (h)	Dried apricot concentration %		
		0	10	20
Cow	0	6.8[a1]	6.8[a]	6.4[a]
	4	7.0[a]	6.8[a]	6.4[a]
	8	6.8[a]	6.8[a]	6.8[a]
	12	7.1[a]	6.9[a]	7.1[a]
	16	7.6[a]	7.2[a]	7.6[a]
Goat	0	6.3[a]	6.8[a]	6.7[a]
	4	6.8[a]	6.8[a]	6.8[a]
	8	6.8[a]	6.8[a]	6.5[a]
	12	6.8[a]	6.8[a]	6.4[a]
	16	6.8[a]	6.8[a]	6.8[a]

[1]Means in the same rows and columns with the same letter are not significantly different at (P < 0.05)

Table 2: *Bifidobacterium* counts (log cfu/ml) in cow's and goat's milks containing different concentrations of raisin extract and inoculated with 1% culture of *B. infantis*

Milk	Time (h)	Dried raisin concentration %		
		0	10	20
Cow	0	6.0[a1]	6.0[a]	6.0[a]
	4	6.0 [a]	5.93[a]	6.0[a]
	8	6.3[a]	6.3[a]	6.4[a]
	12	6.4[a]	6.5[a]	6.3[a]
	16	6.6[a]	6.7[a]	7.1[a]
Goat	0	6.0[a]	6.8[a]	6.5[a]
	4	6.8[a]	6.8[a]	6.5[a]
	8	6.8[a]	6.8[a]	6.5[a]
	12	6.8[a]	6.8[a]	6.7[a]
	16	6.8[a]	6.8[a]	6.8[a]

[1]Means in the same rows and columns with the same letter are not significantly different at (P < 0.05).

The bacterial counts (log cfu/ml) in cow's milk raisin extract increased from 6.0 to 6.6, 6.0 to 6.7 and 6.0 to 7.1 at 0 ,10 and 20% concentrations, respectively after incubation period of 16 hr at 37 ±1.0°C.

The bacterial counts (log cfu/ml) in goat's milk raisin extract increased from 6. 0 to 6.82, and 6.5 to 6.8 at 10 and 20% raisin extract , whereas, no increase in the bacterial count at 10% remained constant throughout the period of incubation.

Viability and activity of *Bifidobacterium infantis* during refrigerated storage at 4 ±1.0°C

Milks containing dried apricots extract: Results in Table 3 show changes in *Bifidobacterium* counts (log cfu/ml) in milks from cow and goat containing different concentration of dried apricots extract and inoculated with 1% culture of *B. infantis*. The changes in the *Bifidobacterium* counts (log cfu/ml) in milk from cow containing 0, 10 and 20% dried apricots extract were significantly different after 15 days of storage at 4 ±1.0°C. The changes in the *Bifibacterium* counts (log cfu/ml) between 0 and 10% concentration of dried apricots extract were also not significantly different.

The *Bifidobacterium* count (log cfu/ml) slightly decreased from 7.0 to 6.4, and from 6.8 to 6.4 at 10 and 20% concentrations, respectively but it slightly increased from 6.5 to 6.8 at 0% concentration of dried apricots extract at 15th day of storage at 4±1.0°C.

Table 3: Bifidobacterium counts (log cfu/ml) in cow's and goat's milk containing different concentrations of dried apricots extract and inoculated with 1% culture of *B. infantis* when incubated at 4 ±1.0ºC for 15 days

Milk	Time (day)	Dried apricots concentration %		
		0	10	20
Cow	0	6.5[a1]	6.8[a]	7.0[b]
	3	6.7[a]	6.5[a]	6.7[b]
	6	6.5[a]	6.8[a]	6.5[b]
	9	6.4[a]	6.1[a]	6.5[b]
	12	6.7[a]	6.7[a]	6.5[b]
	15	6.8[a]	6.4[a]	6.4[b]
Goat	0	6.3[a]	6.8[a]	6.8[ab]
	3	6.4[a]	6.80[a]	5.6[ab]
	6	6.4[a]	6.4[a]	5.8[ab]
	9	6.2[a]	6.3[a]	6.3[ab]
	12	6.5[a]	6.6[a]	6.4[ab]
	15	6.5[a]	6.4[a]	6.3[ab]

[1]Means in the same rows and columns with the same letter are not significantly different at (P < 0.05).

Table 4 : *Bifidobacterium* counts (log cfu/ml)in cow's and goat's milk containing different concentrations of raisin extract and inoculated with 1% culture of *B. infantis* when incubated at 4± 1.0ºC for 15 days

Milk	Time (day)	Raisin concentration %		
		0	10	20
Cow	0	6.0[a1]	6.1[a]	6.0[a]
	3	6.6[a]	6.3[a]	6.8[a]
	6	6.1[a]	6.2[a]	6.8[a]
	9	6.4[a]	6.6[a]	6.4[a]
	12	6.5[a]	6.3[a]	6.4[a]
	15	6.5[a]	6.5[a]	6.3[a]
Goat	0	6.3[a]	6.5[a]	6.8[a]
	3	6.8[a]	6.5[a]	6.8[a]
	6	6.8[a]	6.8[a]	6.8[a]
	9	6.8[a]	6.8[a]	6.8[a]
	12	6.8[a]	6.8[a]	6.5[a]
	15	6.8[a]	7.0[ab]	6.3[a]

[1]Means in the same rows and columns with the same letter are not significantly different at (P < 0.05)

Table 5: Sensory evaluation[1] of cow's and goat's milk containing 10% of raisin extract

Milk	Fruit Extracts	Aroma	Taste	Color	Overall acceptability
Cow	None	4.0[a]	3.4[a]	3.4[a]	3.6[a]
	Raisins	3.1[2b]	3.0[b]	3.3[a]	3.6[a]
	Apricots	4.2[a]	4.0[a]	3.3[a]	4.0[a]
Goat	None	3.5[a]	3.4[a]	3.5[a]	3.4[a]
	Raisins	2.6[b]	2.8[b]	2.9[a]	2.6[b]
	Apricots	3.6[a]	4.0[a]	3.0[a]	3.4[a]

[1]A hedonic scale presented in 5 categories (1 ; dislike very much, 2; dislike, 3; neither dislike nor like, 4; like and 5; like very much).
[2]Means (n = 40) with different letters within a column are significantly different at ($p<0.05$).

Table 3 shows that changes in *Bifidobacterium* counts (log cfu/ml) in goat's milk containing different concentrations of dried apricots extract and inoculated with 1% culture of *B. infantis* were significant at ($P< 0.05$) throughout the period of storage, the counts (log cfu/ml) decreased from 6.8 to 6.4 and 6.8 to 6.3 at 10%, and 20% concentrations, but the count (log cfu/ml) increased from 6.3 to 6.5 at 0% concentration of dried apricots extract after 15 days of storage at 4 ±1.0ºC .

Milks containing raisin extract: Results in Table 4 show changes in *Bifidobacterium* counts (log cfu/ml) in cow's, and goat's milk containing different concentrations of raisin extract and inoculated with 1% culture of *B. infantis*. The changes in the *Bifidobacterium* counts (log cuf/ml) in milk from cow containing 0, 10 and 20% raisin extract weren't significantly different at $p<0.05$ level at refrigerated storage for 15 days at 4±1.0°C.

The *Bifidobacterium* count (log cfu/ml)in milk from cow at all time intervals tested, slightly increased from 6.0 to 6.5, 6.8 to 7.0 at 0%, 10% concentration, whereas, decreased from 6.8 to 6.3 at 20% concentrations, respectively, of raisin extract after 15 days of refrigerated storage at 4±1.0°C.

Table 4 shows also that changes in *Bifidobacterium* counts (log cfu/ml) in goat's milk containing different concentrations of raisin extract and inoculated with 1% culture of *B. infantis* were not significantly different at all concentration.

Sensory evaluation: The result of the sensory evaluation presented in Table 5 shows that the cow's milk drinks were evaluated as the most acceptable drink as they scored 3.6 and 4.0 for milk with raisin and apricot extract, respectively. This means that milk with apricot extract is more preferable and its acceptance lies between "neither like nor dislike" and "like" in other words they were very close to the like rating and the drink with apricot extract is more favourable by panels than milk or milk with raisins extract.

The goats milk with raisin extract had the lowest overall acceptability, the rating was 2.6 against 3.4 for milk and milk with apricot. It is also, clear from the results obtained that the aroma of the cows milk with raisins for both cow's and goats milk were the lowest as they score 3.1 and 2.6 for cow's and goats, respectively. Furthermore, the aroma, taste and colour of milk drink mixed with raisins extract were the least acceptable as they scored 2.6, 2.8 and 2.9 for aroma taste and color, respectively.

Discussion

The production of bifidobacteria fermented milk is not easy compared to yoghurt fermentation. In addition, the taste and aroma of the products are not favorable (Rasic, 1983; Roy *et al.*, 1990).

The use of dried fruit extracts with milk is a continuation of many previous researches to enhance the growth and viability of bifidobacteria (Klaver *et al.*, 1993; Roy *et al.*, 1990; Shin *et al.*, 2001). Raisins and dried apricots were selected in this study for their popularity in the middle east countries and for their high carbohydrates and mineral contents. Moreover, these products are available all over the year and they are one of the main food items always found on the food banquets during the holly month of Ramadan.

The results of the study of growth of *Bifidobacterium* during refrigerated storage, presented in the Table 3 and 4 generally revealed no significant increase in of bacterial count.

Growth promotion, enhancement of activity and retention of viability were greatest when *Biftdobaacrium* were grown in the presence of fructooligosaccharide (FOS), followed in a descending order by galactooligosacchride (GOS) and inulin. The effects of oligosaccharides and inulin increased with increasing carbohydrate concentration (Shin *et al.*, 2000).

The loss in viability of *Bifidobacteria* occurs in fermented milks could be due to acid formation and presence of oxygen (Shah, 2000). Bifidobactria also could grow well in milk inoculated with cultures prepared in a synthetic medium.

This study agree with Shah *et al.* (1995) who studied the survival of *Bifidobacterium bifidum* in five brands of commercial yogurt during refrigerated storage and found that the number of viable counts *B. bifidum* steadily declined in all the products during refrigerated storage. The loss of viability was attributed to the decrease in pH values during refrigerated storage.

Loss of viability of *Bifidobacteria* is typically more pronounced in fermented milk than in unfermented milk due to acid injury to the organism (Dave and Shah,

1997). Lankaputhra *et al.* (1996) observed that viability of *Bifidobacteria* strains such as *Bifidobacterium infantis* in 12% skim milk at pH 4.3 was decreased by 30% after 12 d of storage at 4°C. After 24 d the same temperature, the counts decreased by more than 82%. Medina and Jordan (1994) observed a 93% reduction in bifidobacterial counts of fermented milk produced in Spain at 7°C .

On the contrary, doubling of bacterial count of *B. Bifidum* cultured in skim milk mixed with honey, fructose or glucose was found by Ustunol and Gandi (2001) after 222 minutes of incubation.

Since most of the cfu numbers in all preparations are 10^6 cfu/ml or higher, it is concluded, according to the Adhikari *et al.* (2003), that all preparations are adequate, provided a minimum of 100 ml is consumed daily.

The results of the sensory quality presented in table Table 4 showed that cow's milk mixed with both fruit extracts (Raisins and apricots) have a moderate acceptance for and the least acceptance for goat's milk mixed with raisins extract .

These results are in agreement with Al-saleh results (2001) that indicated a low sensory acceptability of Bifidus milk. The sensory acceptability of Bifidus milk preparation could be improved by combining Bifidus with acidophillus fermentations (Gomes and Malcata, 1999) or by two steps fermentation (Adhikari *et al.*, 2003), since this will result in increasing the sourness of the product.

Conclusions: The inoculation of milk at levels $\geq 10^6$ cfu/ml is adequate for the production of Bifidus milk with a probiotic effect. Also, a cold storage of inoculated milks with Bifidobacteria showed of a bacterial growth which enhance the formulation of fruit flavoured of bifidus milk by inoculation.

References

Adhikari, K., A. Mustapha and I.U. Grun, 2003. Survival and metabolic activity of microencapsulated *Bifidobacterium longum* in stirred yogurt. J. Food Sci., 68: 275-280.

Al-Saleh, A.A., 2001. Bfidus camel milk powder: Sensory evaluation and viability of *Bifidobacteria*. J. King Saud Univ. Agri. Sci., 13: 45-53.

Bezkoravainy, A., 2001.propioticcs determinants of survival and growth in the gut. Am. J. Clin. Nutr., 73 (suppl): 3995-4055.

Crittenden, R., 1999. In; Probiotics; A Critical Review, Tonnock GW edit. Norfolk, England: Horizon Scientific Press, pp: 141-156.

Dave, R.I. and N.P. Shah, 1997. In Viability of probiotic bacteria in youghrt made from commercial starter cultures. Int. Dairy J., 7: 707- 715.

Gandi, H. and Z. Ustunol, 2001. Growth and viability of commercial *Bifidobacteria Strains* in honey sweetened fermented milk. The IFT Annual Meeting.

Gilliland, S.E., 1990. Health and nutritional benefits from lactic acid bacteria . FEMS Microbiol. Rev., 87: 175-188.

Gomes, A.M. and F.X. Malcata, 1999. Bifidobacterium spp. and *Lactobacillus acidophilus*: biological, biochemical, techonological and therapeutical properties relevant for use as probiotics. Trends in Food Science and Techonology, 10:139-157.

Gurr, M.I., 1987. Nutritional aspects of fermented milk products. FEMS Microbial. Rev., 46: 337-342.

Holzapfel, W.H., P. Habere, J. Snel, U. Schillinger and J.H. Huis, 1998. Overview of gut flora probiotics. Int. J. Food Microbiol., 41: 85-101.

Hughes, D.B. and D.G. Hoover, 1991.*Bifidobacteria*: Their potential for use in American dairy products. Food Tec., 45: 74-83.

Klaver, F.A.M., F. Kingma and A.H. Weerkamp, 1993. Growth and survival of *Bifidobacteria* in milk. Neth. Milk Dairy J., 47: 151-164.

Lankaputhra, W.E.V., N. Shah and M. Britz, 1996. Survival of *Bifidobacteria* during refrigerated storage in presence of acid and hydrogen peroxide. Milchwissenschft., 17. 2: 65-70.

Medina, I.M. and R. Jordan, 1994. Survival of constitutive microflora in commercially fermented milk containing *Bifidobacteria* during refrigerated storage. J. Food Prot., 56: 731-733.

Mitsuoka, T., 1990. Beneficial microbial aspects (probiotics). proc. International Dairy Congress. 2: 1226-1237, Montreal,Oct.8-12.

Mitsuoka, T., 1984.Taxonomy and ecology of bifidobactria. *Bifidobacteria* Microflora, 1: 3-24.

Mitsuoka, T., 1982. Recent trends in research on intestinal flora. *Bifidobacteria* Microflora, 1: 3-24.

Munoz, A.M., G.V. Civille and B.T. Carr, 1992. Sensory Evaluation in Quality Control. VNR, New York, pp:108-139.

Rasic, J.L., 1983. The role of dairy foods containing *Bifidobacteria* and acidophilus bacteria in nutrition and health. North. Eur. Dairy J., 48: 80-88.

Roy, D., F. Dussault and P. Ward. 1990. Growth requirements of *Bifidobacterium* strains in milk. Milchwissenschaft, 45: 500-502.

