

Original Article

## Effect on neurobehavioural and cognitive abilities of F1 progeny following millet-based nutritional composition during protein deficit F0 mother

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### ABSTRACT

**Objectives:** Malnutrition can have significant effects on neurobehavioural development. Childhood malnutrition has been linked to impaired cognitive development and neurodevelopmental effects. Hence, there is a need to prevent malnutrition to prevent impairment in neurobehavioural and cognitive development. Therefore, in the present study, the impact of a maternal folate-rich nutritional supplement on the neurobehavioural and cognitive development of F1 progeny born to protein-deficient female rats was investigated.

**Materials and Methods:** Animals were divided into four groups, the control group received a standard pellet diet with 20% protein; the disease control group, fed with a protein-deficient diet with 2% protein throughout the study period; the treatment group 1 and 2 receives 2% protein-deficient diet for initial 10 weeks, followed by non-fermented nutritional diet and fermented nutritional diet for next 10 weeks, respectively. At the end of the 20<sup>th</sup> week of study, all the animals were kept for breeding, and the diet was continued to the mother till 21 days after delivery (F1 progeny). These F1 offspring were subjected to various neurobehavioural assessments. These assessments included evaluating social interaction, anxiety levels, learning and memory abilities and repetitive behaviour tendencies in the F1 progeny.

**Results:** The F1 offspring born to protein-deficient mothers who received either fermented or non-fermented nutritional diets exhibited a reversal in anxious behaviour, characterised by an increase in the number of entries and time spent in open arms in the elevated plus maze apparatus and decreased locomotor activity. Furthermore, these offspring displayed enhanced learning and memory capabilities and also improved social interaction.

**Conclusion:** These findings reveal that a diet primarily comprising millets, combined with bovine colostrum, whether fermented or not, can provide protection against neurological issues resulting from protein deficiency. Notably, the fermented version of this dietary composition exhibited a significant neuroprotective effect when compared to the non-fermented version. These results are supported by the observed elevated folate levels in the fermented composition throughout the study.

**Keywords:** Malnutrition, Memory capabilities, Millets, Neurobehavioural development, Protein deficiency

### INTRODUCTION

A diet with a lack of essential nutrients, particularly protein, has a profound impact on the development and functioning of the central nervous system. Early-life protein deficiencies have been proven to have a negative impact on the brain's cellular development.<sup>[1]</sup> Malnutrition is a condition that occurs mainly due to insufficiency, excesses or imbalances in a person's intake

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of nutrients.<sup>[1]</sup> Malnutrition can have significant effects on neurobehavioural development, particularly in children as well as adults. Childhood malnutrition has been linked to impaired cognitive development and neurodevelopmental effects.<sup>[2]</sup> It has been reported that malnutrition induced through restriction of the maternal diet in the last week of pregnancy led to decreased methylation in the offspring, which could have long-term effects on neurobehavioural development.<sup>[3]</sup>

Folate is an essential nutrient that plays a key role in neurobehavioural development of offspring, and maternal folic acid (Vitamin B<sub>9</sub>) levels during pregnancy have been shown to have a significant impact on offspring neurological function in clinical populations.<sup>[4]</sup> Studies have also reported that the neurobehavioural development of offspring is very closely related to the maternal folate level.<sup>[5]</sup> Therefore, optimal intake of folic acid during pregnancy is crucial to ensure proper cognitive function and motor development in offspring.<sup>[6]</sup> Complementary diets have been developed to combat child malnutrition; however, in poor nations, poverty has impeded this development. As a result, researchers are concentrating on developing complementary diets utilising local staple crops.<sup>[7]</sup> Cereals and other regional foods are the first line of food in underdeveloped nations. The majority of plant-based supplemental diets have been shown to meet a developing child's demands for protein and micronutrients.

Several kinds of millets, such as *Eleusine coracana* (finger millet) and *Pennisetum glaucum* (pearl millet), are recognised for their high levels of protein, dietary fibre, minerals (particularly iron and calcium) and vitamins.<sup>[8]</sup> These nutrient-dense grains have shown significant promise in reducing malnutrition by providing essential nutrients. Legumes have gained popularity as 'poor man's meat' due to their significance as a food source in areas with low levels of animal proteins, where poverty is prevalent, or where eating meat is forbidden due to religious or cultural preferences.<sup>[8]</sup> Research on legume seeds revealed the presence of a high concentration of the desired amino acid. Among the several micro and macronutrients that asparagus is rich in are iron and folate.<sup>[9]</sup> It has been revealed that bovine colostrum (BC) supplies a significant amount of easily digestible protein, making it a particularly attractive choice for a calorie-appropriate diet. Colostrum, the first milk a cow makes in the early days after calving, is highly valued for its distinctive composition and bioactive components. It is rich in proteins, carbohydrates, lipids, immunoglobulins, growth factors, vitamins and minerals.<sup>[10]</sup>

It is usually found that millets and legumes contain phytate, which prevents minerals from getting absorbed. For increasing the bioavailability of vitamins in foods, various methods are used, which include sprouting, heating,

fermentation and soaking. It has been reported that fermentation raises the amount of folate in common foods.<sup>[11]</sup>

Thus, considering the significance of millets as a source of micronutrients and BC as a bioactive material, the study was undertaken to investigate the influence of fermentation on the micronutrient levels with respect to the Vitamin B group in millet-blended composition. The study further explores to investigate the effect of those blended compositions (fermented and non-fermented) on neurobehavioural and cognitive abilities of F1 progeny in an experimental model of protein deficit condition in the mother (F0 generation).

