Content is available at: CRDEEP Journals
Journal homepage: http://www.crdeepjournal.org/category/journals/ijls/

International Journal of Life Sciences
(ISSN: 2277-193x) (Scientific Journal Impact Factor: 6.106)

UGC Approved-A Peer Reviewed Quarterly Journal

Full Length Research Paper

In vitro Evaluation of Millets as Prebiotics

Shruthi Sridhar[1],; Surya K, Vivelia S and Padmashree Kulkarni
Department of Life Science, Mount Carmel College Autonomous, Bangalore, India.

ARTICLE DETAILS

Corresponding Author:
Shruthi Sridhar

Key words:
Prebiotic, Proso Millet, Lactobacillus, Pathogen

ABSTRACT

Prebiotics, probiotics, and synbiotics have garnered significant research interest in recent years due to their therapeutic potential and their role in promoting host well-being. Millets, a group of nutrient-rich and readily available grains, are being explored for their prebiotic properties. This study aimed to evaluate the in vitro prebiotic potential of proso and foxtail millets. A modified growth medium incorporating these millets as the carbon source was optimized for the growth of probiotic strains *Lactobacillus plantarum* and *Lactocaseibacillus rhamnosus*. Prebiotic activity scores (PAS) and prebiotic indices (PI) were calculated to assess their efficacy. The highest PAS of 1.9629 was observed for *L. plantarum* on a proso millet substrate against the pathogen *Enterobacter cloacae*, whereas foxtail millet substrates yielded negative PAS values. Both probiotics demonstrated high PI values on proso millet substrates against *Bacillus cereus* and *E. cloacae*, while foxtail millet substrates showed comparatively lower PI values. Crude extracts from the millet-based media exhibited promising results, suggesting that incorporating millets into daily diets could offer substantial health benefits.

1. Introduction

Understanding the microbiota host interactions, and the correlations with diseases, begins with defining the healthy microbiome. The link between the gut flora and human health is becoming more apparent. It is now well accepted that a balanced gut microbiome is substantially responsible for the host's general health [1]. Prebiotics are a substrate which is selectively utilized by host microorganisms rendering a health benefit [2].

Probiotics are live microorganisms that impart health benefits to the host when ingested in sufficient amounts and have been shown in numerous clinical trials to influence the intestinal microbiota [3].Millets are the small-seeded common annual cereals or grasses that are stress-tolerant grains having the ability to grow with extremely fewer nutritional requirements[5] and in moist/ waterlogged as well as drought regions [6].Common quantitative approaches of analysing prebiotic potentials include (i) Prebiotic index and (ii) Prebiotic activity score. Palframan [7] devised a quantitative equation, called prebiotic index (PI), based on the changes in distinctive bacterial groups during fermentation. The bacterial groups incorporated into this PI equation were *Lactobacilli*, *Bifidobacteria*, *Clostridia* and *Bacteroides.*The equation is as follows:

$$PI = (Bif/Total) - (Bac/Total) + (Lac/Total) - (Clos/Total) \quad \textbf{(Equation 1)}$$ [7]

The prebiotic activity score is an equation that is subjected to the capability of a specific substrate to support the proliferation of a specific organism relative to other micro-organisms and relative to its growth on a non-prebiotic substrate, such as glucose. The activity assay is based on the difference in cell biomass after 24 h of growth of the probiotic strain on 1% prebiotic substrate or 1% glucose relative to the difference in the cell biomass of a mixture of enteric strains

[1]Author can be contacted at: *Associate professor, Dept. of Life Science, Mount Carmel College Autonomous, Bangalore*
Received: 16-12-2024; Sent for Review on: 21-12 -2024; Draft sent to Author for corrections: 05-01-2025; Accepted on: 11-01-2025; Online Available from 13-01-2025
DOI: 10.13140/RG.2.2.30267.94249
IJLS-9888/

grown under the identical conditions. The resulting score will be much higher for substrates with more prebiotic activity [8]. Assessing the prebiotic potential of substrates can provide significant details of the extent to which probiotic microorganisms can thrive in that environment as prebiotics will boost their development. The prebiotic activity score is calculated as[8]:

$$\left[\frac{(\text{Probiotic log}\frac{\text{cfu}}{\text{ml}}\text{ on the prebiotic at 24hrs - Probiotic log}\frac{\text{cfu}}{\text{ml}}\text{ on the prebiotic at 0hr})}{(\text{Probiotic log}\frac{\text{cfu}}{\text{ml}}\text{ on glucose at 24hrs - Probiotic log}\frac{\text{cfu}}{\text{ml}}\text{ on the prebiotic at 0hr})}\right] -$$

