Evaluation of in vitro survival and bioavailability of cobalamin-containing *Limosilactobacillus reuteri* incorporated into finger millet-based cereal bar

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Summary

In an era of emphasising diverse dietary needs, customised nutrition is important for optimal well-being. Embracing this approach, the nutritional richness of millets promotes sustainable agriculture and enhances dietary diversity. This study aimed to assess the in vitro survival and cobalamin bioavailability of cobalamin-containing *Limosilactobacillus reuteri* incorporated into a finger millet-based cereal bar. *Limosilactobacillus reuteri* was lyophilised in cryoprotective matrices to achieve enhanced viability. The final product was selected based on sensory properties, hardness and water activity. Further nutritional analysis revealed that the product has an energy value of 1 474 kJ per 100 g, while exhibiting low-fat composition, high-fibre content and low moisture levels, confirming its ability to maintain shelf stability. Initially, the product was reported to contain 1.83 μg of cobalamin per serving. However, upon digestion, the bio-accessible cobalamin was quantified to be 77 % of the original level. Additionally, the in vitro probiotic viability assay validated the claim of containing viable probiotics for targeted establishment in the regions of the small intestine. The findings of this study confirm the possibility of incorporating probiotics into a millet-based cereal bar while enhancing nutritional availability and an easy, non-genetically engineered approach for targeted delivery of cobalamin.

Keywords

finger millet; *Limosilactobacillus reuteri*; cobalamin bioavailability; probiotic survivability

Probiotic-enriched cereal bars hold significant importance in the realm of nutrition and overall wellbeing, as these bars are designed to combine the convenience of a snack with the potential health benefits of probiotics, promoting a healthy gut microbiome [1]. One of the major advantages of probiotic-enriched cereal bars is their ability to enhance digestive health and support holistic nutrition [2]. The human gut is home to trillions of bacteria, and maintaining a balanced microbiome is crucial for optimal digestion and nutrient absorption [3]. Probiotics help replenish and diversify the beneficial bacteria in the gut, which can improve digestion and alleviate digestive issues such as bloating, gas, and constipation [4].

Limosilactobacillus reuteri is one of the indigenous inhabitants of the human gastrointestinal (GI) tract and is also found in most vertebrates and chickens. It is a heterofermentative probiotic that proliferates in oxygen-limited conditions and employs various mechanisms to greatly inhibit pathogenic microorganisms and secrete antimicrobial metabolites [5].

Probiotics must be able to live and grow in the host in order to have these positive impacts on health. In this regard, probiotics must be metabolically stable, active, and present in sufficient quantities to pass through the stomach and enter the intestines in adequate numbers to confer the beneficial effects [6]. Techniques like the use of oxygen-impermeable containers, two-step fermentation, stress adaptation, and the incorporation of micronutrients like peptides and amino acids are some of the methods that have been suggested to maintain the viability of organisms in the digestive tract [7]. Lyophilisation provides protection from various adverse conditions and from bacteriophages during storage, transportation and processing. Moreover, the powder form is more convenient for use and easier to incorporate into other

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food products, enabling homogeneous distribution [8].

Incorporating probiotics into a cereal bar is an outstanding option for the common population to conveniently add probiotics to the daily diet, even when on the go. These bars have the potential to boost the immune system by modulating the body's immune responses and strengthening the defence against invasive pathogenic organisms. By consuming a cereal bar fortified with probiotics, individuals can support their immune system and potentially reduce the risk of infections and illnesses [9].

