IN-VIVO AND IN-VITRO ANALYSIS FOR THE DEVELOPMENT OF COST-EFFECTIVE PROBIOTIC AND MUFA RICH PROTEIN DIETARY SUPPLEMENT FOR ADULT AGE GROUPS

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1. INTRODUCTION

Malnourished individuals of various age groups are often victims of various infections like weight loss, iron, iodine, mineral, vitamin B complex deficiency etc. As with underweight, the predominance of different micro and macro nutritional deficiencies vary extensively across different states (Rosy, K., *et al*. 2017); where our product can be very effective in nutrition as it is formulated by using locally sourced and organically procured raw materials. Prevention of malnourishment can be started by providing nutrient rich supplementary foods to the individuals of all age groups. According to FAO standards (FAO 1995), suggestion, to meet the recommended dietary allowances of infants, preschool children, adolescent girls, pregnant, lactating women and men, low-cost supplementary foods could be processed domestically by simple, inexpensive processing technology. The use of protein-calorie sources of vegetables or other origins as a supplementation on regular diet has been proposed a possible solution to this problem (Farzana T& Mohajan., 2015). Hence, the present study is designed to develop a low cost, novel nutritionally rich probiotic supplementary product, fortified with various natural products to make it PUFA and MUFA rich for malnourished individuals using locally available milky mushroom, flaxseeds and palm jaggery along with probiotic cultures.

Among the edible mushrooms *Calocybe indica* commonly known as milky white mushrooms is rich in protein, lipid, fiber, carbohydrate and vitamin and contains an abundant amount of essential amino acid and low-fat product (Subbiah *et al.,* 2015). Among its many nutrients are thiamine, riboflavin, nicotinic acid, pyridoxine, biotin, and ascorbic acid. These qualities make it suitable for food supplement in diet and also used in the form of medicines to alleviate various human disorders and diseases. In addition to this it provides a lot of digestible protein, they are low in fat (Shashikant *et al.*, 2022). They primarily contain unsaturated fatty acids, which poses lower health risks than saturated fatty acids, found in animal fats. They also contain Polyphenols and flavonoids which offer protection from oxidative damage from Relative oxygen species (ROS) and free radicals. This activity thus prevents the onset of diseases and other conditions such as aging, neurodegenerative disorders, carcinogenesis, cardiovascular diseases, obesity, and diabetes. (Roy & Prasad., 2016)

Flaxseed contains amino acids like Lysine, Threonine, and Tyrosine, which help in the synthesis of proteins, and also for repairing the cells, tissues, and organs. Dietary fiber helps control appetite and blood glucose promotes laxation and reduces blood lipids (Kajla *et al.,* 2015). Diets rich in dietary fiber may help reduce the risk of heart disease, diabetes, colorectal cancer, obesity, and inflammation (Katare *et al.,* 2012). Researchers are investigating whether omega-3 fatty acids contained in flaxseed may help protect against certain infections and in treating conditions including ulcers, migraine headaches, hyperactivity disorder, eating disorders, preterm labor, emphysema, psoriasis, glaucoma, Lyme disease, lupus, and panic attacks. (Bernacchia *et al.*, 2014).

Nutritional Composition per 100 g of Flax Seed: -

Nutrients	**Amount per 100g Edible Flaxseed**
Moisture	6.5g
Protein	20.3g
Fat	37.1g
Minerals	2.4 g
Crude fibre	4.8 g
Total dietary fibre	24.5 g
Carbohydrates	28.9 g
Energy (kcal)	530.0 g

Potassium	750.0 g
Calcium	170.0 mg
Phosphorous	370.0 mg
Iron	2.7 mg
Vitamin A	30.0 mg
Vitamin B	0.6 mg
Thiamine(B1)	0.23 mg
Riboflavin(B2)	0.07 mg
Niacin	1.0 mg
Pyriodoxine	0.61 mg
Pantothenic acid	0.57 mg
Biotin	0.6 mg
Folic acid	112 mg

