**Study on chemical breakdown and increase in nutritive value of Barnyard millet (*Echinochloa spp*) by fermentation in earthen pot**

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**ASTRACT**

# Barnyard millet (*Echinochloa spp*)has lot of health promoting factors. So the aim of this study was to evaluate the fermentation process related to its endogenous microorganism and effect of *Lacto bacillus* and yeast (*Saccharomyces*) which can enhance bioavailability of nutrients. Hence the study was carried out using Earthen pot for fermentation of Barnyard millet flour, it is aimed to assess the microbial changes and simultaneously biochemical changes. The microbial changes was observed in primary (usingraw millet) and secondary (cooked millet ) fermented Barnyard millet flour. A comparative Study using *Lacto bacillus* and yeast and its biochemical changes were carried out. Increase in total titrable acid in fermented millet is the result of increase in reducing sugar and protein. Quantifiable amount Vitamin B2, B5 and B9 were detected.Ca and Fe was also reported. Fatty acid of C16 and C18 were detected.

# Keywords : Barnyard millet, Endogenous microorganism, Fermentation, Nutritive value

**INTRODUCTION**

Millets are a storehouse of nutrients in large quantities. They include macro and micronutrients needed by the human body. Hence they can help withstand malnutrition (1).Compared to other cereals,millet is considered as rich source of energy, the nutrient content of millet is better than rice or wheat and contains higher proportion of complex carbohydrates, resistant starch and slow rising sugar. They are high in fibre contentof which soluble fibre is 3.4 to 6.5 percent and low in fat from 1.1 to 5.0 percent. Rich in B vitamins especially niacin, pyridoxine and folic acid. Millets offer good amount of calcium, iron, potassium, magnesium and zinc. Millets contain about 8.0 percent protein and 4.0 percent fat. Prolamines and glutelins form the major portion of their proteins and the fats from millet contain a higher portion of unsaturated fatty acids and supply essential fatty acids. Millets are especially rich in calcium. In traditional fermentation processes, microorganisms play an important role in preparation and preservation of different types of food. Fermentation extends the shelf life thereby increasing the nutritive value of foods as well as enhancingflavour and other desirable qualities associated with digestibility and edibility (2).Lactic acid bacteria and their by-products have been shown to be more effective and flexible in several applications. Most inhibitory substances produced by microorganisms are safe and effective natural inhibitors of pathogenic and food spoilage bacteria in various foods(3).

Traditional fermented foods have received extensive scientific attention and many traditional preparations have been analyzed for their microbiological, enzymological and biochemical changes(4, 5 and 6).Traditional fermentation process in finger millet major biochemical changes takes place from 5 - 15 hrs indicating a window period of 10 hrs. Hydrolysis of starch hydrolysis occurs during primary fermentation irrespective of the grain type and also observed in other millets, *viz*. fox tail millet fermentation, where 10% reduction in starch was reported. Among the millets, pearl millet grains had significant reducing sugar content probably due to easily digestible starch type (7).

# The microorganisms may be indigenous to the food, or may be added as a starter culture after pre-treating or cooking the product (8).The use of lactic acid bacteria (LAB) increases the acidity and decreases the pH of the substrate, thereby inhibiting many pathogens (9). A number of lactic acid bacteria (LAB) are used as probiotics, defined as “live microorganisms which, when administered in adequate amounts, confer a health benefit on the host”(10).

Millets can be consumed as breakfast cereal in the form of porridge, soups, stews and bread (11). Volatile compounds in cooked millet impart flavour perception and acceptability of the product by the consumers. Volatile compounds in developed product may vary depending upon the variety used in its preparation (12).A vast number of volatile compounds have been identified from a range of nutrients such as fatty acids and non-volatile palate constituents like phenolic compounds and alkaloids (13).So the present study is focussed on the nutritive value of cooked and fermented millet in earthen pot.

**MATERIALS AND METHODS**

**MILLET- Barnyard Millet - Kuthiraivalli- *Echinochloa spp***

1. **Collection of Sample:**

Dehusked Barnyard millet grain was brought from organic store, Chennai. All samples were segregated, cleaned and stored in air tight containers till further use.

1. **Preparation of Samples.**

Millets used for study was steeped in water and the impurities were removed. The millet grains were dried under shade and the seeds were grinded in a mixer sieved and then stored in air tight container in the laboratory refrigerator for use in experiments.

**3. Evaluation of Microbial Profile :**

**3.1. Isolation and morphological study of bacteria**

**Nutrient agar-** (Yeast Extract -2g, Peptone-5g, Sodium Chloride -5g, Agar Agar-20g in 1000ml Distilled water) were used. 1 ml of serially diluted sample solution from the desired test tube was added to each petridish and poured with the respective media as pour plate technique. The dilution of 10-9dilution were made for bacteria. The plates were maintained in triplicates. The plates with Nutrient agar were incubated at 37o C for 24 hours. . The number of bacteria colonies were assessed in the first and third days of incubation, and the number of Colony forming Unit (CFU) per gm of filter was determined. The desired organism was sub cultured for obtaining pure culture.

**3.3. IDENTIFICATION OF BACTERIA**.

The isolated bacteria were identified up to generic level by using Cowan and Steel’s Manual for the identification of medical bacteria (14) and Bergey’s Manual of Determinative Bacteriology (15) by performing the biochemical tests.

**GRAM STAINING:**

A thin smear was prepared on a grease free slide using the individual colony grown on the medium. The smear was flooded with crystal violet and allowed to stand for one minute. Then the slide was washed with water and then flooded with Gram’s iodine solute and left for one minute. The slide was drained and decolorized with 95% ethanol which was then washed with tap water. The smear was counterstained with safranin for one minute and washed. The slide was blot dried and examined under oil immersion.

**MANNITOL MOTILITY TEST :**

Suspend 28.0 g of powder in 1 litre of distilled or deionized water. Heat to boiling and shake until completely dissolved. Dispense in final tubers. Sterilize at 121°C for 15 minutes. Inoculate tubes with a pure culture by stabbing the centre of the column of medium to greater than half the depth. Incubate tubes for 24-48 hours at 35 ± 2°C in an aerobic atmosphere.

Bacterial motility can be observed directly from examination of the tubes following incubation. Growth spreads out from the line of inoculation if the organism is motile. Highly motile organisms provide growth throughout the tube. Growth of non-motile organisms only occurs along the stab line.

**CATALASE TEST:**

A clean glass slide was placed in the petridish saline suspension of the organism was made on the slide. Immediately drop of 3% H2O2 was added using dropper.

**OXIDASE TEST:**

Oxidase disc was placed on a clean glass slide which is placed in the petridish. The dish was moistened with distilled water. The colony to be tested is picked up using a tooth pick and smeared over moist area.

