Production and Physico-Chemical Evaluation of Non-Dairy Probiotic Beverages

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ABSTRACT

Background: Probiotics are live and beneficial bacteria for gut health. The purpose of this study was to develop non-dairy probiotic-rich beverages.

Methodology: a fixed quantity of brown sugar was dissolved in distilled water and autoclaved and brown rice was added. All samples were incubated for three days (36 hours) at 37 °C. After every 12 hours, the absorbance of each sample and pH were noted. Likewise, after 36 hours of incubation, all samples were filtered to separate the brown rice. Then filtered beverages were divided into three bottles and two different synthetic flavors and honey were added to one beverage type and stored at -4 °C. All beverages were evaluated for their sensory characteristics, pH, and titratable acidity (storage days).

Results: Slight changes in physical and sensory properties of probiotic beverages with synthetic flavor i.e. orange and strawberry were observed while significant changes were seen in the probiotic drink with honey. Probiotic growth continued even after refrigeration after 3rd day of storage which resulted in decreased pH over time. Overall acceptability of all beverages was satisfactory on day 3.

Conclusion: The production of nondairy beverages was a successful venture. The drinks had fairly acceptable storage quality.

Contribution to literature: Very few studies had examined probiotics of plant origin in the past, particularly, rice. So, this study was designed to fill this gap and to evaluate the potential of rice-based probiotics. Our study revealed that non-dairy probiotic beverages can be produced with grain-based probiotics such as rice.

Keywords: Probiotics, Beverages, Sensory analysis, Shelf-life, Absorbance, Growth rate, pH, Incubation time

1. INTRODUCTION

The link between diet, colonic microbiota, and human health has long been recognized. Numerous studies have confirmed the association between disturbed microbiota and a wide array of metabolic diseases (Chizhayeva et al., 2020). Probiotics are live and beneficial bacteria for gut health as they are capable of producing a physiological response in the gut via mechanisms that underlie the slowing growth of pathogenic bacteria, elicit synthesis of immune-regulatory factors, and neurogenic substances (Roselló-Soto et al., 2019), and other physiological molecules such as peptides, vitamins & short chain fatty acids and polyamines, leading to modified metabolic response (Pessione & Cirrincione, 2016), for example, digestion, minerals, and cholesterol regulations, to mention a few. Other metabolic diseases where probiotics were proven effective may include but are not limited to diarrhea, constipation, irritable bowel syndrome, allergies, lactose intolerance, cancer, respiratory infections, urinary tract infections, and helicobacter pylori infection (Min, Bunt, Mason, & Hussain, 2019). Also, it is important to note that the health-promoting effects of probiotics may depend on their specific numbers (Sharma, 2012). For instance, one study recommends 10⁶ (CFU/g) to not more than 10⁹ (Min et al., 2019).

Probiotics are recently evaluated for their immune enhancement and normal neuro-functioning. Furthermore, neurological, behavioral, and metabolic disorders might develop in case the balance between probiotics and pathogenic bacteria is disturbed (Valero-Cases, Cerdá-Bernad, Pastor, & Frutos, 2020). Presently the demand for functional foods such as beverages enriched with healthy microbiota (probiotic) is on the rise throughout the globe. In this regard, numerous dairy-based functional products containing probiotics are produced worldwide. Predominantly, two dairy-based probiotics such as *Lactobacillus spp.* and *Bifidobacterium spp.*, have been added to beverages. In parallel, a variety of plant-based foods containing probiotics are reported in recent years due to the fact that a large number of individuals are either vegetarians or lactose intolerant, and allergic to milk proteins. For these reasons, plant-based probiotics are currently under investigation and may

include Saccharomyces cerevisiae, S. Boulardii, and Lactobacillus acidophilus, Lactobacillus casei, Lactobacillus plantarum, Lactobacillus rhamnosus and Bifidobacterium lactis.