$$\left[\frac{(\text{Enteric log}\frac{\text{cfu}}{\text{ml}}\text{ on the prebiotic at 24hrs – Enteric log}\frac{\text{cfu}}{\text{ml}}\text{ on the prebiotic at 0hr})}{(\text{Enteric log}\frac{\text{cfu}}{\text{ml}}\text{ on glucose at 24hrs – Enteric log}\frac{\text{cfu}}{\text{ml}}\text{ on the prebiotic at 0hr})}\right] \textbf{(Equation 2)}$$

2. Materials and methods

2.1 Sample collection

Millets (namely barnyard, browntop, foxtail, finger, kodo, little, pearl, proso, and sorghum) were procured from the local market in Bengaluru. Cultures of *Lactobacillus plantarum* (MTCC 1407), *Lactocaseibacillus rhamnosus* (MTCC 1408), *Bacillus cereus* (MTCC 1307) and *Enterobacter cloacae* (MTCC 509) were obtained from MTCC, Chandigarh, India.The lyophilised cultures were suspended in the recommended growth medium. The suspension was streaked on respective agar plates as well as in broths and incubated for 24-48hrs at 37^{o} C. The master plates were sub-cultured in the recommended intervals and maintained.

2.2 Screening of potential prebiotic media for the growth of probiotic species

A random screening for the growth of the selected probiotic strains on millet agar was done following a method given byDaniel [9]. About 0.5g of each millet was added to 20ml water and boiled for 10-15mins. The solution was filtered made up to 25ml using distilled water. In a conical flask, about 0.65g agar was added to the filtrate. The suspension was sterilized by autoclaving at 121^{o} C for 15 mins. The media was then poured onto petri plates. After solidification, two samples of curd were streaked onto the plates and incubated at 37^{o} C for 48hrs in the incubator. The millet media showing good growth were considered for further experiments.

2.3 Optimisation of modified MRS media for the growth of selected prebiotic strains

Based on the primary screening results two millet media – proso (*Panicum miliaceum*) and foxtail (*Setaria italica*) – supporting good growth of the probiotic strains were selected for further studies. Probiotic strainswere grown on standard MRS medium with glucose as the source of carbohydrate and modified MRS media namely proso mMRS (PmMRS) and foxtail mMRS (FmMRS) containing respective millet extracts as the source of carbohydrate replacing glucose in MRS media. Composition of modified MRS (mMRS) (g/L):Peptone – 10g; Beef extract – 10g; Yeast extract – 8g; Extract from 20g millets; Sodium acetate trihydrate – 5g; Polysorbate – 1g; Tri-ammonium citrate – 2g; Magnesium sulphate – 0.2g; Manganese sulphate – 0.05g; Agar – 15g. The components were dissolved in distilled water (1L) and pH was adjusted to 6.2 and sterilized by autoclaving at 121°C for 15 minutes.

2.4 Inoculum preparation for the study of growth of the probiotic cultures in the modified media

The cultures of *Lactobacillus plantarum* and *Lactocaseibacillus rhamnosus*were inoculated in MRS broth and incubated on a shaker incubator at 37^{o} C at 100rpm for 16 hrs. A set of 25 test tubes containing 10ml of each broth namely, MRS, PmMRS and FmMRS were separately inoculated with the inoculum in their exponential phase at the rate of 10% and incubated at 37^{o} C for 24 hours in a shaker incubator at 100 rpm. Optical density was measured at 600nm every 1 hr, starting at 0hr, using a UV-visible spectrophotometer. A graph with OD values against time and the growth curves of the lactobacilli in all 3 media was plotted and compared.