**INDOLE PRODUCTION TEST:**

The presence of indole is detectable by adding Kovac’s reagent composed of p-dimethylaminobenzaldehyde which produces a cherry red colour. 5 ml of sterile peptone broth was inoculated with the test culture and incubated at 37oC for 48 hours. Following incubation, 0.2 ml of Kovac’s reagent was added.

**METHYL RED TEST:**

5 ml sterile glucose broth was inoculated with the test culture and incubated at 37oC for 48 hours. Following incubation,5-6 drops of methylred solution was added.

**VOGES-PROSKAUER TEST:**

5 ml of sterile glucose broth was inoculated with the test culture and incubated at 37oc for 48 hours. Following incubation 1 ml of 40% potassium hydroxide and 3 ml of 5% solution of alpha naphthol in absolute ethanol was added.

**CITRATE UTILIZATION TEST:**

Simmons citrate medium was streaked with broth culture of test organism and incubated at 37oC for 24 hours.

**TRIPLE SUGAR IRON (TSI) AGAR TEST:**

The TSI slants contain 1% each of lactose and sucrose, and glucose in a concentration of 0.1%. The phenol red, the acid base indicator is incorporated in the medium to detect carbohydrate fermentation. The medium was inoculated with the test culture by first stabbing the butt down to the bottom and then streaking the surface of the slant and incubated at 37oC for 24 hours.

**UREASE TEST:**

The production of the enzyme urease is detectable when the organisms are grown in urea broth medium containing phenol red, the pH indicator which shows a change in colour. The medium was inoculated with the test culture and incubated for 24 hours at 37oC.

**CARBOHYDRATE FERMENTATION TEST:**

In fermentation, substrate such as carbohydrates and alcohols undergo anaerobic dissimilation and produce an organic acid that may be accompanied by gases such as hydrogen or carbon-dioxide. The broth was inoculated with the test culture and incubated at 37oC for 18-24 hrs.

**OXIDATIVE FERMENTATION TEST:**

This method depends upon the use of a semi-solid tubed containing the carbohydrate (usually glucose) together with a pH indicator. If acid is produced only at the surface of the medium, where conditions are aerobic, the attack on the sugar is oxidative. If acid is found throughout the tube, including the lower layers where conditions are anaerobic, the breakdown is fermentative. Duplicate tubes of medium are inoculated by stabbing; one tube is covered with liquid paraffin to a depth of 5-10 mm and both are incubated at 37oC for 48 hours or longer.

* 1. **ISOLATION AND IDENTIFICATION OF FUNGI :**

**Potato dextrose agar (PDA) media** ( Potato -200 g, Dextrose -20 g, Agar - 20 g in 1000ml of distilled water). was prepared for the isolation of fungi. Antibiotic (Streptomycin) was added to the media before solidification to inhibit the bacterial growth. Pour plate technique was followed to which 1 ml of sample solution is added to each petridish and PDA was poured.

**3.2.1 IDENTIFICATION OF FUNGI**

Identification of culturable molds was done using their macroscopic appearance and their microscopic appearance. The fungi were identified with the help of standard text-books and mono graphs (16,17).

**Macroscopic appearance**

Morphological characteristics of the culture viz, colour, shape, pigmentation, reverse pigmentation were studied by using the hand lens.

**Microscopic appearance**

Microscopic characters were studied by preparing the slides and observing under light microscope. The characters of conidia bearing structure, shape, size, septation, colour and ornamentation were observed.

**Slide preparation**

Lactophenol and lactophenol with cotton blue [for hyaline molds] were used for examination. The preparation of lacto phenol with cotton blue is as follows:

Phenol - 10 g

Lactic acid - 10 g

Glycerol - 10 g

Distilled Water - 10 g

Lacto phenol is prepared by warming phenol with water and then adding lactic acid and glycerol. To prepare lacto phenol with cotton blue, 0.05 g of cotton blue stain is added to 100 ml of lacto phenol.

**Presentation of Data:**

Percentage contribution = Total no. of colonies for an individual species X 100

Total no. of colonies recorded for all species

**4.COMPOSITION OF SKIM MILK MEDIA**

52.15% lactose, 38.71% protein (31.18% casein, 7.53% whey protein), 1.08% fat, and 8.06% ash.

**5. FERMENTATION TREATMENTS IN EARTHEN POTS:**

**Formulation of Millet Beverage taken for biochemical test:**

* 1. **Flour-based millet -water-based;**
  2. **Flour-based millet** -**water-based,**  inoculum-;***Lactobacillus sps***
  3. **Flour-based millet** - **water-based** with substrate **sugar,** inoculum- ***Lactobacillus sps***
  4. **Flour-based millet** - **water-based** with substrate milk inoculum-***Lactobacillus sps*.**
  5. **Flour-based millet** - **water-based**  with substrate**milk and sugar inoculum-; *Lactobacillussps***
  6. **Flour-based millet** - **water-based**  inoculum-;Yeast (*Saccharomyces cerevisiae*)
  7. **Flour-based millet** - **water-based**  with substrate **sugar- inoculum- Yeast**(*Saccharomyces cerevisiae*)
  8. **Flour-based millet** - **water-based**  with substrate **milk- inoculum-;Yeast** (*Saccharomyces cerevisiae*)
  9. **Flour-based millet** - **water-based**  withsubstrate **sugar and milk- inoculum-;Yeast**(*Saccharomyces cerevisiae*) **.**

**5.1 FLOUR-BASED MILLET -WATER-BASED;**

To prepare water-based fermented millet ,–primary and secondary fermentation is done. In primary fermentation take 20 gm of Barnyard millet *i.e*dehusked millet is steeped in water for 8 hours in 250 ml of distilled water in earthen pot. In secondary fermentation the soaked, the primary fermented millet is cooked in the earthen pot at 90–95 °C for 20 min till it is completely cooked. Cover the earthen pot with sterile muslin cloth and leave it for fermentation for 8 -12hrs .This fermented millet is taken for biochemical analysis

**5.2 FLOUR-BASED MILLET** - WATER-BASED INOCULUM-*LACTOBACILLUS SPS*

To prepare water-based fermented millet ,–primary and secondary fermentation is done. In primary fermentation take 20 gm of Barnyard millet *i.e* dehusked millet is steeped in water for 8 hours in 250 ml of distilled water in earthen pot. In secondary fermentation the soaked i. the primary fermented millet is cooked in the earthen pot at 90–95 °C for 20 min till it is completely cooked.The mixture was cooled to 40 °C before adding inoculum bacterial culture.i.e *Lactobacillus sps.* Cover the earthen pot with sterile muslin cloth and leave it for fermentation for 8 hrs. This fermented millet is taken for biochemical analysis.