Zinov'eva, Shnaider, and Zaripova (2021) recently reported juice-based probiotic beverages and tiger nut tubers-based beverages. There are limited studies on probiotics from rice so to fill this gap the present study was designed. The main objective of this research was to produce a plant-based probiotic beverage from the fermentation of brown rice without the use of a starter culture. Our second objective was to evaluate the shelf life and physico-chemical properties of prepared beverages. The hypothesis was to test whether or not non-dairy probiotic beverages can be produced with grain-based probiotics such as rice has reportedly been shown to contain natural probiotics which can be propagated (growth) within certain media.

2. METHODS

2.1. Materials

Brown basmati rice, brown sugar, distilled Water, screw-cap bottles (100 mL capacity), and food flavoring (orange, strawberry, and honey). Similarly, the following tools and equipment were used, for example, digital weight balance, spectrophotometer, glass cuvettes, pH meter, titration burette, beaker, sterilized glass bottles, Whatman filter paper, cotton, bio-safety cabin, incubator, and autoclave.

2.2. Methods and Procedures

All necessary safety and hygiene procedures were followed using a bio-safety cabin. Briefly, brown sugar (60 g) was added to 500 mL of distilled water in a glass bottle followed by autoclaving. Upon cooling, brown basmati rice (120 g) was added, and bottles were closed with an airtight lid and kept at a temperature of 37°C in an incubator for three days. The samples were shaken every day (60-80 times) to facilitate the mixing and to release the probiotic bacteria from the rice to the sugar media. The increase in the number of probiotics was monitored (every 12 hours for a total of 36 hours) by checking the samples' absorption through a spectrophotometer at wavelength 260 nm (Homayouni, Azizi, Ehsani, Yarmand, & Razavi, 2008; Homayouni, Ehsani, Azizi, Razavi, & Yarmand, 2008). For the spectrophotometric reading, 1.0 mL of liquid from the incubated sample was aseptically taken in a glass cuvette then diluted with 2.0 mL of distilled water and the absorbance was read at 260nm. After 3 days, rice was removed from the samples with filter paper and the fermented beverages were put in the refrigerator at 4.0 °C to prevent further production of probiotic bacteria. The stock beverages were then divided into three further samples. Then orange, strawberry, and honey were added as flavoring agents. Each sample was randomly coded i.e. O-001, S-002, and H-003 respectively, and sensory evaluation was conducted on day 3, day 6, and day 9.

2.3. Physical Analysis

For physical analysis, the pH and titratable acidity of beverages were checked regularly. The pH of samples was monitored using a pH meter. For determining the titratable acidity (TA) samples were titrated against the titrant i.e. 0.1 M sodium hydroxide (NaOH) was used along with 2-3 drops of phenolphthalein as an indicator for the estimation of the percentage of lactic acid (Kurt, Cakmakci, & Caglar, 1996). TA was calculated by using the formula:

TA= $\frac{M(NaOH)(mol/L) \times Volume of NaOH used (mL) \times Mol. wt. of Lactic acid \times 100}{Volume of sample (mL) \times 1000}$

2.4. Sensory Evaluation

Sensory evaluation was conducted on days 3, 6, and 9 as described previously with little modification (Chen, Zhu, Zhang, Niu, & Du, 2010). All three beverages were subjected to sensory evaluation by trained faculty members, both male and female aged 22 and above. The three types of beverages were then served in the cold form to the sensory panelists in disposable cups containing 25 mL of the beverage. The sensory panelists evaluated the samples based on sensory attributes such as taste, color, flavor, and overall acceptability. Sensory evaluation was performed by using a hedonic scale where 1 on the scale represents 'extremely dislike' and 9 represents 'extremely liked'. The data obtained from the hedonic scale were then reported mean± SD.

2.5. Statistical Analysis

The growth rate of the probiotics (absorbance data), pH after every 12 hrs & pH over storage time, and titratable acidity are the average means of the triplicate samples while the sensory analysis data is reported in mean ± S.D on day 3, day 6 and day 9.