Additionally, probiotic-enriched cereal bars have a potential impact on mental health. Emerging research indicates a strong connection between the gut and the brain, often referred to as the gut-brain axis, positively influencing mental health conditions such as anxiety and depression [10]. Subsequently, they offer a convenient and effective means of incorporating probiotics into one's diet. As the demand for functional foods continues to rise, the significance of probiotic-enriched cereal bars lies in their ability to provide a beneficial and accessible option for individuals seeking to improve their overall health and well-being [11]. Since *L. reuteri* is a prominent probiotic bacteria producing vitamin B12 in situ, the technique of incorporating this organism into food helps enhance the bioavailability of the vitamin upon digestion [12]. Millet has also been found to have several other health benefits. For example, studies have shown that consuming millet can help lower cholesterol levels, reduce the risk of heart disease, and improve blood sugar control in people with diabetes. It has also been found to have anti-inflammatory properties and may be beneficial for those with inflammatory conditions such as rheumatoid arthritis [13]. The study aimed to develop and evaluate a *Limosilactobacillus reuteri*-enriched finger millet-based cereal bar and to study the probiotic survival under in vitro digestive conditions.

MATERIALS AND METHODS

Microbial culture

L. reuteri DSM 20016 was procured from the Metabolic Engineering Laboratory, Department of Biotechnology, Anna University (Chennai, India). About 100 μl of De Man, Rogosa and Sharpe (MRS) broth culture was used for 10 ml of fresh MRS broth (HiMedia, Mumbai, India) and incubated overnight at 37 °C. The cells were cultivated in non-stirred batch cultures under anaerobic conditions (95% N_2 and 5% CO_2) unless stated otherwise. The obtained culture was used as the inoculum for the study.

Lyophilisation of *L. reuteri*

Two cytoprotective media (to protect the probiotic cells during the lyophilisation process) were investigated in this study. Conventionally lyophilised culture contained 15% skim milk and 5% saccharose (SKM), while casein-based medium (CSM) contained 20% casein, 15% skim milk and 5% saccharose. Cells were harvested from 24 h culture by centrifugation at 10 000 $\times g$ for 4 min at 4 °C, then washed twice with distilled water (sterilised). The collected cell pellets were resuspended in 500 ml round bottom flasks containing 50 ml of cytoprotective media (cell count adjusted to 10^8–10^9 CFU·ml^{-1}) as specified earlier. The flasks were frozen overnight at –20 °C. Subsequently, frozen probiotic cultures were placed into the lyophiliser (Mini Lyodel LYO0555, Delvac Pumps, Chennai, India), which was operated at –40 °C (40 Pa pressure) until complete drying was achieved. The freeze-dried cells were collected in a powder form and stored in dry, airtight falcon tubes till use.

Cell viability studies

Lyophilised *L. reuteri* powder was investigated for cell viability before incorporation into the cereal bar. The standard plate count method was performed at an interval of 20 days to enumerate the viable probiotic cells during storage. The lyophilised powder was rehydrated with 0.9% saline solution for 15 min aseptically and serially diluted. The aliquots were plated on MRS agar medium and incubated at 37 °C for 24 h, after which the numbers of colonies were enumerated and expressed as colony-forming units per gram [14].

Ingredients and pre-processing

The ingredients used for the preparation of millet cereal bars were as follows: finger millet flour, powdered sugar, cocoa powder, skimmed milk powder, maltodextrin, peanuts, oil and water. All the ingredients (except peanut, oil and water) were sieved to obtain uniform size (particle cut-off: 150–300 μm). After sieving, finger millet flour was roasted at 150 °C for 5 min. Peanuts were roasted at 180 °C for 12 min.

Product development and optimisation

The oven was pre-heated to 150 °C for 30 min. Varying ratios of finger millet to powdered sugar were optimised by preparing and analys-

Tab. 1. Optimisation of ingredients.