**5.3 FLOUR-BASED MILLET** - **WATER-BASED** WITH **SUGAR** INOCULUM**- *LACTOBACILLUS SPS***

To prepare water-based fermented millet ,–primary and secondary fermentation is done. In primary fermentation take 20 gm of Barnyard millet *i.e* dehusked millet is steeped in water for 8 hours in 250 ml of distilled water in earthen pot. In secondary fermentation the soaked i. the primary fermented millet is cooked in the earthen pot at 90–95 °C for 20 min till it is completely cooked**.** 5% sugar (sucrose) was added in the last five minutes of the pre-treatment. After the pre-treatment, the mixture was cooled to 40°C before adding inoculum bacterial culture *i.e Lactobacillus sps.* Cover the earthen pot with sterile muslin cloth and leave it for fermentation for 8 hrs. This fermented millet is taken for biochemical analysis

**5.4 FLOUR-BASED MILLET** - **WATER-BASED WITH MILK INOCULUM-*LACTOBACILLUS SPS*.**

To prepare water-based fermented millet ,–primary and secondary fermentation is done. In primary fermentation take 20 gm of Barnyard millet *i.e* dehusked millet is steeped in water for 8 hours in 250 ml of distilled water and milk (1:1) in earthen pot. In secondary fermentation the soaked the primary fermented millet is cooked in the earthen pot at 90–95 °C for 20 min till it is completely cooked. The mixture was cooled to 40 °C before adding inoculum bacterial culture *i.e Lactobacillus sps.* Cover the earthen pot with sterile muslin cloth and leave it for fermentation for 8 hrs. This fermented millet is taken for biochemical analysis

**5.5 FLOUR-BASED MILLET - WATER-BASED WITH MILK AND SUGAR INOCULUM-; *LACTOBACILLUS SPS***

To prepare water-based fermented millet ,–primary and secondary fermentation is done. In primary fermentation take 20 gm of Barnyard millet *i.e* dehusked millet is steeped in water for 8 hours in 250 ml of distilled water and milk (1:1) in earthen pot. In secondary fermentation the soaked i. the primary fermented millet is cooked in the earthen pot at 90–95 °C for 20 min till it is completely cooked. 5% sugar (sucrose) was added in the last five minutes of the pre-treatment. After the pre-treatment, the mixture was cooled to 40 °C before adding inoculum bacterial culture *i.e Lactobacillus sps.* Cover the earthen pot with sterile muslin cloth and leave it for fermentation for 8 hrs. This fermented millet is taken for biochemical analysis

**5.6 Flour-based millet - water-based inoculum-;*Yeast*** (*Saccharomyces cerevisiae*)

To prepare water-based fermented millet ,–primary and secondary fermentation is done. In primary fermentation take 20 gm of Barnyard millet *i.e* dehusked millet is steeped in water for 8 hours in 250 ml of distilled water in earthen pot. In secondary fermentation the soaked i. the primary fermented millet is cooked in the earthen pot at 90–95 °C for 20 min till it is completely cooked. The mixture was cooled to 40°C before adding inoculum fungi culture -yeast*.* Cover the earthen pot with sterile muslin cloth and leave it for fermentation for 8 hrs. This fermented millet is taken for biochemical analysis

**5.7 Flour-based millet - water-based with sugar- inoculum- Yeast** (*Saccharomyces cerevisiae*)

To prepare water-based fermented millet ,–primary and secondary fermentation is done. In primary fermentation take 20 gm of Barnyard millet- *i.e* dehusked millet is steeped in water for 8 hours in 250 ml of distilled water in earthen pot. In secondary fermentation the soaked i. the primary fermented millet is cooked in the earthen pot at 90–95 °C for 20 min till it is completely cooked**.** 5% sugar (sucrose) was added in the last five minutes of the pre-treatment. After the pre-treatment, the mixture was cooled to 40 °C before adding inoculum fungi culture -yeast*.* Cover the earthen pot with sterile muslin cloth and leave it for fermentation for 8 hrs. This fermented millet is taken for biochemical analysis

**5.8 Flour-based millet - water-based with milk- inoculum-Yeast** (*Saccharomyces cerevisiae*)

To prepare water-based fermented millet ,–primary and secondary fermentation is done. In primary fermentation take 20 gm of Barnyard millet *i.e* dehusked millet is steeped in water for 8 hours in 250 ml of distilled water and milk (1:1) in earthen pot. In secondary fermentation the soaked i. the primary fermented millet is cooked in the earthen pot at 90–95 °C for 20 min till it is completely cooked. The mixture was cooled to 40°C adding inoculum fungi culture –Yeast. Cover the earthen pot with sterile muslin cloth and leave it for fermentation for 8 hrs. This fermented millet is taken for biochemical analysis

**5.9 Flour-based millet - water-based with sugar and milk- inoculum- Y*east*..**

To prepare water-based fermented millet ,–primary and secondary fermentation is done. In primary fermentation take 20 gm of Barnyard millet *i.e* dehusked millet is steeped in water for 8 hours in 250 ml of distilled water and milk (1:1) in earthen pot. In secondary fermentation the soaked i. the primary fermented millet is cooked in the earthen pot at 90–95 °C for 20 min till it is completely cooked. 5% sugar (sucrose) was added in the last five minutes of the pre-treatment. After the pre-treatment, the mixture was cooled to 40 °C adding inoculum fungi culture –Yeast. Cover the earthen pot with sterile muslin cloth and leave it for fermentation for 8 hrs. This fermented millet is taken for biochemical analysis

**6. MILLET –BIOCHEMICAL TEST**

**6.1. CARBOHYDRATE:-**

**Reagents:**

* **Standard for carbohydrate is prepared using D- Glucose (0.1mg/ml)**
* **Anthrone reagent 33%**

**Estimation of reducing sugars:** Cooked fermented Barnyard Millet (CF-BM) broth was taken to estimate the reducing sugar. To a clean dry test tubes0.1ml of millet broth is taken and the volume is made to 1 ml by adding distilled water. For blank 1 ml of distilled water is taken. Then add 4 ml of anthrone reagent to each test tube and mix thoroughly. Keep it in boiling water for 8 min. Cool it rapidly and green colour formed was measured at 620 nm using colorimeter(18). For quantitative analysis, reducing sugars was calculated from the regression equation of the standard plot **y = 2.325x - 0.0005; R² = 0.9709** and is expressed as gm of equivalent of D-glucose per gram of dry weight.

Fig 1:

**PROTEIN (MODIFIED LOWRY’S METHOD)** (48)

**Reagents:**

* **Standard for protein:** Bovine Serum Albumin (BSA) is taken as standard (1mg/ml)
* **Alkaline reagent :**0.3 N NaOH with 2.9% Na2CO3.
* **FolinCiocalteau reagent-** This reagent has to be diluted with two times its volume with distilled water.

**ESTIMATION OF PROTEIN:**

Cooked fermented Barnyard Millet (CF-BM) broth was taken to estimate the reducing sugar. To a clean dry test tubes 0.1ml of millet broth is taken and the volume is made to 1 ml by adding distilled water. For blank 1 ml of distilled water was taken. To these test tubes add 2.5 ml of alkaline reagent and 0.75 ml Folin’s reagent was add. Blue colour formed was measured at 640 nm using colorimeter. For quantitative analysis, protein was calculated from the regression equation of the standard plot **of BSA is y = 0.0009x + 0.008, R² = 0.9908** and is expressed as gm of equivalent of BSA per gram of dry weight.