3. RESULTS

3.1. Growth Rate of The Probiotics and pH

Figure 1 shows the absorbance of the samples at 3-time points. We noted an increased absorbance of probiotics over time (Figure 1), with linear growth at day 1 (0.711), day 2 (0.862), and day 3 (1.014), which affirmed the increase in the number of probiotics. Bajpai et al. (2017) reported bacterial growth (absorbance 1.0 plus) at 260 nm for the control samples. The growth rate of probiotics in a liquid media has frequently been reported. The absorbance of a given sample through a spectrophotometer is an easy procedure to monitor the performance of the fermentation process. However, a number of studies have reported different wavelengths for the absorbance as a proxy for the number of probiotics in the media. Homayouni et al. (2008); Homayouni et al. (2008) investigated the growth of probiotic strain at a wavelength set at 580 nm. Similarly, Kebbi et al. (2020) computed microbes at three different wavelengths such as 260, 280, and 580 nm. Likewise, Takeda et al. (2013) determined the microbial growth at OD set at 596 for the enumeration of LAB and bifidobacteria strains. Moreover, Martens-Habbena and Sass (2006) evaluated the growth at 436 nm (OD 436) on spectrophotometer. In parallel, Ačai, Valík, and Medveďová (2021) introduced growth enumeration of Staphylococcus aureus and Escherichia coli, being the model microbes. This study made a cell counting device named Kit-8 based on the redox reaction between water-soluble tetrazolium salt i.e. WST-8 [2-(2-methoxy-4nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium] and the enzyme dehydrogenase (bacterial enzyme). The WST-8 solution is thus reduced by NADPH to orange-yellow formazan by the action of bacterial enzyme dehydrogenases. Further, the number of live bacteria is related to the absorbance value of formazan at 450nm.

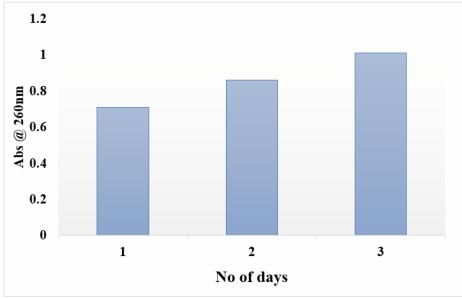


Figure 1. The growth rate of the probiotics i.e. absorbance after every 12 hrs for a period of 3 days.

Figure 2 described the pH variations on three (3) different days. A great work that classifies foods, on the basis of their pH, is presented by Dionisio et al. (2017). Accordingly, low-acid foods would have a pH > 4.5, acidic foods will have a pH of 4.0-4.5 while very acidic food will present a pH < 4.0. As can be seen from our study the pH decreased gradually (more acidic), demonstrating strong bacterial growth due to more acidic content in the fermented media. Agregán-Pérez, Alonso-González, Mejuto, and Pérez-Guerra (2021) reported that pH is reduced during 0–48 h) then increases (48–60 and 60–96 h) and finally decreased (96–120 and 120–348 h). These fluctuations are due to the synthesis of lactic and acetic acids, then their utilization, and subsequently, the production of new acids (lactic and acetic acids). Our study has not noted pH beyond 36 hours; however, in conclusion, the decrease in pH indicated probiotics growth in the beverages.

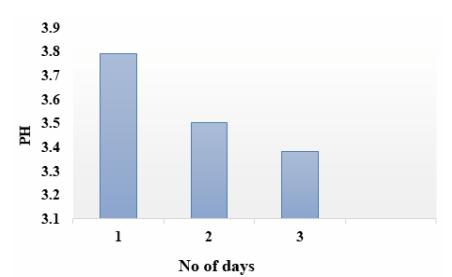


Figure 2. pH of beverages after every 12 hrs for a period of 3 days.

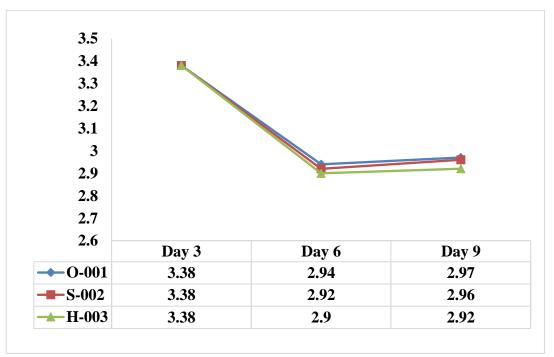


Figure 3. pH over storage times.

