Nutritional and toxicological evaluation of double starter cultured fermented *Canavalia ensiformis* in mice

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Abstract

The nutritional value and toxicological effects of seeds of *Canavalia ensiformis*, an under-utilized tropical leguminous plant and a possible alternative protein source was investigated. The seeds were pre-treated before fermenting with pure double strains inoculum comprising of three sets of bacteria and four sets of moulds. The treatment diets were formulated with crude protein content from all the samples. Additionally, positive (casein) diet and negative (basal) diet serves as controls. Each group had four mice. Feeding was for 14 days to evaluate the net protein utilization while the toxicological evaluation was by haematological, serum enzymes changes and histopathology. There was significant ($p<0.05$) difference in terms of feed/ nutrient utilization by the mice on the different diets. In all, only diet formulated from *Bacillus subtilis* plus *Pediococcus pentosaceus* fermented sample (0.30 ± 0.01g/mice/day and 79.11 ± 2.37%) was close to the nitrogen intake and net protein of the casein diet (0.83 ± 0.02g/ mice/day and 94.18 ± 4.08%). The serum transferases increased significantly in treatment groups when compared with the casein diet. The PCV and RBC values decreased significantly while the WBC counts increased in all the mice fed with fermented diets except for those on *B. subtilis* plus *P. pentosaceus* fermented sample (46.40% - PCV, 11.40 x 10^6mm^{-3} -RBC). The histopathology of the livers of mice in some treated groups showed moderate to severe hepatic degeneration. In terms of nutritional constituent and assessment of protein quality only diet formulated from *B. subtilis* plus *P. pentosaceus* fermented diet showed great promises.

Introduction

The preference for legume based fermented foods is due to desirable changes in the legumes that include texture, organoleptic characteristics (especially elimination of beany flavours and improvement in digestibility), enhancement in keeping quality of the product, improved safety, absence of toxins, partial and/or complete elimination of antinutritional factors, increase in nutrition and reduced cooking time. The organoleptic characteristics of fermented foods make them more attractive to the consumers than those of raw beans or legumes coupled with its ability to provide adequate amounts of food; energy, protein, and vitamins to the people of poor resource countries (Sahlin, 1999).

Legume based fermented products are used in the human diets for various purposes such as a main course like Tempe and ontjom in Indonesia and Idli in India (Wood, 1998). They also serves as flavouring agent for foods like soy sauce in Japan, kecap in Indonesia and Dawadawa in Central and West Africa; and as soup base like miso in Japan and China.

Underutilized legumes such as jack beans *(C. ensiformis)* have been reported to be rich in protein and could serve as potential protein source, with a high potential as protein replacer (Seena *et al*., 2006). Since nutrition has been defined as food at work in the body, which is everything that happens to food from the time it is eaten until it is used for various functions in the body, it is important to know the possible toxic effects of what animals and human feed on.

To evaluates toxic effects of food substances, various investigations has shown that changes in the haematological values especially packed cell volume (PCV) and haemoglobin concentration (Hbc) plays significant role, hence improvement in diets will surely enhance haematological values under disease free conditions (Aletor and Egberongbe, 1992).

Various studies has been conducted to batten the nutritional value of jack beans using different methods, Oyawoye *et al.* (1998) employed treatments with rumen fluid which composed mainly of bacteria for 48 hours followed by boiling for one

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hour, this treatment resulted in 84.77% reduction of canavanine, an antinutritional factor when compared to the raw unprocessed jack bean. A previous work of Gabriel (2002) also revealed the effectiveness of fermentation using bacteria and fungi which resulted in the reduction of some antinutritional factors (tannin, phytic acid, cyanide and haemaglutinins) and increase in the nutritional content of jack beans. However, the combined effect of the bacteria and fungi when used on jack bean has not been given due attention. Although these molds produce toxin in the process of this solid fermentation, the toxic effect of such has not been previously evaluated.

The present work evaluates the combined effects of starter cultures on jack beans using the following microorganisms: *Alcaligenes faecalis, Bacillus subtilis, Bacillus polymyxa, Pediococcus pentosaceus, Aspergillus niger, Neurospora crassa, Mucor mucedo* and *Penicillium italicum*. The study was to determine the effect of the fermented jack beans on the nutritional performance of albino mice and the effect on biochemical markers, haematology and tissue pathology of mice fed with the fermented jack bean.