2.5 Prebiotic activity score

Cultures (100µl) in their exponential phase (16-18hr) were inoculated into 2 test tubes incubated at 37°C for 0hr & 24hr. The former was plated immediately onto agar plates (MRS and mMRS) while the latter was incubated in a shaker incubator at 37^{o} C at 100 rpm for 24hrs and then plated. The MRS plates act as the control (glucose substrate), while mMRS plates (millet substrate) are the modified prebiotic media. OD of the broths was measured at 0hr and 24hrs using a UV-visible spectrophotometer. On the millet agar media lawn growth was observed and the colonies obtained were too numerous to count. The same process was repeated for pathogens using M9 and modified M9 (mM9) media. To prepare mM9, the glucose component was substituted with equivalent millet extract and the other components were retained. The composition of mM9 broth is as follows (gL^{-1}):Sodium hydrogen phosphate – 6.4g; Dipotassium phosphate – 1.5 g; Sodium chloride – 0.25g; Ammonium chloride – 0.5g; Extract from 10g millets prepared as mentioned above; Distilled water – 489ml. The modified M9 media was autoclaved. Once cooled, the filter sterilized solutions as given in Appendix _ was added. The autoclaved media were inoculated and OD of broths at 0hr and 24hrs were measured. The M9 broth was used

as a control (glucose substrate). Optical densities were read, and PAS of different samples was calculated, tabulated and compared.

2.6 Prebiotic index

The optical density of the 2 probiotic and 2 pathogenic species, at 24hrs, was obtained using a UV-visible spectrophotometer at 600nm. Using the absorbance values, number of organisms was calculated for *B. cereus* [10],*E. cloacae* [11] and *Lactobacillus*species [12]. The value of prebiotic index on proso modified media and foxtail modified media were calculated using the equation.

3. Results and discussion

3.1 Revival and maintenance of pure cultures

The prebiotic cultures were successfully revived and maintained at 37° C.

3.2 Screening of growth on millet media

Of the nine different millet media screened only foxtail and proso millet agar showed good growth after 48hrs of incubation (Plate 1 (a) and (b)). No growth was observed in other millet media. On staining the isolates from re-inoculation in MRS plates Gram positive cocci were observed.

Plate 1: Growth of the selected probiotic strains on different millet media (a) Foxtail millet agar (b) Proso millet agar (c) Smooth white colonies of curd isolate obtained on MRS plates

3.3 Study of growth of the selected probiotic strains on PmMRS and FmMRS

Good growth of the probiotic strains was observed on PmMRS and FmMRS at 37°C after 24 to 48 hours of incubation (Plate 2). Growth of *L. plantarum* and *Lactocaseibacillus rhamnosus* on MRS was comparatively less. In a similar study conducted using modified MRS media without carbon source substituted by konjac(east Asian plant) hydrolysate, inulin, inulo-hydrolysate, pectin-hydrolysate or xylo-hydrolysate showed good growth of *L. casei* NCFB 161, *L. delbruckii* NCFB 1489 and *L. acidophilus* NCFB 1748[13]. MRS media containing 1% FOS used for *Lactobacillus* species had shown good growth in another study [14]. In the present study, proso millet proved to be a good prebiotic source. Fibre and resistant starch that is present in the proso acts as a probiotic and beneficial in many ways and can bring about an effective reduction in the levels of glucose and insulin with up-regulated expression of adiponectin and down regulating effect of tumour necrosis factor-α (TNF-α) and abetting type 2 diabetes, obesity, and cardiovascular diseases [15].

3.4 Study of growth curve of the probiotic strains and pathogens

Optical densities of cultures were measured every 1hr. Figures 1 and 2show **t**he growth curves of *L. plantarum* and *L. rhamnosus* in FmMRS, PmMRS and MRS. The standard growth curve of *L. plantarum* at 37° C was found to exceed an OD value of 2.0 and found to reach stationary phase by 30 hr [16]. The values are slightly differed in the present study as indicated in Figure1. Growth conditions and media components might be reasons. Further, it is observed that the growth at 18hr in PmMRS shows a higher value than MRS which indicates that PmMRS enhances growth of the spp. FmMRS shows a relatively lower growth. Growth of *L. rhamnosus* is found to reach OD of almost 6.0 in MRS broth at 37° C in anaerobic conditions within 10hrs[17]. The values are much lesser in the present study. The reason could be aerobic conditions maintained in the laboratory. The growth in FmMRS is lower than the other two in case of *L. rhamnosus* (**Figure2).** A more stabilised growth is observed in PmMRS and MRS indicating that PmMRS could prove to be a good alternate to the standard MRS media for the growth of *Lactobacillus* spp.