Shah, N.P., 2000. Probiotics, prebiotic bacteria: selective enumeration and survival in dairy foods. J. Dairy Sci., 83: 894-907.

Shah, N.P., W.E. Lankapulhara, M.L. Britz and W.S. and A. Kyle, 1995. Survival of *Lactobacillus acidophilus* and *Bifidobacterium bifidum* in commercial yogurt refrigerated storage. Int. Dairy J., 5: 515.

Shin, H.S., J.H. Lee, J.J. Pesttka and Z. Ustunol, 2000. Growth and viability of commercial *Bifidobacterium* spp in skim milk containing oligosaccharides and inulin. J. Food Sci., 65: 520.

Ustunol, Z. and H. Gandi, 2001. Growth and viability of commercial *Bifidobacterium* spp. in honey-sweetend skim milk. J. Food Protec., 64: 1775-1779.

Pakistan Journal of Nutrition 4 (3): 175-182, 2005
ISSN 1680-5194

Association of Serum C-Reactive Protein (CRP) with Some Nutritional Parameters of Maintenance Hemodialysis Patients

Azar Baradaran[1] and Hamid Nasri[2]*
[1]Department of Biochemistry, Center of Research and Reference Laboratory, Iran
[2]Hemodialysis Department, Hajar Medical, Educational and Therapeutic Center,
Shahrekord University of Medical Sciences, Shahrekord, Iran

Abstract: Malnutrition and inflammation are common in hemodialysis patients, and are usually closely associated. Serum C-reactive protein (CRP) concentrations have been found to be significantly elevated in hemodialysis patients and reflects chronic inflammation, and as an acute-phase reactant, is a sensitive and independent marker of malnutrition. To investigate the association of serum CRP level with some nutritional variables in diabetic and non diabetic end-stage renal failure patients undergoing regular hemodialysis, we designed a study of 36 maintenance hemodialysis patients (f = 15 m = 21), consisting of 25 non-diabetic HD patients and 11 diabetic HD patients. In this study we found a near significant difference of CRP between diabetic and non-diabetics of total patients with more values of CRP in diabetics, a significant difference of CRP between diabetic and non-diabetic of female HD patients with more values in diabetics and a significant difference of CRP between males and female of non-diabetic population with more values of CRP in males. An inverse correlation of serum CRP with serum cholesterol and triglyceride levels and a near significant positive correlations of CRP with serum ALP and with serum iPTH were found. An inverse correlation of serum CRP with dialysis efficacy was seen too. No significant association between serum CRP and serum albumin was seen. Compatible with some studies and in contrast to some other studies, the association of serum albumin with serum CRP levels in this study was insignificant. The positive correlation of high serum PTH with inflammation implies further need to control of hyperphosphatemia and secondary hyperparathyroidism in HD patients, also inverse correlation of serum CRP with cholesterol and triglyceride further support the malnutrition-inflammation complex syndrome (MICS) which frequently seen in HD patients.

Key words: End-stage renal failure, hemodialysis, C-reactive protein (CRP), malnutrition-inflammation

Introduction

C-reactive protein (CRP), is an acute phase protein whose synthesis in the liver is regulated by different cytokines, particularly interleukin 6 (IL-6). Plasma levels of CRP in the absence of active disease are low, but can rise up to 1000-fold in patients with an inflammatory reaction. Besides being a marker of inflammation, CRP itself may have proinflammatory properties since it can activate the complement system (Wolbink *et al*., 1996; Morrow and Ridker, 2000). Thus elevated plasma concentrations of C-reactive protein (CRP), a sensitive marker of underlying systemic inflammation. (Clyne and Olshaker, 1999; McIntyre *et al*., 1997; Haubitz *et al*., 1996). Serum CRP concentrations have also been found to be significantly elevated in hemodialysis patients (Stenvinkel *et al*., 2000; Panichi *et al*., 2000) and reflects chronic inflammation, and as an acute-phase reactant, is a sensitive and independent marker of malnutrition (Ortega *et al*., 2002). An elevated serum C-reactive protein has been shown to be strongly predictive of morbidity and mortality in dialysis patients, specially is a strong predictor of cardiovascular mortality in hemodialysis patients and (Kim *et al*., 2005; Caglar *et al*., 2002). Uremic malnutrition is characterized by insidious loss of somatic protein stores (reflected by lean body mass or serum creatinine) and visceral protein concentrations (reflected by serum albumin or serum prealbumin) (Fernandez-Reyes *et al*., 2000). The etiology of malnutrition in renal failure is complex and may include many factors, e.g. poor food intake because of anorexia, nausea and vomiting due to uraemic toxicity, hormonal derangements, acidosis and increased resting energy expenditure (Fernandez-Reyes *et al*., 2000; Fujino *et al*., 2005).Malnutrition and inflammation are common in hemodialysis patients, and are usually closely associated (Marckmann, 1988). Indeed the prevalence of protein-energy malnutrition in hemodialysis (HD) patients is high (23 to 73%) in various studies (Enia *et al*., 1993; Cianciaruso *et al*., 1995; Leavey *et al*., 1998; Lowrie and Lew, 1990).Several recent reports have focused on the association between nutritional status and clinical outcome, providing support for the hypothesis that malnutrition may cause or contribute to mortality and predict poor clinical outcome in end-stage renal disease patients. (Leavey *et al*., 1998; Lowrie and Lew, 1990;

Table 1: Mean±SD, Minimum and Maximum of age, duration and dosage hemodialysis and also laboratory results of total hemodialysis patients

Total patients n=36		Min.	Max.	Mean±SD
Age	years	16	80	46.6±17
DH*	months	2	156	32±36
Dialysis dose	sessions	36	1584	294±3393
URR	%	39	76	59±8.9
BMI	kg/m^2	16	34	22±4.4
iPTH	Pg/ml	16	1980	434±455
Ca	mg/dl	5	10	7.7±0.94
P	mg/dl	3	10	6.4±2
Alp	IU/L	175	5487	628±891
HCO3	mE/l	14	25	20±2.6
CRP	mg/l	3	40	8.7±6.7
BUN	mg/dl	30	180	83±33
Creat	mg/dl	3	18	9.5±3.6
Alb	g/dl	2.4	4.8	3.8±0.5
25-OH Vit D	nMol/l	1.3	105	10.5±18
Chol	mg/dl	59	211	117±39
TG	mg/dl	29	461	130±95
LDL	mg/dl	12	122	61.6±22
HDL	mg/dl	2	70	37±11

*Duration of hemodialysis treatment

Table 2: Mean±SD, Minimum and Maximum of age, duration and dosage hemodialysis and also laboratory results of non-diabetic hemodialysis patients

Total patients n=25		Min.	Max.	Mean±SD
Age	years	16	80	44±17
DH*	months	2	156	40±40.8
Dialysis dose	sessions	36	1584	370±452
URR	%	60	76	61±7.5
BMI	kg/m^2	16	36	21±4.6
iPTH	Pg/ml	22	1980	537±483
Ca	mg/dl	6	9	7.8±0.70
P	mg/dl	4	10	6.6±1.8
ALP	IU/L	190	5487	760±1044
HCO3	mE/l	14	25	20±2.8
CRP	mg/l	2	20	7.4±3.8
BUN	mg/dl	30	180	81±32
Creat	mg/dl	4	15	9.8±2.9
Alb	g/dl	2.4	4.7	3.8±0.50
25-OH Vit D	nMol/l	1.3	105	12.6±21
Chol	mg/dl	59	171	110±33
TG	mg/dl	61	461	130±85
LDL	mg/dl	12	99	61±2
HDL	mg/dl	25	70	38±11

*Duration of hemodialysis treatment

Goldwasser *et al.*, 1993). Low serum albumin as a parameter of malnutrition in HD patients is principally associated with infection/inflammation but not with malnutrition alone (Stenvinkel *et al.*, 2000). Preliminary studies showed the association of CRP level and the serum albumin level (Teruel *et al.*, 2005). Recently in a study on a group of hemodialysis patients no correlation between baseline CRP and serum albumin was found (Nascimento *et al.*, 2004), in this regard we sought to investigate the association of serum CRP level with some nutritional variables consisting serum albumin in our diabetic and non diabetic end-stage renal failure patients undergoing regular hemodialysis patients.

Table 3: Mean±SD, Minimum and Maximum of age, duration and dosage hemodialysis and also laboratory results of diabetic hemodialysis patients

Total patients n=11		Min.	Max.	Mean±SD
Age	years	27	75	53±15.8
DH*	months	6	24	14.5±6
Dialysis dose	sessions	54	216	123±54
URR	%	39	75	53.5±9.8
BMI	kg/m^2	20	34	23±3.9
iPTH	Pg/ml	16	860	202±277
Ca	mg/dl	5	10	7.5±1.3
P	mg/dl	3	10	6±2
ALP	IU/L	175	584	327±148
HCO3	mE/l	18	25	20±1.8
CRP	mg/l	4	40	12±10
BUN	mg/dl	30	140	87±36
Creat	mg/dl	3	18	9±4.8
Alb	g/dl	3	4.8	3.8±0.50
25-OH Vit D	nMol/l	1.5	3.6	5.8±10
Chol	mg/dl	60	211	133±49
TG	mg/dl	29	456	130±120
LDL	mg/dl	25	122	62±26
HDL	mg/dl	20	46	35±9.5

*Duration of hemodialysis treatment

Materials and Methods

This cross-sectional study was conducted on patients with end-stage renal disease (ESRD), who were undergoing maintenance hemodialysis treatment with acetate basis dialysate and polysulfone membranes. According to the severity of secondary hyperparathyroidism, each patient being treated for secondary hyperparathyroidism was given oral active vitamin D3 (Rocaltrol), calcium carbonate, and Rena-Gel capsules at various doses. According to the severity of anemia ,patients were under IV iron therapy with Iron Sucrose (venofer) at various doses after each dialysis session, all patients were under treatments of 6mg folic acid daily, 500mg L-Carnitine daily, oral Vitamin B-complex tablet daily and also 2000U IV Eprex (recombinant human erythropoietin (rHuEPO) unique for each patient after each dialysis session routinely. Exclusion criteria were active or chronic infection. For patients levels of serum predialysis creatinine (Creat), post and predialysis blood urea nitrogen(BUN), Serum calcium (Ca), phosphorus (P), albumin (Alb) and also lipid profile containing serum Triglyceride (TG), Cholesterol (Chol) and high density lipoprotein (HDL-C) as well as serum C-reactive protein (CRP) were measured using standard kits. Also Intact serum PTH (iPTH) was measured by the radioimmunoassay (RIA) method using DSL-8000 of USA (normal range of values is 10-65 pg/ml). Serum 25-hydrox VitD (25-OH Vit D) level (normal range of values is 25 to 125 nmol/l) was

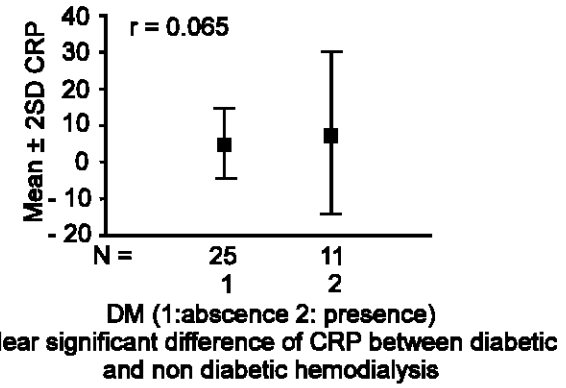


Fig. 1: Near significant difference of CRP between diabetic and non-diabetics.

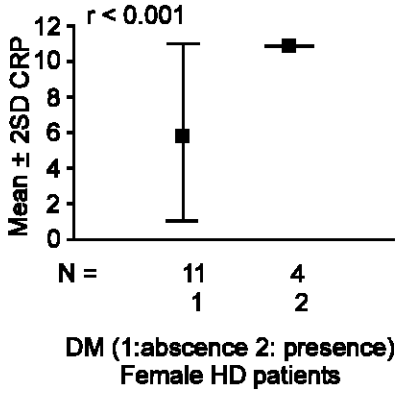


Fig. 2: Significant difference of CRP between diabetic and non-diabetic of female HD patients.

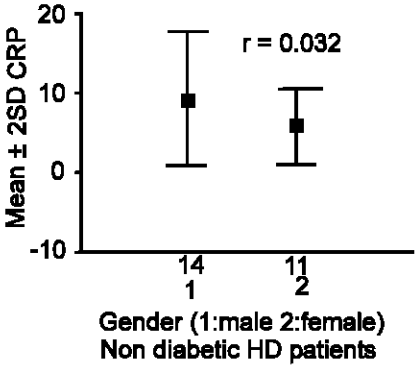


Fig. 3: Significant difference of CRP between males and female of non-diabetic HD patients.

measured by enzyme-linked immunosorbent assay (ELISA) method using DRG kit of Germany. Plasma HCO_3^- and blood P^H was measured by arterial blood gas. For the efficacy of hemodialysis the urea reduction rate (URR) was calculated from pre- and post-blood urea nitrogen (BUN) data (Boag, 1994). The body mass index(BMI) was calculated using weight and height (kg/m^2) (http://www.halls.md/body-mass-index/av.htm). Serum LDL-C was calculated using friedewal's formula (Friedewald *et al.*, 1972). Duration and dosages of hemodialysis treatment were calculated from the patients' records. The duration of each hemodialysis session was 4 hours. Statistical analysis were performed on total hemodialysis (HD), females, males, diabetics and non diabetics populations separately. For statistical analysis, the data are expressed as the mean ± SD. Comparison between the groups was done using Student's t-test. Statistical correlations were assessed using partial correlation test. All statistical analyses were performed using SPSS (version 11.5.00). Statistical significance was determined at a p-value <0.05.