Fig :2

**6.3 QUANTITATIVE ESTIMATION OF ACIDTY IN FERMENTED MILLET**

**6.3.1. TOTAL TITRABLE ACID AND PH DETERMINATION:**

**Reagents :**

* + 0.1 M NaOH. -Dilute 4g of NaOH to 1 litre.
  + 0.5 % Phenolphthalein indicator :(0.5 % Phenolphthalein in 5 % alcohol).

The pH and titrable acid of cooked fermented Barnyard millet (CF-BM) during secondary fermentation was taken after 12 hrs of fermentation. The titrable acid of cooked fermented Barnyard millet CF-BM was determined by transferring 10ml of broth cultures of test organisms into 100 ml conical flasks and 1ml of phenolphthalein indicator was added to it. This was titrated with 0.1 M NaOH until consistent pink colour appeared. Readings of titre values were obtained in triplicates. The acidity was expressed as based on the conversion of 1m of 0.1M NaOH being equivalent to 9.008 x10-3 g (90.08 mg ) of lactic acids. The titratable acidity was then calculated as stated in A.O.A.C (1980). (20,21)

**6.3.2. TOTAL TITRABLE ACID AND pH DETERMINATION AT DIFFERENT INCUBATION TIME**

The quantity of lactic acid and volatile acid was taken for quantification using cooked fermented Barnyard millet (CF-BM) and with inoculum (*Lactobacillus* and yeast-*Saccharomyces cerevisiae*, ) with and without substrate was incubated for 24hrs, 48hrs and 72hrs. The determination of titrable acid was carried out by taking out sample for analysis at interval of 24hrs for a period of 72 hrs by transferring 10ml of broth cultures of test organisms into 100 ml flasks. The titrable acid of cooked fermented Barnyard millet (CF-BM) was determined by transferring 10ml of broth cultures of test organisms into 100 ml conical flasks and 1ml of phenolphthalein as indicator was added to it. This was titrated with 0.1 M NaOH until consistent pink colour appeared. Readings of titre values were obtained in triplicates the acidity was expressed as based on the conversion of 1M of 0.1M NaOH being equivalent to 9.008 x10-3 g (90.08 mg ) of lactic acids. The titratable acidity was then calculated as stated in A.O.A.C (1980) (20,21)

Where; ml NaOH = volume of NaOH used

**Lactic acid and Volatile acid:**

**6.4. QUANTITATIVE ESTIMATION OF DIACETYL**

Diacetyl production at 24hrs, 48hrs and 72hrs was determined by transferring 25ml of broth cultures of test organisms into 100 ml flasks. Hydroxylamine solution (7.5 ml) of 1 molar was added to the flask. The flasks were titrated with 0.1 M HCl to a greenish yellow end point using bromothymol blue as indicator**.**

The equivalence factor of HCl to diacetyl is 21.52 mg. The concentration of diacetyl produced was calculated using the A.O.A.C. (1980). Where Ak = % of diacetyl b- s = volume of HCl used E = equivalence factor (21.52/mg) W = volume of broth 100 = constant (22)

**7. CALCIUM, IRON, ZINC-ICPMS PROCEDURE:**

**7. 1. Closed-system microwave mineralization:**

Place a portion of the sample weighing between 0.5 g liquid and solid samples, Samples 1.0 g taken in the digestion vessel. Add 5 mL of deionized water, then 5 mL of nitric acid to the digestion vessel and close. To ensure that there has been no contamination, each series of analyses should include a blank test (a matrix-free test performed with the same amounts of reagents that undergoes digestion at the same time as the samples).To control the analysis method, each series of analyses should contain a reference material containing a known amount of the elements of quantify. This reference material should undergo digestion under the same conditions as the sample under examination.

**7.2. Digestion**

Install the digestion flask on the rotor, and then apply the appropriate digestion programmed. Oven programming (power/time, for example) should be performed according to manufacturer’s recommendations. For microwave digestion times are 45 minutes. A gradual increase between selected phases is recommended so as to avoid pressure spikes inside the vessel. To reduce the temperature and pressure inside the digestion vessel, a cooling phase is included at the end of the program. The final state of digestion of the sample depends on the digestion temperature. In general, the higher the temperature, the less residual carbon is left in the solution and the better the quality of the mineral deposit. The digestion solution should be limpid, without any suspended particles, and its volume should be practically the same as before digestion. After digestion, open the vessels, then rinse the covers and walls with deionized water, take up in polypropylene flasks, and dilute to 25 mL with deionized water.

**8. VITAMINS-LC MS MS PROCEDURE:**

**Instrument:- LC/MS/MS**

Agilent: LC: (G1312B) HPLC-1260 Binary pump.

MS MS:G6430A Triple Quad MS,

EclipsPlus C18 column 100 X 4.6, 3.5 um

**8.1. Instruments Conditions for LC/MS/MS :**

**8.1.1. HPLC conditions :**

The LC-program for the Vitamins compounds. Eclipse Plus C18 column 100 X 4.6, 3.5 um was used with a methanol and 0.1 % Formic acid in gradient. The flow rate was 0.400 ml/min. The column was kept 40˚C. The injection volume was 25 micro liter and run time 12 min.

**8.1.2. Sample Preparation :**

Weigh 10 gm of sample in a screw capped conical flask and dissolve with 10-15 ml of water and then cover the flask with aluminum foil. Add 10 ml of 0.1N hydrochloric acid and then shake it. The sample was heated for half an hour over a boiling water bath and stirred frequently. The sample was next cooled in a basin of water and then adjust pH to 4- 4.5 with 2.5M sodium acetate solution. The extract was then transferred into a 100 ml volumetric flask and then made up to the mark with water. After shaking well, a portion of the extract was filter through 0.22 µm membrane filter. Inject 20 µl of the filtrate into LC MS MS and compare against known concentration of mixture of standards.