Materials and Methods

Experimental site and animals

Four-week old male and female Albino mice obtained from the animal house of the University of Jos Teaching Hospital, Jos, Nigeria were used for the feeding experiment. The mice were provided with clean drinking water and fed ad libitum with standard rat commercial pelleted feed (Vital feed®, Nigeria) until they were five weeks old. They were housed individually in an environment of normal ambient temperature and the lighting period of 13h daily and relative humidity of 30-50% in wire mesh cages at the Nutrition House of the Department of Animal Production and Health, Federal University of Technology, Akure, Nigeria. Ethical conditions governing the conduct of experiments with life animals were strictly adhered to as stipulated by Ward and Elsea (1997) and all animal experiments were conducted in compliance with NIH Guide for Care and Use of Laboratory Animals (pub. No 85-23, Revised 1985). The institution's Ethical Committee for the use of laboratory animals approved the experimental protocol.

Fermentation

The seed coat of clean healthy seeds of *C. ensiformis* were removed by soaking with boiled distilled water for 3 hours and then rinsed with sterile distilled water. The dehulled seeds were dried at 55°C for 48h in the drying cabinet and ground with the Marlex Excella grinder (Kanchan International, Mumbai, Maharashtra 400034, India) and stored in sterile transparent polythene bags, tightened and kept in the deep freezer for further usage. The jack beans were pressure cooked for 40 minutes, inoculated and incubated in controlled temperatures of 30 and 40°C for fermentation by moulds and bacteria respectively, by placing the covered transparent plastic vessel used for pressure cooking in the water bath at these temperatures.

The inoculums were prepared by first performing two successive transfers of the test organisms in Nutrient agar (Fluka; Buchs, Switzerland) and Potato dextrose agar (Difco; Sparks, MD, USA) at 37 and 30ºC for 24 and 72h (bacteria and fungi respectively). The viable culture was inoculated into Nutrient broth (Fluka) and Potato dextrose broth (Difco) at 37 and 30ºC for 16 and 48h, respectively. The broths were then centrifuged at 3000 rpm for 1min with the sediment resuspended in 5 ml of sterile water, to give a viable population of 10^8cfu/ml and 10^6sfu/ml respectively. The suspensions were then used to inoculate prepared jack beans aseptically.

The double strains inoculums comprising pure strains of *Bacillus subtilis* plus *Alcaligenes faecalis, Bacillus subtilis* plus *Bacillus polymyxa, Bacillus subtilis* plus *Pediococcus pentosaceus, Neurospora crassa* plus *Aspergillus niger, Neurospora crassa* plus *Mucor mucedo, Penicillium italicum* plus *Aspergillus niger* and *Penicillium italicum* plus *Mucor mucedo*, were used to inoculate 1 kg sample of oven dried ground jack beans as starter culture. The pH of the substrates were taken using the portable Hanna pH meter and the total titratable acidity were measured by titrating with 0.1M NaOH, at every 48 hours interval for 14 days of solid state fermentation (Steinkraus, 1997). The control was set-up with 1kg oven dried; pressure cooked ground jack beans for 40 minutes, and was aseptically transferred into dry sterile 1-litre flask and covered with non-absorbent cotton wool and aluminum foil for 14 days (Njoku *et al.*, 1990).

Proximate analysis

All the samples (both the fermented and unfermented) were dried in the oven at 55⁰C, ground and sieved. The proximate compositions of the samples such as the moisture, crude protein, total ash, crude fibre and crude fat contents were determined by the AOAC (2000) techniques.

Experimental diets formulation

The diets were formulated based on the method reported by Agbede and Aletor (2005). The diets consisted of: Diet 1(positive control, which had casein as the source of protein): Diet 2 (negative control, which is the basal non-protein): Diet 3 (unfermented jack beans as the source of protein), while Diets 4 to 10 (test diets of the different composition of fermented jack beans), respectively. The baked diets provided 16.12 – 16.23% crude protein, 5% crude fiber and 2.9 kcal digestible energy/g.

Experimental model

After 1 week of physiological adjustment, 40 mice were randomly allocated to each of the ten diets (n = 4 rats per treatment) with their weight ranged between 20 and 24 g. During the experimental period, feed consumption was determined daily as a difference between supply (10 g) and leftovers. Feed was supplied at 24 h interval while the changes in body weights of the mice were recorded using sensitive weighing balance at 2-day interval. Feed intake, protein intake, weight gain, carcass nitrogen and faecal nitrogen were obtained to calculate biological parameters. After the 14-day feeding experiment, performance indices including were daily weight gain, daily feed intake, nitrogen intake and feed: gain ratio were determined. The criteria used in the assessment of the diets were: protein efficiency ratio (PER), net protein utilization (NPU), true digestibility (TD), and biological value (BV). Protein efficiency ratio determination employed the concept introduced by Edem *et al.* (2001). The carcass nitrogen technique of Edem *et al.* (2001) was used to determine NPU and the TD of the dietary nitrogen. The BV was computed by dividing NPU by TD (Edem *et al.*, 2001).