As a natural sweetener, palm jaggery is much more nutritious than cane sugar. Due to its medicinal properties, it is highly-priced. It is extensively used in cooking and has many health benefits. An intense, earthy taste reminiscent of chocolate characterizes its taste and has a mild salty flavor. Jaggery obtained after processing is darker and richer in color. This product is highly valued because of its cooling effects on the body. It does not contain bone meal, which is used to whiten processed sugar. With its low glycemic index (35 GI), it is extremely useful in reducing obesity and preventing diabetes, as well as providing a sustained and uniform energy supply to the body. It is also rich in vitamins B1, B2, B3, B6, B12, and C. (Vengaiah *et al.*, 2017, Vengaiah *et al.*, 2013; Krishnaveni *et al.*, 2020).

Nutrients	**Content in 100g of Palm Jaggery**
Total Minerals	3.15%
Sucrose	76.86%
Protein	1.41%
Phosphorus	0.05%
Glucose	1.66%
Fat	0.91%
Calcium	0.86%
Ascorbic Acid	15.74 mg
Copper	0.767 mg
Iron	11.01 mg
Niacin	674.4 mg

Nicotinic Acid	5.24 mg
Riboflavin	44.4 mg
Thiamine	82.3 mg
Vitamin B1	24 mg
Vitamin C	11 mg

This research intends to develop a formulation using flax seeds, milky mushroom, and palm jaggery and carry out sensory analysis, proximate analysis, in-vitro and in-vivo analysis of the developed formulation. The formulation **will be** evaluated for its total polyphenol content (TPC) and total flavonoid content (TFC) assays as a part of proximate analysis. *In-vitro* analysis **will be** performed for cytotoxicity testing by MTT assay (Cell viability and metabolic activity), (ROS) Reactive Oxygen Species (cell differentiation and apoptosis), Nitrite Production assays (bacterial infection). (Popescu *et al.*, 2021).

Further, it includes in-vitro protein and starch digestibility analysis. *In-vivo* studies **will be carried out** on albino rats for Protein efficiency ratio (PER) analysis and also by measuring the levels of HDL-c (High-Density Lipoprotein - Cholesterol), TAG (Triacylglycerols), cholesterol, and blood pressure level by orally administering the formulated product.

The specific objectives of the study are:

1. To develop low cost, novel functional probiotic supplement product especially designed for malnourished individuals of adult age groups.
2. To fortify the product to make it MUFA and PUFA rich by incorporating organically sourced indigenous natural produce.
3. To assess organoleptic quality and cytotoxicity by in-vitro and in-vivo analysis of the prepared products.

1.1 Comparative study for cost effectiveness

Ingredients	Kilograms (Kg)	Cost (Rupees)
Flax seeds	1Kg	170/-
Milky mushroom	1kg	550/-
Palm jaggery	1kg	120/-

Total	840/-

Ingredients	FMP-1		FMP-2		FMP-3	
	gm	Rs.	gm	Rs.	gm	Rs.
Flax seeds	48	8.16	44	7.48	38	6.4
Milky Mushrooms	27	14.85	32	17.6	34	18.4
Palm jaggery	20	2.4	19	2.28	23	2.7
Total	95	25	95	27	95	27

2.

METHODOLOGY:

2.1 Procurement of raw materials

Organically produced mushroom, flaxseeds, palm jaggery were collected and processed by thorough cleaning, drying and powdering following appropriate standard protocols. (Hule, S., Landge, P., & Maharana, S. 2021), (Hashmi, S.I., Raheem, A., & Gandhi, R. 2010), (Toan, N.V., & Thu, L.N. 2018).

2.2 Identification, Screening and maintenance of probiotic cultures

Probiotic cultures **were** obtained or isolated, purified and identified using suitable media, and tested for probiotic potential. The cultures are Freeze-dried and were stored in appropriate temperature for further use. (Shekh, S. L., *et al.*2020)

2.3 Development and formulation of supplement powder

The supplement powder was prepared by mixing all the individual ingredients in different ratios and divided into 3 samples. The prepared powder was stored in airtight containers or vacuum sealed in translucent or coloured polythene zip lock packets and used for further physico-chemical analysis, sensory evaluation, in-vitro and in-vivo analysis. (Farzana, T., *et al.* 2017).