3.2. Titratable Acidity (TA; G/L) Over Time

Titratable acidity is the measurement of the sum of the free and un-dissociated hydrogen ions (acids). It determines the overall acidity as a result of fermentation. In our study titratable acidity was taken as % Lactic acid production by *Lactobacillus* during the fermentation of brown rice (Figure 4). The titratable acidity was determined just after the addition of flavor essence so it remained unchanged i.e. 0.063 g/ L for all three types of beverages on day 3. However, on day 6 and day 9 all beverage types exhibited an increased titratable acidity. It has been shown that samples stored at room temperature or under refrigeration temperature can present total TA values between 0.19 and 0.18 g/100 mL, given that there is no or limited oxidation of organic acids. The lower values of TA in our case could be due to the short storage time leading to the production of fewer acids or a higher oxidation rate of the organic acids at day 6 and 9, respectively. In conclusion, the total TA increased gradually over the storage time.

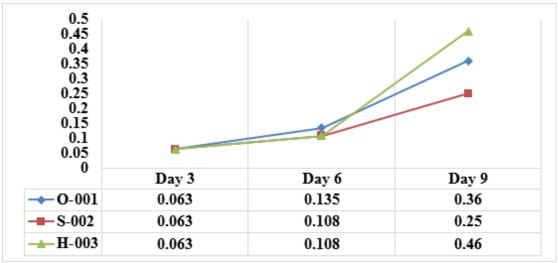


Figure 4. Differences in titratable acidity (g/L) over storage time.



Figure 5. Samples for sensory evaluation.

3.3. Sensory Evaluation

Data related to sensory analysis, presented in terms of mean values along with standard deviations on different days, are given in Tables 1, 2, and 3. Among sensory attributes included were color, flavor, taste, appearance, and overall quality. According to the results, color was rated 5 for all beverage types on day 3. The appearance of all samples ranged between 6 (like slightly) to 7 (liked moderately). Overall acceptability of all beverages was satisfactory on day 3. On day 6 overall quality of all beverages remained between 6 and 7. However, out of all three samples, sample O-001 was rated high in terms of taste, flavor, and overall quality. On day 9, there were prominent changes with respect to the flavor, taste, and overall quality of all three samples. The panelist liked sample O-001 (orange), which had the highest score in terms of taste, flavor, and overall quality. Based on the results obtained for the sensory indices it is concluded that the sample O-001 had the best scores and overall acceptance by the sensory panelists. Sample H-003 had the least score because honey might have provided the substrate for bacterial fermentation which increased acidity while other synthetic flavors did not.

Table 1. Sensory evaluation on day 3.						
Sample	Color	Flavor	Taste	Appearance	Overall quality	
O-001	5.0±0.00	7.6±0.54	7.4±0.54	6.4±0.54	7.4±0.54	
S-002	5.0±0.00	7.0±0.70	7.0±0.70	6.4±0.54	7.2±0.44	
H-003	5.0±0.00	7.0±0.70	6.6±0.89	6.2±0.44	6.8±0.83	

Table 2. Sensory evaluation on day 6.						
Sample	Color	Flavor	Taste	Appearance	Overall quality	
O-001	5.6±0.54	7.4±0.83	7.2±0.70	6.4±1.14	7.2±0.83	
S-002	5.4±0.54	6.8±1.51	6.2±1.30	6.2±0.83	6.6±1.1	
H-003	5.6±0.54	5.8±1.30	6.4±1.51	6.4±1.51	6.8±0.83	

Table 3	Sensory evaluation on day 9

Sample	Color	Flavor	Taste	Appearance	Overall quality
O-001	5.4±0.54	7.2±0.83	7.0±0.83	6.0±0.54	7.0±0.70
S-002	5.4±0.54	6.4±0.84	5.4±0.54	5.8±1.14	6.2±0.54
H-003	3.4±0.54	2.4±1.14	2.2±1.14	3.4±0.54	2.0±1.09

4. DISCUSSION

The basic purpose of this experiment was to produce a probiotic drink by fermentation of non-dairy sources. Brown rice was used for this purpose as according to previous studies, non-dairy matrices such as cereals and legumes are potential carriers of probiotics, prebiotics, and other bioactive compounds (Valero-Cases et al., 2020). The physicochemical and sensory evaluations were used during production as well as the storage phase to assess the quality of the beverages and to check the growth of probiotic bacteria (i.e. *Lactobacillus*), pH, and total acidity.