## MATERIALS AND METHODS

### Procurement of nutritional food materials

Red lentils (*Lens culinaris*), Moong beans (*Vigna radiata*), Red rajma (*Phaseolus vulgaris*), Pearl millet (*P. glaucum*), Finger millet (*E. coracana*) and Flaxseed (*Linum usitatissimum*) were procured from farmers. Shatavari (*Asparagus racemosus*) was procured from the local market.

### Collection and lyophilisation of BC

BC is collected within the first 24 h period of postpartum and was procured from farmers. After collection of BC, it was immediately stored at  $-20^{\circ}\text{C}$  for 72 h. The frozen BC was then lyophilised at  $-120^{\circ}\text{C}$  in a freeze dryer (OPERON, Korea) for 72 h under vacuum conditions. After that, the BC was collected in dry crystal and powder form and stored at normal refrigeration temperature before being utilised for oral supplementation.

### Preparation of fermented and non-fermented blend

The millet, red lentils, moong beans and red rajma underwent a visual inspection to ensure there were no visible impurities. Subsequently, they were soaked in water for 12 h. Following the soaking period, the damp seeds were placed in a cotton cloth and placed in a dark room to facilitate proper germination. Once the germination process is finished, all the seeds are dehydrated by sunlight and air. Once dried, they were milled into fine powders. The quantities of each seed powder were measured as specified in Table 1 and combined in a Mason jar, followed by the addition of 500 mL of distilled water. Mixing was done thoroughly to uniformly distribute the ingredients. Subsequently, the lid is tightly sealed, and the jar is allowed to ferment for a period of approximately 72 h. During the fermentation process, the pH was measured at intervals of 24 h. The process continues until the pH reaches a value of 4.0. After the fermentation process, the mixture is formed into pellets using a 1:1 ratio, specifically combining 50% of the nutritional blend with 50% of the protein-deficient diet. For a non-fermented diet, a similar procedure as above

is followed except for the fermentation procedure. The pellets are made in a 1:1 ratio, specifically combining 50% of the nutritional blend with 50% of the protein-deficient diet.

### Vitamin B estimation using high performance liquid chromatography (HPLC)

Vitamin B estimation was performed using HPLC (NABL Accredited, Equinox lab, Navi Mumbai). Briefly, 1 g of a sample was added to 4.5 mL of water and 4.5 mL of ethanol and sonicated and shaken for 10 min. Then the sample was filtered through 0.45 µm filter tips, and aliquots of 20 µL from the solution were injected into the HPLC. A COSMOSIL column (25 × 4.6 mm, 5 µm) was used for HPLC analysis, with a linear gradient of 20 Mm KH<sub>2</sub>PO<sub>4</sub> at pH 2.5 at a constant flow rate of 0.80 mL/min with 2300 psi pressure using waters pump (1515 isocratic) and a ultraviolet (2487) detector was employed for the detection of peaks, using channels at a wavelength of 210 nm. The respective Vitamin B standard was used.

### Preparation of modified American institute of nutrition (AIN)-93G protein-deficient diet (2%)

The nutritional composition of the protein-deficient diet in this study adhered to the specifications outlined in the National Research Council (US) Subcommittee on Laboratory Animal Nutrition. The protein content was deliberately limited to 2% based on the documented results of comparable studies.<sup>[12]</sup> The ingredients were combined and pellets were made, and dried [Table 2].

### Ethical approval

The research was carried out in compliance with the guidelines set out by the New Delhi-based Committee for Control and Supervision of Experiments with Animals. The research protocol was approved by Institutional Animal Ethics Committee and the protocol number is CPCSEA/IAEC/ PT-15/02-2k22.

### Experimental design

Thirty-two female Wistar rats with body weights ranging from 200 to 230 g were sourced from the Animal House. The rats underwent a 7-day acclimatisation period in controlled laboratory conditions. During the acclimatisation period, the temperature was maintained at 25 ± 2°C and appropriate humidity. Following acclimatisation period, the rats were then randomly divided into four groups ( $n = 8$ ): The control group, which received a standard pellet diet with 20% protein throughout the entire study; the disease control group, fed with a protein-deficient diet with 2% protein throughout the study, the treatment group 1 animals fed with protein-

**Table 1:** Composition of fermented blend.

Ingredients	Quantity (per kg)
<i>Lens culinaris</i>	180 g
<i>Vigna radiata</i>	100 g
<i>Phaseolus vulgaris</i>	180 g
<i>Pennisetum glaucum</i>	200 g
<i>Eleusine coracana</i>	70 g
<i>Linum usitatissimum</i>	100 g
<i>Asparagus racemosus</i>	70 g
Bovine colostrum	100 g

**Table 2:** Composition of protein-deficient diet.

Ingredient	Standard diet American Institute of Nutrition 93 G diet (g/kg)	Protein and folate deficient (2%) diet composition (g/kg)
Casein	200	23
Corn starch	397.486	489.7
Dextrinized corn starch	132	132
Sucrose	100	200
Cellulose	50	65
Soybean oil	70	70
Vitamin mix	10	0
Mineral mix	30	0
Methionine	3	0.3
Choline	2.5	2.5
Bactrim	0.014	0.014

deficient diet for 10 weeks and then fed with non-fermented nutritional diet for next 10 weeks and the treatment group 2, fed with protein-deficient diet for 10 weeks and then fed with fermented nutritional diet for next 10 weeks. At the end of the 20<sup>th</sup> week period of study, all the animals were kept for breeding, and the diet was continued to the mother till 21 days after delivery (F1 progeny). On a daily basis, behavioural experimentation was performed on pups within the light phase between 09:00 am and 03:00 pm. The experimental design is given in Figure 1.