Toxicological investigation

After 14 days of experimental feeding; the mice in each group were anaesthetized with diethyl ether and whole blood was collected by heart puncture, into 2 sets of plastic tubes; one set containing EDTA as anticoagulant and the other set without EDTA for the determination of biochemical parameters. The serum was aspirated and further centrifuged at 3000 rpm for 10 min. The sera were decanted and deep-frozen for serum marker enzymes analyses using appropriate kits at the Department of Biochemistry of the Federal University of Technology, Akure, Nigeria.

The animals were quickly dissected; the liver and kidney were removed into 10% formalin for histopathological analysis. Incisions were made into the skull, thoracic and body cavities. The carcass of each mouse was dried in a hot air-circulating oven (Stuart Scientific HT Oven, England) at 70°C. It was then ground in a mortar and digested in concentrated sulphuric acid. After about 1 hr, the slurry was cooled and made up to 250 ml with distilled water. 25 ml of slurry were taken in triplicate for nitrogen determination (of the carcass) by the Kjeldahl procedure (AOAC, 2000).

Haematological and biochemical analyses

The haematological parameters determined included the packed cell volume (PCV), White blood cell (WBC) and Red blood cell (RBC). The PCV and WBC values were determined according to the procedure of Mirale (1982). The RBC value was determined as described by Schalm *et al.* (1975) using the Neabeauer counting chamber.

The serum albumin was evaluated using the method of Tietz (1999); serum total protein in serum was assayed using direct biuret method (Gornall *et al.*, 1948), while serum bilirubin was determined according to the method of Jendrasik and Grof (1938) using their respective absorbance. The solutions were mixed thoroughly and allowed to stand at room temperature (25 - 27^{0}C) for 10, 20, 15 mins respectively and the absorbance were then read against reagent blank. The globulin concentration was determined by subtracting the albumin value from total protein value.

The activities of Aspartate aminotransferase (AST) was evaluated spectrophotometrically using the method of Piesova *et al.* (2012) according to the description of the Biosystem laboratories, S.A. Barcelona, Spain using Biosystem kits; Alanine aminotransferases (ALT) was measured by monitoring the concentration of pyruvate hydrazone formed with 2,4-dinitrophenyl hydrazine as narrated in Biosystem Kits (Piesova *et al.*, 2012). In the case of determining both the alkaline phosphatases and acid phosphatases, the evaluations were carried out using phenolphthalein monophosphate method of Tietz (1999) as described by Biosystem test kits.

Histopathological analyses

At autopsy the internal organs including the livers and kidneys were examined grossly for any pathological changes. The organs tissues were fixed in 10% formalin for 24 h, dehydrated in ascending gradients of ethanol, cleared in xylene and embedded in paraffin wax for 2 h. The embedded organs were sectioned using microtome and stained with haematoxylin – eosin (Bancroft and Stevens, 1977). All the sections were examined under a light microscope under different (x100 and x 400)

Table 1. Proximate composition of Jack beans (*Canavalia ensiformis,* L) fermented with
double starter cultures (Mean ± SEM)

Fermented Substrates	Moisture (g/100g)	Crude Protein (g/100g)	Fat (g/100g)	Crude Fibre (g/100g)	Ash (g/100g)	Carbohydrate (g/100g)
Bacillus subtilis + *Alcaligenes faecalis*	6.52±0.8 [b]	32.13±1.16 [c]	6.14±0.88 [e]	3.36±0.82 [h]	1.12±0.57 [a]	50.72±1.16 [e]
Bacillus subtilis + *Bacillus polymyxa*	6.68±0.58 [b c]	33.47±5.77 [e]	9.85±0.12 [g]	2.53±0.82 [c]	2.69±0.12 [c]	44.79±8.82 [a]
Bacillus subtilis + *Pediococcus pentosaceus*	7.11±0.12 [c]	34.81±8.82 [f]	6.83±0.58 [f]	3.17±0.77 [g]	1.28±0.12 [b]	46.80±8.82 [b]
Neurospora crassa + *Aspergillus niger*	7.58±0.88 [d]	32.83±8.82 [d]	4.97±0.88 [a]	2.71±0.88 [d]	3.11±0.88 [d]	48.80±5.77 [d]
Neurospora crassa + *Mucor mucedo*	6.21±0.88a	35.90±8.82 [g]	5.63±0.88 [d]	2.83±0.12 [e]	3.19±0.88 [d]	46.23±1.16 [b]
Penicillium italicum + *Aspergillus niger*	10.77±0.12 [f]	29.99±4.04 [b]	5.55±0.58 [c]	1.97±0.88 [a]	2.65±0.58 [c]	50.07±8.82 [e]
Penicillium italicum + *Mucor mucedo*	9.99±0.88 [e]	31.78±8.81 [c]	5.27±0.12 [b]	2.00±0.57 [b]	3.44±0.12 [e]	47.52±5.77 [c]
Pressure cooked unfermented	7.83±0.12 [d]	26.20±6.67 [a]	11.95±0.12 [h]	3.00±0.12 [f]	3.50±0.88 [f]	47.52±6.67 [c]