Ingredients	Formulations								
	T1	T2	T3	T4	T5	T6	T7	T8	T9
Finger millet flour (roasted) [g]	30	30	30	35	35	35	40	40	40
Powdered sugar [g]	15	20	25	15	20	25	15	20	25
Cocoa powder [g]	3	3	3	3	3	3	3	3	3
Maltodextrin [g]	6	6	6	6	6	6	6	6	6
Skimmed milk powder [g]	6	6	6	7	7	7	8	8	8
Oil [ml]	0.3	0.3	0.3	0.4	0.4	0.4	0.5	0.5	0.5
Water [ml]	11	11	11	11.5	11.5	11.5	12	12	12
Roasted-chopped peanuts [g]	5	5	5	5	5	5	5	5	5

ing nine varying cereal bar formulations (Tab. 1). The sieved ingredients were measured as specified in Tab. 1, and 5 g of chopped peanuts were added. The blended mixtures were levelled and cut manually using a knife to obtain bars, each weighing 30 g. These bars were placed on a greased baking tray and allowed to rest for 15 min. Afterwards, the bars were baked in a preheated oven for 15 min at 160 °C. After baking, the products were allowed to cool down and the lyophilised *L. reuteri* was sprinkled over (3 g per 100 g of product). These finished products were placed in between the sheets, heat-sealed and stored at room temperature.

Product characterisation

Colour

The product was analysed for tristimulus colour values: L^* (black-white component, lightness or luminosity), a^* (green to red component) and b^* (blue to yellow component). The colours were recorded on a scale of the Commission internationale de l'éclairage (CIE) colour model in triplicates using a colorimeter (Ultrascan VIS, Model 45/0-S, Hunter Associates Laboratory, Reston, Virginia, USA), which was earlier calibrated using white and black. The chroma (C) value was calculated using the Eq. 1:

$$C = \sqrt{a^2 + b^2} \tag{1}$$

Texture and water activity

The texture of the product was determined using TA-XT Plus Texture Analyser (Godalming, United Kingdom). A 36 mm-cylinder probe with a radiused edge was used to assess the hardness and firmness of the sample. The height and force calibration were performed prior to the sample testing. The required texture profile was uploaded, and the sample was placed on the platform. The force was expressed in newtons. For water activity, 1 g of the cereal bar product was weighed and placed into the sample analysis chamber. The water activity of the product was estimated using the Aqualab water activity meter (Decagon Devices, Pullman, Washington, USA).

Sensory evaluation

The sensory evaluation was carried out by a panel of 15 semi-trained panellists using a 9-point hedonic scale. On the scale, 1 meant dislike extremely, 5 – neither like nor dislike, and 9 meant like extremely. The desirable characteristics such as colour, flavour, taste, aroma, appearance and overall acceptability were evaluated [15].

Moisture and nutritional analysis

The nutritional parameters, such as the total energy value, moisture, ash, fibre, crude protein, and fat, were analysed for the product.

A hot air oven-drying method was done to estimate the moisture content of the product. Mortar and pestle were used to crush 5 g of the product and placed inside the preheated oven maintained at 105 ± 2 °C until a constant weight was reached. The moisture content (M) was calculated using Eq. 2 and expressed as a percentage:

$$M = \frac{w_i - w_f}{w_i} \times 100 \tag{2}$$

where w_i is the initial weight and w_f is the final weight.

The crude protein content was estimated using the Kjeldahl method. The total ash content was estimated by the incineration method using a muffle furnace. The carbohydrate content was estimated using the total difference method. The total energy value was calculated using the Eq. 3:

$$TE = 4CA + 4P + 9F \tag{3}$$

where *TE* stands for total energy (expressed in kilocalories). The abbreviation *CA* denotes carbohydrates, *P* are proteins and *F* are fats (all of them expressed in grams). For expressing the energy value in kilojoules, the conversion factor of 4.184 kJ per 1 kcal was used.

In vitro digestion and probiotic stability

In vitro gastrointestinal digestion was done to mimic the human digestive system and consisted of two important electrolyte solutions, as done by ZHOU et al. [16]. To prepare the gastric electrolyte solution (GES), 3.1 g NaCl, 1.1 g KCl, 0.6 g $NaHCO_3$, and 0.15 g $CaCl_2.H_2O$ were dissolved in 1 000 ml of distilled water and autoclaved at 103 421 Pa pressure and 121 °C for 15 min. Afterwards, 150 ml of the prepared GES was supplemented with 37.5 mg gastric lipase, 35.4 mg pepsin and 1.5 ml sodium acetate (HiMedia, Mumbai, India). Using the 0.1 mol·l^{-1} HCl solution, the pH of the mixture was adjusted to 3.0 to form the simulated gastric juice (SGJ).