### Neuro-behavioural parameters testing

Pups (F1 progeny) were subjected to a series of tests for the following numerous behavioural evaluations.

### Assessment of social interaction

The three-chambered social interaction testing apparatus (57 cm × 36 cm × 30 cm) was used to access social interaction in F1 progeny as described briefly previously,<sup>[10,13]</sup> the three

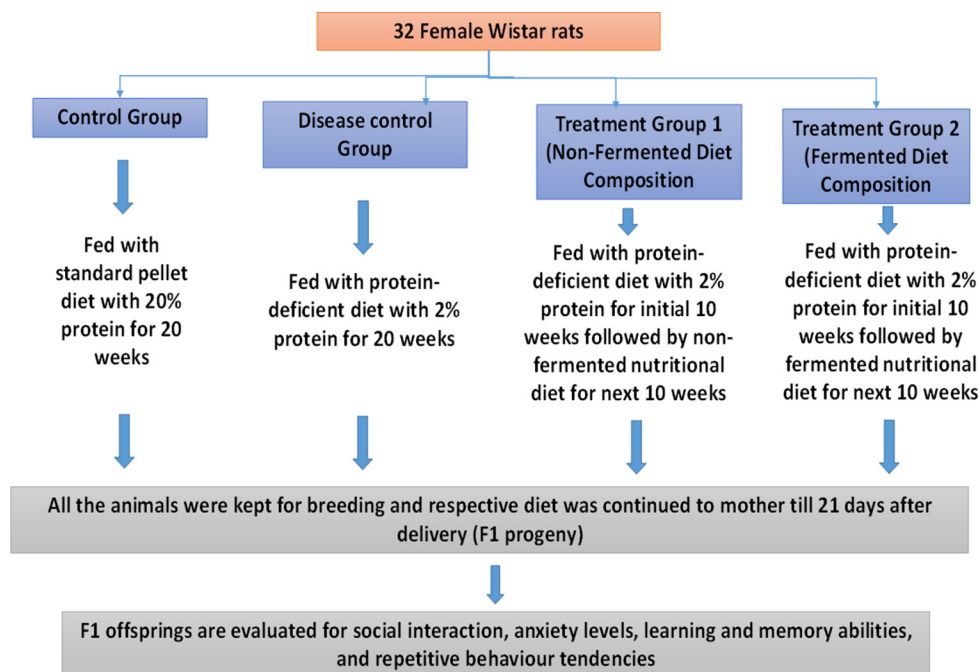


Figure 1: Experimental design.

sessions are involved in the testing method. The Wistar rats are acclimatised for a period of 5-min during the first session, whereas during the second session of the sociability testing of 10 min duration, a stranger rat was placed at any side of the chamber randomly designated as the 'Stranger chamber' while the other chamber was called as 'empty chamber'. During the third session of 10 min duration in this testing method, a novel rat was introduced into an empty chamber, which was known as the 'Novel chamber'. The chamber, which was called 'stranger' during the earlier session, was now known as 'Familiar'.

The amount of time spent by rats in both the side arms of the elevated plus maze (EPM) was measured. The sociability index (SI) was used to express sociability, whereas the social preference index was used to express social preference.

$$\text{Sociability index (SI)} = \frac{\text{Time spent in stranger chamber}}{\text{Time spent in empty chamber}}$$

$$\text{Social preference index (SPI)} = \frac{\text{Time spent in novel chamber}}{\text{Time spent in familiar chamber}}$$

#### Assessment of locomotor activity

The digital actophotometer was used to determine the locomotor activity in F1 progeny. The animals were placed for 20 minutes in a digital actophotometer. The activity was recorded in two different periods at 0–10 min and 10–20 min.

The number of beams broken by the rat is called locomotor activity.<sup>[13]</sup>

#### Assessment of repetitive behaviour

The behavioural abilities, which include active memory, spatial reference memory and repetitive behaviour, were investigated using the Y-maze apparatus.<sup>[14,15]</sup> In the current investigation, we have used the Y-maze apparatus (42 cm × 15 cm × 14 cm) to assess repetitive behaviour. The Y-maze comprises three arms. The sequences of rat entries in three separate arms in continuation were measured to investigate the number of spontaneous alterations. The reduction in the number of spontaneous alterations denotes repetitive behaviour. The testing is done for a duration of 8 min.<sup>[16]</sup>

$$\% \text{Spontaneous alterations} = \frac{\text{Total alterations}}{(\text{Total arms entered} - 2)} \times 100$$