Values with the same superscript letter(s) down a column are not statistically significantly (P>0.05) different

magnifications with an Olympus 20744 (Tokyo, Japan). Lesions were taken using the Axioskop and TSview imager.

Statistical analysis

The results were presented as means SEM and data were analyzed using one way analysis of variance (ANOVA) procedures of Gomez and Gomez (1984). Statistical difference among the fermented samples and dehulled raw jack beans were established by the Duncan's multiple range tests at 5% level of probability between the attributes in the control and the products. With values of p<0.05being considered statistically significant. SPSS for windows version 17 was used for the statistical analysis.

Results and Discussion

Proximate composition of the starter cultures fermented samples

The proximate compositions of the samples of Jack beans fermented with different combined starter cultures are presented in Table 1. There was a general significant (P<0.05) increase in the crude protein content of the starter cultures fermented substrates when compared to the control that is, the pressure cooked unfermented sample; while the fat and ash contents generally decreased compared to the control. The crude protein of 26.20 mg/100 g of the unfermented jack beans was about 12.64 – 27.02% lower than the crude protein contents of those fermented with the double starter cultures; while the fat and ash contents of the unfermented bean generally reduced by 41.59 – 82.43% and 32.00 – 98.00% respectively. The ash contents also showed a significant (P<0.05) decrease when compared

with the control (3.50 g/100 g), with the fermented substrate of *P. italicum* plus *M. mucedo* having the highest value of 3.44 g/100 g and the substrate of *B. subtilis* plus *A. faecalis* (1.12 g/100 g) with the lowest value. The carbohydrate contents of the beans fermented with *B. subtilis* plus *A. faecalis*, *N. crassa* plus *A. niger* and *P. italicum* plus *A. niger* were significantly (P<0.05) higher than the 47.52 g/100 g of the unfermented bean, those fermented with *B. subtilis* plus *B. polymyxa*, *B. subtilis* plus *P. pentosaceus* and *N. crassa* plus *M. mucedo* had carbohydrate contents significantly (P<0.05) lower than the unfermented bean, while fermentation with *P. italicum* plus *M. mucedo* did not have any effect on the carbohydrate content of jack beans.

Nutrient utilization and assessment of protein quality of the diets

Table 2 showed that mice fed diet fermented with *P. italicum* plus *A. niger* had a daily weight gain of 0.54, daily feed intake of 3.69, and nitrogen intake of 0.17 g/mice/day, with feed: gain ratio of 6.81 while that of *N. crassa* plus *M. mucedo* fermented diet had a daily weight gain of 0.44, daily feed intake of 3.67, and nitrogen intake of 0.20 g/mice/day, with feed: gain ratio of 8.70. These are not significantly different from the positive control that is, the casein diet. The mice on diet fermented with *B. subtilis* plus *P. pentosaceus* had daily weight gain of 0.35, daily feed intake of 7.27, and nitrogen intake of 0.30 g/mice/day, with feed: gain ratio of 20.73, and the one fermented with *B. subtilis* plus *A. faecalis* having daily weight gain of 0.30, daily feed intake of 6.35, and nitrogen intake of 0.23 g/mice/day, with feed: gain ratio of 21.33; were the double bacteria starter cultures inoculated substrates diets that were closer

Table 2. Nutrient utilization and assessment of the protein quality of Jack beans fermented with double starter cultures by albino mice (Mean ± SEM)