Results

Total patients were 36 (f = 15 m = 21), consisting of 25 (f = 11 m = 14) non-diabetic HD patients and 11 (f = 4 m = 7) diabetic HD patients .Table 1, 2 and 3 show the patients' mean ± SD age, the length of time they were on hemodialysis, the dialysis dosage, and the results of laboratory tests. The mean patient's age was 46.6 (±17) years. In total patients, no significant difference of CRP between males and females of total patients was seen, also no significant difference of CRP between diabetic and non-diabetic male HD patients was seen(p N.S.), however a near significant difference of CRP between diabetic and non-diabetics of total patients was seen (p= 0.056; Fig. 1), also a significant difference of CRP between diabetic and non-diabetic of female HD patients was seen (p<0.001; Fig. 2), moreover a significant difference of CRP between males and female of non-diabetic population was seen (p=0.032; Fig. 3). In this study the associations between serum CRP levels and variables consisting of age, duration of hemodialysis, hemodialysis doses, serum lipids, parameters of bone activity, Serum 25-hydroxy Vit D levels, dialysis adequacy and also with plasma HCO_3^- in all groups containing total, non-diabetic, diabetic, female and male HD patients were as follows; In total HD patients, a near significant inverse correlation of serum CRP with serum cholesterol (r = -0.29, p=0.090; Fig. 4) and a significant positive correlation of serum CRP with serum phosphorus (r = 0.33, p=0.050; Fig. 5) were found (adjusted for duration of dialysis for two correlations) .In this group also a near significant positive correlation of serum CRP with serum BUN (r = 0.33, p=0.050) (adjusted for dialysis dosage) was found too. In non-diabetic HD patients a significant positive correlation of serum CRP with serum ALP (r = 0.77, p <0.001; Fig. 6) (adjusted for age) and a near significant inverse correlations of serum CRP with serum Creat (r = -0.38, p=0.065; Fig. 7) and with dialysis efficacy as determined by URR (r = -0.37, p=0.074; Fig. 8) were seen too) (adjusted for dialysis doses for both two correlations). In diabetic HD patients a significant positive correlation of serum CRP with serum iPTH (r =

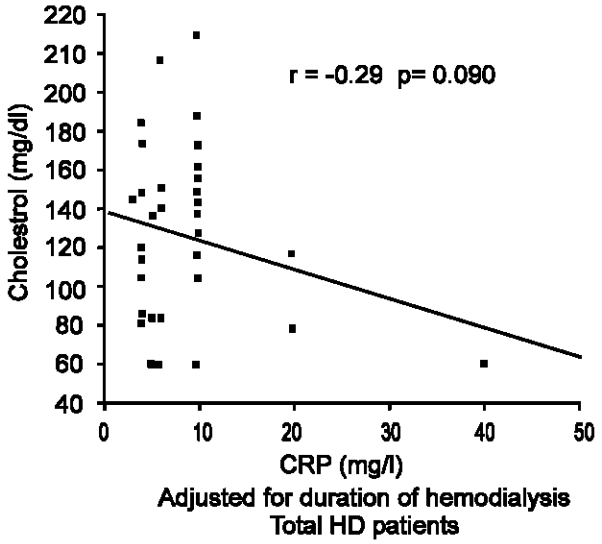


Fig. 4: Near significant inverse correlation of serum CRP with serum cholesterol in total HD patients

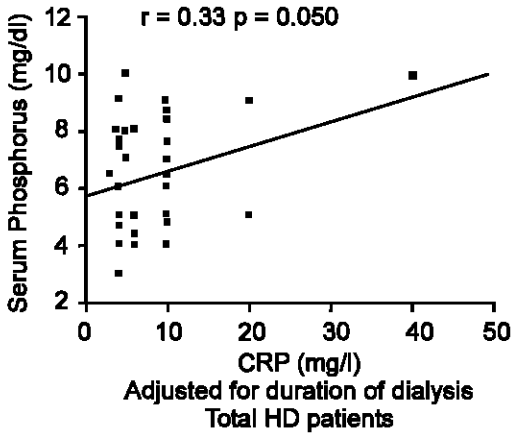


Fig. 5: Significant positive correlation of serum CRP with serum phosphorus

0.68, p=0.030; Fig. 9) (adjusted for dialysis doses), and a near significant inverse correlation of serum CRP with plasma HCO3 (r = -0.52, p=0.054; Fig. 10) (adjusted for duration of dialysis) were found too. More over in this group we found a near significant positive correlation of serum CRP with age (r = 0.50, p=0.065) (adjusted for duration of dialysis). In male HD patients there was a near significant inverse correlations of serum CRP with serum Tg (r = -0.40, p=0.079) (adjusted for age), more over we found that the previous near significant inverse correlation of serum CRP with cholesterol in total patients was significantly negative in this population (r = -0.45, p=0.046) (adjusted for dialysis doses), in this group we also found a near significant positive correlation of serum albumin and plasma HCO_3^- (r = 0.42, p=0.065, Fig. 11) (adjusted for dialysis duration). In this study no significant association between serum CRP and serum albumin was seen (p N.S.).

Discussion

In this study we found a near significant difference of CRP between diabetic and non-diabetics of total patients with more values of CRP in diabetics, a significant difference of CRP between diabetic and non-diabetic of female HD patients with more values in diabetics and a significant difference of CRP between males and female of non-diabetic population with more values of CRP in males. we also found inverse correlation of serum CRP with serum cholesterol and triglyceride levels. A significant positive correlation of serum CRP with serum phosphorus and also near significant positive correlations of serum CRP with serum BUN, with serum ALP and serum iPTH, and also with age of patients were found too. Inverse correlations of serum CRP with serum Creat, and with dialysis efficacy and also with plasma HCO_3^- were found too. No significant association between serum CRP and serum albumin was seen. It has recently been recognized evidence of inflammation that predicts serum albumin concentration in dialysis patients (Fernandez-Reyes et al., 2000). Uremic malnutrition and chronic inflammation often coexist in ESRD patients. A recent study by Stevinkel *et al.*, 1999. demonstrated this relationship in advanced chronic kidney disease patients not yet on maintenance dialysis. Of 109 patients with advanced chronic renal failure, 44% had moderate to severe protein-calorie malnutrition, and 32% had signs of inflammation, as determined by elevated CRP concentrations (Stenvinkel *et al.*, 1999). Most importantly, 53% of the patients with malnutrition had signs of inflammation and 72% of the patients with inflammation had signs of malnutrition. A similar association is observed in chronic hemodialysis patients. Qureshi *et al.*, (1998) have demonstrated that chronic hemodialysis patients with severe malnutrition display signs of chronic inflammation (CRP >20 mg/L) approximately four times that of well-nourished chronic hemodialysis patients (Qureshi *et al.*, 1998). Recently, markers of chronic inflammation have also been associated with adverse clinical outcome in chronic renal failure patients. CRP is a significant predictor of mortality as well as morbidity in both chronic hemo- and peritoneal dialysis patients (Ikizler *et al.*, 1999; Zimmermann *et al.*, 1999; Yeun *et al.*, 2000). Similarly, increased levels of proinflammatory cytokines are associated with increased risk of mortality in ESRD patients (Stenvinkel *et al.*, 2002; Bologa *et al.*, 1998). Whereas these two unfavorable conditions independently predispose ESRD patients to increased risk of morbidity and mortality (Warner *et al.*, 2002), when uremic malnutrition and chronic inflammation coexist, they have an exponential relationship to morbidity and mortality, i.e., a small increase in the severity of either condition leads to significant worsening of morbidity and mortality (Caglar *et al.*, 2002) .In a study conducted by

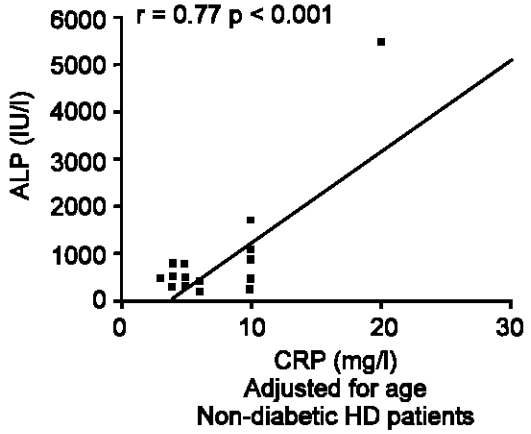


Fig. 6: Significant positive correlation of serum CRP with serum ALP

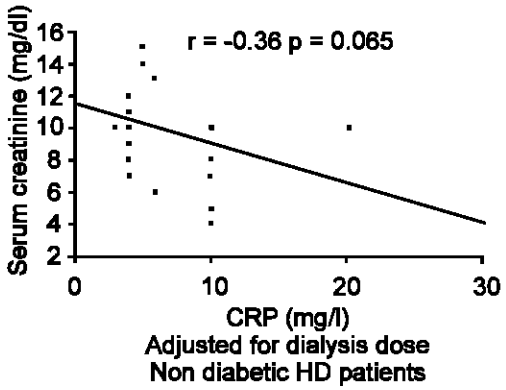


Fig. 7: Near significant inverse correlation of serum CRP with serum Creatinine

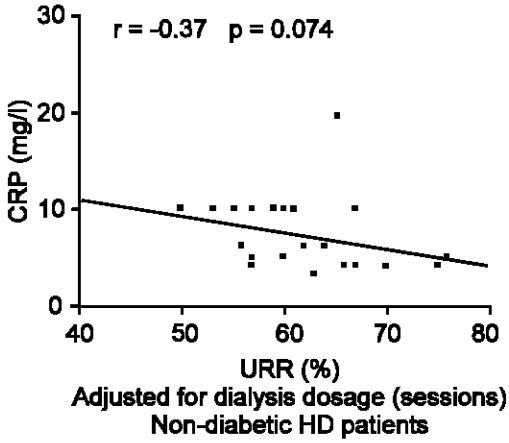


Fig. 8: Near significant inverse correlation of serum CRP with dialysis efficacy as determined by URR.

Fujino et al. on 389 HD patients, significant negative correlations were seen between fat mass changes and CRP was mentioned (Fujino *et al.*, 2005). In our study no significant correlation between serum CRP and serum albumin nether in total nor in subgroups were found .In accordance with our finding, Nascimento *et al.*, 2004 found that baseline C-reactive (CRP) was not correlated with serum albumin level in the group of haemodialysis (HD) patients that they examined (Nascimento *et al.*, 2004) also Tsirpanlis *et al.*, 2005 in a longitudinal study on a similar patient population, reached to similar finding (Warner *et al.*, 2002). In contrast to previous studies Iseki *et al.* (1999) on 163 dialysis patients that divided into two groups according to their baseline CRP levels, with group 1 consisting of CRP<10 mg/l (n=128) and group 2 of CRP ≥10 mg/l (n=35), found significant lower serum albumin in group two Furthermore, the risk of death was significantly higher in group 2, Iseki concluded that CRP is a significant predictor of death in chronic dialysis patients, independent of serum albumin and other possible confounders (Tsirpanlis *et al.*, 2005). In contrast to the finding of this work, Panichi *et al.* (2001) conducted a study on 102 chronic pre-dialysis patients whose mean age was 53±5.8 years with a mean creatinine clearance of 52±37 ml/min. While serum CRP was greater in patients with lower creatinine clearance ,the serum albumin levels were not significantly different between the groups of patients with a creatinine clearance lower than 20 mL/min (n=32) and patients with a creatinine clearance higher than 20 mL/min (n = 70) (Iseki *et al.*, 1999). In hemodialysis patients serum triglyceride and cholesterol are considered as nutritional parameters (Fernandez-Reyes *et al.*, 2000), their inverse correlation with CRP showed the effect of inflammatory state of uremia and its negative effects on lipids (Panichi *et al.*, 2001). To support of our results, Fernandez-Reyes *et al.* (2000) in a cross sectional study which was performed in 64 patients (35 males, 13% diabetics; mean age 64±12), who had beep on HD for 64±58 months showed the correlation of low serum cholesterol and high level of CRP (Fernandez-Reyes *et al.*, 2000). We also showed negative correlation of dialysis adequacy with CRP, which show that in an adequate dialysis, will be accompanied with lower stimulation of proinflammatory mediators. Parathyroid hormone (PTH) promotes IL-6 secretion by osteoblasts, and may also up-regulate IL-6 production in the liver and adipose tissue; this may explain why serum IL-6 is markedly elevated in primary hyperparathyroidism, and low in hypoparathyroidism. IL-6 is the chief stimulus to hepatic production of many acute phase reactants, notably fibrinogen and C-reactive protein as mentioned (Liu *et al.*, 2004). In hemodialysis, hyperphosphatemia is accompanied by uncontrolled secondary hyperparathyroidism(SHPTH) and high serum PTH level, hence association of hyperphosphatemia and high iPTH with serum CRP in this study further support the previous confirmed data (Liu *et al.*, 2004), which explain the need to better control of SHPTH in these patients. In this regard, *in vitro*

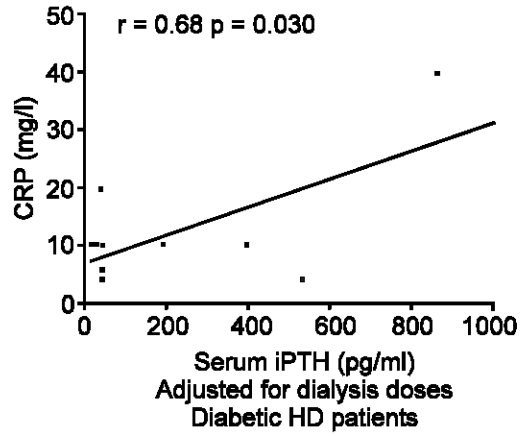


Fig. 9: Significant positive correlation of serum CRP with serum iPTH.

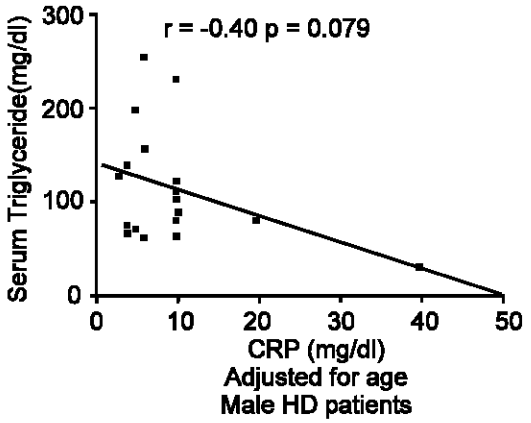


Fig. 10: Near significant inverse correlation of serum CRP with plasma HCO_3

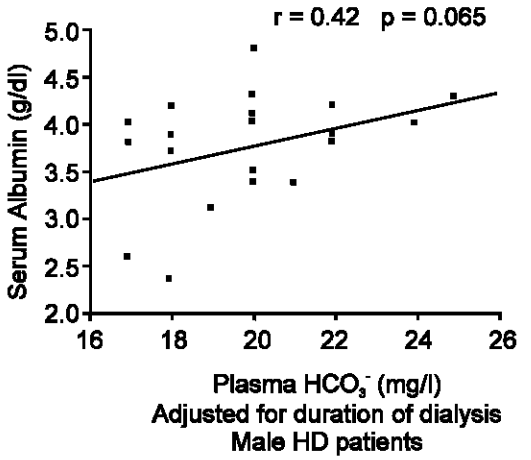


Fig. 11: Near significant positive correlation of serum albumin and plasma HCO_3^-

studies have shown that 1, 25 dihydroxyvitamin D3 [1,25$(OH)_2D_3$] decreases cytokine production by monocytes and lymphocytes. In addition, intravenous or oral pulse calcitriol treatment suppresses interleukin 6 (IL6) and interleukin1beta (IL1 beta) in hemodialysis patients (McCarty, 2005).In this study we also found an inverse association of plasma HCO_3^- with serum CRP. Metabolic acidosis has been suggested to be an important factor responsible for protein and caloric malnutrition (Gannage-Yared *et al*., 2003; Mitch, 1996; Gennari, 1985). In contrast to the metabolic studies, many epidemiologic studies in maintenance dialysis patients have indicated a paradoxically inverse association between mildly decreased serum bicarbonate and improved markers of protein-energy nutritional state (Movilli *et al*., 1998; Kalantar-Zadeh *et al*., 2004). Hence metabolic acidosis may be considered as yet another element of the reverse epidemiology in ESRD patients, to support this finding we could show the positive association of plasma HCO_3^- with serum albumin in our male population. Taken together, we could not show the association of serum albumin with serum CRP level, we showed the positive correlation of SHPTH with inflammation implies further need to control of hyperphosphatemia in HD patients, also inverse correlation of serum CRP with cholesterol and triglyceride further support the malnutrition-inflammation complex syndrome (MICS) which frequently seen in HD patients.

Acknowledgements

We would like to thank Dr. K. Kalantar-Zadeh, Assistant Professor of Medicine of UCLA School of Medicine for sending his articles to our center and his comments .

