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Nutritional Enhancement of Wheat Bread by Probiotic *Lactobacillus consortium*

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Abstract: The *Lactobacillus acidophilus* and *L. rhamnosus* are used as probiotic strains, supplement food for better human health. Initially, the wheat flour was fermented for 1 to 3 days. A higher increase in carbohydrates, proteins, and lipids was observed in flour fermented for 48 hours. It was compared both in the presence and the absence of *Lactobacillus* strains. Elevated levels of proteins, carbohydrates, and fats were noted in bread with probiotic strains. *Lactobacillus* spp. proved to be deficient in fat as the fat content was found to have decreased in bread made with the same—selenium and folate, "vitamin B9," the essential vitamins for all (pregnancy) women. Bread with the probiotic strains is notably enriched with it. Selenium possesses an antioxidant property estimated using the FRAP assay technique. Bread with and without the *Lactobacillus* spp. was given for assessment via sensory analysis. The bread with the *Lactobacillus* was further tested and compared with normal wheat bread, with enhanced flavor and texture. This study shows that a combination of LAB (lactic acid bacteria) with wheat bread can be produced; it exists without any fungus for more than 10 days, while the wheat bread made without LAB wouldn't attain its staleness period.

Keywords: *Lactobacillus strains*; selenium; folate; deficiency diseases; probiotic; lactic acid fermentation.

1. Introduction

In underdeveloped countries, there are massive casualties due to malnutrition-related deficiency illnesses, where poverty and impoverished living conditions also play a vital role in these cases [1,2]. Malnutrition arises primarily due to a lack of availability or distribution of nutrition-enriched food. India ranks among the countries with the highest number of children suffering from malnutrition worldwide. People suffering from deficiency diseases do not receive the ample amount of nutrition they require daily [3]. To overcome such deficiencies, a fortification method is done on these foods. It is the process of adding micronutrients to foods, consequently increasing the vitamin or mineral content of the food. It is introduced instantly into the human body and subsequently improves the body's health [4-7]. It is acceptable from a socio-cultural point of view as it solves human health issues and prevents the population from facing deficiency diseases. Food fortification is achieved through using/adding a micronutrient which is, in fact, a probiotic organism. It delivers extra nutrients to the food to eliminate deficiencies.

Probiotics are yeast and live bacteria. The probiotic strain utilized in this instance is Lactobacillus, i.e., lactic acid bacteria (LAB), the most commonly used probiotic. Amongst

different types of LAB, the most probiotic strain belongs to the genus *Lactobacillus*. *Lactobacillus acidophilus* and *Lactobacillus rhamnosus* were used in this study, which are the most beneficial strains among the different species of *Lactobacillus*. They are termed rod-shaped bacteria [8-10]. They assist in digestion and suppress disease-causing bacteria.

Furthermore, they possess remedial benefits for cardiovascular diseases by decreasing cholesterol levels, urinary tract infections, and skin-related disorders. The commensal flora of lactobacilli has been recognized as the inhibitor of pathogenic bacteria as it develops antimicrobial bacteria substances such as lactic acid, bacteriocins, and hydrogen peroxide [11-13]. Such probiotic bacterial strains are often termed beneficial/helpful bacteria. The human body itself hosts a large number of good bacteria. But they get lost in an overdose of antibiotics. These probiotics help retain the good bacteria in the human body, thereby increasing metabolic function.

In this study, whole wheat bread is fortified using *Lactobacillus* species. It was proved that the nutrient levels (carbohydrates, proteins, vitamins, and minerals) had increased, and the antioxidant levels were also significantly higher when comparing the wheat bread made using lactobacillus species (*Lactobacillus acidophilus* and *Lactobacillus rhamnosus*) with the wheat bread made without lactobacillus species [14-18]. A vast number of people suffer from vitamin deficiency, and one of the major vitamin deficiencies is “vitamin B9” deficiency. There are several types of vitamin B deficiencies, such as B1 (thiamine), B2 (riboflavin), B3 (niacin), B6 (pyridoxine), and B9 (folate) deficiency. It is observed that folate deficiency is quite common among pregnant individuals today. The function of this folate is to aid in making and repairing DNA and producing red blood corpuscles (RBC). A deficiency in folates causes anemia which in turn decreases the RBC count. The folate deficiency in pregnant individuals affects them and their infants. In this study, the folate content is noted to have increased considerably through the use of lactobacillus species. The intake of folate vitamin (vitamin B9) is of extreme necessity as it reduces the risk of newborns afflicted with neural tube defects (Incomplete development of the spinal cord and brain) [19-21]. Whole wheat is notably rich in iron, calcium, selenium, and other minerals. Another mineral of vital importance is selenium. Researchers have concluded that the intake of selenium helps to decrease cardiovascular and cancer-related problems due to its antioxidant activity. Selenium's presence helps fight the aging process in humans and helps in anti-obesity properties by helping to reduce weight [8].

2. Materials and Methods

2.1. Materials.

In the current study, probiotic strains *Lactobacillus acidophilus* and *Lactobacillus rhamnosus* were utilized. YEPD and MRS mediums were employed for the purpose of yeast and LAB culture. Reagents such as DNSA, BIURET, and VANILLIN as biochemical tests have been made use of for nutritional assays. Lacto phenol and crystal violet were also utilized to strain fungi and bacteria. Phosphate buffer, potassium ferricyanide, trichloro acetic acid, and ferric chloride solutions were used to estimate the antioxidant activity. Additionally, pure wheat flour, sugar, and rock salt were used for baking bread.