**RESULTS AND DISCUSSION**

**MICROBIAL STUDIES AND FERMENTATION.**

**Table :1Microbiological analysis - Raw Barnyard millet- Primary fermentation and Cooked and fermented Barnyard millet- Secondary fermentation**

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
|  | **Bacterial species isolated from primary and secondary fermented millet** | | | | |
| **S.NO** | **SPECIES** | **Raw Barnyard millet- Primary fermentation** | | **Cooked and fermented Barnyard millet- Secondary fermentation** | |
| **Colony Forming Unit (CFU)** | **Percent contribution** | **Colony Forming Unit** | **Percent contribution** |
| **(CFU)** |
| **1** | ***Bacillusalvei*** | **Nil-** | **Nil** | **2.33×109** | **6.4** |
| **2** | ***Bacillus sp*** | **4.66×109** | **21.1** | **Nil** | **Nil -** |
| **3** | **Micrococcus *lylae*** | **7×109** | **31.48** | **9.33×109** | **25.9** |
| **4** | ***Micrococcuskristinae*** | **10.33×109** | **46.9** | Nil | **Nil** |
| **Fungal species isolated from primary and secondary fermented millet** | | | | | |
|
|
| **S.NO** | **SPECIES** | **Raw Barnyard millet- Primary fermentation** | | **Cooked and fermented Barnyard millet- Secondary fermentation** | |
| **Colony Forming Unit** | **Percent contribution** | **Colony Forming Unit (CFU)** | **Percent contribution** |
| **(CFU)** |
| **1** | ***Aspergillus flavus*** | **0.33× 102** | **0.26** | **0.33× 102** | **0.24** |
| **2** | ***Penicillium citrinum*** | **126x102** | **99.7** | **133x 102** | **99.5** |
| **3** | ***Saccharomyces cerevesis*** | Nil | **Nil** | **0.33** | **0.24** |

Microbial fermentation was carried out in earthen pot using Barnyard Millet.

**Primary fermentation in bacteria:**

In primary fermentation of raw Barnyard millet was soaked with distilled water in earthen pot for 8hrs. The total number of bacterial Colony Forming Units (CFU) were 66 colonies in primary fermentation(uncooked millet). Of these *Micrococcus kristinae* 31 colonies was reported more number of colonies followed by *Micrococcus lylae* 21 colonies and *Bacillus sps* 14 colonies.

**Secondary fermentation in bacteria:**

In secondary fermentation Barnyard millet was cooked and fermented in earthen pot for 8hrs. The total number colony forming unit of bacteria is 112 colonies . Among these *Micrococcus lylae* 28 colonies followed by *Bacillus alvei* 7 colonies. Similar the percentage contribution of bacterial species (67.4%) and followed by *Micrococcus lylae* (25.9%) and *Bacillus alvei* (6.4%).

**Primary fermentation in fungi:**

In primary fermentation of raw Barnyard millet was soaked with distilled water in earthen pot for 8hrs. The total number of fungal colony forming units is 379 colonies. Among these colonies 378 colonies of *Penicillium citrinum* had maximum number of colonies and least number of colonies in *Aspergillus flavus* of 1colony. Similar to these isolated fungi species, the percentage contribution fungal isolate in primary fermentation is *Penicillium citrinum* (99.7%) and the *Aspergillus flavus* (0.24%).

**Secondary fermentation in fungi:**

In secondary fermentation Barnyard millet was cooked and fermented in earthen pot for 8hrs. The total number of fungal colony forming unit is 401. Among these *Penicillium citrinum* maximum of 399 colonies, whereas *Aspergillus flavus* and *Saccharomyces cerevesiae* reported 1 colony only. The percentage contribution of fungal isolates in secondary fermentation is maximum in *Penicillium citrinum* (99.5%) followed by *Aspergillus flavus* and *Saccharomyces cerevesiae* ( 0.24%)

Yeasts and Molds were isolated and identified as *Saccharomyces cerevisiae*, *Candida sp. Aspergillus niger, Aspergillus flavus* and *Penicillium sp.* The bacteria were *Lactobacillus plantarum, Lactobacillus casie, Lactobacillus fermentum, Lactobacillus lactis, Klebisella pnemoniae, Escherichia coli, lavobacterium sp., Proteus vulgaris* (23) from five starchy- based food product.

In millet and defatted Soybean blends, decrease in pH from 9.2-9.3 to a range of 6.6-6.8 was recorded from day 1 (0 hours) to day 4 (72 hours) *Bacillus cereus, Bacillus subtillis*, *Enterococcus* sp*., Micrococcus* sp*.* and *Lactobacillus* sp were isolated during fermentation. In fermentation of flour some microorganism such as *Pseudomonas* sp., *Proteus* sp., and coliforms (*Enterobacter* sp. and *Klebsiella* sp.) may not have played any role in the process of fermentation. The microbiological load as measured by the total plate count per gram of the extrudates was generally low in all the extrudates *st*ored at room temperature (35+2°C) (24).

* 1. **Biochemical test on formulated Barnyard millet**
* **UNSOAKED** = Raw millet
* **SOAKED**= Steeped in water for 8 hrs –Primary fermentation
* **CF-BM**= Cooked and fermented- Barnyard millet for 8 hrs- Secondary fermentation
* **LB** = CF-BM with *Lactobacillus sps*
* **LB+S**-= CF-BM with *Lactobacillus sps* with substrate sugar
* **LB+M**= CF-BM with *Lactobacillus sps* with substrate milk
* **LB+S+M**= CF-BM with *Lactobacillus sps* with both substrate sugar and milk
* **Y =** CF-BM with Yeast (*Saccharomyces cerevisiae*)
* ***Y*+S**-= CF-BM with Yeast (*Saccharomyces cerevisiae*) with substrate sugar
* **Y+M**= CF-BM with Yeast (*Saccharomyces cerevisiae*) with substrate milk
* **Y+S+M**= CF-BM with Yeast (*Saccharomyces cerevisiae*) with both substrate sugar and milk

**1.2.1. Reducing sugar in Barnyard millet grains**

**Table:2 Reducing sugar in Barnyard millet grains**

|  |  |  |
| --- | --- | --- |
| **S.NO** | **Barnyard Millet** | **gm of Reducing sugar/gm of millet** |
| 1 | Unsoaked | 0.004 |
| 2 | Soaked | 0.016 |
| 3 | CF-BM | 0.004 |
| 4 | LB | 0.017 |
| 5 | LB+S | 0.012 |
| 6 | LB +M | 0.010 |
| 7 | LB +S+M | 0.006 |
| 8 | Y | 0.004 |
| 9 | Y+S | 0.009 |
| 10 | Y+M | 0.004 |
| 11 | Y+S+M | 0.008 |

It is evident from the Table2 that due to fermentation the polysaccharides get reduced to reducing sugar . The concentration of reducing sugar in unsoaked Barnyard millet is less, but the concentration of reducing sugar in soaked gram of millet is 0.004gm/g of Barnyard Millet there is a sharp decrease in the reducing sugar of carbohydrate in cook and fermented Barnyard millet of 0.004 gm / g of Barnyard millet. The change in carbohydrate value with fermentation was reported in fermented pumpkin seeds and fermented cowpea respectively ( 25, 26).

The effect of two substrate milk and sugar and the combined effect of both substrate on the carbohydrate content due to fermentation was studied using *Lactobacillus* bacteria and yeast separately cooked and fermented Barnyard millet (CF-BM) when inoculated with *Lactobacillus* bacteria only, showed increase in reducing sugar of 0.017 gm but there is a study decrease in carbohydrate value when added substrate of sugar 0.012 gm, milk 0.010 gm of reducing sugar separately and the level of carbohydrate value 0.006 gm showed sharp decrease with the combined effect of both the substrate with sugar and milk to CF-BM with Lactobacillus. Lactic acid bacteria do not ferment polysaccharides like starch or dextrins because they lack the necessary hydrolytic enzymes but can ferment sucrose, maltose, galactose (27).