Fermented Substrates	Daily weight gain (g/mice/day)	Daily feed intake (g/mice/day)	Feed: Gain ratio	Nitrogen intake (g/mice/day)	Protein Efficiency Ratio	Net Protein Utilization (%)	Biological Value (%)	True Digestibility (%)
Bacillus subtilis + *Alcaligenes faecalis*	0.30±0.01^c	6.35±0.20^d	21.33±0.65^d	0.23±0.01^d	1.50±0.25^e	80.50±3.25^e	78.84±4.56^g	75.27±3.09^d
Bacillus subtilis + *Bacillus polymyxa*	-0.43±0.01^a	3.31±0.04^b	-7.75±0.40^a	0.10±0.00^b	-4.30 ±0.27^a	64.30±2.15^d	68.66±2.58^f	77.15±2.40^e
Bacillus subtilis + *Pediococcus pentosaceus*	0.35±0.02^c	7.27±0.35^e	20.73±0.06^d	0.30±0.01^f	1.17 ±0.12^{cd}	79.11±2.37^e	52.82±1.34^d	80.00±2.16^f
Neurospora crassa + *Aspergillus niger*	-0.56±0.02^a	5.74±0.88^c	-10.59±1.13^a	0.30±0.05^f	-1.87 ±0.14^b	51.50±2.15^a	54.48±2.07^{bc}	55.16±2.59^a
Neurospora crassa + *Mucor mucedo*	0.44±0.04^d	3.67±0.04^b	8.70±0.87^{bc}	0.20±0.01^{cd}	2.22 ±0.18^f	59.17±2.47^c	61.20±2.55^d	55.00±2.24^a
Penicillium italicum + *Aspergillus niger*	0.54±0.01^f	3.69±0.09^b	6.81±0.09^b	0.17±0.01^c	3.18±0.27^g	53.00±2.82^b	65.82±2.38^e	70.29±3.24^c
Penicillium italicum + *Mucor mucedo*	-0.59±0.01^a	5.37±0.94^c	-9.19±0.67^a	0.27±0.04^{ef}	-2.19±0.06^b	53.67±2.46^b	60.38±2.43^d	54.94±2.68^a
Pressure cooked unfermented	0.26±0.01^b	5.52±0.12^c	21.53±1.58^d	0.23±0.01^d	1.31±0.12^d	50.78±3.65^a	36.74±1.58^a	58.04±2.07^b
Basal diet (non-protein) control	-0.54±0.06^a	2.09±0.12^a	-3.28±0.52^a	0.01±0.00^a	ND	ND	ND	ND
Casein diet (protein) control	0.49±0.02^e	5.72±0.19^c	11.73±0.18^c	0.83±0.02^g	0.94±0.12^c	94.18±4.08^f	83.34±3.55^h	74.64±3.14^d

Values with the same superscript letter(s) down a column are not statistically significantly (P>0.05) different.
ND-Not Done.

in values to the positive control (casein) diet.

The protein efficiency ratio of the diets produced from substrates fermented with *B. subtilis* plus *B. polymyxa*, *P. italicum* plus *M. mucedo* and *N. crassa* plus *A. niger* had negative values. The diets produced from substrates with the other double starter cultures had significantly (P < 0.05) higher protein efficiency ratio values when compared to the positive control (Casein) diet (Table 2). The net protein utilization for all the diets produced from substrates fermented with double starter cultures had significant (P < 0.05) reduction in value when compared with the positive control. The NPU of 79.11 and 80.50% recorded for mice on diets produced from substrates fermented with *B. subtilis* plus *A. faecalis* and *B. subtilis* plus *P. pentosaceus*, respectively, were the only closest ones to the NPU of 94.18% for positive control (casein) diet. The biological values of 68.66 and 78.84% obtained from the diets from substrates fermented with *B. subtilis* plus *B. polymyxa* and *B. subtilis* plus *A. faecalis*, respectively, were significantly nearer to the 83.34% for control (casein) diet. There was, however, a generally there was a significant decrease in the biological values when compared to the positive control. The true digestibility values of almost all the diets from substrates fermented with the double starter cultures were significantly lower than that of the control (74.63%), except those of diets produced from substrates fermented with *B. subtilis* plus *B. polymyxa* (77.15%), *B. subtilis* plus *P. pentosaceus*

(80.00%) and *B. subtilis* plus *A. faecalis* (75.27%).