The increase in absorption of beverages (Figure 1) as determined by the spectrophotometer, demonstrated an increase in the number of probiotic bacteria. We observed a linear growth over time (Homayouni et al., 2008), reflecting the growth of probiotics bacteria. This facilitated production of organic acids and as a result pH changed from 3.79 to 2.9 over time indicating an effective fermentation Figure 2-3. Similar observations have been reported previously, e.g. post-acidification due to lactic acid production (Aportela-Palacios, Sosa-Morales, & Vélez-Ruiz, 2005). This is further confirmed through the titratable acidity (day 3 to day 9, Figure 4) experiment where an increase in titratable acidity was noted. As mentioned earlier, lactic acid accumulation over time could be the reason for such change (Cairns, Watson, Creanor, & Foye, 2002).

Sensory evaluation was performed on day 3, day 6, and day 9, and their means were recorded (Tables 1, 2 & 3). According to the results two samples i.e. O-001 and S-002 were accepted because there were slight changes in sensory properties and overall quality of the accepted samples (O-001 & S-002) during cold storage. These results were corresponding well with a prior study in which changes in probiotic drinks (made from soy and rice by-products) were evaluated during cold storage. Slight physical and sensory changes were noted in fermented drinks during the storage period. However, they did not affect product quality (Costa, Júnior, Rosa, Caliari, & Pimentel, 2017). In our case, the sample with honey (H-003) was not suitable because there were significant changes in sensory evaluation. A research study conducted to evaluate the effect of the addition of honey on the fermentation of *Lactobacillus casei* showed that honey significantly stimulated the growth of *Lactobacillus casei* as well as lower pH (Slačanac et al., 2011).

5. CONCLUSION

The development of non-dairy drinks using brown rice and brown sugar was a successful venture. The organoleptic properties of such drinks can remain acceptable for three days of storage and can be an alternative to dairy-based fermented beverages. There should be more studies like this so that new, fermented, and non-dairy probiotic drinks can be produced for those who cannot consume dairy sources in order to improve gut health.

6. LIMITATIONS

We have checked the absorbance of the sample at 260 nm, however, we were unable to determine the absorbance at 596, 580, and 280 nm. Furthermore, a negative control should have been prepared lacking brown rice or containing boiled rice. Similarly, positive control samples containing brown sugar plus

streptomycin (10.0 μ g/mL) should have been used. Likewise, we were unable to quantify organic acid types/metabolites and types of bacteria in the beverages.

7. FUTURE DIRECTIONS

Future studies may determine total soluble solids (via a suitable tool such as a refractometer) and then divide the total soluble solids by the total titratable acidity. This could be a new variable. Similarly, levels of ascorbic acids can be carried out through simple, quick, and cost-effective procedures such as the reduction of dichlorophenol-indofenol as an indicator through the ascorbic acid assay. More studies are required in the future to confirm the present procedure's applicability as an innovative, straightforward, and most importantly cost-efficient process for probiotic production and their metabolites. There should be more studies like this so that new, fermented, and non-dairy probiotic drinks can be produced for those who cannot tolerate dairy sources.

FUNDING

This study received no specific financial support.

ETHICAL STATEMENT

All sample beverages were prepared in a sterilized environment to avoid any contamination and to make them safe for human use. Sensory analysis was performed after taking consent from the panelists. Details of the study, composition of samples, and procedure were explained to the panelists before the analysis. Panelists were asked if they have an allergy to rice or probiotics. No side effects were recorded during and after sensory evaluation by the panelists. This study was approved by the Research Forum Committee of the School of Health Sciences, University of Management & Technology (UMT), Lahore.

CONFLICT OF INTEREST

The authors declare that they have no competing interests.

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AUTHORS' CONTRIBUTIONS

All authors contributed equally to the conception and design of the study.

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