To prepare the intestinal electrolyte solution (IES), 5.4 g NaCl, 0.65 g KCl and 0.35 g $CaCl_2.H_2O$ were dissolved in 1 000 ml of distilled water. The pH was adjusted to 7.0 with 0.1 mol·l^{-1} NaOH and the solution was autoclaved.

Simulated small intestinal juice (SSIJ) was prepared by mixing 20 g of IES, 20 g pancreatin solution (7%, w/w), 40 g bile salt solution (4%, w/w) and 2.6 mg trypsin. The pH was adjusted to 7.5 with 0.2 mol·l^{-1} NaOH (HiMedia).

All enzymes used in the study were obtained from HiMedia.

The cereal bar product was crushed using the mortar and pestle. The gastric digestion was performed by combining the product and SGJ solution in a 1 : 1 ratio (1 g of product and 1 ml SGJ) in a sterile beaker. The beaker was placed in a water bath with a stirrer set at 10.5 rad·s^{-1} and the temperature was maintained at 37 °C. The 2 ml samples were collected at 0, 1, 2, 3 and 4 h to check the viability of the cells. After 5 h of gastric digestion, SSIJ solution was added to the gastric digested solution in the ratio of 3 : 10. The pH was adjusted to 7.0 and mixed. The samples were withdrawn every 4 h until the 16-th hour to check the probiotic viability. The collected aliquots were serially diluted using 0.9% saline solution, plated on MRS agar and incubated at 37 °C for 24 h.

Extraction and analysis of vitamin B12

Vitamin B12 (cyanocobalamin) was quantified using high performance liquid chromatography (HPLC). Hydroxocobalamin, a natural form of cobalamin found in bacterial cells, was converted to cyanocobalamin using KCN [17]. For the HPLC analysis, the cell culture broth (40 ml) was centrifuged at 9 000 ×*g* for 10 min at 4 °C to harvest the cells. After discarding the supernatant, the pellet was washed with 10 ml of 0.2 mol·l^{-1} potassium phosphate buffer (pH 5.5), then centrifuged at 10 000 ×*g* for 15 min and re-suspended in 1 ml buffer containing 0.1% potassium cyanide. To achieve the cell lysis and the release of vitamin B12, the re-suspended cells were vortexed and autoclaved at 121 °C for 15 min. Subsequently, the samples were centrifuged at 10 000 ×*g* for 15 min. The resulting supernatant was filtered through a 0.22 μm syringe filter and analysed using HPLC. A Shimadzu chromatography system (Shimadzu, Kyoto, Japan) with UV-Vis detector and Shodex C18-4E column (internal diameter 4.6 mm, length 250 mm, Resonac, Tokyo, Japan) was employed. The UV detection wavelength was set to 361 nm, with a mobile phase flow rate of 1 ml·min^{-1}, an oven temperature of 30 °C, and an injection volume of 20 μl. The mobile phase consisted of a 50 : 50 mixture of HPLC water and methanol. Calibration was performed using cyanocobalamin as the external standard [18].

Statistical analysis

All the experiments were carried out in triplicates and the analytical results were expressed as mean ± standard deviation of the triplicates of individual treatments. Statistical analysis was performed using IBM SPSS Software, version 25 (IBM, Chicago, USA). The data obtained were analysed using univariate Duncan's multiple range test and significant differences were tested using ($P < 0.05$).