Cooked fermented Barnyard millet (CF-BM) when inoculated with yeast and supplemented with non-reducing sugar sucrose slight increase in reducing sugar value, which was also noticed on pearl millet that on hydrolysis of starch resulted in high concentration of total soluble, reducing, non-reducing sugars when compared to unfermented millet (28).

**Table: 3 Protein content in Barnyard millet grains**

|  |  |  |
| --- | --- | --- |
| **S.NO** | **SAMPLE** | **PROTEIN gm/gm of millet** |
| 1 | **UNSOAKED** | **5.83** |
| 2 | **SOAKED** | **11.39** |
| 3 | **CF-BM** | **30.83** |
| 4 | **LB** | **16.94** |
| 5 | **LB+S** | **12.78** |
| 6 | **LB +M** | **29.44** |
| 7 | **LB +S+M** | **11.39** |
| 8 | **Y** | **14.17** |
| 9 | **Y+S** | **19.72** |
| 10 | **Y+M** | **14.17** |
| 11 | **Y+S+M** | **11.39** |

It is evident from the about Table 3 that the concentration of protein per gram of Barnyard millet (BM) in unsoaked is less 5.83 gm/g of millet, but there is a gradual increase in the concentration of protein in soaked gram and cooked fermented Barnyard millet (CF –BM) of 11.39 to 30.83 gm / g of millet respectively. The increase in protein content after fermentation than in raw Barnyard millet was also reported in finger millet (29). This may be due to utilization of carbohydrate content by the action of enzyme produced by fermentation (30).The effect of two substrate milk and sugar and the combined effect of both substrate on the protein content due to fermentation was studied using *Lactobacillus* bacteria and yeast separately

Cooked and fermented millet (CF-BM) when inoculated with *Lactobacillus sps* bacteria, in presence substrate milk showed increase in protein of 29.44 gm /gm of millet, but there is a steady decrease in protein value with only *Lactobacillus sps*, with sugar and with both milk and sugar to cooked and fermented millet (CF-BM). Fermentation of pearl millet lead to increase in protein content (31).

Cooked and fermented Barnyard millet (CF-BM) when inoculated with yeast and supplemented with sucrose there was a slight increase in protein of 19.72 gm, but with only yeast and substrate milk showed the same in the value of 14.7 gm of protein , but there was decrease in protein value 11.39 gm with of both the substrate of milk and sucrose was used in yeast inoculated CF-BM . Increase in protein content in finger millet was reported when inoculated with yeast (29).

* 1. **ACIDITY OF FERMENTED MILLET**

**2.3.1 Titrable Acid, Diacety and pH of Barnyard millet produced on fermentation**

**TABLE 4- Titrable acid, Diacety and pH of Barnyard millet produced on fermentation**

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| **S.NO** | **SAMPLE** | **TOTAL ACIDITY OF LACTIC ACID** | **VOLATILE ACID** | **% TOTAL TITRABLE ACID** | **DIACETY** | **pH** |
| 1 | **UNSOAKED** | **0.019** | **0.015** | **45.0** | **0** |  |
| 2 | **SOAKED** | **0.0152** | **0.012** | **36.0** | **0** | **6** |
| 3 | **CF-BM** | **0.38** | **0.3** | **90.1** | **0** | **6** |
| 4 | **CF-BM +LB** | **0.0228** | **0.018** | **54.0** | **0** | **5** |
| 5 | **CF-BM +LB+S** | **0.076** | **0.06** | **18.0** | **0** | **5** |
| 6 | **CF-BM +LB +M** | **0.019** | **0.015** | **45.0** | **0** | **5** |
| 7 | **CF-BM +LB +S+M** | **0.0228** | **0.018** | **54.0** | **0** | **5** |
| 8 | **CF-BM +Y** | **0.228** | **0.18** | **54.0** | **0** | **5** |
| 9 | **CF-BM +Y+S** | **0.0304** | **0.024** | **72.1** | **0** | **5** |
| 10 | **Y+M** | **0.0228** | **0.018** | **54.0** | **0** | **5** |
| 11 | **Y+S+M** | **0.0228** | **0.018** | **54.0** | **0** | **5** |

To determine the acid production of soaked , cooked and fermented with inoculum *Lactobacillus sps*, and Yeast (*Saccharomyces cerevisiae*); substrate of milk, sucrose and combined effect of milk and sucrose with the inoculum lactic acid , volatile acid and percent titrable acid was measured using 0.1 N NaOH it showed different level of acidity**.**

It is evident from the about Table 4 that not much significant drop pH, pH6 but shows corresponding increase in the lactic acid , volatile acid and percent titrable acid in unsoaked millet 0.019, 0.015 and 45 percent, is less as compared with soaked millet pH 5.6 0.015, 0.012, 36 percent, and cooked and fermented Barnyard millet CF-BM pH 6 has maximum acidity of 0.038, 0.3 and 90.1 percent, respectively.

In CF-BM the pH6, has dropped to pH 5 when inoculated with *Lactobacillus sps* bacteria having no substrate , but subsequently showed increase lactic acid , volatile acid and percent titrable acid 0.023, 0.018 and 54 percent, and with substrate milk 0.19, 0.015 and 45 percent, but with sucrose as substrate and substrate milk and sucrose at pH 5 decrease in acidity was noted as 0.023, 0.018 and 54 percent, with only sucrose, 0.076, 0.076 and 18 per cent respectively.

There was decrease in pH from 8.50 at 0 hr to pH 7.60 for pearl millet and pH 7.90 for finger millet, while total titrable acid (TTA) increased from 0.0038 to 0.18 g/L during germination. The decrease in pH and increase in total titrable acid (TTA) might be due to degradation of some complex organic molecules such as lipids, phytin, and protein to simpler compounds (30)

*Lactobacillus* generate energy only during breakdown of carbohydrate but *Lactobacillus* do not ferment polysaccharides that starch or dextrin because they lack hydrolytic enzyme. Lactose are absent in plant (27).Homo fermentative species of *Lactobacillus* convert sugars in milk mostly into lactic acid, whereas the hetero fermentative species convert lactose into lactic acid, acetic acid, ethanol and CO2. Production of lactic acid by *Lactobacillus* is strain dependent (31).

Lactose present in milk utilised by *Lactobacillus* have a role in milk fermentation to produce acid which is important as preservative agents and generating flavour of the products. Exopolysaccharides are also produced which are essential as texture formation and has several health promoting properties and is widely used in developing new fermented products (32).