2.4 Sensory analysis

Sensory evaluation of the product has been conducted based on a 6 points hedonic scale for appearance, colour, odour, texture, flavour and flavour intensity. A group of 30 members are

partially trained in were randomly selected to evaluate the sensory properties of the developed formulation. (Farzana, T., *et al.*2017), (Hule, S., *et al*.2021)

2.5 Evaluation of Key Physico-Chemical Parameters

2.5.1 Proximate analysis

All the analysis of the sample **were** carried out in triplicates. For moisture, ash, crude fiber, fat, protein, carbohydrate, Iron and energy content estimation, respective standard protocols specified in FSSAI manual (2012) and AOAC (2012) will be followed with certain modifications in procedure if required. Total polyphenol content (TPC), Total flavonoid content (TFC). (Popescu, I., *et al* .2021), (Farooq, M., *et al*.2021). Bradford's method was carries out to estimate protein content. (Maehre, et al., 2018).

2.5.2 In-vitro analysis

In vitro analysis **must be carried out** by procurement of cell lines, animal models, Standard drug and chemicals are to be done in related research laboratories. Cell line culture and maintenance is to be performed according to a previously described method (Choi, Kim, et al. 2021) in a cell culture laboratory facility. Reactive Oxygen Species (ROS) are natural byproducts of cellular oxidative metabolism and play important roles in the modulation of cell survival, cell death, differentiation, cell signaling. MTT assay (cell viability and metabolic activity assay) was performed to assess the metabolic viability. Inflammation-related factor production and Nitrite Production (Griess reagent) were also performed. (Popescu, I.,*et al*. 2021),(Celebioglu, H. 2020), Millan-Linares, M. C., *et al*.2019), (Yasmin, I., *et al.* 2020). Further, **it includes** protein digestibility analysis and starch digestibility analysis. (Ojokoh A.O.& Yimin W.2011), (Chinma, C. E., *et al.* 2022).

2.5.3 In-vivo analysis

The previously described approach for analyzing the protein efficiency of a product in-vivo analysis **must include** healthy albino rats. (Serrem, *et al.*, 2011), (Ashwath Kumar, K., *et al*. 2019). The **study must also include** measuring the levels of HDL-c (High-Density Lipoprotein - Cholestrol), TAG (Triacylglycerols), cholesterol, and blood pressure (Shalaby, *et al.* 2019).

Analysis of the faecal microbiota and the percentage of apparent mineral absorption **are also considered necessary**. (Patrignani, M., *et al.* 2015).

2.5.4 Statistical analysis

Data analysis **will be carried out** in triplicates using Statistical Package for the Social Sciences (SPSS version 16.0 SPSS Inc. Chicago, Illinois, and U.S.A). The statistical significance among averages will be analyzed and the data **will be interpreted** in the form of mean ($\bar{x}$) ± standard deviation (SD). (Bertéli, M. B. D., *et al* .2021)

2.5.5 Ethical Approval:

The *in-vitro* and *in-vivo* protocols using animals and cell lines **must be** performed according to the standard guidelines, reviewed and approved by a certified institutional Ethical Committee. (Chuah, L. O., *et al.* 2019), (Qiao, Y.,*et al* .2021).