#### Assessment of anxiety

Using the EPM apparatus, the anxious behaviour of all the rats was investigated. The EPM apparatus is raised upwards from floor level, and it consists of two open arms, which are perpendicular to two closed arms (50 cm × 10 cm × 40 cm). Initially, the rats were placed in the centre of the EPM in such a manner that the rats were facing towards the open arms. The test is based on the natural tendency of rats to explore new environments and to stay apart from bright-elevated arms in EPM. The 5 min is the test duration. The parameters

that were measured during testing include the total number of entries and time spent in both open and closed arm. The anxious behaviour was denoted by the percentage of lower time spent and entries in the open arm in comparison to the closed arms. In general, two anxiety indices were investigated in the test, which include (1) % of time spent in the open arm as compared to total time spent by the rat on the EPM apparatus and (2) % of entries in the open arm as compared to total number of entries. After every test, the EPM apparatus was washed with 90% ethanol.<sup>[17,18]</sup>

$$\% \text{Time spent in open arms} = \frac{\text{Time spent in open arm}}{\text{Total time spent}} \times 100$$

$$\% \text{Entries in open arms} = \frac{\text{Entries in open arm}}{\text{Total number of entries}} \times 100$$

### Assessment of learning and memory

The experiment was conducted within a 50 × 50 cm<sup>2</sup> square open enclosure, which comprises four walls of 50 cm<sup>2</sup> height. At the initial stage of the experiment, all rats are allowed to explore the entire apparatus in the absence of any object for a period of 5 min. After 24 h of post-initial exposure, the rats were reintroduced into the apparatus; at that time, two identical objects, designated as A1 and A2, were kept at two adjacent corners in such a manner that a separation of 10 cm from the enclosure walls was achieved. Following that, all the rats were allowed to investigate these identical objects for a period of 5 min. This phase was known as the 'acquisition' session. The rats were again placed into the experimental apparatus after an additional 24 h duration. At this time, one of the two identical objects (either A1 or A2) was substituted with a completely dissimilar object, denoted as B. Following that, the rats are allowed to investigate these two disparate objects for a duration of 5 min. The prime purpose of this study is to investigate the rats' aptitude for distinguishing between A1 and A2 (well-known stimuli) and B (unfamiliar stimulus) following a time delay. The rat's memory retention and object recognition capacity are determined by this method. The discrimination index is a quantitative measure used to predict the animal's discriminatory performance and cognitive abilities in this test.<sup>[13,19]</sup>

$$\% \text{Recognition index} = \frac{\text{Time spent by rat towards Novel object}}{\text{Total time spent by rat in NOR apparatus}} \times 100$$

### Haematological assessment

The non-heparinised collection tubes were used to collect animal blood samples, whereas heparinised collection

tubes were used for serum chemistry and blood parameters analysis. The collected blood was subjected to centrifugation at 3000 rpm for a period of 10 min, and the resultant supernatant (serum) was then used for the investigation of haematological parameters such as haemoglobin, red blood cells, total protein, globulin and albumin.

### Statistical analysis

The Graph Pad In-Stat version 3.10 was used to analyse data statistically. The statistical test, one-way analysis of variance, was used to analyse all parameters, which was followed by the Tukey-Kramer multiple comparison test. The data are reported as mean ± standard deviation.  $P < 0.05$  was considered statistically significant.

## RESULTS

### Protein estimation of nutritional composition

The non-fermented blend was found to have 10.3% protein, whereas the fermented blend had 10.8% protein. The protein content found was similar in both compositions. The protein content is estimated using the Kjeldahl method, designed for food.

### Vitamin B estimation of nutritional composition

Vitamin B estimation was performed using HPLC. These results were outsourced from an NABL-accredited lab. The results of Vitamin B estimation are given in Table 3.

### Effect on social interaction

In the field of neuroscience and animal research, the most widely used behavioural assay includes the social novelty test and three-chamber sociability, particularly in the study of social behaviour in rodents, such as mice and rats. This test is used to assess a rodent's social behaviour, including its preference for social interaction and its ability to differentiate

**Table 3:** Estimation of Vitamin B in nutritional composition.

Parameters	Non-fermented nutritional composition (mg/100 g)	Fermented nutritional composition (mg/100 g)
Folate	3.1	21.01
Thiamine	0.075	0.046
Riboflavin	0.19	0.14
Niacin	0.0064	0.0075
Pantothenic acid	0.31	0.36
Pyridoxine	0.016	0.016
Biotin	0.0021	0.0024

between familiar and novel social stimuli. The test is also used to examine neurodevelopmental disorders in animal models of social deficits.

### Sociability and SI

The reality is that control rats during the sociability phase of a social testing apparatus spent more proportion of time in the stranger room as compared to the empty chamber supports classic social behaviour, whereas the diseased rats show different behaviour, spending more proportion of time in the empty chamber as compared to the unfamiliar chamber ( $P < 0.001$ ). Treatment with fermented and non-fermented diets was able to change this behaviour. Rats in treatment groups spent more time in the stranger chamber compared to the empty chamber, whereas this difference in time duration spent was not statistically significant for the non-fermented diet, that is ( $P < 0.05$ ) but significant for the fermented diet, it was ( $P < 0.001$ ). Figures 2 and 3 show the effect of the millet-based nutritional supplement on SI.