References

Boag, J.T., 1994. Basic Truths in Optimal Hemodialysis, Dialysis & Transplantation, Volume 23, Number 11, Page 636.

Bologa, R.M., D.M. Levine and T.S. Parker, 1998. Interleukin-6 predicts hypoalbuminemia, hypocholesterolemia, and mortality in hemodialysis patients. Am. J. Kidney Dis., 32: 107-114.

Caglar, K., R.M. Hakim and T.A. Ikizler, 2002. Approaches to the reversal of malnutrition, inflammation, and atherosclerosis in end-stage renal disease. Nutr. Rev., 60: 378-87.

Cianciaruso, B., G. Brunori, J.D. Kopple, G. Traverso, G. Panarello, G. Enia, P. Strippoli, A. De Vecchi, M. Querques, G. Viglino, E. Vonesh and R. Maiorca, 1995. Cross-sectional comparison of malnutrition in continuous ambulatory peritoneal dialysis and hemodialysis patients. Am. J. Kidney Dis., 26: 475-486.

Clyne, B. and J.S. Olshaker, 1999. The C-reactive protein. J. Emerg. Med., 17: 1019 -1025.

Enia, G., C. Sicuso, G. Alati and C. Zocalli, 1993. Subjective global assessment of nutrition in dialysis patients. Nephrol. Dial. Transplant., 8: 1094-1098.

Fernandez-Reyes, M.J., F. Alvarez-Ude, R. Sanchez, C. Mon, P. Iglesias and A. Vazquez, 2000. Nutritional status, comorbidity, and inflammation in hemodialysis. Nefrologia, 20: 540-9.

Friedewald, W.T., R. Levy and D.S. Fredrickson, 1972. Estimation of the concentration of Low-density lipoprotein cholesterol in plasma without use of the preparative ultracentrifuge. Clin. Chem., 18: 799-502.

Fujino, Y., E. Ishimura, S. Okuno, N. Tsuboniwa, K. Maekawa and T. Izumotani, 2005. C-reactive protein is a significant predictor of decrease in fat mass in hemodialysis patients. Biomed. Pharmacother., 13.

Gannage-Yared, M.H., M. Azoury, I. Mansour, R. Baddoura, G. Halaby and R. Naaman, 2003. Effects of a short-term calcium and vitamin D treatment on serum cytokines, bone markers, insulin and lipid concentrations in healthy post-menopausal women. J Endocrinol. Invest., 26: 748-53.

Gennari, F., 1985. Acid-base balance in dialysis patients. Kidney Int., 28: 678-688.

Goldwasser, P., N. Mittman, A. Antignani, D. Burrell, M.A. Michel, J. Collier and M.M. Avram, 1993. Predictors of mortality in hemodialysis patients. J. Am. Soc. Nephrol., 3: 1613-1622.

Haubitz, M., R. Brunkhorst, E. Wrenger, P. Froese, M. Schulze and K.M. Koch, 1996. Chronic induction of C-reactive protein by hemodialysis, but not by peritoneal dialysis therapy. Perit. Dial. Int., 16: 158-162.

Ikizler, T.A., R.L. Wingard, J. Harvell, Y. Shyr and R.M. Hakim, 1999. Association of morbidity with markers of nutrition and inflammation in chronic hemodialysis patients: a prospective study. Kidney Int., 55: 1945-1951.

Iseki, K., M. Tozawa, S. Yoshi and K. Fukiyama, 1999. Serum C-reactive protein (CRP) and risk of death in chronic dialysis patients. Nephrol. Dial. Transplant., 14: 1956-1960.

Kalantar-Zadeh, K., R. Mehrotra, D. Fouque and J.D. Kopple, 2004. Metabolic acidosis and malnutrition-inflammation complex syndrome in chronic renal failure. Semin Dial., 17: 455-65.

Kim, B.S., D.S. Jeon, M.J. Shin, Y.O. Kim, H.C. Song and S.H. Lee, 2005. Persistent Elevation of C-Reactive Protein May Predict Cardiac Hypertrophy and Dysfunction in Patients Maintained on Hemodialysis. Am. J. Nephrol., 29: 25: 189-195.

Leavey, S.F., R.L. Strawderman, C.A. Jones, F.K. Port and P.J. Held, 1998. Simple nutritional indicators as independent predictors of mortality in hemodialysis patients. Am. J. Kidney Dis., 31: 997-1006.

Lowrie, E.G. and N.L. Lew, 1990. Death risk in hemodialysis patients: The predictive value of commonly measured variables and an evaluation of death rate differences between facilities. Am. J. Kidney Dis., 15: 458-482.

Liu, Y., J. Coresh, J.A. Eustace, J.C. Longenecker, B. Jaar and N.E. Fink, 2004. Association between cholesterol level and mortality in dialysis patients: role of inflammation and malnutrition. JAMA., 28; 291: 451-9.

Marckmann, P., 1988. Nutritional status of patients on hemodialysis and peritoneal dialysis. Clin. Nephrol., 29: 75-78.

McCarty, M.F., 2005. Secondary hyperparathyroidism promotes the acute phase response -- a rationale for supplemental vitamin D in prevention of vascular events in the elderly. Med Hypotheses, 64: 1022-6.

McIntyre, C., I. Harper, I.C. Macdougall, A.E. Raine, A. Williams and L.R. Baker, 1997. Serum C-reactive protein as a marker for infection and inflammation in regular dialysis patients. Clin. Nephrol., 48: 371-374.

Mitch, W.E., 1996. Metabolic acidosis stimulates protein metabolism in uremia. Miner Electrolyte Metab., 22: 62-65.

Morrow, D.A. and P.M. Ridker, 2000. C-reactive protein, inflammation, and coronary risk. Med. Clin. North Am., 84: 149-161.

Movilli, E., R. Zani and O. Carli, 1998. Correction of metabolic acidosis increases serum albumin concentrations and decreases kinetically evaluated protein intake in haemodialysis patients: a prospective study. Nephrol. Dial. Transplant., 13: 1719-1724.

Nascimento, M.M., R. Pecoits-Filho, A.R. Qureshi, S.Y. Hayashi, R.C. Manfro and M.A. Pachaly, 2004. Prognostic impact of fluctuating levels of C-reactive protein in Brazilian haemodialysis patients: a prospective study. Nephrol. Dial. Transplant., 19: 2803-9.

Ortega, O., I. Rodriguez, P. Gallar, A. Carreno, M. Ortiz and B. Espejo, 2002. Significance of high C-reactive protein levels in pre-dialysis patients. Nephrol. Dial. Transplant., 17: 1105-1109.

Panichi, V., M. Migliori, S. De Pietro, M.R. Metelli, D. Taccola and R. Perez, 2000. Plasma C-Reactive Protein in Hemodialysis Patients: A Cross-Sectional, Longitudinal Clinical Survey. Blood Purification, 18: 30-36.

Panichi, V., M. Migliori, S. De Pietro, D. Taccola, A.M. Bianchi and M. Norpoth, 2001. C reactive protein in patients with chronic renal diseases. Ren. Fail., 23: 551-62.

Qureshi, A.R., A. Alvestrand and A. Danielsson, 1998. Factors predicting malnutrition in hemodialysis patients: a cross-sectional study. Kidney Int., 53: 773-782.

Stenvinkel, P., P. Barany, O. Heimburger, R. Pecoits Filho and B. Lindholm, 2002. Mortality, malnutrition, and atherosclerosis in ESRD: what is the role of interleukin-6? Kidney Int., 61: 103-108.

Stenvinkel, P., O. Heimburger and F. Paultre, 1999. Strong association between malnutrition, inflammation, and atherosclerosis in chronic renal failure. Kidney Int. 55: 1899-1911.

Stenvinkel, P., O. Heimburger, B. Lindholm, G.A. Kaysen and J. Bergstrom, 2000. Are there two types of malnutrition in chronic renal failure? Evidence for relationships between malnutrition, inflammation and atherosclerosis (MIA syndrome). Nephrol. Dial. Transplant., 15: 953-60.

Stenvinkel, P., O. Heimburger, B. Lindholm, G.A. Kaysen and J. Bergstrom, 2000. Are there two types of malnutrition in chronic renal failure? Evidence for relationships between malnutrition, inflammation and atherosclerosis (MIA syndrome) Nephrol. Dial. Transplant., 15: 953-960.

Teruel, J.L., R. Marcen, J. Ocana, M. Fernandez-Lucas, M. Rivera, G. Tabernero and J. Ortuno, 2005. Clinical Significance of C-Reactive Protein in Patients on Hemodialysis: A Longitudinal Study. Nephron. Clin. Pract., 25; 100: c140-c145.

Tsirpanlis, G., P. Bagos, D. Ioannou, A. Bleta, I. Marinou and A. Lagouranis, 2005. Serum albumin: a late-reacting negative acute-phase protein in clinically evident inflammation in dialysis patients. Nephrol Dial. Transplant., 20: 658-9.

Warner, C., J. Zimmermann, S. Schwedler and T. Metzger, 2002 Inflammation and cardiovascular risk in dialysis patients. Kidney Int., 61: 99-102.

Wolbink, G.J., M.C. Brouwer, S. Buysmann, I.J. ten Berge and C.E. Hack, 1996. CRP-mediated activation of complement *in vivo*: assessment by measuring circulating complement-C-reactive protein complexes. J. Immunol., 157: 473-9.

Yeun, J.Y., R.A. Levine, V. Mantadilok and G.A. Kaysen, 2000. C-Reactive protein predicts all-cause and cardiovascular mortality in hemodialysis patients. Am. J. Kidney Dis., 35: 469-476.

Zimmermann, J., S. Herrlinger, A. Pruy, T. Metzger and C. Wanner, 1999. Inflammation enhances cardiovascular risk and mortality in hemodialysis patients. Kidney Int., 55: 648-658.

Pakistan Journal of Nutrition 4 (3): 183-186, 2005
ISSN 1680-5194

Survey of Starch Amylose Content in Naked Barley (*H. vulgare*. Nudum)

A. Mohammadkhani
Cereal Research Department, Seed and Plant Improvement Institute, Karaj, Iran

Abstract: Starch of two lines of naked barley were extracted by three different methods of washing. The apparent amylose content were determined with the same standard method which recently has been modified for use with samples of 2-3 seeds of cereal. In the present experiment factorial design (2x3x3) was used. The starch extracted by soaking the seeds overnight in dilute ammonia solution grinding in NaCl solution in a microfuge tube with an appropriate pestle and decanting the starch slurry. Then it was washed in 4M NaCl, SDS and acetone. The results of apparent amylose content of this method statistically were better than the two others. With present selected method the apparent amylose content ranged from 18 to 34% was determined for 145 accessions of naked barley. The lowest amylose content was related to the line of 4062 from Pakistan and the highest one was related to the line of 743 from Turkmenstan. This range is considered sufficiently broad to allow amylose content to be further diversified through the working with more lines and breeding.

Key words: Amylose, starch, cereal seed, naked barley

Introduction

Barley cultivars with a wide range of different characteristics have been developed, including barley with low amylose starch, which has been shown to have interesting properties for human and animal food as well as for industrial uses. Variation in the amounts of amylose can affect the physico chemical and functional properties of starch, which may turn affect its utilization in food products or industrial applications (Kobayashi *et al*., 1986; Yan *et al*., 1993). An excellent freeze/ thaw stability of waxy barley starches has been reported (Bahatty and Rossangel, 1997), indicating that they may become a substitute for chemically modified maize starch, which is presently used in frozen foods. High-amylose starches from hydrogen-bonded, in soluble aggregates, which are suited for use as a source of dietary fibere, whereas waxy starches from clear, stable gels with little retrogradiation and particularly suited for use as thickeners or gel-foaming agents (Whistler, 1984).

Numerous reports have shown that in humans and other monogastric animals, amylose is more slowly digested than amylopectin, so blood glucose and insulin levels are lower after a meal high in amylose, satiety is maintained longer and the next meal is likely to be smaller (Heijnen *et al*., 1995; Holt and Brand Miller, 1995). Increasing the amylose content of the diet is thus likely to be beneficial for many members of society.

A substantial level of genetic variability in amylose content has been reported in both barley (Morrison *et al*., 1986; Salomonsson and Sundberg, 1994) and rice (Sano *et al*., 1986; Nakamura *et al*., 1995) the amylose content varied from 0 to 40%.

The amylose content of starch may be determined in several ways. The most economical and rapid, and hence probably the most commonly used, is based on the colorimetric measurement of the blue amylose-iodine complex. This has been applied widely in rice and wheat breeding programmes (Juliano, 1971; Welsh and Blakeney, 1992; Mohammadkhani *et al*., 1998; Mohammadkhani *et al*., 1999). It is; however, subject to interference from lipids bound to the amylose and true amylose content is determined only following a lengthy defatting process without which it is more correct to describe the result as " apparent" amylose (South and Morrison, 1990).

Here to find a method for the evaluation of apparent amylose in samples of 2-3 grains of barley, we compare three different methods of washing during extraction of starch. The selected method was used to survey naked barley for variation in amylose content that could be applied in plant breeding programmes.

Materials and Methods

Standard samples were maize starch as 0% amylose (waxy), 27% amylose (normal) were supplied through the courtesy of Goodman Fielder Ltd., and 95% amylose (high amylose) was from ICN Biomedicals Inc., (lot number 55172) Aurora, Ohio, USA.

A revised method was necessary for extraction and purification on the starch granules; therefore the factorial design (3x2x3) with three different methods of washing and two lines of naked barley were used with three replication . The indicated methods are as follow:

Method 1: Two to three grains of barley were soaked overnight in 0.2 Mammonia solution, drained, resuspended in 0.5M NaCl and ground in a Waring blender. The slurry was filtered through muslin (approximate pore size 160 um) and centrifuged. The

Factorial ANOVA for the factors: Grand Mean=0.204 Grand Sum =3.670 Total Count =18 Rep. (Var 3: r) with values from 1 to 3. Factor A (Var 1: a) with values from 1 to 3. Factor B (Var 2: b) with values from 1 to 2

Table 1: Mean of absorbance at 620nm of starch

3	1	2	4	Total
1	*	*	0.201	1.204
2	*	*	0.197	1.180
3	*	*	0.214	1.286
*	1	*	0.206	1.235
*	2	*	0.233	1.397
*	3	*	0.173	1.038
*	*	1	0.204	1.837
*	*	2	0.204	1.833
*	1	1	0.209	0.626
*	1	2	0.203	0.609
*	2	1	0.234	0.703
*	2	2	0.231	0.694
*	3	1	0.169	0.508
*	3	2	0.177	0.530

Table 2: Summary analysis of variance for amylose absorbance at 620nm following different extraction of starch

S.O.V	df	MS	F	
Rep.	2	0.515	1.66	n.s
A	2	5.387	17.32	**
B	1	0.0009	0. 0029	n.s
AB	2	0.071	0.54	n.s
Error	10	0.311		
Total	17			

C V : 0. 86%

Table 3: Mean of amylose absorption wavelength at 620 nm resulted from working with different treatment of starch washing and two lines of naked barley

Nm	Treatment
0.206	First method
0.233	Second method
0.173	Third method
0.204	First cultivar
0.204	Second cultivar
0.209	First method x First cultivar
0.203	First method x Second cultivar
0.234	Second method x First cultivar
0.231	Second method x Second cultivar
0.169	Third method x First cultivar
0.177	Third method x Second cultivar

pellet was resuspended and washed in a sequence of 0.1M acetic acid, distilled water, 95% ethanol, distilled water and acetone and dried in a desiccator over silica gel overnight.