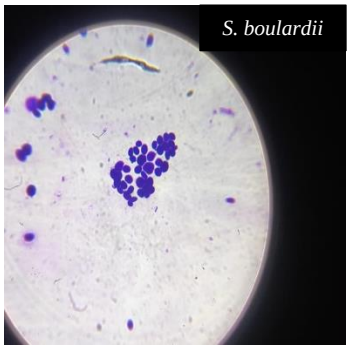
2. RESULTS:

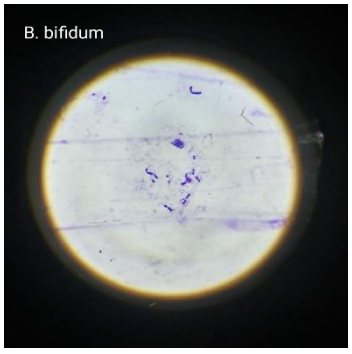
3.1 Procurement of raw materials

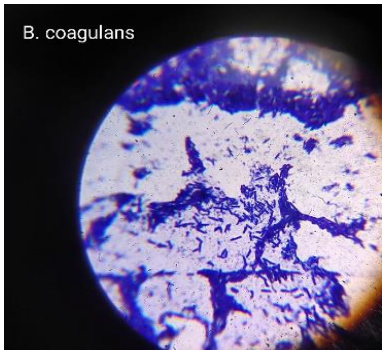
Organically produced mushroom, flaxseeds, palm jaggery were collected and processed by thorough cleaning, drying and powdering following appropriate standard protocols.

3.2 Identification, Screening and maintenance of probiotic cultures

On MRS isolation agar medium, the isolates *Lactobacillus acidophilus, Lactobacillus rhamnosus, Bacillus coagulans, Bifidobacterium bifidum* and *Saccharomyces boulardii* were sub cultured, coded and the genus of the isolated bacteria was determined by employing colony morphology, Gram Character and biochemical tests. All the cultures were Freeze-dried and stored in appropriate temperature for further use.







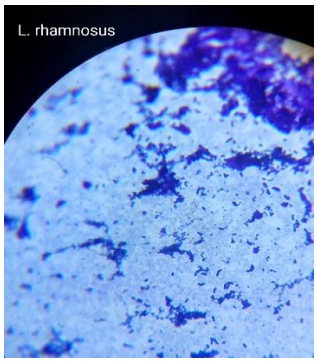


Fig1: Gram's staining of Probiotic bacteria

2.3 Development and formulation of supplement powder

The supplement powder was prepared by mixing all the individual ingredients in different ratios and will be divided into 3 formulations. 5g of Probiotic freeze-dried powder was mixed to each formulation. The prepared powder is to be stored in airtight containers or vacuum sealed in translucent or coloured polythene zip lock packets and used for further physico- chemical analysis, sensory evaluation, in-vitro and in-vivo analysis. (Farzana, T., et al. 2017).

Ingredient	FMP-1	FMP-2	FMP-3
Flax seeds	48g	44g	38g
Milky mushrooms	27g	32g	34g
Palm jaggery	20g	19g	23g
Probiotics	5g	5g	5g