Toxicological assessment

There was no significant (P>0.05) difference in the serum ALP activities of all the mice fed with diets produced from double starter cultures inoculated samples except for those fed with diets from substrates fermented with *B. subtilis* plus *A. faecalis* and *P. italicum* plus *A. niger* (36.85 ± 3.21 IU/L); that were at par with those fed diets consisting the unfermented pressure cooked jack beans which significantly (P<0.05) increased when compared with the positive control (casein) diet (23.03 ± 4.61 IU/L) as revealed in Table 3. The serum ACP activities of mice fed with diets from substrates fermented with *B. subtilis* plus *P. pentosaceus, N. crassa* plus *A. niger* and *P. italicum* plus *M. mucedo* significantly (P<0.05) increased compared to the positive control diet (9.85 ± 1.41 IU/L) and higher than others. All the mice fed with diets produced from substrates fermented with double starter cultures had a significant (P<0.05) increase in their serum ALT levels when compared to the positive control (2.33 ± 0.58 IU/L). Also the serum AST activities of these mice increased significantly (P<0.05) except for those fed with diets from substrates fermented with *B. subtilis* plus *B. polymyxa* (124.75 ± 16.75 IU/L), *B. subtilis* plus *P. pentosaceus* (104.76 ± 10.91 IU/L) and *P. italicum* plus *A. niger* (110.58 ± 5.82 IU/L) which showed no

Table 3. Serum proteins and enzyme activities of albino mice fed with Jack beans fermented with double starter cultures (Mean ± SEM)

Fermented Substrates	ALP (IU /L)	ACP (IU /L)	ALT (IU /L)	AST (IU /L)	Bilirubin (µmol/L)	Albumin (g/L)	Globulin (g/L)	Total Protein (g/L)
Bacillus subtilis + Alcaligenes faecalis	36.85±3.21[b]	11.72±1.69[ab]	11.06±0.58[b]	384.12±17.46[c]	15.00±0.96[d]	15.47±0.94[b]	2.95±0.06[a]	18.42±0.99[a]
Bacillus subtilis + Bacillus polymyxa	23.03±4.61[a]	10.32±1.88[ab]	13.54±1.16[b]	124.75±16.75[a]	5.56±2.08[a]	15.47±0.94[b]	1.98±0.04[a]	17.45±0.98[a]
Bacillus subtilis + Pediococcus pentosaceus	27.64±0.00[ab]	14.07±1.81[b]	9.31±0.58[b]	104.76±10.91[a]	6.12±2.94[ab]	21.81±1.56[c]	35.70±2.58[d]	57.50±4.14[d]
Neurospora crassa + Aspergillus niger	23.03±4.61[a]	14.07±1.81[b]	16.88±1.16[c]	250.26±20.74[b]	11.67±2.89[c]	15.47±0.94[b]	27.51±1.29[c]	42.98±2.23[b]
Neurospora crassa + Mucor mucedo	18.43±4.61[a]	11.25±1.81[ab]	58.20±2.33[e]	424.86±5.82[cd]	8.90±0.56[bc]	30.47±0.62[d]	17.71±0.28[b]	48.18±0.90[c]
Penicillium italicum + Aspergillus niger	36.85±4.61[b]	12.66±1.44[ab]	27.35±1.16[d]	110.58±5.82[a]	6.67±0.96[ab]	17.17±1.07[bc]	2.30±0.21[a]	19.47±1.28[a]
Penicillium italicum + Mucor mucedo	23.03±4.61[a]	14.07±1.41[b]	13.97±1.75[b]	273.54±11.64[b]	8.90±1.11[bc]	19.95±0.46[c]	15.44±0.45[b]	35.40±0.92[b]
Pressure cooked Unfermented	36.85±5.21[b]	9.85±1.41[ab]	2.33±0.58[a]	93.12±15.37[a]	3.89±1.47[a]	27.53±1.68[d]	46.07±1.80[e]	73.60±3.48[e]
Basal diet (non-protein) control	46.07±5.21[c]	7.03±1.41[a]	4.66±1.09[a]	157.14±20.16[a]	6.11±0.56[ab]	2.18±0.17[a]	18.21±1.25[b]	20.40±1.43[a]
Casein diet (protein) control	23.03±4.61[a]	9.85±1.41[ab]	2.33±0.58[a]	116.40±15.40[a]	7.79±2.42[bc]	17.17±1.07[bc]	35.83±1.57[d]	53.00±2.64[d]

KEY: AST-Aspartate amino transferase; ALT-Alanine amino transferase; ALP-Alkaline phosphatase; ACP-Acid phosphatase. Values with the same superscript letter(s) down a column are not statistically significantly (P>0.05) different.