### Sociability preference and sociability preference index

During the sociability preference phase of a social testing apparatus, the rats from the control group spent more time in the stranger room as compared to the empty chamber, which is consistent with standard social behaviour. The rats from the disease control group exhibited unusual behaviour;

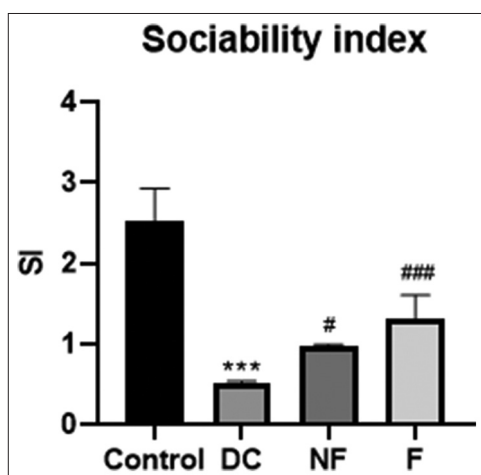
they spent considerably more amount of time in the empty chamber in comparison to the stranger chamber ( $P < 0.001$ ). The administration of fermented and non-fermented food throughout the study alters this behaviour in disease control rats. Rats present in treatment groups spent more amount of time in the stranger chamber compared to the empty chamber. This difference in time length was  $P < 0.01$  for the non-fermented diet, whereas it was  $P < 0.001$  for the fermented diet. Figures 4 and 5 show the effect of the millet-based nutritional supplement on the sociability preference index.

### Effect on locomotor activity

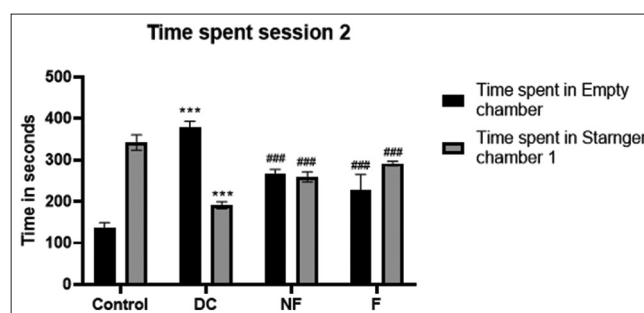
In the field of behavioural biology and pharmacology, the actophotometer is a widely used device for the measurement of locomotor activity of organisms. The aim of this study was to determine the locomotor activity of a group of experimental animals. The number of counts increased significantly in both the 0–10 min ( $P < 0.001$ ) and the 10–20 min ( $P < 0.001$ ) period of time compared to control rats, indicating more locomotor activity. The number of counts in non-fermented diet treated rats was significantly decreased by the 0–10 min interval ( $P < 0.05$ ) and the 10–20 min interval ( $P < 0.001$ ). In addition, treatment with the fermented diet shows a decrease in locomotor activity, which was noted as  $P < 0.001$  for both 0–10 min and 10–20 min intervals. Figure 6 shows the effect of a millet-based nutritional supplement on locomotor activity.

### Effect on repetitive behaviour and learning and memory

The novel object recognition test is a behavioural test commonly used in neuroscience and psychology research to assess the ability of animals, typically rodents such as mice and rats, to recognise and remember new or novel objects.

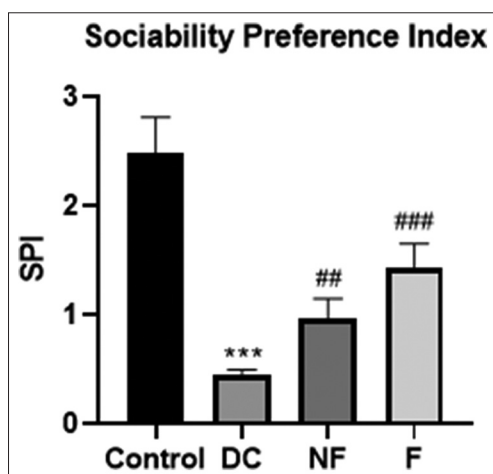


**Figure 2:** Effect of millet-based nutritional supplement on sociability index (SI). Results are expressed as mean  $\pm$  standard deviation ( $n = 6$ ). \*\*\*,##  $P < 0.001$ ; #  $P < 0.05$ . Disease control group compared with normal vehicle control group. Disease control group compared with NF and F group. Comparison between the groups was made by one-way analysis of variance followed by Tukey-Kramer multiple comparisons test. DC: Disease control, NF: Non fermented and F: Fermented.

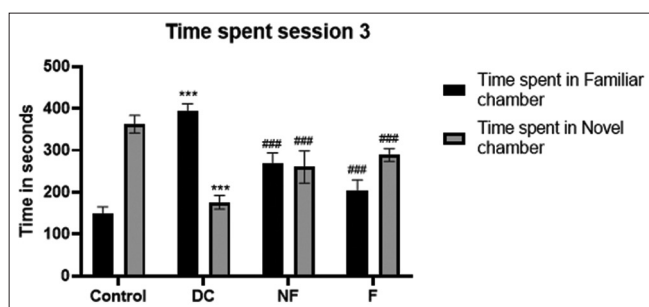


**Figure 3:** Effect of millet-based nutritional supplement on time spent (represented in seconds) in empty chamber and stranger chamber in session 2 using three-chambered sociability testing apparatus. Results are expressed as mean  $\pm$  standard deviation ( $n = 6$ ). \*\*\*,##  $P < 0.001$ ; Disease control group compared with NF and F group. Comparison between the groups was made by one-way analysis of variance followed by Tukey-Kramer multiple comparisons test. DC: Disease control, NF: Non fermented and F: Fermented.