Method 2: Two or three grains were soaked overnight 0.2M ammonia solution, drained resuspended in 0.5M NaCl and ground. The slurry was filtered and centrifuged (as above). The pellet was resuspended and washed in a sequence of 4M NaCl, 5% sodium dodecyl sulphate (SDS), distilled water, 95% ethanol and acetone and dried overnight.

Method 3: Two or three grains were soaked overnight 0.5M NaCl and ground. The slurry was filtered and centrifuged (as above). The pellet was resuspended and washed in a sequence of 4M NaCl, 6M NaCl/50% sucrose (105 g NaCl + 300 g sucrose + 300 ml distilled water), distilled water, 2% SDS, distilled water and acetone and dried.

The apparent amylose content were determined with the same standard method which recently was modified (Mohammadkhani *et al.*, 1998) from 100 mg rice starch procedure (Juliano, 1971), therefore 5 mg of starch was weighed to the nearest 0.1 mg into a 25 ml beaker. The starch was dispersed in 1 ml of ethanol and then 2.7 ml of 1M NaOH was added with swirling to improve dispersion. The contents were heated to boiling on a sand bath at 175'C for 15 min during which the starch completely gelatinized. The beaker was cooled and the starch washed 2 to 3 times with distilled water into 25-ml volumetric flask. The flask vortex mixed and then duplicate 2.5-ml samples were taken into separate test tubes, neutralized with 2 ml 0.3N citric acid and 1 ml of fresh iodine solution was added. 14.5 ml distilled water were added and the sample was refrigerated for 20 minutes, then the tubes were vortex mixed and duplicate subsamples were read in the spectrophotometric at 620 nm. The samples covered 145 accession of naked barley was from Iran and the other Countries.

Results

The analysis of variance showed that the main effects of starch extraction (washing method) were significant (Table 2). Among three methods of washing, method 2 showed a statistically better result than the others. When the method 2 of washing with the 5-mg procedure of starch analysis applied to the naked barley considerable variation revealed (Fig. 1). In present work apparent amylose content ranged from 18 to 34% with the highest frequency, including more than about one-seventh of the collection, at 25% (Table 4). The distribution among the lines was approximately normal (Fig. 1). Ten lines of the lowest and ten highest amylose content from working with 145 lines of naked barley with Country of origin were demonstrated in Table 5.

Combination of different concentration of amylose from standard starch with related absorbance wave length were shown (Table 6).

Table 4: Range of amylose% in starch of 145 naked barley lines from different Country

Amylose%	No. line	Country	Amylose%	No. line	Country
19.3-28.8	9	China	23.4-32.6		England
25.4	1	Germany	28.5	1	Egypt
18.8-29.2	16	Pakistan	21.2-31.5	3	Japan
21.3-28.8	17	India	19.2-27.8	8	Iran
20.3-28.8	9	Ethiopia	20.6-33.4	30	Russia
21.1-32.7	20	Unknown	21.1-32.7	14	USA
30.3	1	Sweden	33.9	1	Turkmenstan
19.5-28.9	9	Afghanistan	20.1	1	Australia
			32.7-33.1	2	Moroco

Table 5: Ten lines of Min. and Max. of amylose% from 145 lines of naked barley collection with certificate no. and Country of origin

Amylose%	Line No.	Country	Amylose%	Line No.	Country
31.5	2080	USA	18.3	4062	Pakistan
31.5	5899	Japan	19.2	80173	Iran
31.7	5624	USA	19.3	4206-2	China
31.9	5204	Russia	19.5	4150-2	Afghanistan
32.1	5701	USA	19.8	4078	Pakistan
32.6	587	England	20.1	6022	Australia
32.7	5675	USA	20.3	6049-5	Ethiopia
32.7	5698	USA	20.5	3909-1	Ethiopia
33.4	3978-3	Russia	20.6	3995-1	Russia
33.9	743	Turkmenstan	20.8	7554	Russia

Table 6: Combination of different concentration of amylose from standard starch with related absorbance wave length

No.	ml am.27%	ml am.0%	Amylose%	Abs at 620nm
1	2.5	0	0	0.041
2	2	0.5	5.4	0.08
3	1.5	1	10.8	0.127
4	1	1.5	16.2	0.173
5	0.5	2	21.6	0.224
6	0	2.5	27	0.268
no.	ml am.95%	ml am.0%	Amylose%	Abs at 620nm
7	2	0.5	19	0.18
8	1.5	1	38	0.324
9	1	1.5	57	0.462
10	0.5	2	76	0.626
11	0	2.5	95	0.769
no.	ml am.95%	ml am.27%	Amylose%	Abs at 620nm
12	2	0.5	40.5	0.364
13	1.5	1	54.2	0.463
14	1	1.5	67.8	0.565
15	0.5	2	81.4	0.662
16	0	2.5	95	0.77

Amylose% = 131.21 *Abs-5.952

Discussion

A starch extraction method was adapted for use with small (2-3 grain) samples of naked barley. The superiority of the new cleaning method (extraction method 2) was demonstrated in the 13% and 34% higher absorbance values achieved for the similar materials compare with method 1 and 3 respectively.

The ammonia replaced with a weak NaCl left the proteins in a suitable condition to be worked into a ball of gluten during of grounding and sieving stage of starch extraction. The earlier sequence of reagents for washing the starch (method 1), namely acetic acid and ethanol, was similar to the Osborne series (Osbome, 1907) for dissolving protein from wheat, but SDS is well known to be an effective solvent for a wide range of proteins. South and Morrison (South *et al*., 1990).

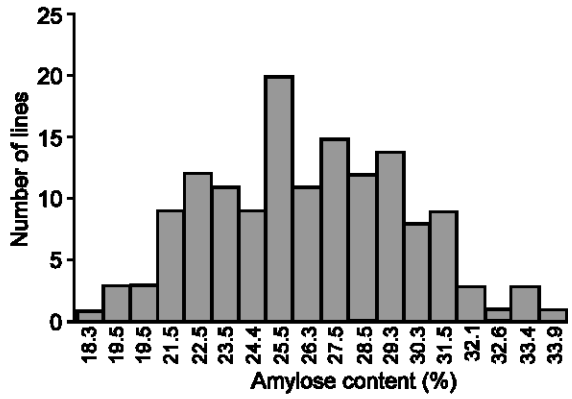


Fig. 1: Frequency distribution of amylose%in naked barley

demonstrated a considerable reduction in the amount of protein bound to starch when it has been washed with SDS. They also showed that washing with SDS and ethanol (as were used in method 2) was an effective way of removing some of the lipids bound to the surface of the starch, although it did not reach the lipids bound within the starch granules.

Fig.1 demonstrated that between the lowest and the highest amylose content of 145 lines of naked barley it was found 89% differences. The distribution among the lines was approximately normal.

References

Bahatty, R.S. and B.G. Rossangel, 1997. Zero amylose lines of hull-less barley. Cereal Chemistry 74: 190-191.

Heijnen, M.L.A., J.M.M. van Amelsvoort and J.A. Westrate, 1995. Interaction between physical structure and amylose:amylopectin ratio of food on postprandial glucose and insulin responses in healthy subjects. Eu. J. Clin. Nutr., 49: 446-457.

Holt, S.A. and J. Brand Miller, 1995. Increased insulin responses to ingested foods are associated with lessened satiety. Appetite, 24: 43-54.

Juliano, B.O.A.,1971. simplified assay for milled-rice amylose. Cereal Science Today, 16: 334-340,360. The method is also published as ISO 6647 by the International Organization for Standardization, ISO.

Kobayashi, S., S.J. Schwartz and D.R. Lineback, 1986. Comprison of the structures of amylopectins from different wheat varieties. Cereal Chem., 63: 71-74.

Mohammadkhani, A., F.L. Stoddard and D.R. Marshall, 1998. Survey of amylose content in Secale cereale, Triticum monococcum, T. turgidum and T.tauschii. J. Cereal Sci., 28: 273-280.

Mohammadkhani, A., F.L. Stoddard, D.R. Marshall, M. Nizam Uddin and X. Zhao, 1999. Starch extraction and amylose analysis from half seeds. Starch, 51, Nr.2-3, S.62-66.

Morrison, W.R., D.C. Scott and J. Karkalas, 1986. Variation in the composition and physical properties of barley starches. Starch, 38: 374-379.

Nakamura, T., M. Yamamori, H. Hirano, S. Hidaka and T. Nagamine, 1995. Production of waxy (amylose-free) wheats. Molecular and General Genetics, 248: 253-259.

Osborne, T.B., 1907. The proteins of the wheat kernel. Publication no.84, Carnegie Institute of Washington, Washington DC, USA.

Salomonsson, A.C. and B. Sundberg, 1994. Amylose content and chain profile of amylopectin from normal, high amylose and waxy barleys. Starch, 46: 325-328.

Sano, Y., M. Katsumata and K. Okuno, 1986. Genetic studies of speciation in cultivated rice. 5. Inter- and intra-specific differentiation in the waxy gene expression of rice. Euphytica, 35: 1-9.

South, J.B. and W.R. Morrison, 1990. Isolation and analysis of starch from single kernels of wheat and barley. J. Cereal Sci., 12: 43-51.

Welsh, L.A. and A.B. Blakeney, 1992. Choosing an amylose standard for cereal starch analysis. In Proceeding of the 42nd Australian Cereal Chemistry Conference, pp: 347-350.

Whistler, R.L., 1984. History and future expectation of starch use. In Starch: Chemistry and Technology, 2nd Edn.'(R.L. whistler, J.N. BeMiller and E.F. Paschall, eds), Academic Press Orlando, FL, U.S.A., pp: 1-9.

Yan, R.C., D.B. Thompson and C.D. Boyer, 1993. Fine structure of amylopectin in relation to gelatinization and retrogradation behavior of maize starches from three wx-containing genotypes in two inbred lines. Cereal Chem., 70: 81-89.

Pakistan Journal of Nutrition 4 (3): 187-196, 2005
ISSN 1680-5194

Chemical Composition and the Effect of Salts on the Food Properties of *Triticum durum* Wholemeal Flour

Emmanuel I. Adeyeye[1]* and Pius A. Aye[2]
[1]Department of Chemistry, University of Ado Ekiti, P.M.B. 5363, Nigeria
[2]Department of Animal Production and Health, University of Ado-Ekiti, P.M.B 5363, Ado-Ekiti, Nigeria
E-mail: eiadeyeye@yahoo.com

Abstract: The proximate composition, nutritionally valuable minerals and the effect of some salts (NaCl, $NaNO_3$, Na_2SO_3, Na_2CO_3, CH_3COONa) on the food properties of *Triticum durum* wholemeal flour were investigated on dry weight basis. Both the protein and carbohydrate values were high with respective values of 20.21g/100g and 65.7g/100g. Many minerals were either not detected or low but phosphorus was high with a value of 4285.71mg/100g. The lowest gelation concentration varied between 2.0 in Na_2CO_3 (0.5% w/v) and 10.0 in CH_3COONa and Na_2SO_3 (15.0% w/v). The highest value of water absorption capacity was 230.33g/100g at 2.0% w/v CH_3COONa. The oil emulsion capacity varied from 20.0g/100g at 1.0% w/v Na_2CO_3 to 80.0g/100g at 0.5%w/v NaCl. The oil emulsion stability (cm^3) was generally good in most of the salts and in all their concentrations with rate of change (cm^3/h) ranging from 1.54 to 3.75 at 24h. The foaming capacity ranged between 4.0% at 20.0% w/v CH_3COONa and 72.0% at 0.5% w/v $NaNO_3$. The foaming stability values were high in $NaN0_3$ and Na_2SO_3 while the rate of change (%min^{-1}) ranged between 0.06 and 1.67 at 1050 minutes. The protein solubility recorded two maxima under the pH and salt concentrations suggesting that the sample might be having two distinct proteins. These results indicated that *T. durum* would be potentially useful in some food formulations.

Key words: *Triticum durum,* chemical composition, food properties

Introduction

Wheat is the world's most widely cultivated plant. It is grown from the equator to 60° N and 40°S, with the greatest concentration in the warm temperate regions where the winters are cool followed by relatively dry and warm summers for ripening and with moderate rainfall between 30 and 90cm (Kochhar, 1986). In West and Central Africa wheat has apparently been grown for several centuries on small irrigated plots during the dry season in the Sudan and Sahel zones. Its importance is increasing with the development of irrigation schemes, particularly in Nigeria and also in the Lake Chad Basin in the Niger, Tchad, Cameroun Republics and in Ghana (Phillips, 1977). All wheats, whether wild or cultivated belong to the genus *Triticum* of the tribe Triticeae in the family Poaceae and subfamily Pooideae (Kochhar, 1986). Of all the wheats, bread wheat, *T. aestivum* L. is by far the most important and widely grown food crop. *T. aestivum* is an hexaploid wheat. Some varieties of *Triticum durum* Desf, used for making macaroni, have yielded well in Nigeria (Phillips, 1977). *T.durum* is a tetraploid wheat (Willis, 1973). The uses of wheat in the baking industry have been enumerated particularly in *T. aestivum* but very little is known about the composition and food properties of *T. durum*. This work aims to draw attention to the nutritional value of *T. durum,* its mineral composition and functional properties. Such information will enhance food composition tables and might lead to more nutrient applications of *T. durum*.

Materials and Methods

Collection and treatment of samples: The wheat grains were collected from the farm located in Zaria, Kaduna State, Nigeria. First of all, the shaft was removed by threshing, sorting of the grains was done to separate bad grains, stones and other non-wheat particles.
The dried whole wheat grains (without shaft) were dry-milled into flour and sieved with a screen mesh of aperture 425 microns. Sieved samples were put in McCartney bottles and kept in the laboratory deep freezer (-10°C) pending analyses.

Analysis of the samples: Moisture, total ash, ether extract and crude fibre were determined by the methods of the Association of Official Analytical Chemists (AOAC) (1990) while nitrogen was determined by the micro-Kjeldahl method described by Pearson (1976) and the percentage nitrogen was converted to crude protein by multiplying by 5.7. Carbohydrate was determined by difference. The minerals were analyzed from solutions obtained by first dry-ashing the wholemeal flour at 450°C and dissolving the ash in 0.1MHCl and transferring to 100 ml standard flask using distilled de-ionized water to make it up. Fe, Zn, Cu, Ni, Na, K, Ca, Mg, Pb and Co were determined by means of atomic absorption spectrophotometer (PYE Unicam Sp 9, Cambridge, UK)

Table 1: Chemical composition of *Triticum durum*

Parameter	Value
Moisture	7.93 ± 0.2 g/100g
Protein	20.21 ± 0.04 g/100g
Crude fat	2.79 ± 0.0 g/100g
Total ash	0.92 ± 0.04 g/100g
Crude fibre	2.46 ± 0.04 g/100g
Carbohydrate	65.70 ± 0.16 g/100g
Metabolizable energy	1564.64 kJ
Iron	4.93 ± 0.1mg/100g
Zinc	2.83 ± 0.02mg/100g
Copper	ND[a]
Nickel	ND
Sodium	19.41± 0.2mg/100g
Potassium	21.67 ± 0.01mg/100g
Calcium	14.79 ± 0.3mg/100g
Magnesium	16.69 ± 0.04mg/100g
Chromium	ND
Manganese	0.24 ± 0.01mg/100g
Lead	0.24 ± 0.02mg/100g
Cobalt	ND
Phosphorus	4285.71 ± 0.5mg/100g

[a]ND. = Not detected

while phosphorus was determined colorimetrically by Spectronic 20 (Gallenkamp, UK) using the phosphovanado molybdate method of AOAC (1990).