significant (P>0.05) difference in comparison with the positive control (116.40 ± 15.40 IU/L). The serum bilirubin and albumin of all the mice fed with diets produced from substrates fermented with the double starter cultures showed no significant difference, except those fermented with *B. subtilis* plus *P. pentosaceus* (bilirubin-6.12 ± 2.94 µmol/L) and *N. crassa* plus *M. mucedo* (albumin-30.47 ± 0.62 g/L) which showed a significant increase when compared to the positive control (bilirubin-7.79 ± 2.42 µmol/L; albumin-17.17 ± 1.07 g/L). The total protein of the serum of mice fed with the unfermented beans diet (73.60 ± 3.48 g/L) increased significantly compared to all the other diets while those of the diets from the fermented beans were significantly (p < 0.05) lower than that of the diet from the positive casein diet (53.00 ± 2.64 g/L) with the exception of the diet from *B. subtilis* plus *P. pentosaceus* (57.50 ± 4.14 g/L).

The PCV and RBC values of mice fed diets produced from *B. subtilis* plus *A. faecalis, N. crassa* plus *M. mucedo, P. italicum* plus *A. niger,* and *P. italicum* plus *M. mucedo* were significantly (p < 0.05) lower than those fed the unfermented beans, mice fed diets produced from *B. subtilis* plus *P. pentosaceus, N. crassa* plus *A. niger* and basal non-protein had higher PCV and RBC values compared with those fed the unfermented beans. Diet from substrates produced from *B. subtilis* plus *B. polymyxa* had no influence on the PCV.

All the animals fed diets from the fermented substrates had higher WBC counts than those fed with the unfermented beans. The PCV and RBC values of mice fed with diets produced from all the samples fermented with the double starter cultures decreased significantly (Table 4), except for those fed with diet from sample fermented with *B. subtilis* plus *P. pentosaceus* (46.40 ± 0.58%-PCV, RBC-11.4 ± 0.03 x 10^6 mm^{-3}) which showed no significant (p > 0.05) difference in both, when compared with the casein control diet (PCV-41.60 ± 0.58%, RBC-10.32 ± 0.06 x 10^6 mm^{-3}). The WBC counts of mice fed almost all the diets produced from substrates fermented with the double starter cultures showed a significant increase except those from diets from samples fermented with *B. subtilis* plus *B. polymyxa* (12.16 ± 8.56 x 10^6 mm^{-3}), *B. subtilis* plus *P. pentosaceus* (11.02 ± 1.53 x 10^6 mm^{-3}) and *N. crassa* plus *A. niger* (11.02 ± 1.40 x 10^6 mm^{-3}) which showed no significant difference when compared with the control (12.16 ± 1.15 x 10^6 mm^{-3}).

Histopathological analysis

Figures 1 showed that there were observable pathological changes in the livers of all the mice groups fed with diets produced from substrates fermented with *N. crassa* plus *M. mucedo* and *P. italicum* plus *M. mucedo* which showed moderate to severe fatty hepatic degeneration (David and Johnston, 1999; Thapa and Walia, 2007). The livers of animals fed with the other diets shows no visible pathological changes.

Fermentation of *C. ensiformis* with starter cultures generally improved the crude protein contents over

Table 4. Haematological studies on mice fed with Jack beans fermented with double starter cultures (Mean ± SEM)

Fermented Substrates	Packed Cell Volume (%)	Red Blood Cell (x10^6 mm^{-3})	White Blood Cell (x10^6 mm^{-3})
Bacillus subtilis + Alcaligenes faecalis	30.40±1.76[ab]	6.88±0.21[ab]	14.44±1.31[d]
Bacillus subtilis + Bacillus polymyxa	33.60±0.88[bc]	7.74±0.09[ab]	12.16±8.56[c]
Bacillus subtilis + Pediococcus pentosaceus	49.40±0.58[f]	11.4±0.03[e]	11.02±1.53[b]
Neurospora crassa + Aspergillus niger	38.40±0.38[d]	9.25±0.07[c]	11.02±1.40[b]
Neurospora crassa + Mucor mucedo	28.80±0.88[ab]	6.95±0.06[ab]	16.72±2.30[e]
Penicillium italicum + Aspergillus niger	25.60±0.78[a]	5.81±0.08[a]	16.72±2.96[e]
Penicillium italicum + Mucor mucedo	27.20±1.76[a]	6.24±0.19[a]	19.38±2.91[f]
Pressure cooked unfermented	33.60±1.00[bc]	7.74±0.08[ab]	8.36±1.41[a]
Basal diet (non-protein) control	35.20±0.68[bc]	8.17±0.03[bc]	11.40±1.41[bc]
Casein diet (protein) control	41.60±0.58[e]	10.32±0.06[cd]	12.16±1.15[c]