It is used as a tool to study aspects of learning and memory, particularly non-spatial recognition memory. Recognition memory is disturbed in a range of neurobehavioural disorders. Rats fed with a protein-deficient diet showed a significant decline in memory recognition, that is ( $P < 0.001$ ) as compared to the control group. The group given with fermented and non-fermented diet shows an increase



**Figure 4:** Effect of millet-based nutritional supplement on sociability preference index (SPI). Results are expressed as mean  $\pm$  standard deviation ( $n = 6$ ). \*\*\*,### $P < 0.001$ ; \*\* $P < 0.01$ ; Disease control group compared with normal vehicle control group. Disease control group compared with NF and F group. Comparison between the groups was made by one-way analysis of variance followed by Tukey-Kramer multiple comparisons test. DC: Disease control, NF: Non-fermented and F: Fermented.

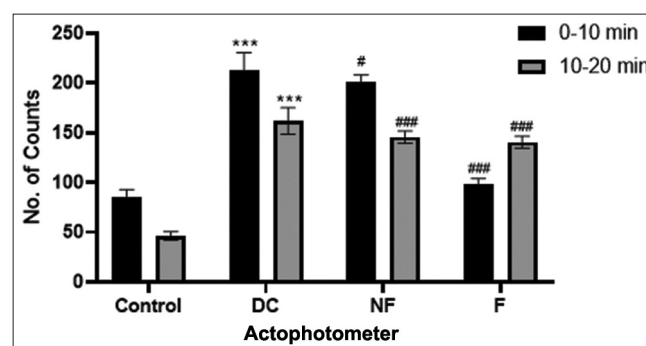


**Figure 5:** Effect of millet-based nutritional supplement on time spent (Represented in seconds) in familiar chamber and novel chamber in session 2 using three-chambered sociability testing apparatus. Results are expressed as mean  $\pm$  standard deviation ( $n = 6$ ). \*\*\*,### $P < 0.001$ ; Disease control group compared with normal vehicle control group. Disease control group compared with NF and F group. Comparison between the groups was made by one-way analysis of variance followed by Tukey-Kramer multiple comparisons test. DC: Disease control, NF: Non-fermented and F: Fermented.

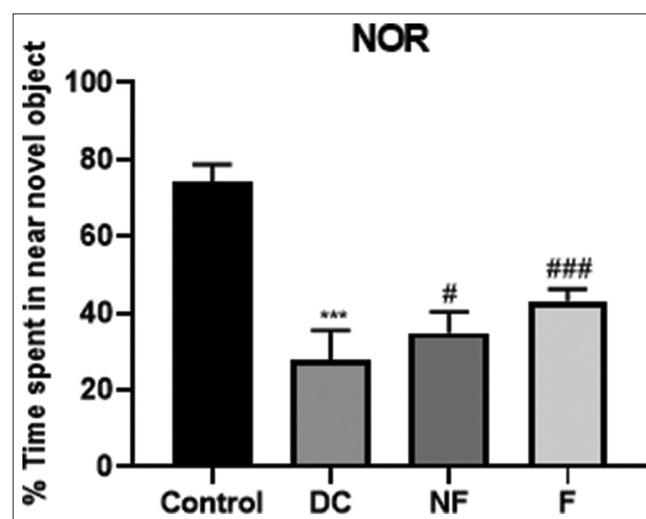
in recognition memory, that is,  $P < 0.001$  and  $P < 0.05$ , respectively. Figure 7 shows the effect of a millet-based nutritional supplement on learning and memory.

#### Effect on anxiety

The effect of millet-based nutritional supplement on anxiety in F1 progeny of protein deficit mother was evaluated



**Figure 6:** Effect of millet-based nutritional supplement on locomotor activity in actophotometer testing. Results are expressed as mean  $\pm$  standard deviation ( $n = 6$ ). \*\*\*,### $P < 0.001$ ; # $P < 0.05$ ; ns- not significant; Disease control group compared with normal vehicle control group. Disease control group compared with NF and F group. Comparison between the groups was made by one-way analysis of variance followed by Tukey-Kramer multiple comparisons test. DC: Disease control, NF: Non-fermented and F: Fermented.



**Figure 7:** Effect of millet-based nutritional supplement on learning and memory using novel object recognition (NOR) apparatus. Results are expressed as mean  $\pm$  standard deviation ( $n = 6$ ). \*\*\*,### $P < 0.001$ ; # $P < 0.05$ ; Disease control group compared with normal vehicle control group. \*Disease control group compared with NF and F group. Comparison between the groups was made by one-way analysis of variance followed by Tukey-Kramer multiple comparisons test. DC: Disease control, NF: Non-fermented and F: Fermented.

using the EPM method. F1 progeny of mother fed with standard diet spent a greater amount of time in the open arm of EPM than they did in the enclosed arm, which was consistent with their usual behaviour. On the other hand, F1 progeny of mother fed a protein-deficient diet (2%) showed a marked fall in the proportion of time spent ( $P < 0.001$ ) and the percentage of entries ( $P < 0.001$ ) in the open arm, both of which are signs of anxiety. The administration of both fermented and non-fermented millet-based diets, however, significantly enhances the proportion of time spent in the open arm by decreasing this anxious behaviour,  $P < 0.01$  and  $P < 0.01$  for the non-fermented and fermented diets, respectively. In addition, there was an increase in the probability of entry into the open arm, with values of  $P < 0.001$  for the non-fermented diet and  $P < 0.05$  for the fermented millet-based diet. These results highlight the anxiety-reducing properties of both millet-based diets. Figures 8 and 9 show the effect of millet-based nutritional supplement on anxiety.