*FMP- Flax seeds, Milky mushroom, Palm jaggery

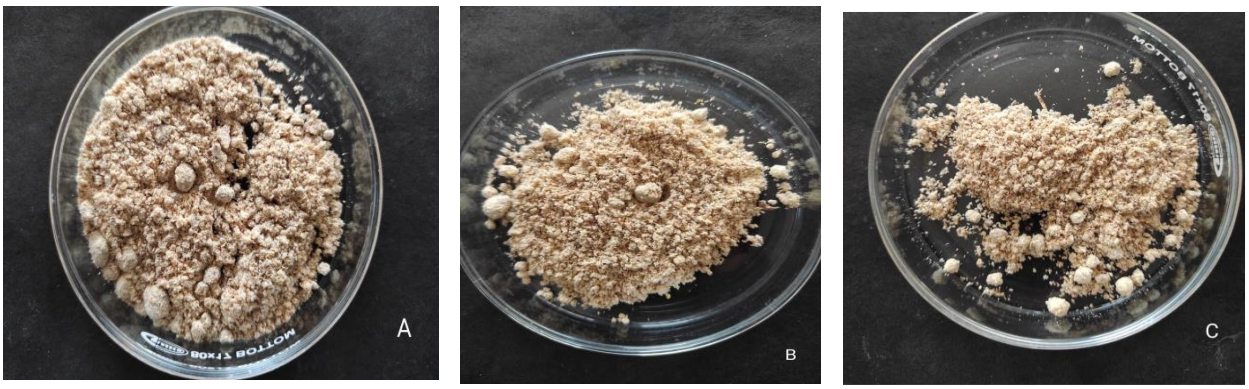
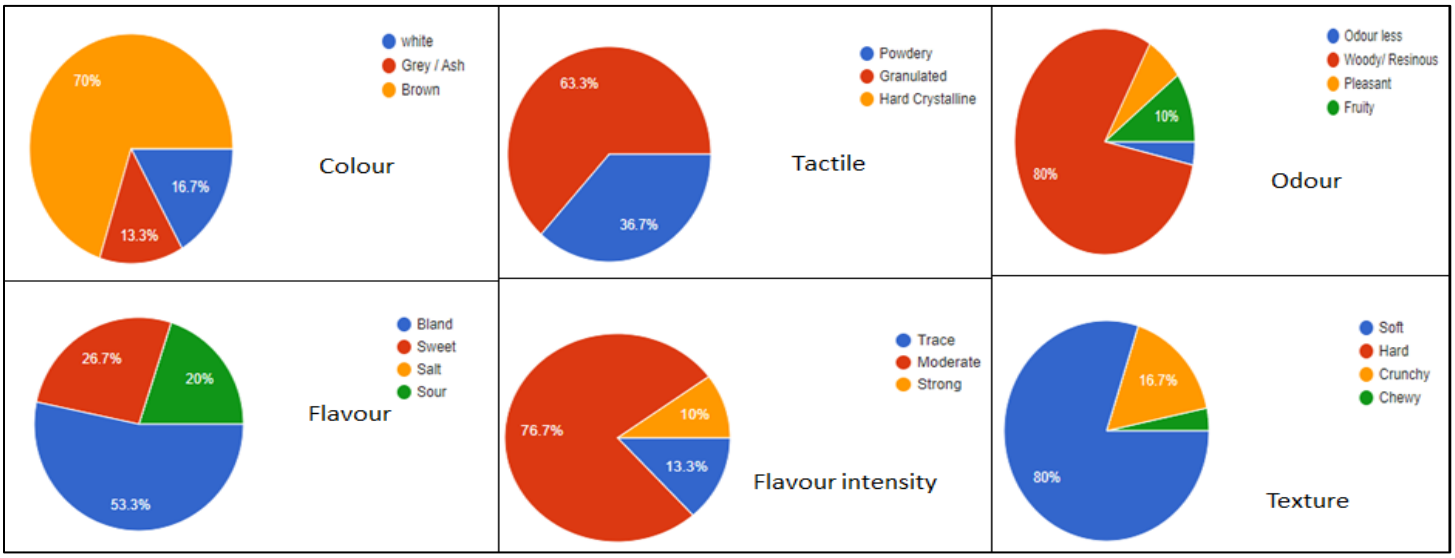


Fig2: Formulations comprising flax seeds, milky mushroom, palm jiggery and probiotic bacteria

3.4 Sensory analysis

Sensory evaluation of the product was conducted based on a 6 points hedonic scale for appearance, colour, odour, texture, flavour and flavour intensity. Semi-trained panels of 30 members were selected to evaluate the sensory properties of the formulated product. According to the analysis, FMP-3 was better suitable for consumption and gave good results.