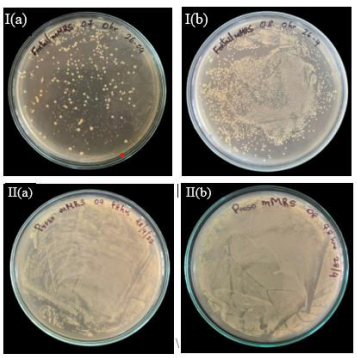

Plate 2: Growth of probiotic strains (a) *L. plantarum* and (b) *L. rhamnosus* on I. FmMRS, II. PmMRS

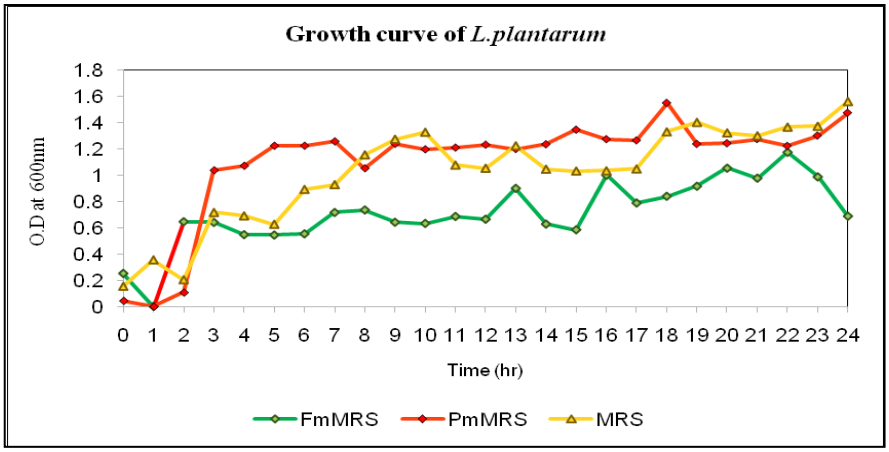

Fig1: Growth curves of *L. plantarum* in FmMRS, PmMRS and MRS

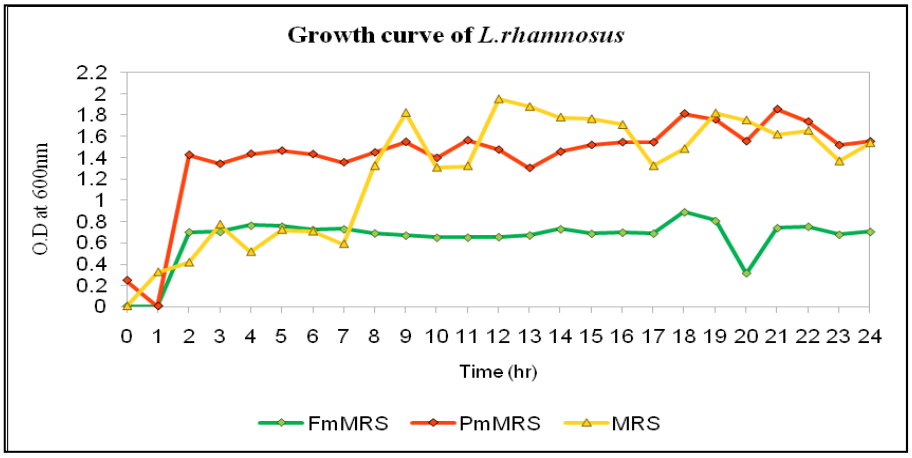

Fig 2: Growth curves of *L. rhamnosus* in FmMRS, PmMRS and MRS

3.5 Prebiotic activity score

When the 0hr and 24hr cultures of probiotic and pathogens were plated on modified as well standard media, the colonies obtained were too numerous to count. The bacterial population was calculated from optical densities read at 0hr and 24hr of incubation (Table 1 and Table 2). The prebiotic activity scores of selected millets were calculated using the PAS equation (Table 3).

A maximum PAS scores of 1.9629 was obtained for *L. plantarum* on proso. Best positive results were obtained for probiotic spp. on proso against *E. cloacae.*In a similar study conducted byPalaniappan [18] the activity score of *L. plantarum* was found to be between 0.7 to 0.8 on xylo-oligosaccharide substrate. Here, almost 2 times of the same value has been obtained, proving that proso contains a prebiotic oligosaccharide better that XOS. Accordingto a study conducted [19], the highest prebiotic activity scores were obtained in *L. acidophilus* grown on inulin (2.22), garlic (2.15), shallot (2.09), and onion (1.94) whereas *L. acidophilus* had prebiotic activity scores close to zero (0.17 and 0.23) when grown on low molecular weight carbohydrates extracted from germinated rice. The PAS for *L. plantarum* Mut7 on sweet potato fibre extract was 1.62[20]. Negative or low scores indicate that the test strain less growth on the prebiotic than it did on glucose, and/or it grew less than the enteric strain did on the prebiotic sugar according to Huebner [8]. A negative value was obtained on foxtail millet indicating that it is not an efficient prebiotic substrate.