#### Effects on haematological and biochemical parameters

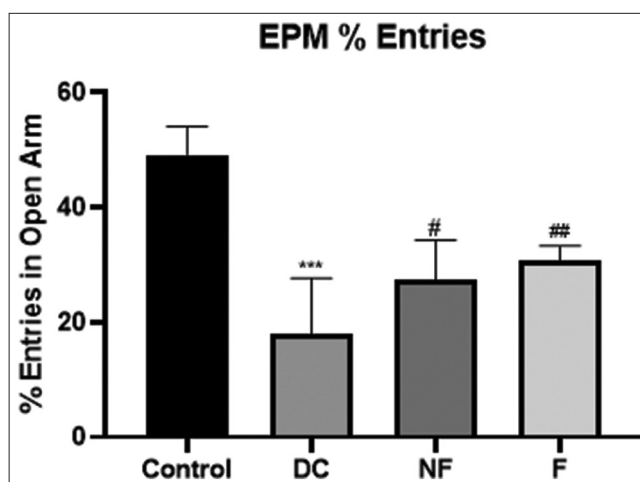
The albumin, globulin, total protein, haemoglobin and haematocrit levels were decreased in the disease control group compared to the vehicle control group ( $**P < 0.01$ ,  $***P < 0.001$ ). While the groups fed with fermented and non-fermented nutritional composition showed an increase in the levels compared to the disease control group ( $\# P < 0.05$ ,  $\#\# P < 0.01$  and  $\#\#\# P < 0.001$ , respectively). Table 4 shows the effect of millet-based nutritional supplement on haematological and biochemical parameters.

#### DISCUSSION

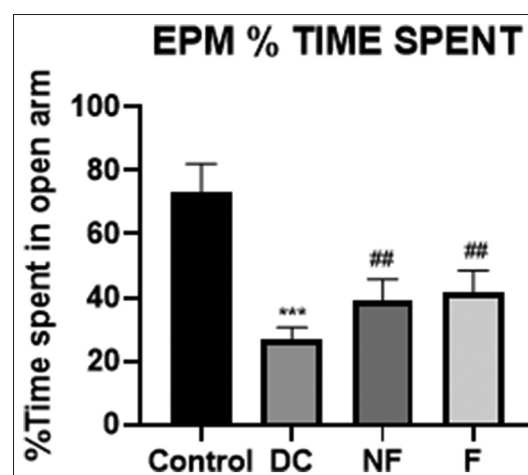
Malnutrition can have significant effects on individuals and society. These effects can be physical, mental and economic. Malnutrition is a condition that occurs mainly due to insufficiencies, excesses or imbalances in a person's intake of nutrients.<sup>[1]</sup> Malnutrition can result in psychosocial effects such as apathy, depression, anxiety and self-neglect.

Malnutrition can also adversely affect the physiological as well as mental well-being of individuals, which causes a decrease in productivity, making that individual and their respective country more vulnerable to poverty.

Malnutrition causes an increase in the cost of healthcare, decreases productivity and reduces economic progress, which can maintain a state of deprivation and ill-health. Malnutrition can compromise physical and intellectual development, reduce productivity levels and make individuals more vulnerable to disease.<sup>[20]</sup> Undernutrition is an important causal factor of maternal and child health because it possesses significant deteriorating effects on the



**Figure 8:** Effect of millet-based nutritional supplement on % entries in open arm of elevated plus maze (EPM) apparatus. Results are expressed as mean  $\pm$  standard deviation ( $n = 6$ ).  $***P < 0.001$ ; Disease control group compared with normal vehicle control group.  $\#P < 0.05$ ; Disease control group compared with NF and F group. Comparison between the groups was made by one-way analysis of variance followed by Tukey-Kramer multiple comparisons test. DC: Disease control, NF: Non-fermented and F: Fermented.



**Figure 9:** Effect of millet-based nutritional supplement on % time spent in open arm of elevated plus maze (EPM) apparatus. Results are expressed as mean  $\pm$  standard deviation ( $n = 6$ ).  $***P < 0.001$ ;  $##P < 0.01$ ; Disease control group compared with normal vehicle control group. Disease control group compared with NF and F group. Comparison between the groups was made by one-way analysis of variance followed by Tukey-Kramer multiple comparisons test. DC: Disease control, NF: Non-fermented and F: Fermented.

development of the brain and cognitive ability of children.<sup>[21]</sup> Undernutrition alone is responsible for causing almost half