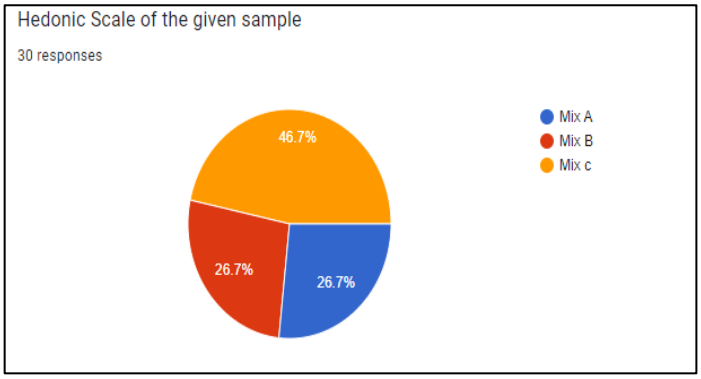


Fig3: Results of sensory analysis

3.5 Evaluation of Key Physico-Chemical Parameters

3.5.1 Proximate analysis- Protein estimation by Bradford's method

An assay originally described by Bradford's has become the preferred method for quantifying protein. The highest protein content (1.82±0.02 mg/g) was found in FMP-3. Protein content of individual components was estimated to compare it with formulated products. During this comparison, we observed that the protein concentration increased in each formulation and FMP-3 had better protein content respectively.

Table 1: Results for protein estimation using Bradford's **method**

Sample	Protein content (mg/g)			
	Control	FMP-1	FMP-2	FMP-3
Flax seeds	1.84±0.05mg/g	1.26±0.07mg/g	1.51±0.02mg/g	1.82±0.02mg/g
Mushroom powder	1.74±0.04mg/g			
Palm jaggery	0.24±0.04mg/g			

3. REFERENCE:

1. Ashwath, Kumar, K., Sharma, G. K., Anilakumar, K. R.(2019). Influence of multigrain premix on nutritional, in-vitro and in-vivo protein digestibility of multigrain biscuit. Journal of Food Science and Technology. 56(2):746-753. https://doi.org/10.1007/s13197- 018-3533-z
2. Bernacchia, R., Preti, R., & Vinci, G. (2014). Chemical composition and health benefits of flaxseed. Austin J Nutri Food Sci, 2(8), 1045.
3. Bertéli, M. B. D., Oliveira Filho, O. B. Q., Freitas, J. D. S., Bortolucci, W. C., Silva, G. R., Gazim, Z. C., Lívero, F. A. R., Lovato, E. C. W., Valle, J. S., Linde, G. A., Barros, L., Reis,

F. S., Ferreira, I. C. F. R., Paccola-Meirelles, L. D., & Colauto, N. B. (2021). Lentinus crinitus basidiocarp stipe and pileus: chemical composition, cytotoxicity and antioxidant activity. European Food Research and Technology, 247(6), 1355–1366. https://doi.org/10.1007/s00217-021-03713-1

4. Chinma CE, Ibrahim PA, Adedeji OE, Ezeocha VC, Ohuoba EU, Kolo SI, Abdulrahman R, Ogochukwu Anumba NL, Adebo JA, Adebo OA. (2022). Physicochemical properties, *In vitro* digestibility, antioxidant activity and consumer acceptability of biscuits prepared from germinated finger millet and Bambara groundnut flour blends. Heliyon, 8(10):e10849. https://doi.org/10.1016/j.heliyon.2022.e10849.
5. Celebioglu, H. (2020). Probiotic bacteria grown with chestnut honey enhance *In vitro* cytotoxicity on breast and colon cancer cells. Archives of Biological Sciences, 72(3), 329–338. https://doi.org/10.2298/abs200114027c 5. Choi, J., Lee, H., Park, S., Kim, S., Seo, K., & Kim, K. (2021).
6. Cytotoxicity, metabolic enzyme inhibitory, and anti-inflammatory effect of Lentinula edodes fermented using probiotic lactobacteria. Journal of Food Biochemistry, 45(8). KSCST: Student Project Programme: 46 th series: 2022-2023 6 https://doi.org/10.1111/jfbc.13838
7. Chuah, L. O., Foo, H. L., Loh, T. C., Mohammed Alitheen, N. B., Yeap, S. K., Abdul Mutalib, N. E., Abdul Rahim, R., & Yusoff, K. (2019). Postbiotic metabolites produced by *Lactobacillus plantarum* strains exert selective cytotoxicity effects on cancer cells. BMC Complementary and Alternative Medicine. 19(1). https://doi.org/10.1186/s12906-019- 2528-2
8. Farooq, M., Rakha, A., Hassan, J. U., Solangi, I. A., Shakoor, A., Bakhtiar, M., Khan, M. N., Khan, S., Ahmad, I., Ahmed, S., & Yunyang, W. (2021). Physicochemical and Nutritional Characterization of Mushroom Powder Enriched Muffins. Journal of Innovative Sciences, 7(1). https://doi.org/10.17582/journal.jis/2021/7.1.110.120
9. Farzana T, Mohajan S. Effect of incorporation of soy flour to wheat flour on nutritional and sensory quality of biscuits fortified with mushroom. Food Sci Nutr. 2015 Sep;3(5):363-9. doi: 10.1002/fsn3.228.
10. Hashmi, S.I., Raheem, A., & Gandhi, R. (2010). Effect of Different Particle Sizes of Jaggery Powder on Storability. International Journal of Processing and Post harvest Technology, 1, 73-76.