Cooked and fermented Barnyard millet (CF-BM) when inoculated with yeast and supplemented with sucrose pH 6 was recorded increase in lactic acid , volatile acid and percent titrable acid of 0.75, 0.59 and 1.78 was noted. With only yeast and substrate milk and with both substrate of milk and sucrose supplemented along with the inoculum yeast showed almost the same value but the pH 5 was noted 0.023, 0.18 and 54 percent. But with sucrose was used in yeast inoculated cooked and fermented (CF-BM) had pH 5 with decrease in acidity of 0.030, 0.024 and 72.1 percent. Glucose is the critical nutrient which stimulates a variety of growth-related events in the yeast *Saccharomyces cerevisiae* (33). Diacety in cooked and fermented (CF-BM) was negative at pH 6-5 since gram negative was not observed during fermentation. It is reported that he optimum pH for diacetyl production is pH 4.5–5.5 (34) and also the test carried out using Diacetyl compound showed lactic acid bacteria was uneffected even at the concentration of 100 and 350 µg/ml at pH 5 to 7, gram-positive non-lactic acid bacteria, were inhibited by 300 ,ug/ml at pH s7.0 and in yeasts and gram-negative bacteria that grew at pH 5.5 were inhibited by 200 µg/ml (35).

**1.2.3.1 Quantity of Lactic Acid, Volatile Acid, Total Titrable Acid, Diacetyl produced on fermentation at different incubation times**

Table :5 **Quantity of Lactic Acid, Volatile Acid, Total Titrable Acid on fermentation at different incubation times**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **SAMPLE** | **HOURS** | **TOTAL ACIDITY OF LACTIC ACID** | **VOLATILE ACID** | **% TOTAL TITRABLE ACID** |
| **COOKED AND FERMENTED** | **24 HRS** | **0.038** | **0.03** | **90.1** |
| **48 HRS** | **0.064** | **0.051** | **153.1** |
| **72 HRS** | **0.083** | **0.066** | **198.2** |
| **LACTO BACILLUS** | **24 HRS** | **0.023** | **0.018** | **54.0** |
| **48 HRS** | **0.045** | **0.036** | **108.1** |
| **72 HRS** | **0.075** | **0.06** | **180.2** |
| **MILK+ LACTO BACILLUS** | **24 HRS** | **0.019** | **0.015** | **45.0** |
| **48 HRS** | **0.049** | **0.039** | **117.1** |
| **72 HRS** | **0.079** | **0.063** | **189.2** |
| **MILK+SUGAR+ LACTO BACILLUS** | **24 HRS** | **0.023** | **0.018** | **54.0** |
| **48 HRS** | **0.049** | **0.039** | **117.1** |
| **72 HRS** | **0.071** | **0.057** | **171.2** |
| **Yeast** | **24 HRS** | **0.023** | **0.018** | **54.0** |
| **48 HRS** | **0.056** | **0.045** | **135.1** |
| **72 HRS** | **0.083** | **0.066** | **198.2** |
| **MILK+ YEAST** | **24 HRS** | **0.023** | **0.018** | **54.0** |
| **48 HRS** | **0.034** | **0.027** | **81.1** |
| **72 HRS** | **0.056** | **0.045** | **135.1** |
| **MILK+SUGAR+ YEAST** | **24 HRS** | **0.023** | **0.018** | **54.0** |
| **48 HRS** | **0.045** | **0.036** | **108.1** |
| **72 HRS** | **0.079** | **0.063** | **189.2** |

It is evident from Table 5 that the Cooked and fermented Barnyard millet (CF-BM) was incubated i.e. fermented for 24hours, 48 hours and 72 hours showed increase in lactic acid from 0.038 to 0.083 ,volatile acid 0.03 to 0.066 and percent of total titrable acid (TTA) 0.09 to 0.198. Cooked and fermented Barnyard millet (CF-BM) when inoculated with Lactobacillus and incubated for 24hours, 48 hours and 72 hours there was increase in lactic acid from 0.023 to 0.075 ,volatile acid 0.18 to 0.06 and percent of total titrable acid(TTA) 54 to 180.2.

Cooked and fermented Barnyard millet (CF-BM) when inoculated with *Lactobacillus* and supplemented with substrate milk and incubated for 24hours, 48 hours and 72 hours there was increase in lactic acid from 0.019 to 0.079 ,volatile acid 0.015 to 0.063 and percent of total titrable acid (TTA) 45 to 189.2. Cooked and fermented Barnyard millet (CF-BM) when inoculated with *Lactobacillus sps* and supplemented with substrate milk and sugar and incubated for 24hours, 48 hours and 72 hours there was increase in lactic acid from 0.023 to 0.071 ,volatile acid 0.018 to 0.057 and percent of total titrable acid (TTA) 54 to 171.2.

Cooked and fermented Barnyard millet (CF-BM) when inoculated with yeast and incubated for 24hours, 48 hours and 72 hours there was increase in lactic acid from 0.023 to 0.083 ,volatile acid 0.018 to 0.066 and percent of total titrable acid(TTA) 54 to 198.2.

Cooked and fermented Barnyard millet (CF-BYM) when inoculated with yeast and supplemented with substrate milk and incubated for 24hours, 48 hours and 72 hours there was increase in lactic acid from 0.023 to 0.056 ,volatile acid 0.018 to 0.045 and percent of total titrable acid (TTA) 54 to 135.1.

Cooked and fermented Barnyard millet (CF-BM) when inoculated with yeast and supplemented with substrate milk and sugar and incubated for 24hours, 48 hours and 72 hours there was increase in lactic acid from 0.023 to 0.079 ,volatile acid 0.018 to 0.063 and percent of total titrable acid (TTA) 54 to 189.2.

With increase in incubation time the nutritional, anti‑nutritional, and bioactive components of finger millet (*Eleusine coracana L*.) (29). Fermentation treatments were found effective in increasing the nutritional value and decreasing the anti-nutritional components in finger millet.

Eighteen volatile compounds were identified in Barnyard millet which included cyclohexasiloxane, dodecamethyl (7.22%), cycloheptasiloxane, tetradecamethyl (5.82%), benzene, 1,3-bis-1,1-dimethylethyl (5.65%), dodecane (5.52%), eicosane (4.61%) and cyclooctasiloxane, hexadecamethyl (4.55%). The notable amount present in the form of decanal, heptanes, 2,4-dimethyl, benzene, 1-ethyl-3-methyl and mesitylene etc.**(36).**

Volatile compound that heptanal was produced in higher amounts (2-heptaone and 2-nonaone touched 25.56 and 10.67 µg L-1 , respectively) by *L. delbrueckii subsp.bulgaricus* during storage period was reported in fermented milk **(37).**These compounds were reported in dairy products, including milk, fermented milk and cheese (38).