RESULTS AND DISCUSSION

Probiotic viability on storage

Successful incorporation of probiotic microorganisms into ready-to-eat foods is relatively difficult due to their sensitivity to growth conditions, limited survivability and various factors affecting the strains during product manufacturing and storage until consumption [19]. Two cryoprotective matrices were studied for efficiency in preserving the cell viability during lyophilisation and subsequent storage (Fig. 1). Bacteria incorporated into CSM lyoprotectant matrix exhibited 3 log CFU·g^{-1} reduction in viability after 60 d storage time while, SKM showed 6 log CFU·g^{-1} reduction. After the completion of the 120 d time period, the CSM matrix samples showed 10 times greater viability than the SKM matrix samples. The loss in cell

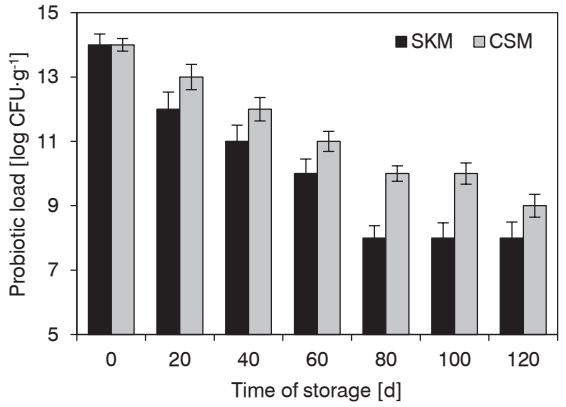

Fig. 1. Stability of lyophilised *Limosilactobacillus reuteri*.

SKM – skimmed milk media, CSM – casein skimmed milk media.

viability can be attributed to cell shrinkage from sublimation, collapse of cell structures and protein molecules [20]. Reports demonstrating the efficiency of skim milk as a cryoprotectant for preserving probiotic viability [21] have advocated for further modifications in the cryoprotective matrices for enhanced results [22]. The high content of milk solids from the CSM matrix supports bacterial growth. Additionally, lactose catalyses the generation of β-galactosidase enzyme that assists the proliferation of *Lactobacillus* sp., while saccharose holds up the bacterial cell membrane under dehydrated conditions.

Colour

During the CIE system-based colour investigation of the nine cereal bar formulations, the L^* parameter showed a variable pattern of slightly increasing luminosity with an increasing proportion of the finger millet flour and sugar, resulting in brighter products (Fig. 2). The formulations T9, T8 and T6 had significantly higher L^* values than the rest. The a^* values were positive, indicating redness. All the a^* values were in the range of 11 to 12 with no significant differences. Accordingly, all the products showed the reddish-brown tint acquired from the finger millet base. The positive b^* values pertaining to yellowness showed deviations of no specific pattern. T1 and T9 showed the lowest and the highest b^* values, 19.49 ± 0.55 and 21.25 ± 0.34, respectively. While more darkness (lower L^*) is expected with the increasing proportion of millet, as the recent study evaluating millet-based biscuits has shown [23], there was a possible counter effect, when adding more millet diluted the proportion of dark cocoa pigments and increased the brightness in this way.

Texture and water activity analysis

The hardness was compared between the investigated cereal bar formulations. The hardness values ranged around 270–280 N for all the formulations (Fig. 3), with the lowest value recorded for T7. T1 was found to be the hardest formulation with 280.69 ± 0.55 N. The estimated hardness is proportional to the force applied to cause deformation. The greater the force needed to penetrate the food, the greater its hardness, being an important parameter for the acceptance of the product. The correlation of hardness of the product to moisture content is high. In a low-moisture product, the hardness needs to be managed for the product to be palatable. Otherwise, it may negatively impact consumer acceptance [24].