Values with the same superscript letter(s) down a column are not statistically significantly (P>0.05) different.
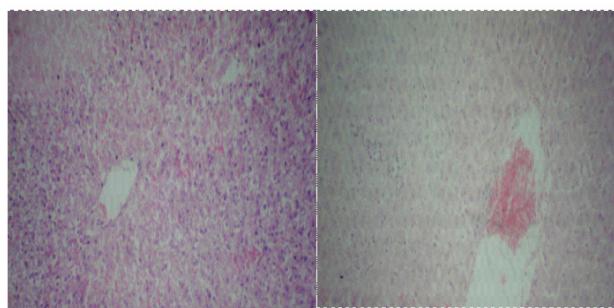

Figure 1. Hepatic fatty degeneration observed in mice livers fed with moulds fermented *C. ensiformis* seeds diets (A) Severe hepatic fatty degeneration observed with *Neurospora crassa* plus *Mucor mucedo* fermentation (H&E X100). (B) Moderate hepatic fatty degeneration observed with *Penicillium italicum* plus *Mucor mucedo* fermentation (H&E X100).

the unfermented *C. ensiformis* might be due to the protelytic enzyme activities and hydrolyses of free amino acids in the substrates. This is in agreement with the reports of Terlabie *et al.* (2006) that observed that recorded the highest protease and amylolytic activities, 101 U/ml and 26.68 mg/ml, respectively, and liberated the most amino acids.

The ALP activities of mice fed diets produced from *B. subtilis* plus *A. faecalis, P. italicum* plus *A. niger,* unfermented beans and basal non-protein control which were significantly higher than the ALP activities of mice fed the casein diet and were also higher than the physiological range of 10.5 – 27.6 IU/L for normal mice as reported by Mitruka and

Rawnsley (1981). The implication of this is that mice fed with *B. subtilis* plus *A. faecalis, P. italicum* plus *A. niger,* unfermented beans and basal non-protein control might have suffered hepatocellular injury caused by cell proliferation, which is in line with the findings of Barakat *et al.* (2012) and Cui *et al.* (2011). The AST activities of mice fed diets produced from *B. subtilis* plus *A. faecalis, N. crassa* plus *M. mucedo* and *P. italicum* plus *M. mucedo* which were higher than the 50 – 250 IU/L range recommended by Evans (2009) as the physiological range of AST for normal mice might be attributed to hepatotoxic effect of these diets to the animals. Only mice fed diets *N. crassa* plus *M. mucedo* and *P. italicum* plus *A. niger* had ALT activities above the range of 2.10 – 23.80 IU/L for normal mice (Mitruka and Rawnsley, 1981), while the ALT activities of all the experimental mice were within the normal physiological range of 4.5 – 21.7 IU/L recommended by Mitruka and Rawnsley (1981). The 2.18 g/L albumin concentration observed for mice fed basal non-protein diet, which was lower than the expectable physiological range of 2.8 – 4.0 g/dL (Evans, 2009).

Only mice fed diets produced from *P. italicum* plus *A. niger* and *P. italicum* plus *M. mucedo* had PCV and RBC values below the recommended PCV ranges of 33.1 – 49.9% and 6.86 – 11.7 x 10^6 mm^{-3} by Mitruka and Rawnsley (1981) for adult normal mice. The lower PCV from mice fed diets *P. italicum* plus *A. niger* and *P. italicum* plus *M. mucedo* indicated that the diets might contain toxins which might have affected the blood forming tissues in the animals

indicating the mice might have suffered significantly from the synthesis (erythrocytes) and concentration of erythrocytes. The significantly higher WBC counts in mice fed diets containing beans fermented with double starter cultures compared with those fed the unfermented beans, indicated that the mice fed the diets might have suffered lencocytosis, as a result of intoxication. The increased in the WBC values of mice fed diets containing beans fermented with double starter cultures, although with all within the range of $12.1 - 15.6$ x 10^6 mm^{-3} for normal mice (Mitruka and Rawnsley,1981) is physiological response by the mice to the possible mold toxins in the feed.

Conclusion

Diets from substrates produced from *B. subtilis* plus *B. polymyxa* and *B. subtilis* plus *P. pentosaceus* showed great promises to be used for controlled fermentation of *C. ensiformis* seeds.

Acknowledgements

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