11. Hule, S., Landge, P., & Maharana, S. (2021). Nutritional enhancement of vegan biscuits using moringa and finger millet. Annals. Food Science and Technology, 22(1). http://www.afst.valahia.ro/images/documente/2021/issue_1/I.2_Hule.pdf
12. Krishnaveni, T. R. S., Arunachalam, R., Chandrakumar, M., Parthasarathi, G., & Nisha, R. (2020). Potential Review on Palmyra (*Borassus flabellifer* L.). Advances in Research, 29–40. https://doi.org/10.9734/air/2020/v21i930229
13. Kajla P, Sharma A, Sood DR. Flaxseed-a potential functional food source. J Food Sci Technol. 2015 Apr;52(4):1857-71. doi: 10.1007/s13197-014-1293-y.
14. Katare, C., Saxena, S., Agrawal, S., & Prasad, G. (2012). Flax Seed: A Potential Medicinal Food. Journal of Nutrition &Amp; Food Sciences, 02(01). https://doi.org/10.4172/2155-9600.1000120
15. Maehre HK, Dalheim L, Edvinsen GK, Elvevoll EO, Jensen I-J. Protein Determination—Method Matters. Foods. 2018; 7(1):5. https://doi.org/10.3390/foods7010005
16. Millan-Linares, M. C., Martin, M. E., Rodriguez, N. M., Toscano, R., Claro, C., Bermudez, B., Pedroche, J., Millan, F., & Montserrat-de La Paz, S. (2019). Nutraceutical Extract from Dulse (Palmaria palmata L.) Inhibits Primary Human Neutrophil Activation. Marine Drugs, 17(11), 610. https://doi.org/10.3390/md17110610
17. Nguyen, P. H., Scott, S., Headey, D., Singh, N., Tran, L. M., Menon, P., & Ruel, M. T. (2021). The double burden of malnutrition in India: Trends and inequalities (2006–2016). Plos one, 16(2), e0247856.
18. Ojokoh A.O., Yimin W. Effect of fermentation on chemical composition and nutritional quality of extruded and fermented soya products. International Journal of Food Engineering, 2011;7(4)
19. Patrignani, M., Rinaldi, G. J., Lupano, C.E. (2016). *In-vivo* effects of Maillard reaction products derived from biscuits. Food Chemistry; 196: 204-10. https://doi.org/10.1016/j.foodchem.2015.09.038.
20. Popescu, I., Codrici, E., Mihai, S., Luntraru, C. M., Neagu, M., & Tanase, C. (2021). *In vitro* assessment of the cytotoxicity and anti-inflammatory properties of a novel dietary supplement. Experimental and Therapeutic Medicine, 22(4). https://doi.org/10.3892/etm.2021.10604