Food products with water activity ranging from 0.6 to 0.9 are termed to be intermediate mois-

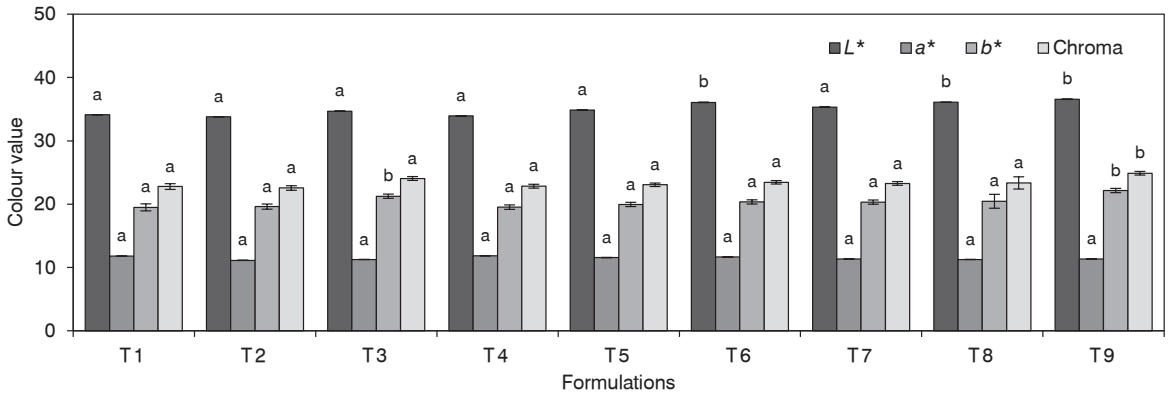

Fig. 2. Colour analysis of the cereal bar formulations.

Columns with different letters within a set are significantly different ($P < 0.05$).

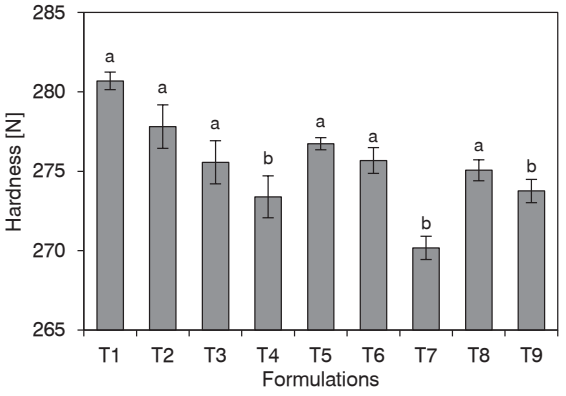

Fig. 3. Hardness of the cereal bar formulations.

Columns with different letters within a set are significantly different ($P < 0.05$).

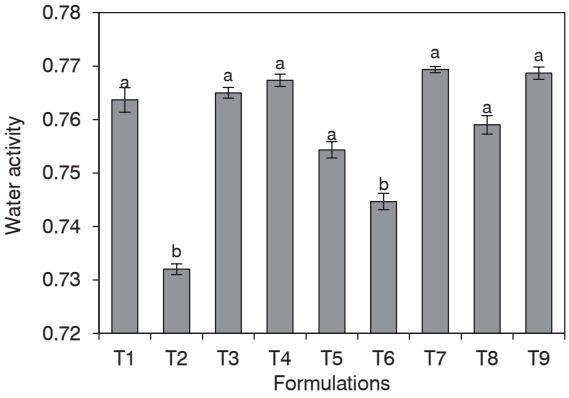

Fig. 4. Water activity of the cereal bar formulations.

Columns with different letters within a set are significantly different ($P < 0.05$).

ture food (IMF). These foods are regarded as shelf-stable without refrigeration or further heat treatment and are found to be refractory towards mould growth and mycotoxin formation. The recorded values of all the tested products were between 0.7 and 0.8 (Fig. 4), with T2 being the lowest.