Table 1: Colony forming units of probiotic strains at 0hr and 24hr

Time	*L. plantarum*			*L. rhamnosus*		
	MRS	FmMRS	PmMRS	MRS	FmMRS	PmMRS
0hr	2.49×10^8	4.1×10^8	7.4×10^7	6.4×10^6	3.2×10^6	3.92×10^8
24hr	2.44×10^9	1.1×10^9	2.3×10^9	2.46×10^9	1.12×10^9	2.48×10^9

Table 2: Colony forming units of pathogenic species at 0hr and 24hr

Time	*B. cereus*			*E. cloacae*		
	M9	FmM9	PmM9	M9	FmM9	PmM9
0hr	4.06×10^5	7.8×10^6	4.3×10^6	2.4×10^7	3.6×10^7	4.9×10^8
24hr	2.1×10^7	2.37×10^7	1.5×10^7	3.3×10^8	2.2×10^8	1.5×10^8

Table 3: Prebiotic activity scores of probiotic spp. in millet media against pathogenic spp.

	B. cereus		***E. cloacae***	
	Proso	Foxtail	Proso	Foxtail
L. plantarum	1.1878	0.1546	**1.9629**	-0.2541
L. rhamnosus	-0.008	0.7063	0.7671	0.2976

3.6 Prebiotic index

The number of organisms at 0hr and 24hrs were taken as given in Table 1 and Table 2. The PI values calculated using the equation and the values obtained are tabulated (Table 4).

Table 4: Prebiotic Index of millets using probiotic strains against pathogenic species

Prebiotic Index	
Proso substrate	Foxtail substrate
0.9339	0.8032

From the results shown in Table 4, it could be concluded that highest index was obtained for proso millet. Overall values of proso were found to be higher for both probiotic spp. as compared to foxtail substrate. Thus, proso millet is a better prebiotic than foxtail. It is seen that disaccharides with linkages of 1−2, 1−4, and 1−6 have a higher PI in the range of 18-21, whereas mannose-containing disaccharides generate lower PI scores due to increase in *Bacteroides* [21]. Proso contains more pentoses than hexoses, and galactose was present as an additional hexose. The viscosity of starches in proso millet was found to be the highest as well [22]. Highest carbohydrate content of 75.06 ± 7.3 was found to be present in proso millets [23]. These aspects of proso millets contribute to higher prebiotic values compared to foxtail millet.

4. Conclusion

The gut microbiome plays an important role in regulating overall health of the host. Prebiotics are extensively being researched for their ability to restore a healthy system coupled with probiotics. They are selectively utilized by the beneficial gut microorganisms to confer a positive effect on the gut health. Millets are a class of extremely under-rated grains with immense nutritional benefits. Based on the results obtained in the present study, it could be concluded that proso millet and foxtail millet are suitable to be used as prebiotic for the selected *Lactobacillus spp.* strains. A good prebiotic activity score and high prebiotic index suggests that the prebiotics have enhanced the growth of the probiotic strains.