Functional properties: The protein solubility was examined from pH 2 - 12 by the method of Adeyeye *et al.* (1994). The sample (0.2g) was thoroughly stirred with distilled water (10ml) at room temperature, and the pH was adjusted using either 0.1M HCl or 0.1M NaOH and a supernatant was obtained whose protein content was determined by the Wiechselboven method (1946). For protein solubility in salt solutions, the salts used were NaCl, $NaN0_3$, Na_2SO_3, Na_2C0_3 and CH_3C00Na, all British Drug Houses products. The concentrations of the various salt solutions used were prepared by weighing 0.5, 1, 2, 5, 10,15 and 20g of the salts which were dissolved in 99.5, 99, 98, 95, 90, 85 and 80g of distilled, deionised water, respectively. Similar weights of 0.2g of sample were used in 10ml salt solution and the supernatant treated as above. The lowest gelation concentration, water absorption and foaming properties of the wheat wholemeal flour were determined using the methods of Sathe *et al.* (1982), replacing water with appropriate salt solutions. The emulsion capacity and stability were determined by the method described by Sathe and Salunkhe (1981). The results were means of triplicate determinations.

Statistical evaluation: The statistical calculations included: percentage value, grand mean, standard deviation, coefficient of variation percent and rate of change as appropriate.

Results and Discussion

Proximate composition: The chemical composition values of *Triticum durum* is shown in Table 1. The moisture content was low thereby affording a longer keeping quality of the flour. The crude fibre and crude fat were also low, but the crude protein and the available carbohydrate were both high. From the values of the proteins, crude fat and the available carbohydrate, the value of 1564.64kJ (1.56MJ) was obtained for the metabolizable energy. The value reported here was generally higher than the values reported for whole wheat grain by Oke and Ojofeitimi (1984) but very close to the value reported in Kilgour (1986). The protein could have been very high because of the likely application of fertilizer. The high metabolizable energy indicated that *T.durum* was a concentrated source of energy. Using a conversion factor of 0.72, the crude fat content of 2.79 was converted to total fatty acids, that is, 2.79 x 0.72 = 2.0g fatty acids (Paul and Southgate, 1978). With these chemical characteristics, *T. durum* will be useful in making pastas or alimentary pastes; macaroni, farfals, noodles and spaghetti.

Minerals: The results of the mineral analysis are also shown in Table 1. Metals not detected in the sample were Cu, Ni, Cr and Co. The following metals were of reasonable levels: Fe, Zn, Na, K, Ca, Mg and Mn. Phosphorus level was very high probably because of the use of fertilizers. Lead is not needed at any level in the body physiology and it is gratifying that its level was low in the current report. Of concern is the availability of Zn and Ca from wheat to the human body for biochemical use. The levels of phytate (287mg/100g), Phy/Zn (43.0), Ca/Phy (1.7) and [Ca][Phy]/[Zn] (0.23) have been reported in the wheat grown in Nigeria (Adeyeye *et al.*, 2000). It was therefore concluded that these minerals would be bioavailable. The consumption of animal protein will also enhance the absorption of iron in the intestine (Bender, 1992).

Functional properties: The water absorption capacity (WAC) values are shown in Table 2. The value of WAC in distilled, deionised water was 140.63% but ranged between 89.29 - 230.33 in the various salt solutions. All the coefficients of variation (CV%) were low. The best salt for the WAC property was CH_3C00Na particularly at 2.0% (w/v) and Na_2C0_3 at 2.0% (w/v) salt concentrations. The values compared favourably with WAC of 138% reported for pigeon pea flour (Oshodi and Ekperigin, 1989); 130% for soy flour, 107.1% for sunflower and 60.2% for wheat flour (Lin *et al.*, 1974); three varieties of melon by Ige *et al.* (1984) having values ranging from 200.0 -288.8%. This means that *T. durum* could be a useful replacement in viscous food formulations such as soups or baked goods.

The values for the lowest gelation concentration (LGC)

Table 2: Water absorption capacity (g/100g) of *T. durum* flour

Concentration of salts (%)[a]	Water absorption capacity							
	CH_3COONa	$NaNO_3$	Na_2SO_3	NaCl	Na_2CO_3	Mean	SD	CV%
0.0	140.63	140.63	140.63	140.63	140.63	140.63	0.0	0.0
0.5	124.66	99.58	117.20	102.27	114.64	111.67	10.5	9.4
1.0	117.70	94.36	104.50	89.29	124.13	106.0	14.9	14.0
2.0	230.33	108.53	112.27	102.71	227.15	156.20	66.3	42.5
5.0	122.07	106.54	98.09	102.04	134.89	112.73	15.4	13.6
10.0	137.83	99.75	124.91	114.59	148.72	125.16	19.2	15.3
15.0	119.45	123.22	137.0	127.93	151.29	131.78	12.7	9.7
20.0	131.35	129.47	123.75	142.82	150.75	135.63	10.9	8.1
Mean	140.50	112.76	119.78	115.29	149.03	-	-	-
SD	37.3	16.49	14.83	19.78	34.19	-	-	-
CV%	26.5	14.62	12.38	17.16	22.94	-	-	-

CV = Coefficient of variation percent, SD = Standard deviation. [a]% = w/v.

Table 3: Lowest gelation concentration (g/100g) *of T. durum* flour

Concentration of salts	Lowest gelation concentration							
	CH_3COONa	$NaNO_3$	Na_2SO_3	NaCl	Na_2CO_3	Mean	SD	CV%
0.0	8.0	8.0	8.0	8.0	8.0	8.0	0.0	0.0
0.5	4.0	6.0	6.0	4.0	2.0	4.4	1.7	38.0
1.0	4.0	6.0	6.0	6.0	4.0	5.2	1.1	21.2
2.0	8.0	6.0	4.0	2.0	4.0	4.8	2.3	47.5
5.0	4.0	6.0	6.0	4.0	6.0	5.2	1.1	47.5
10.0	10.0	4.0	10.0	4.0	6.0	6.8	3.0	44.6
15.0	8.0	4.0	6.0	2.0	4.0	4.8	2.3	47.5
20.0	10.0	8.0	8.0	4.0	6.0	7.2	2.3	31.7
Mean	7.0	6.0	6.8	4.3	5.0	-	-	-
SD	2.6	1.5	1.8	2.0	1.9	-	-	-
CV%	37.4	25.2	27.1	46.6	37.0	-	-	-

Table 4: Oil emulsion capacity (g/100g) of *T. durum* flour in various salt concentrations

Concentration of salts	Emulsion capacity							
	CH_3COONa	$NaNO_3$	Na_2SO_3	NaCl	Na_2CO_3	Mean	SD	CV%
0.0	100	100	100	100	100	100	0.0	0.0
0.5	60	60	68	80	56	64.8	9.6	14.7
1.0	60	72	56	40	20	49.6	20.1	40.6
2.0	80	40	60	60	40	56.0	16.7	29.0
5.0	60	80	60	60	60	64.0	8.9	14.0
10.0	72	56	40	72	60	60.0	13.3	22.1
15.0	80	60	60	60	20	56.0	21.9	39.1
20.0	60	40	56	56	40	50.4	9.6	19.1
Mean	71.5	63.5	62.5	66	49.5	-	-	-
SD	14.6	20.2	17.1	18.0	26.0	-	-	-
CV%	20.4	31.8	27.4	27.3	52.6	-	-	-

are shown in Table 3 for all the salts. The salt free value was 8.0% while the various salt concentration values ranged from 4.0 - 10.0% (CH_3C00Na), 4.0 - 8.0% ($NaNO_3$), 4.0 - 10.0% (Na_2S0_3), 2.0 - 6.0% (NaCl) and 2.0 - 6.0% (Na_2CO_3) showing that the best salt concentrations (w/v) were 0.5%, 1.0%, 2.0% and 15.0% in most of the salts used. Most of these values were lower or within the range of most literature values for leguminous seeds (Oshodi and Ekperigin, 1989; Ige *et al.*, 1984; Adeyeye and Aye, 1998). The variation in the gelling properties of the sample under different salt concentrations and anions might be their different

Table 5: Oil emulsion stability (cm^3) of *T. durum* flour using CH_3C00Na

Time (h) [a]	Salt concentrations (%)[b]										
	0.0	0.5	1.0	2.0	5.0	10.0	15.0	20.0	Mean	SD	CV%
0	5	56	60	60	62	62	60	63	59.8	2.9	4.8
1	12	14	15	17	17	18	18	21	16.5	2.8	16.9
2	12	14	15	16	17	18	18	21	16.4	2.8	16.9
3	12	14	15	16	17	18	18	20	16.3	2.6	15.7
4	12	13	14	16	17	18	17	20	15.9	2.7	17.0
5	12	13	14	16	17	18	17	20	15.9	2.7	17.0
20	11	13	14	15	16	18	17	20	15.5	2.9	18.6
22	11	13	14	15	16	18	17	20	15.5	2.9	18.6
24	11	13	14	15	16	18	17	20	15.5	2.9	18.6
Mean	16.4	18.1	19.4	20.7	21.7	22.9	22.1	25	-	-	-
SD	14.5	14.2	15.2	14.8	15.1	14.7	14.2	14.3	-	-	-
CV%	88.0	78.5	78.3	71.4	69.8	64.1	64.3	57.0	-	-	-
Rate[c]	1.83	1.79	1.92	1.88	1.92	1.83	1.79	1.79	-	-	-

[a]h = hour, [b]% = w/v, [c]Rate of change per hour

Table 6: Oil emulsion stability (cm^3) of *T. durum* flour using $NaNO_3$

Time (h)	Salt concentrations (%)										
	0.0	0.5	1.0	2.0	5.0	10.0	15.0	20.0	Mean	SD	CV%
0	55	63	70	67	67	68	62	68	65.0	4.8	7.5
1	12	28	29	26	21	22	16	20	21.8	5.9	27.0
2	12	27	28	24	20	22	15	20	31.0	5.5	26.3
3	12	27	28	24	20	21	15	20	20.9	5.5	26.4
4	12	26	28	24	20	20	15	20	20.6	5.4	26.0
5	12	26	27	23	20	20	15	20	20.4	5.1	25.0
20	11	26	27	23	20	20	15	19	20.1	5.4	26.6
22	11	26	27	23	20	20	15	19	20.1	5.4	26.6
24	11	26	27	23	20	20	15	19	20.1	5.4	26.6
Mean	16.4	30.6	32.3	28.6	25.3	25.9	20.3	25.0	-	-	-
SD	14.5	12.2	14.1	14.5	15.6	15.8	15.6	16.1	-	-	-
CV%	88.0	39.9	43.7	50.6	61.7	61.1	76.9	64.5	-	-	-
Rate	1.83	1.54	1.79	1.83	1.96	2.0	1.96	2.04	-	-	-

Table 7: Oil emulsion stability (cm^3) of *T. durum* flour using $NaNO_3$

Time (h)	Salt concentrations (%)										
	0.0	0.5	1.0	2.0	5.0	10.0	15.0	20.0	Mean	SD	CV%
0	55	66	72	71	70	69	70	68	67.6	5.4	8.0
1	12	25	36	24	25	23	23	22	23.8	6.5	27.4
2	12	25	36	24	25	23	23	22	23.8	6.5	27.4
3	12	25	36	24	25	23	23	22	23.8	6.5	27.4
4	12	25	36	24	25	23	23	22	23.8	6.5	27.4
5	12	25	36	24	25	23	23	22	23.8	6.5	27.4
20	11	22	35	22	23	22	23	22	22.5	6.4	28.6
22	11	22	35	22	23	22	23	22	22.5	6.4	28.6
24	11	22	35	22	23	22	23	22	22.5	6.4	28.6
Mean	16.4	28.6	39.7	28.6	29.3	27.8	28.2	27.1	-	-	-
SD	14.5	14.1	12.1	16.0	15.3	15.5	15.7	15.3	-	-	-
CV%	88.0	49.4	30.6	55.9	52.1	55.7	55.5	56.6	-	-	-
Rate	1.83	1.83	1.54	2.04	1.96	1.96	1.96	1.92	-	-	-

Table 8: Oil emulsion stability (cm^3) of *T. durum* flour using NaCl

Time (h)	Salt concentrations (%)										
	0.0	0.5	1.0	2.0	5.0	10.0	15.0	20.0	Mean	SD	CV%
0	55	73	70	66	73	71	73	67	68.5	6.1	8.9
1	12	28	25	25	26	25	26	21	23.5	5.0	21.5
2	12	27	25	24	26	25	25	21	23.1	4.8	20.8
3	12	27	25	24	25	25	25	20	22.9	4.8	21.1
4	12	27	24	24	25	24	25	20	22.6	4.7	20.9
5	12	27	24	23	25	24	25	20	22.5	4.7	20.8
20	11	26	24	23	25	24	25	20	22.3	4.9	22.0
22	11	26	24	23	24	24	24	20	22.3	4.9	22.0
24	11	26	24	23	24	24	24	20	22.3	4.9	22.0
Mean	16.4	31.9	29.4	28.3	30.3	29.6	30.2	25.4	-	-	-
SD	14.5	15.4	15.2	14.1	16.0	15.5	16.1	15.6	-	-	-
CV%	88.0	48.3	51.7	49.82	52.8	52.4	53.3	61.4	-	-	-
Rate	1.83	1.96	1.92	1.79	2.04	1.96	2.04	1.96	-	-	-

Table 9: Oil emulsion stability (cm^3) of *T. durum* flour using Na_2CO_3

Time (h)	Salt concentrations (%)										
	0.0	0.5	1.0	2.0	5.0	10.0	15.0	20.0	Mean	SD	CV%
0	55	90	100	100	87	77	73	62	80.5	16.7	20.8
1	12	16	15	10	55	27	28	17	22.5	14.7	65.1
2	12	15	15	10	51	26	27	17	21.6	13.4	61.7
3	12	15	15	10	49	26	27	17	21.4	12.7	59.5
4	12	15	15	10	49	26	27	17	21.4	12.7	59.5
5	12	15	15	10	49	26	27	17	21.4	12.7	59.5
20	11	15	15	10	43	25	26	15	20.0	11.0	55.0
22	11	15	15	10	43	25	26	15	20.0	11.0	55.0
24	11	15	15	10	43	25	26	15	20.0	11.0	55.0
Mean	16.4	23.4	24.4	20.0	52.1	31.4	31.9	21.3	-	-	-
SD	14.5	25.0	28.3	30.0	13.7	17.1	15.4	15.3	-	-	-
CV%	88.0	106.7	115.9	150.0	26.3	54.4	48.4	71.6	-	-	-
Rate	1.83	3.13	3.54	3.75	1.83	2.17	1.96	1.96	-	-	-

Table 10: Foaming capacity (%) of *T. durum* flour in various salt concentrations

Salt	Salt concentrations (%)										
	0.0	0.5	1.0	2.0	5.0	10.0	15.0	20.0	Mean	SD	CV%
CH_3COONa	56.0	38.0	40.0	40.0	18.0	12.0	6.0	4.0	26.8	19.2	71.7
$NaNO_3$	56.0	72.0	58.0	54.0	52.0	26.0	36.0	36.0	48.8	14.9	30.6
Na_2SO_3	56.0	38.0	38.0	28.0	20.0	20.0	22.0	26.0	31.0	12.4	40.1
NaCl	56.0	60.0	60.0	54.0	38.0	44.0	38.0	12.0	45.3	16.2	35.8
Na_2CO_3	56.0	58.0	66.0	74.0	50.0	34.0	12.0	14.0	45.5	23.2	51.0
Mean	56.0	53.2	52.4	50.0	35.6	27.2	22.8	18.4	-	-	-
SD	0.0	14.9	12.6	17.3	16.1	12.4	14.2	12.6	-	-	-
CV%	0.0	28.0	24.1	34.5	45.2	45.5	62.2	68.5	-	-	-

effects on the relative ratios of different constituents - proteins, lipids and carbohydrates (Sathe *et al.*, 1982). The low LGC values of *T. durum* wholemeal flour might likely lead to good setting of stews prepared from it. The CV% of LGC ranged between 21.2 - 47.5 among the various salts but ranged between 25.2 - 46.6 among salt concentrations. Both range values were close showing the results were not seriously varied.