Sensory analysis

Sensory evaluation was taken as a key factor in identifying the most preferred formulation from the consumer's perspective (Fig. 5). The products were evaluated based on parameters such as colour, appearance, taste, aroma, mouthfeel, texture and overall acceptability using a 9-point hedonic scale. Varying opinions were recorded on the aroma, taste and overall acceptability of the product. T7 was the most accepted tested product, which surpassed other formulations in all the parameters except of the aroma. T8 was the next most successful product. Hence, T7 was studied further to estimate the nutritional values and probiotic stability. The scores on the hedonic scale were comparable to reports on sensory evaluation of gluten-free extruded snacks with added fibre and protein sources [25, 26].

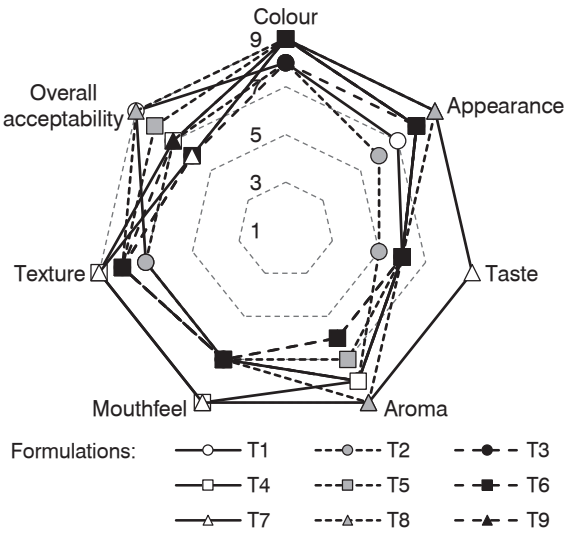

Fig. 5. Sensory analysis of cereal bar formulations.

Nutritional analysis

Food products with lower moisture content (less than 15 %) are less susceptible to microbial contamination and have a prolonged shelf life. The moisture content was estimated to be around 13.8 %. The relatively low ash content (1.8 %) was considered a desirable attribute of the product. If a food product has 30 % or less of the calories contributed by fat, it can be considered a low-fat product [27]. From the estimated crude fat content (5.4 %), it was evident that the cereal bar in this study was a low-fat food product. The carbohydrates estimated by the total difference method were found to be approximately 64 %. The total energy obtained from the product was calculated to be around 1 474 kJ (Tab. 2). While studying the energy value of pearl millet-based nutrition bars, SAMUEL and PEERKHAN [28] reported similar levels, 1 390–1 586 kJ, and stated that the 15.7–18.3 % of protein could mainly be attributed to the pearl millet fractions contained in the product.

In vitro digestion - probiotic survivability

By adhering to and colonising the intestinal mucosa, probiotics inhibit the growth of harmful

Tab. 2. Proximate analysis of cereal bar.

Component	Value
Moisture [g]	13.8 ± 0.5
Ash [g]	1.8 ± 0.3
Crude fat [g]	5.40 ± 0.03
Protein [g]	11.6 ± 2.8
Crude fibre [g]	3.06 ± 0.01
Total carbohydrate [g]	64 ± 3
Total energy [kcal]	352.3
Total energy [kJ]	1 474

Values are expressed per 100 g of cereal bar.

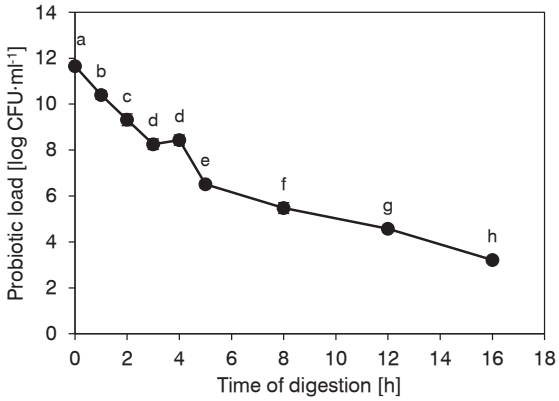

Fig. 6. Probiotic viability during the simulated digestion in vitro.