5. References

1. Jandhyala, S. M., Talukdar, R., Subramanyam, C., Vuyyuru, H., Sasikala, M., & Nageshwar Reddy, D. (2015). Role of the normal gut microbiota. *World journal of gastroenterology*, 21(29), 8787–8803.
2. de Paulo Farias, D., de Araújo, F. F., Neri-Numa, I. A., & Pastore, G. M. (2019). Prebiotics: Trends in food, health and technological applications. *Trends in Food Science & Technology*, *93*, 23-35.
3. Kim, S. K., Guevarra, R. B., Kim, Y. T., Kwon, J., Kim, H., Cho, J. H., Kim, H. B., & Lee, J. H. (2019). Role of Probiotics in Human Gut Microbiome-Associated Diseases. *Journal of microbiology and biotechnology*, 29(9), 1335–1340.
4. Thakur M, Tiwari P (2019). Millets: the untapped and underutilized nutritious functional foods. *Plant Arch* 19(1):875–883
5. Malathi B, Appaji C, Reddy GR, Dattatri K, Sudhakar N (2016) Growth pattern of millets in India. Indian J Agric Res 50(4):382–386
6. Michaelraj PSJ, Shanmugam A (2013) A study on millets based cultivation and consumption in India. *Int J Mark Financ Serv Manag Res* 2(4):49–58.
7. Palframan, R., Gibson, G. R., & Rastall, R. A. (2003). Development of a quantitative tool for the comparison of the prebiotic effect of dietary oligosaccharides. *Letters in applied microbiology*, 37(4), 281–284.
8. Huebner, J., Wehling, R.L., Hutkins, R.W. (2007). Functional activity of commercial prebiotics. *International Dairy Journal*. 17(7), 770-775.
9. Daniel, Ashefo & Oboh, Emmanuel. (2021). FORMULATION OF MICROBIAL GROWTH USING MILLET AND SWEET POTATO. *Pharmaceutica Analytica Acta.* 6. 53-57.
10. Vojcic, L., Despotovic, D., Martinez, R., Maurer, K. H., & Schwaneberg, U. (2012). An efficient transformation method for Bacillus subtilis DB104. *Applied microbiology and biotechnology*, 94(2), 487-493
11. Valkova, N., Lépine, F., Valeanu, L., Dupont, M., Labrie, L., Bisaillon, J. G., ... & Villemur, R. (2001). Hydrolysis of 4-hydroxybenzoic acid esters (parabens) and their aerobic transformation into phenol by the resistant Enterobacter cloacae strain EM. Applied and environmental microbiology, 67(6), 2404-2409.
12. Lin, S. Y., Ayres, J. W., Winkler Jr, W., & Sandine, W. E. (1989). Lactobacillus effects on cholesterol: in vitro and in vivo results. *Journal of dairy science*, 72(11), 2885-2899.
13. Al-Ghazzewi, F. H., Khanna, S., Tester, R. F., & Piggott, J. (2007). The potential use of hydrolysed konjac glucomannan as a prebiotic. *Journal of the Science of Food and Agriculture*, 87(9), 1758-1766.
14. Sriphannam, W., Lumyong, S., Niumsap, P., Ashida, H., Yamamoto, K., & Khanongnuch, C. (2012). A selected probiotic strain of Lactobacillus fermentum CM33 isolated from breast-fed infants as a potential source of β-galactosidase for prebiotic oligosaccharide synthesis. The Journal of Microbiology, 50(1), 119-126.

15. Karkannavar, S. J., Shigihalli, S., Nayak, G., & Bharati, P. (2021). Physico-chemical and nutritional composition of proso millet varieties.
16. Smetanková, J., Hladíková, Z., Valach, F., Zimanová, M., Kohajdová, Z., Greif, G., & Greifová, M. (2012). Influence of aerobic and anaerobic conditions on the growth and metabolism of selected strains of Lactobacillus plantarum. *Acta Chimica Slovaca,* 5(2), 204.
17. Serra, S., & De Simeis, D. (2018). Use of Lactobacillus rhamnosus (ATCC 53103) as whole-cell biocatalyst for the regio-and stereoselective hydration of oleic, linoleic, and linolenic acid. *Catalysts,* 8(3), 109.
18. Palaniappan, Ayyappan; Balasubramaniam, V. Geetha; Antony, Usha (2017). Prebiotic potential of xylooligosaccharides derived from finger millet seed coat. *Food Biotechnology*, 31(4), 264–280.
19. Moongngarm, A., Trachoo, N., & Sirigungwan, N. (2011). Low molecular weight carbohydrates, prebiotic content, and prebiotic activity of selected food plants in Thailand. *Adv J Food Sci Technol,* 3(4), 269-274.
20. Lestari, L. A., Soesatyo, M. H. N. E., Iravati, S., & Harmayani, E. (2013). Characterization of Bestak sweet potato (Ipomoea batatas) variety from Indonesian origin as prebiotic. *International Food Research Journal*, 20(5), 2241.
21. Sanz, M. L., Gibson, G. R., & Rastall, R. A. (2005). Influence of disaccharide structure on prebiotic selectivity in vitro. *Journal of agricultural and food chemistry,* 53(13), 5192-5199.
22. Muralikrishna, G., Paramahans, S. V., & Tharanathan, R. N. (1982). Carbohydrate Make-up of Minor Millets. *Starch-Stärke*, 34(12), 397-401.
23. Shankaramurthy, K. N., & Somannavar, M. S. (2019). Moisture, carbohydrate, protein, fat, calcium, and zinc content in finger, foxtail, pearl, and proso millets. *Indian Journal of Health Sciences and Biomedical Research (KLEU),* 12(3), 228.