The oil emulsion capacity varied from 20.0% in Na_2CO_3 at concentrations 1.0% and 15.0% (w/v) to 80% in NaCl (0.5% w/v), $NaNO_3$ (5.0% w/v) and CH_3C00Na (2.0 and 15.0% w/v). The results in Table 4 showed that emulsion

Table 11: Foaming stability (%) of *T. durum* flour using CH_3COONa

Time (min)[a]	Salt concentrations (%)										
	0.0	0.5	1.0	2.0	5.0	10.0	15.0	20.0	Mean	SD	CV%
0.0	100	100	100	100	100	100	100	100	100.0	0.0	0.0
30	64.3	26.3	25.0	60.0	66.7	50.0	33.3	50.0	47.0	16.8	35.7
60	35.7	5.3	20.0	40.0	33.3	33.3	33.3	0.0	28.7	12.0	41.9
90	7.1	5.3	20.0	40.0	33.3	33.3	33.3	0.0	24.6	13.9	56.6
120	7.1	5.3	15.0	10.0	33.3	33.3	33.3	0.0	19.6	13.2	67.0
150	7.1	5.3	15.0	10.0	33.3	33.3	33.3	0.0	19.6	13.2	67.0
180	7.1	5.3	15.0	10.0	33.3	33.3	33.3	0.0	19.6	13.2	67.0
1,020	7.1	0.0[b]	10.0	0.0	0.0	0.0	0.0	0.0	nd[c]	nd	nd
1,050	7.1	0.0	10.0	0.0	0.0	0.0	0.0	0.0	nd	nd	nd
Mean	27.0	21.8	25.6	38.6	47.6	45.2	42.9	75.0	-	-	-
SD	33.8	35.4	28.3	33.4	26.2	24.9	25.2	35.4	-	-	-
CV%	125.4	162.2	110.8	86.5	55.1	55.1	58.8	47.2	-	-	-
Rate	0.09	0.53	0.09	0.50	0.37	0.37	0.37	1.67	-	-	-

[a]min = minute. [b]O = Not used in calculating Mean, SD, CV% and Rate. [c]nd = Not determined.

Table 12: Foaming stability (%) of *T. durum* flour using $NaNO_3$

Time (min)	Salt concentrations (%)										
	0.0	0.5	1.0	2.0	5.0	10.0	15.0	20.0	Mean	SD	CV%
0.0	100	100	100	100	100	100	100	100	100	0.0	0.0
30	64.3	63.9	58.6	66.7	11.5	36.5	27.8	27.8	44.6	21.3	47.7
60	35.7	16.7	58.6	66.7	11.5	23.1	16.7	27.8	35.8	23.7	66.0
90	7.1	16.7	58.6	33.3	11.5	23.1	16.7	27.8	24.4	16.2	66.7
120	7.1	16.7	41.4	14.8	11.5	23.1	16.7	27.8	19.9	10.8	54.3
150	7.1	16.7	37.9	14.8	11.5	23.1	16.7	27.8	19.5	9.8	50.5
180	7.1	16.7	37.9	14.8	11.5	23.1	16.7	27.8	19.5	9.8	50.5
1,020	7.1	16.7	37.9	14.8	11.5	23.1	16.7	27.8	19.5	9.8	50.5
1,050	7.1	16.7	37.9	14.8	11.5	23.1	16.7	27.8	19.5	9.8	50.5
Mean	27.0	31.2	49.9	37.9	21.4	33.1	27.2	35.8	-	-	-
SD	33.8	30.2	23.1	32.0	29.5	25.5	27.6	24.1	-	-	-
CV%	125.4	96.8	46.3	84.6	138.0	76.9	101.5	67.2	-	-	-
Rate	0.09	0.08	0.06	0.08	0.08	0.07	0.08	0.07	-	-	-

capacity depended mostly on salt concentration and the type of salt under consideration; NaCl, $NaNO_3$ and CH_3C00Na favoured good emulsion capacity property while Na_2CO_3 antagonized emulsion formation. The Table also showed that the CV% were highly varied at both the horizontal and the vertical levels. However the current report was better than 11.0% reported for wheat flour and 18.0% for soy flour (Lin *et al.,* 1974), hence, *T. durum* might be useful in the production of sausages, soups and cakes (Altschul and Wilcke, 1985).

The oil emulsion stability (OES) (cm^3) of *T. durum* wholemeal flour are shown in Table 5 (CH_3C00Na), 6 ($NaN0_3$), 7 (Na_2S0_3), 8 (NaCl) and 9 (Na_2C0_3) between 0.5% - 20.0% salt concentrations and stability period of 24h. The capacity of protein to aid the formation and stabilization of emulsions is important for many applications in cake batters, coffee whiteners, milks, mayonnaise, salad dressings, comminuted meats and frozen desserts (Kinsella *et al.,* 1985). The OES values were best in Na_2S0_3, Na_2C0_3 and NaCl in decreasing order respectively at various salt concentrations. The enhanced OES for some of the salt concentrations might be due to the fact that the oil binding domain had been more exposed. The change in OES as time increased (as shown in the CV%) was high in all the salts with values in CH_3COONa (57.0 - 78.5), $NaNO_3$ (39.9 - 76.9), Na_2CO_3 (30.6 - 56.6), NaCl (48.3 - 61.4) and Na_2CO_3 (26.3 - 150.0). Also the rate of change in cm^3/h was high in all the salts with values in CH_3C00Na (1.79 - 1.92), $NaNO_3$ (1.54 - 2.04), Na_2SO_3 (1.54 - 2.04), NaCl (1.79 - 2.04) and Na_2C0_3 (1.83 - 3.75). This meant that OES of the Na_2CO_3 was the least stable. The decrease in emulsion stability as time increased, might be due to increased contact leading to coalescence which thereby reduced stability (Parker, 1987). Most of the values for the rate of change here were higher than the values reported for many varieties of African yam bean seeds (AYB) whose results ranged between 0.98 - 1.60 cm^3/h

Table 13: Foaming stability (%) of *T. durum* flour using Na_2SO_3

Time (min)	Salt concentrations (%)										
	0.0	0.5	1.0	2.0	5.0	10.0	15.0	20.0	Mean	SD	CV%
0.0	100	100	100	100	100	100	100	100	100	0.0	0.0
30	64.3	52.6	63.2	85.7	60.0	70.0	70.0	69.2	66.9	9.6	14.4
60	35.7	47.4	57.9	50.0	60.0	60.0	60.0	38.5	51.2	8.0	19.5
90	7.1	47.4	57.9	50.0	50.0	30.0	60.0	15.4	39.7	19.9	50.0
120	7.1	47.4	57.9	50.0	50.0	30.0	20.0	15.4	34.7	19.0	54.8
150	7.1	47.4	57.9	50.0	50.0	0.0	20.0	15.4	31.0	22.7	73.3
180	7.1	47.4	57.9	50.0	50.0	0.0	20.0	15.4	31.0	22.7	73.3
1,020	7.1	10.5	0.0	28.6	0.0	0.0	20.0	0.0	8.3	10.9	131.5
1,050	7.1	10.5	0.0	0.0	0.0	0.0	20.0	0.0	4.7	7.4	157.3
Mean	27.0	45.6	64.7	58.0	60	58.0	43.3	38.5	-	-	-
SD	33.8	26.2	15.7	23.1	18.3	29.5	30.0	33.8	-	-	-
CV%	125.4	57.4	24.3	39.7	30.4	50.9	69.2	87.9	-	-	-
Rate	0.09	0.09	0.23	0.07	0.28	0.58	0.08	0.47	-	-	-

Table 14: Foaming stability (%) of *T. durum* flour using NaCl

Time (min)	Salt concentrations (%)										
	0.0	0.5	1.0	2.0	5.0	10.0	15.0	20.0	Mean	SD	CV%
0.0	100	100	100	100	100	100	100	100	100	0.0	0.0
30	64.3	70.0	66.7	79.3	21.1	18.2	21.1	12.5	44.1	28.2	63.9
60	35.7	70.0	66.7	79.3	21.1	32.6	15.8	12.5	43.1	29.6	68.7
90	7.1	70.0	66.7	29.6	21.1	30.6	15.8	6.3	28.8	25.6	88.8
120	7.1	26.7	23.3	29.6	21.1	13.6	15.8	6.3	17.9	8.7	48.4
150	7.1	26.7	23.3	25.9	15.8	13.6	15.8	6.3	16.8	2.9	17.4
180	7.1	23.3	20.0	25.9	15.8	13.6	15.8	6.3	16.0	7.1	44.1
1,020	7.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	nd	nd	nd
1.050	7.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	nd	nd	nd
Mean	27.0	55.2	52.4	52.8	30.8	26.6	28.6	21.4	-	-	-
SD	33.8	29.7	30.6	32.0	30.6	32.4	31.6	34.8	-	-	-
CV%	125.4	53.8	58.4	60.6	99.3	121.7	110.5	162.3	-	-	-
Rate	0.09	0.43	0.44	0.41	0.47	0.48	0.47	0.52	-	-	-

(Adeyeye and Aye, 1998). This meant that *T. durum* might not be a good oil emulsion stabilizer when compared to AYB.

The oil absorption capacity (OAC) of *T. durum* was 72.44% which was lower than the values obtained for wheat and soy flours (84.2%) and (84.4%) respectively (Lin *et al.,* 1974). Contrary to the absorption of water, *T. durum* flour bound less to oil than water. In this regard, structurally, the *T. durum* wholemeal flour could be more hydrophilic in nature. The OAC obtained for *T. durum* was much lower than the values obtained for many leguminous seeds (Adeyeye and Aye, 1998; Oshodi and Adeladun, 1993; Fagbemi and Oshodi, 1991). OAC is important since oil acts as a flavour retainer and increases the mouth feel of foods (Kinsella, 1976). This meant that *T. durum* flour may not be a good flavour retainer compared with those samples cited from literature.

The foaming capacity (FC) of *T. durum* under various salts and salt concentrations are shown in Table 10. High foaming capacity variation existed between salts and within salt concentrations as depicted by the CV%. The values of FC ranged from 4.0 - 38.0% (CH_3COONa), 26.0 - 72.0% ($NaNO_3$), 20.0 - 38.0% (Na_2SO_3), 12.0 - 60.0% (NaCl) and 12.0 - 74.0% (Na_2CO_3). While the highest FC was reported for Na_2CO_3 at 2.0% (w/v) salt concentration, lowest FC was recorded for CH_3COONa at 20.0% (w/v) salt concentration. Some of the FC values were better than the values earlier reported like the hulled seed flours of AYB (39.91 - 55.43%) and the dehulled AYB seeds (21.34-48.44%) (Adeyeye and Aye, 1998) but much lower than 600.0% reported for sunflower (Lin *et al,* 1974). The high foaming capacities of *T. durum* flour will enhance its functionality in its uses for the production of cakes (Johnson *et al.*, 1979; Lee *et al.,* 1993) and whipping toppings where foaming is an important property (Kinsella, 1979).

The foaming stability (FS) values are shown in Tables

Table 15: Foaming stability (%) of *T. durum* flour using Na_2CO_3

Time (min)	Salt concentrations (%)										
	0.0	0.5	1.0	2.0	5.0	10.0	15.0	20.0	Mean	SD	CV%
0.0	100	100	100	100	100	100	100	100	100	0.0	0.0
30	64.3	24.1	57.6	56.8	48.0	47.1	66.7	57.1	52.7	13.4	25.5
60	35.7	24.1	30.3	32.4	32.0	23.5	66.7	28.6	34.2	13.8	40.3
90	7.1	17.2	24.2	24.3	20.0	23.5	66.7	28.6	26.5	17.5	66.1
120	7.1	17.2	21.2	16.2	20.0	23.5	66.7	14.3	23.3	18.2	78.3
150	7.1	17.2	21.2	16.2	16.0	11.8	50.0	14.3	19.2	13.1	68.1
180	7.1	17.2	15.2	16.2	16.0	11.8	50.0	14.3	18.5	13.1	71.1
1,020	7.1	13.8	15.2	16.2	16.0	11.8	50.0	14.3	18.0	13.2	73.4
1,050	7.1	13.8	15.2	16.2	16.0	11.8	50.0	14.3	18.0	13.2	73.4
Mean	27.0	27.2	33.3	32.7	31.6	29.4	63.0	31.8	-	-	-
SD	33.8	27.6	28.3	28.6	27.8	28.8	16.2	29.3	-	-	-
CV%	125.4	101.3	84.9	87.3	88.2	98.0	25.7	92.2	-	-	-
Rate	0.09	0.08	0.08	0.08	0.08	0.08	0.05	0.08	-	-	-

11 (CH_3C00Na), 12 ($NaNO_3$), 13 (Na_2SO_3), 14 (NaCl) and 15 (Na_2CO_3). The order of decreasing foaming stability among the salts were Na_2CO_3 (rate = 0.05 -0.08% min^{-1}) > $NaNO_3$ (rate = 0.06 -0.08% min^{-1}) > Na_2SO_3 (rate = 0.07 - 0.47% min^{-1}) > NaCl (rate = 0.41 - 0.52% min^{-1}) and CH_3C00Na (rate = 0.09 - 1.67% min^{-1}). The best Na_2CO_3 concentration was 15.0% (w/v), 1.0% (w/v) in $NaNO_3$, 15.0% (w/v) in Na_2SO_3, 2.0% (w/v) in NaCl and 1.0% (w/v) in CH_3COONa. The values of FS% at the end of two hours period had been reported in literature for some legumes. The FS% for hulled AYB seeds ranged between 43.3 - 42.5 (Adeyeye and Aye, 1998), soy flour (14.6%) and sunflower flour (9.0%) (Lin *et al.,* 1974) and pigeon pea (20.0%) (Oshodi and Ekperigin, 1989), most of our results were greater than these quoted values: however our values were lower than 91.0% reported for raw cowpea flour (Padmashree *et al.,* 1987) for the same time interval. Foam stability is important since success of a whipping agent depends on its ability to maintain the whip as long as possible. As seen in Tables 11 - 15, the type of salts and their concentrations have a lot of influence on the foam stability of *T. durum.*

The results of the pH effects on the protein solubility of *T. durum* are depicted in Fig. 1. The sample flour showed maximum protein solubility in both acidic and basic regions of the pH. The isoelectric point (pI) was pH 7 at 6.0% protein solubility. Highest solubility at acidic region was 10.0% (pH 5) while it was 8.0% (pH 9-10) at basic region. Although the solubility values were low, the fact that the sample was soluble at both acid and basic regions meant that the *T. durum* flour might be useful in the formulation of acid foods such as protein rich carbonated beverages and milk analogue products (Kinsella, 1979; Cherry, 1981). The minimum solubilities (pI) were recorded for various salts at various concentrations, viz: $NaNO_3$, 5.6% (0.5% w/v); Na_2SO_3, 6.0% (1.0% w/v); NaCl, 4.0% (0.5%, 2.0%,20% w/v), Na_2C0_3, 1.2% (15% w/v) and CH_3COONa, 0.40% (0.5%, 1.0% w/v). For the maximum solubility, values were also as varied as the above, viz: $NaNO_3$, 23.9% (15.0% w/v); Na_2SO_3, 35.9% (5.0% w/v); NaCl, 35.9% (1.0% w/v); Na_2CO_3, 16.0% (0.5% w/v) and CH_3COONa, 12.0% (20.0% w/v). The lyotropic series here could therefore be in the order: $SO_3^{2-} > Cl^- > NO_3^- > CO_3^{2-} > CH_3COO^-$ (Fig. 2). Shen (1981) studied the effect of various neutral salts on the solubility of soy proteins.