21. Qiao, Y., Qiu, Z., Tian, F., Yu, L., Zhao, J., Zhang, H., Zhai, Q., & Chen, W. (2021). *Pediococcus acidilactici* Strains Improve Constipation Symptoms and Regulate Intestinal Flora in Mice. Frontiers in Cellular and Infection Microbiology, 11. https://doi.org/10.3389/fcimb.2021.655258 KSCST: Student Project Programme: 46 th series: 2022-2023 7
22. Rosy, K., Alka, G., & Sarita, S. (2017). Development of nutrient dense supplementary products for children by using locally available whole wheat flour, ragi flour, green gram flour, soy flour, roasted groundnut flour and jaggery. The Allahabad Farmer, 73(2).
23. Roy, A., & Prasad, P. (2016). Assessment of Antihyperglycemic Potential of Lyophilized and Oven-Dried Extract of *Calocybe indica* in Experimentally Streptozotocin Nicotinamide Induced Diabetic Rats. International Journal of Medical Research & Health Sciences, 5(4), 82–88. https://www.ijmrhs.com/medical-research/assessment-of antihyperglycemic-potential-of-lyophilized-and-ovendried-extract-of-calocybe-indica-in experimentally-stre.pdf
24. Rakha, A., Hassan, J. U., Solangi, I. A., Shakoor, A., Bakhtiar, M., Khan, M. N., Khan, S., Ahmad, I., Ahmed, S., & Yunyang, W. (2021). Physicochemical and Nutritional Characterization of Mushroom Powder Enriched Muffins. Journal of Innovative Sciences, 7(1). https://doi.org/10.17582/journal.jis/2021/7.1.110.120
25. Srivastava, P., Singh, S., & Rajput, H. (2019). Development the nutrimix powder and its quality analysis. Pharma Innovation 2019; 8(7):193-195.
26. Serrem, C.A., de-Kock, H. L., Taylor, J. R. N. (2011). Nutritional quality, sensory quality and consumer acceptability of sorghum and bread wheat biscuits fortified with defatted soy flour. International Journal of Food Science & Technology. 46:74–83.
27. Shekh, S. L., Boricha, A. A., Chavda, J. G., & Vyas, B. R. M. (2020). Probiotic potential of lyophilized Lactobacillus plantarum GP. Annals of Microbiology, 70(1). https://doi.org/10.1186/s13213-020-01556-x
28. Shalaby., Hanan., Mostafa Ahmed., & Elzahar, Khaled. (2019). Evaluation of feeding biscuits enriched with polyunsaturated fatty acids on Hypercholesterolemia Rats. Wulfenia. 26. 36-57.
29. Subbiah K, Balan V. A Comprehensive Review of Tropical Milky White Mushroom (Calocybe indica P&C). Mycobiology. 2015 Sep;43(3):184-94. doi: 10.5941/MYCO.2015.43.3.184.

30. Shashikant M, Bains A, Chawla P, Fogarasi M, Fogarasi S. The Current Status, Bioactivity, Food, and Pharmaceutical Approaches of *Calocybe indica*: A Review. Antioxidants (Basel). 2022 Jun 10;11(6):1145. doi: 10.3390/antiox11061145.
31. Toan, N.V., & Thu, L.N. (2018). Preparation and Improved Quality Production of Flour and the Made Biscuits from Shitake Mushroom (*Lentinus edodes*). Clinical Journal of Nutrition and Dietetics,1(1).
32. Vengaiah, P. C., Ravindrababu, D., Murthy, G. N., & Prasad, K. R. (2013). Jaggery from Palmyrah palm (*Borassus flabellifer* L.)-Present status and scope.
33. Vengaiah, P. C., Ravindrababu, D., Murthy, G. N., & Prasad, K. R. (2017). Vale Added Food Products from Palmyrah Palm (*Borassus Flabellifer* L). Journal of Nutrition and Health Sciences, 4(1). https://doi.org/10.15744/2393-9060.4.105
34. Yasmin, I., Saeed, M., Khan, W. A., Khaliq, A., Chughtai, M. F. J., Iqbal, R., Tehseen, S., Naz, S., Liaqat, A., Mehmood, T., Ahsan, S., & Tanweer, S. (2020). In-Vitro Probiotic Potential and Safety Evaluation (Hemolytic, Cytotoxic Activity) of *Bifidobacterium* Strains Isolated from Raw Camel Milk. Microorganisms, 8(3), 354. https://doi.org/10.3390/microorganisms8030354