**Table 4:** Estimation of haematological and biochemical parameters in animals.

Groups	Albumin (g/dL)	Globulin (g/dL)	Total protein (g/dL)	Haemoglobin (g/dL)	Haematocrit (%)
Control	4.3±0.14	2.95±0.12	7.02±0.44	14.34±1.21	42.36±4.02
Disease control	3.7±0.36**	1.74±0.53**	5.46±0.52**	7.44±0.87***	21.22±2.54***
Non-fermented	4.22±0.13 <sup>#</sup>	2.45±0.31 <sup>#</sup>	6.52±0.34 <sup>#</sup>	12.36±1.21 <sup>###</sup>	38.36±2.67 <sup>##</sup>
Fermented	4.34±0.88 <sup>#</sup>	2.67±0.39 <sup>#</sup>	6.74±0.77 <sup>#</sup>	12.63±1.36 <sup>###</sup>	39.72±3.14 <sup>##</sup>

All values are expressed as Mean±standard deviation, (n=5); \*\*P<0.01, \*\*\*P<0.001 as compared to control; <sup>#</sup>P<0.05, <sup>##</sup>P<0.01, <sup>###</sup>P<0.001 as compared to disease control on 2% protein-deficient diet. Statistically analysed by one-way analysis of variance followed by Tukey-Kramer multiple comparisons test

of all deaths, especially in children under the age of 5 years, because undernutrition in children puts them at a high risk of death, mainly due to common infections enhance the prevalence and severity of those infections and reduce recovery rate.<sup>[22]</sup>

Milletts are a good source of various B vitamins such as B<sub>1</sub> (thiamine), B<sub>2</sub> (riboflavin), B<sub>3</sub> (niacin), B<sub>6</sub> (pyridoxine) and folate. These vitamins are crucial for proper brain function and the synthesis of neurotransmitters. Milletts contain minerals such as magnesium and zinc, which are important for cognitive function and maintaining healthy neurotransmitter balance. Antioxidants found in milletts, such as phenolic compounds and flavonoids, can help protect brain cells from oxidative stress and inflammation.<sup>[23]</sup> The dietary fibre content of milletts can support gut health, which is increasingly recognised as having a connection to brain health through the gut-brain axis. Compared to refined grains, milletts have a lower glycaemic index, which can help stabilise blood sugar levels and potentially support a consistent energy supply to the brain.

BC has also been found to be beneficial for health perspectives and is a good source of several minerals, especially calcium and phosphorus. In addition, it is rich in lactoferrin, immunoglobulins, lysozyme, growth factors, lactoperoxidase and bioactive peptides which help to ameliorate the variety of disease states.<sup>[9]</sup> In an earlier study, it is reported that the BC may decrease apoptotic cell death, which may have neuroprotective benefits and aid in the recovery of brain function after haemorrhagic stroke.<sup>[24]</sup>

In addition, it has been discovered that ingesting BC reduces blood levels of pro-inflammatory cytokines, lessens brain damage, and enhances neurological outcomes following focal cerebral ischemia in rat models. It has also been reported that the BC helps to reduce the oxidative damage and safeguards neurons from ischemic injury. This reveals a potential mode of action for neuroprotection.

In the present investigation, we have investigated the effect of fermentation on micronutrient levels, and the results reflect a rise in folate levels, which is supported by earlier findings

indicating an increase in the level of folate by fermentation with cereals<sup>[25]</sup> and other micronutrients. In the present study, our prime objective was to evaluate the effect of blended composition of milletts with BC-based diet in fermented and non-fermented form on neurobehavioural and cognitive functions of F1 progeny of protein deficit mothers (F0) in an experimental model of undernourished rats.

The results of the neurobehavioural and cognitive testing in F1 progeny of protein deficit mothers show an increase in locomotor activity, anxious behaviour, show decline in learning ability and memory and an increase in repetitive behaviour. These finding reflects the anxiety and memory deficit in protein deficit condition. While the F1 progeny of protein deficit mothers fed with fermented and non-fermented nutritional diets, respectively, shows a decrease in anxious behaviour, which leads to an incredible increase in the number of entries and also per cent of time spent in the open arm as shown in the EPM model and reduced locomotor activity. The results were significant in both fermented ( $P < 0.01$  and  $P < 0.05$ ) and non-fermented ( $P < 0.05$ ) in comparison with the F1 progeny of protein deficit mothers. Social impairments are mainly responsible for neurological disorders. Three-chambered sociability tests are used to determine the SI and sociability preference index. This three-chambered sociability test is considered a gold standard method for examining social behaviour in rodents. Results indicate that SI and sociability preference index were improved considerably in the F1 progeny of protein deficit mother fed with fermented ( $P < 0.01$ ,  $P < 0.001$ ) and non-fermented ( $P < 0.05$ ) nutritional diet composition.

## CONCLUSION

From these findings the study concludes that the nutritional composition, that is, a millet-based diet blended with BC in fermented and non-fermented form, helps to protect from the neurological implications in protein deficit condition. Among these, the fermented nutritional composition showed more significant neuroprotective activity compared to the non-fermented composition. These results are supported

by the high folate level in the fermented composition in the present investigation.

While these nutritional values may vary according to species, health of the animal and season/time of collection of millet source or BC, respectively. During the process of pasteurisation, it is mentioned that there may be considerable loss of nutritional values/loss of temperature sensitive nutrients such as immunoglobulins, Vitamin B<sub>12</sub> and folic acid. The present study was undertaken to use unpasteurised BC, which was collected within 24 hour period postpartum from farmers. The present investigation is not associated with any translation value. For human consumption or trial, it is recommended to use pasteurised BC or milk as per the Food and Drug Administration guideline.

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