Globulins solubility is dependent upon salt concentration. The effect of salts in increasing the solubility of globulins is called the "salting - in" effect. The solubility is a function of the ionic strength, which is readily calculated from the molar concentrations of the ions and their charge, using the expression

$$\mu = \tfrac{1}{2} \sum mZ^2$$

where μ is the ionic strength, m the molarity and Z the charge of the ion, the $\sum$ denotes that the mZ^2 terms are added for each of the ions (White *et al.,* 1973) (see Table 16). The Table shows that the sample was generally more soluble between 0.5% -5% (w/v) of salt concentration.

The solubility in most of the salt solutions was better than in pH. However, there was general decrease in solubility for most of the salts particularly at concentrations 15.0 - 20.0% (w/v), this might be due to sample denaturation. This is also explained by the "salting-out" phenomenon where proteins are precipitated from aqueous solution by high concentrations of neutral salts. Di - and trivalent ions are more effective than univalent ions. This is seen in the results of Na_2S0_3 and Na_2C0_3. However, in the case of CH_3COO^-, which might be a water-structure-enhancing ion here (Kinsella *et al.,* 1985), the solubility was low in virtually all the solutions indicating that CH_3COO^- has only a positive effect on the hydrophobic interactions. As shown in pH effects and the salts effects, each graph showed at least two distinct peaks showing that *T. durum* might be having two distinct proteins.

Table 16: Various salts concentration (percentage and molarity) and ionic strength (μ)

Salt	Percentage concentration	Molarity	Ionic strength
NaCl	0.5	8.2×10^{-4}	8.5×10^{-4}
NaCl	1.0	1.7×10^{-3}	1.7×10^{-3}
NaCl	2.0	3.4×10^{-3}	3.4×10^{-3}
NaCl	5.0	8.1×10^{-3}	8.1×10^{-3}
NaCl	10.0	1.5×10^{-2}	1.5×10^{-2}
NaCl	15.0	2.2×10^{-2}	2.2×10^{-2}
NaCl	20.0	2.7×10^{-2}	2.7×10^{-2}
$NaN0_3$	0.5	5.9×10^{-4}	5.9×10^{-4}
$NaN0_3$	1.0	1.2×10^{-3}	1.2×10^{-3}
$NaN0_3$	2.0	2.3×10^{-3}	2.3×10^{-3}
$NaN0_3$	5.0	5.6×10^{-3}	5.6×10^{-3}
$NaN0_3$	10.0	1.1×10^{-2}	1.1×10^{-2}
$NaN0_3$	15.0	1.5×10^{-2}	1.5×10^{-2}
$NaN0_3$	20.0	1.9×10^{-2}	1.9×10^{-2}
Na_2S0_3	0.5	3.9×10^{-4}	1.2×10^{-3}
Na_2S0_3	1.0	7.9×10^{-4}	2.4×10^{-3}
Na_2S0_3	2.0	1.6×10^{-3}	4.8×10^{-3}
Na_2S0_3	5.0	3.8×10^{-3}	1.14×10^{-2}
Na_2S0_3	10.0	7.1×10^{-3}	2.14×10^{-2}
Na_2S0_3	15.0	1.01×10^{-2}	3.03×10^{-2}
Na_2S0_3	20.0	1.3×10^{-2}	3.9×10^{-2}
Na_2CO_3	0.5	4.7×10^{-4}	1.41×10^{-3}
Na_2CO_3	1.0	9.3×10^{-4}	2.8×10^{-3}
Na_2CO_3	2.0	1.8×10^{-3}	5.4×10^{-3}
Na_2CO_3	5.0	4.5×10^{-3}	1.35×10^{-2}
Na_2CO_3	10.0	8.5×10^{-3}	2.6×10^{-2}
Na_2CO_3	15.0	1.2×10^{-2}	3.6×10^{-2}
Na_2CO_3	20.0	1.51×10^{-2}	4.5×10^{-1}
CH_3C00Na	0.5	6.1×10^{-4}	6.1×10^{-4}
CH_3C00Na	1.0	1.2×10^{-3}	1.2×10^{-3}
CH_3C00Na	2.0	2.4×10^{-3}	2.4×10^{-3}
CH_3C00Na	5.0	5.8×10^{-3}	5.8×10^{-3}
CH_3C00Na	10.0	1.1×10^{-2}	1.1×10^{-2}
CH_3C00Na	15.0	1.6×10^{-2}	1.6×10^{-2}
CH_3C00Na	20.0	1.95×10^{-2}	1.95×10^{-2}

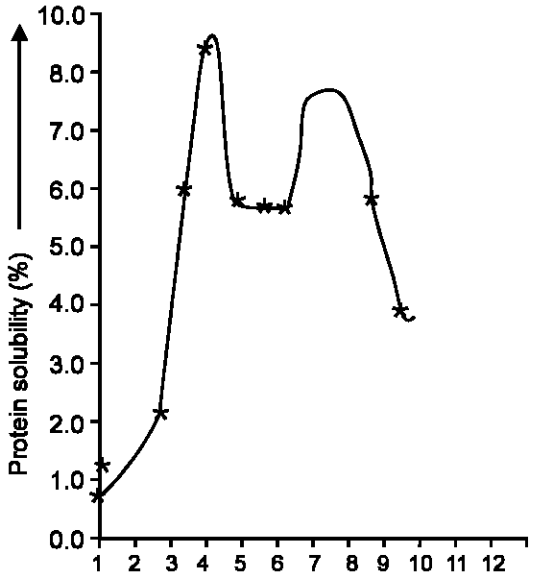


Fig. 1: Protein solubility of Triticum durum as a functionof pH --------->

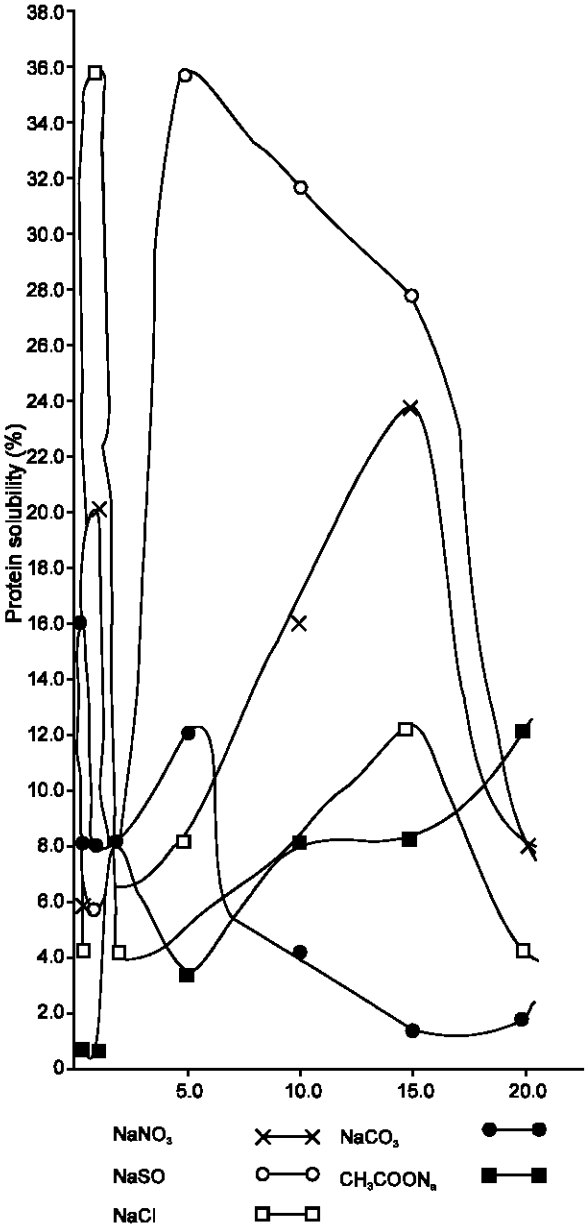


Fig. 2: Protein solubility of *Triticum aestivum* as a function of salts concentrations (%)

Conclusion: *Triticum durum* is a good source of proteins and carbohydrates with a good value of metabolizable energy and average mineral supply having a good Na-K ratio. The following functional properties were also favourable: water absorption capacity, lowest gelation concentration, oil emulsion capacity and stability, foaming capacity and stability, oil absorption capacity and protein solubility, making it potentially useful in many food formulations.

References

Adeyeye, E.I. and P.A. Aye, 1998. The effects of sample preparation on the proximate composition and the functional properties of the African yam bean *(Sphenostylis stenocarpa* Hochst ex A. Rich) flours. Note 1. La Rivista Italiana Delle Sostanze Grasse, LXXV - Maggio: 253 - 261.

Adeyeye, E.I., L.A. Arogundade, E.T. Akintayo, O.A. Aisida, and P.A. Alao, 2000 Calcium, zinc and phytate interrelationships in some foods of major consumption in Nigeria. Food Chem., 71: 435-441.

Adeyeye, E.I., A.A. Oshodi and K.O. Ipinmoroti, 1994. Functional properties of some varieties of African yam bean *(Sphenostylis stenocarpa)* flour II. Int. J. Food Sci. Nutr., 45: 115-126.

Altschul, H.M. and H.L. Wilcke, 1985. New protein foods, Volume 5, Seed storage proteins. New York Academic Press, pp: 107-179.

A.O.A.C., 1990. Official Methods of Analysis. 15th edition. Association of Official Analytical Chemists, Washington, DC, pp: 222-245.

Bender, A., 1992. Meat and Meat Products in Human Nutrition in Developing Countries. FAO Food and Nutrition Paper 53, FAO/UN, Rome, pp: 3-54.

Cherry, J.P., 1981. Protein functionality in foods. American Chemical Society Symposium Series 147, Washington DC, pp: 1-19.

Fagbemi, T.N. and A.A. Oshodi, 1991. Chemical composition and functional properties of full-fat fluted pumpkin seed flour *(Telfairia occidentalis)*. Nig. Food J., 9: 26-32.

Ige, M.N., A.O. Ogunsua and O.L. Oke, 1984. Functional properties of the proteins of some Nigeria oil seeds: Conophor seeds and three varieties of some Nigerian oil seeds. J. Agri. Food Chem., 32: 822-825.

Johnson, L.A., E.F. Havel and R.S. Hoseney, 1979. Bovine plasma as a replacement for egg in cakes. Cereal Chemistry, 56 : 339-343.

Kilgour, O.F.G., 1986. Mastering nutrition. Macmillan Education Ltd., London, pp: 106-132.

Kinsella, J.E., 1976. Functional properties of protein foods. Critical Reviews in Food Science and Nutrition, 1: 219-229.

Kinsella, J.E., 1979. Functional properties of soy proteins. J. Am. Oil Chem. Soc., 56: 242-258.

Kinsella, J.E., S. Damodaran and B. German, 1985. Physicochemical and functional properties of oilseed proteins with emphasis on soy proteins. In: New Protein Foods, Vol. 5. A. M. Altschul and H. L.Wilcke (eds.). Academic Press, New York, pp: 107-179.

Kochhar, S.L., 1986. Tropical Crops - a textbook of economic botany. Macmillan India Ltd., London, pp: 56-109.

Lin, M.J.Y., E.S. Humbert and F.W. Sosulski, 1974. Certain functional properties of sun flower meal products. J. Food Sci., 39: 368-370.

Lee, C.C., J.A. Love and L.A. Johnson, 1993. Sensory and physical properties of cakes with bovine plasma products substituted for egg. Cereal Chem., 70: 18-23.

Oke, O.L. and E.O. Ojofeitimi, 1984. Nutrition for nurses. Churchill Livingstone, London, pp: 89-103.

Oshodi, A.A. and M.O.A. Adeladun, 1993. Proximate composition, some nutritionally valuable minerals and functional properties of three varieties of lima bean *(Phaseolus lunatus* L.). Int. J. Food Sci. Nutr., 43: 181- 185.

Oshodi, A.A. and M.M. Ekperigin, 1989. Functional properties of pigeon pea *(Cajanus cajan)* flour. Food Chemistry, 34: 187-191.

Padmashree, T.S., L. Vijayalashmi and S. Puttaraj, 1987. Effect of traditional processing on the functional properties of cowpea *(Vigna catjang)* flour. J. Food Sci. Tec., 24: 221-225.

Parker, N.S., 1987. Properties and functions of stabilizing agents in food emulsions. Cr. Rev. Food Sci. Nutr., 25: 112-120.

Paul, A.A. and D.A.T. Southgate, 1978. McCance and Widdowson's The composition of foods, 4th edition In: Food composition data production, management and use, H. Greenfield and D.A.T. Southgate (eds.). Elsevier Applied Science, London, pp: 227-228.

Pearson, D., 1976. Chemical analysis of foods, 7th edition. J. & A. Churchill, London: 204-239.

Phillips, T.A., 1977. An agricultural notebook. Longman Group Ltd., London, pp: 34-36.

Sathe, S.K. and D.K. Salunkhe, 1981. Functional properties of the great northern bean *(Phaseolus vulgaris L.)* proteins: emulsion, foaming, viscosity and gelation properties. J. Food Sci., 46: 71-76.

Sathe, S.K., S.S. Deshpande and D.K. Salunkhe, 1982. Functional properties of lupin seed *(Lupinus mutabilis)* proteins and protein concentrates. J. Sci., 47: 491-497.

Shen, J.L., 1981. Solubility and viscosity. In: Protein functionality in foods, J.P. Cherry (ed.). American Chemical Society Symposium Series 147, Washington DC, pp: 89-109.

White, A., P. Handler and E.L. Smith, 1973. Principles of Biochemistry, 5th edition. McGraw Hill, Kogakusha Ltd., Tokyo, pp: 118-126.

Wiechselboven, T.E., 1946. Biuret method of protein determination. Am. J. Clin. Path., 16 : 40-44.

Willis, J.C., 1973. A dictionary of the flowering plants and ferns, 8th edition. Cambridge University Press, London.