The probiotic load is expressed per millilitre of digestate volume.
Different letters within a set indicate significantly different values ($P < 0.05$).

bacteria and compete with them for the nutrients present in the ecological niche, aiding in the restoration of the intestinal microbiota and exhibiting prospective anticarcinogenic properties. To confer the said benefits, the organisms should endure the gastrointestinal transit and reach the large intestine in adequate amounts to allow colonisation and proliferation [29].

During the simulation of the gastric digestion phase that mimics the processes in the stomach cavity, approximately 5 log CFU·ml^{-1} per digestate volume was depleted (Fig. 6) and this decrease in the probiotic count must be attributed to the lethal acidic conditions due to H^+ ion influx that impairs the F_1F_0-ATPase proton pump activity [30]. Subsequently, shifting the digestate matter to the intestinal juice complex led to a further loss of 2 log CFU·ml^{-1} in probiotic viability. Later, there was a further, but less steep decrease in viability between the 5th and 8th hour, when cca 10 CFU·ml^{-1} were lost (Fig. 6). Within the 8th to 16th hour of digestion, the viability loss of probiotics was lower compared to the gastric phase. The viability loss observed during intestinal digestion can be attributed to the harsh impact of bile and digestive enzymes (such as lipases, proteases, and amylases), causing the destruction of the cell membranes and damage to the DNA [31]. In vitro studies using *Lactobacillus salivarius* and *Pediococcus pentosaceus* have reported similar courses during the intestinal digestion phase [31, 32]. In spite of a great reduction of viability from before to after the simulated digestion, we have demonstrated that cca 10^3 bacterial cells per millilitre of digestate survived the whole 16 h long process. Since jejunum and duodenum are the major portions of the small intestine and known as the colonisation region of organisms of *Lactobacillus* spp. [33], the observed probiotic viability proves to be adequate. Thus, the studied product can be labelled as "Added probiotics".

Vitamin analysis

L. reuteri is a notable vitamin B12 producer, so this feature was considered to be an additional quantifiable benefit of consuming the product. Therefore, B12 quantification was performed for the lyophilised cells present in the whole product before and after the simulated digestion. Initially, the content was 1.83 μg of the vitamin per 30 g serving size. After the simulated digestion, the samples were analysed within 2 h to ensure the accuracy of bioavailable vitamin levels. The content of 1.29 μg of the vitamin per 30 g serving size was revealed post-digestion, i.e. cca 70 % of the pre-digestion value. The data indicate efficient encapsulation and sustained release of the compound upon consumption. Studies on rice-based puffed extrudates [34] have indicated about 77 % retention of the vitamin after processing at 140 °C. Encapsulation in sugar-based matrices [35] has also led to similar vitamin release patterns. The resulting levels were found to be in accordance with the prescribed limits of recommended dietary allowance 2.4 μg per day for each suggested serving size [36].

CONCLUSION

With personalised nutrition on the rise, the market demand is fuelled by the need for indigenous and minimally processed products. The expanding demand for sustainable options, such as probiotics and millet, are no exceptions, considering the mental and physical health benefits

their consumption confers. The study focused on developing a probiotic-enriched finger millet-based ready-to-eat cereal bar. *L. reuteri*, a prominent probiotic, was lyophilised in two different cryoprotective matrices, of which casein skimmed milk matrix matrix showed 10 times greater protection of viability. Nine different product formulations were evaluated sensorially for consumer acceptance and instrumentally for product hardness and water activity. The T8 product was preferred sensorially, and further nutritional analysis was performed. The in vitro probiotic viability assay validated the probiotic claim of the product for establishment in the regions of the small intestine. The energy value of 1 474 kJ per 100 g product was recorded. The cereal bar is low-fat and a good source of fibre, while low moisture confirmed the product to be shelf stable. Originally, the product was reported to contain 1.83 μg per serving of cobalamin, while the post-digestion sample contained 77 % of the compound.

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