**1.3 Vitamin B and minerals in cooked fermented Barnyard millet**

**1.3.1 Vitamin B level:**

**Table:6 Vitamin B level**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
|  | **VITAMIN** | **GENERIC NAME** | **UNITS** | **MG/100ML** |
| **S.NO** |
| **1** | Vitamin -B1 | Thiamin | mg/100ml | BLQ (LOQ 0.01) |
| **2** | Vitamin -B2 | Riboflavin | mg/100ml | 0.0653 |
| **3** | Vitamin -B3 | Niacin | mg/100ml | BLQ (LOQ 0.01) |
| **4** | Vitamin -B5 | Pantothenic acid | mg/100ml | 0.3176 |
| **5** | Vitamin -B6 | Pyridoxine | mg/100ml | BLQ (LOQ 0.01) |
| **6** | Vitamin -B7 | Biotin | mg/100ml | BLQ (LOQ 0.01) |
| **7** | Vitamin -B9 | Folic acid | mg/100ml | 0.0103 |
| **8** | Vitamin -B12 | Cyanocobalamin | mg/100ml | BLQ (LOQ 0.01) |
| **Note: BLQ- Below the Limit of Quantification**  **LOQ- Limit of Quantification** | | |  |  |
|  |  |
|  |  |

Water Soluble vitamins the millets are rich sources of B-complex vitamins (except Vitamin B 12). In cooked and fermented (CF-BM) the total vitamin B2 - Riboflavin content present is 0.0653 mg/100 ml, vitamin B5 Pantothenic acid is 0.3176 mg/100ml and vitamin B9- Folic acid is 0.013 mg/100ml of extract. The other vitamin like -. Vitamin -B1- Thiamin, Vitamin -B3- Niacin, Vitamin -B6, B7- Pyridoxine, Biotin and Vitamin -B12- Cyanocobalamin was found Below the Limit of Quantification (BLQ) (Table 6). The presence of water Soluble Vitamins in other millet, Bajara, Sorghum, , Ragi, Kodo and , Foxtail showed the presence vitamin B1, B2, B3 , B5 , B6, B7, B9 , Proso millet reported the presence of vitamin B2, B3, B5, B6, but in Barnyard millet showed the presence vitamin B2, B3 and B5 only (39).

.

**1.3.2 Mineral**

**Table:7 Evaluation of Minerals in fermented in cooked fermented Barnyard millet (CF-BM)**

|  |  |  |  |
| --- | --- | --- | --- |
| **S.NO** | **MINERAL** | **Units** | **Unit- mg/l** |
| **1** | **Calcium as Ca** | **mg/l** | **39.6** |
| **2** | **Iron as Fe** | **mg/l** | **2.4** |
| **3** | **Zinc as Zn** | **mg/l** | **0.396** |
|  | **mg/l - milligrams per litre** | |  |

This study evaluated the extent of variability of micronutrients (Fe, Zn) and macronutrients (Ca,). In Barnyard millet has shown presence calcium content (Ca) is nearly 39.6 mg/l, Iron (Fe) 2.4 mg/l and Zinc (Zn) 0.396 mg/l (Table 7).

In Finger Millet or *Ragi* (*Eleusine coracana*) calcium content is nearly 350 mg for 100g whereas in wheat and rice it is even below 50mg. Pearl Millet or B*ajra* (*Pennisetum glaucum*) contains magnesium, copper, zinc. and is rich in calcium and unsaturated fats which benefits our health. Sorghum or *Jowar* (*Sorghum vulgare*) good amount of calcium with small amounts of iron and sodium. Jowar helps to maintain heart, body weight and arthritis (40). Mineral content of millets such as pearl millet, finger millet, foxtail millet, little millet, proso millet and Kodo millet namely calcium content of the above mentioned millet was reported as 10-46, 240-410,10-30,12-30, 20-33 and 10-31 mg/100g ; Zinc content 2.95-3.1, 2-2.3, 2.14-9,3.5-11, 1.4-2.4,0.7-1.5 mg/100g and iron content 7.49-8, 3.9-7.5, 3.2-19, 13-20, 4-5.2and 0.7-3.6 mg/100g (41).

**2.5. FATTY ACIDS OF BARNYARD MILETT**

**Fig:3 GC-MS OF FATTYACIDS**



**Table:8 FATTY ACID PROFILE OF BARNYARD MILETT**

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| **COMPOUND NAME** | **FORMULA** | **PEAK** | **START** | **RT** | **END** | **AREA SUM PERCENT** |
| **Palmitic Acid (C16:0)** | **C16H32O2** | **1** | **17.003** | **17.069** | **17.131** | **20.53** |
| **Linoleic Acid (C18:2)** | **C18H32O2** | **2** | **18.861** | **18.901** | **18.941** | **41.95** |
| **Stearic Acid (C18:0)** | **C18H36O2** | **3** | **19.942** | **18.969** | **18.969** | **4.81** |
| **Oleic Acid (C18:1)** | **C18H34O2** | **4** | **18.969** | **18.994** | **19.078** | **32.71** |

**Fatty Acid Composition in Barnyard Millet**

The analysis of fatty acid composition is carried out by GC /MS. The fatty acid is converted into fatty acid methyl ester by acid – catalysed esterification and it showed the calibration curve having four major peaks. The first peak *i.e* the fatty acid has the lowest boiling point which convert liquid to gaseous phase and has the retention time of 17.069 is palmitic acid whereas the other three peaks has retention time of 18.901 is linoleic acid, 18.969 is stearic acid and 18.994 is oleic acd. The percent area of the peak with respect to retention time has maximum of 41.95 is linoleic acid followed by 32.71 oleic acid, 20.53 palmitic acid and 4.81 stearic acid (Table 8). Foxtail millet bran oil is rich in linoleic acid (66.5%) and oleic acid (13.0%)of saturated fatty acids- palmitic acid (6.4%) and stearic acid (6.3%) (42). Several fatty acid were detected in pearl millet, the major fatty acid was linoleic acid (47.5%) (43). Linoleic (38-40%), oleic (27-37%), palmitic (16-22%) and linolenic (1-4%) are the major fatty acids found in millets. The presence of unsaturated fatty acids account for more than 85% of the total fatty acid content in millets (44,45). Barnyard millet oil could be a good source of natural oil rich in linoleic acid and tocopherols (46).

**CONCLUSION :**

Fermentation of Barnyard millet was carried out in earthen pot. Biochemical changes accompanied with the fermentation of Barnyard millet by endogenous microflora .Increase in total titrable acid (TTA) *i.e* organic acid with decrease in pH was observed due to fermentation.Starch being the major source of energy is utilized by the microflora and results in increased reducing sugars due hydrolysis.Fermentation resulted in increased protein content.Vitamin B2, B5 and B9 were present in quantifiable amount and rich in Ca and Fe is reported in Barnyard millet.Vitamins and minerals helps for normal functioning of human body. C16 , C18 fatty acid has been observed , which would decreases the risk of heart disease and stroke. Remarkable rise in nutritive value was observed when cooked and fermented in earthen pot.

**Conflict of interest:**  "The authors declare that there is no conflict of interest”

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