2.1.1. Stock culture preparation and growth of *Saccharomyces Cerevisiae.*

YEPD (yeast extract peptone dextrose) medium was prepared by adding 1% of yeast extract, 2% of peptone, 2% of dextrose, and 2.5% of agar to 100 ml of distilled water and then autoclaving it. Serial dilutions were performed with the stock solution, which is, in fact, fermented wine. The autoclaved YEPD medium was poured onto sterile Petri plates, to which the serially diluted inoculum was added steadily and spread evenly with the help of a sterile L-loop, then storing it for incubation virtually 24 hours. The yeast was cultivated on Petri plates after the completion of the incubation period. Pinpointed colonies were obtained. Lacto phenol was employed to perform a straining test for the confirmation of yeast. The presence of yeast was proved by the indication of blue color strain. The slide was then observed under a microscope for the structural characteristics of yeast. YEPD broth containing (*Saccharomyces cerevisiae*) was preserved for future use.

2.1.2. Stock culture preparation and growth of *Lactobacillus* species (*Lactobacillus acidophilus* and *Lactobacillus rhamnosus*).

MRS broth was prepared with 1% of peptone, 1% of beef extract, 0.4% of yeast, 2% of glucose, 0.5 % of sodium acetate trihydrate, 0.1% of polysorbate (tween 80), 0.2% of dipotassium hydrogen phosphate, 0.2% of triammonium citrate, 0.02% of magnesium sulfate-heptahydrate, 0.05% of manganese sulfate tetrahydrate and 1.5% of agar. *Lactobacillus acidophilus* and *Lactobacillus rhamnosus* were the bacterial strains that were cultured and isolated. Serial dilution was performed upon the two strains and then plated with MRS agar afterward. The colonies formed from the cultured plates were added along with MRS agar to the sterile Petri plates and then incubated. The following day, the colonies from the plates were extracted, and streaking was executed and subcultured for the two bacterial strains [22,23]. Crystal violet and safranine were used to carry out straining tests to identify gram-positive/negative bacteria. It was proven that the bacteria were gram-positive through the indication of purple color. Under the microscope, the bacterial shapes were of two kinds, rod-shaped with short ends (strain 1) and rods that appeared like chains (strain 2). The test served its purpose in proving that the bacteria cultured were (*Lactobacillus acidophilus* and *Lactobacillus rhamnosus*). MRS broth of both strains was prepared for future use and frozen [24].

2.1.3. Wheat fermentation in bread preparation.

Wheat is the primary component in producing whole wheat bread. Organic wheat is dried and ground into fine powder for bread making. The baking test was executed with a formula of 20% wheat flour, 4% yeast, 1.5% sugar, 0.7% salt, and 4% bacillus strains. 200 ml of water was added to 2 ml of each prepared broth containing either strain. The yeast and bacillus strains were centrifuged to avoid sourness. An airtight container was utilized to store the strains for fermentation for about (24 hours) [22]. The fermented wheat flour was employed for bread preparation. It was then mixed with dried wheat flour, kneaded, and isolated for nearly 2 hours for the dough to rise. Firstly, the oven was preheated at 200 Celsius, and then the bread was baked at 180 degrees Celsius. The bread baked was fluffier and spongy in texture and gave off a sweet taste and aroma [25].

2.1.4. Nutritional analysis.

Biochemical tests were performed for carbohydrates, proteins, and lipids to estimate the number of nutrients present in the fermented wheat flour in figure 1. The wheat flour was allowed to ferment for 24, 48, and 72 hours respectively. Biochemical tests were carried out on the fermented wheat flour for each hour to calculate its essential nutrients. The DNSA test for carbohydrates, the biuret test for proteins, and the vanillin test were performed to estimate lipids. The color changes were noted, and absorbance was calculated.

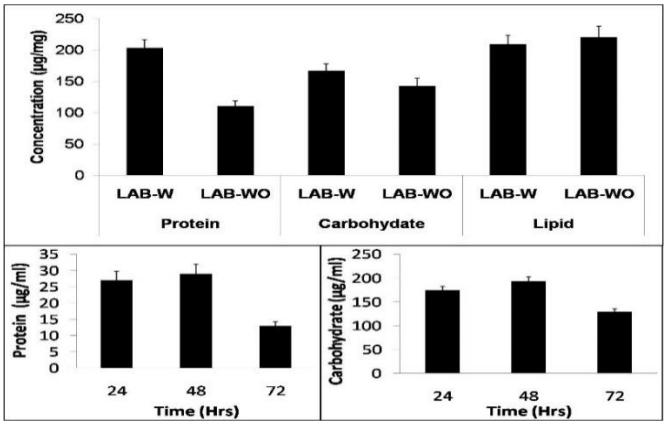

Figure 1. Nutritional analysis of carbohydrates and protein in the samples.

2.1.5. Microbial analysis.

Microbial growth analyses were individually performed for 24, 48, and 72 hours. Serial dilution was then carried out for the hour samples. A quantity of 100 µl was taken in test tubes from each sample, poured onto MRS agar plates, and then spread evenly with the help of the sterile L-shaped loop. The serial dilution was carried out aided by four Petri plates for each hour to analyze the growth of microbial activity (figure 2) [26]. The plates were then stored for incubation for 24–72 hours to support the organism's growth while observing the microbial activity. The colonies were grown on each of the 24, 48, and 72-hour plates after completing the incubation period. The number of colonies was tallied using the CFU machine. The colony forming unit (CFU) gives the precise number of colonies observed on each plate.

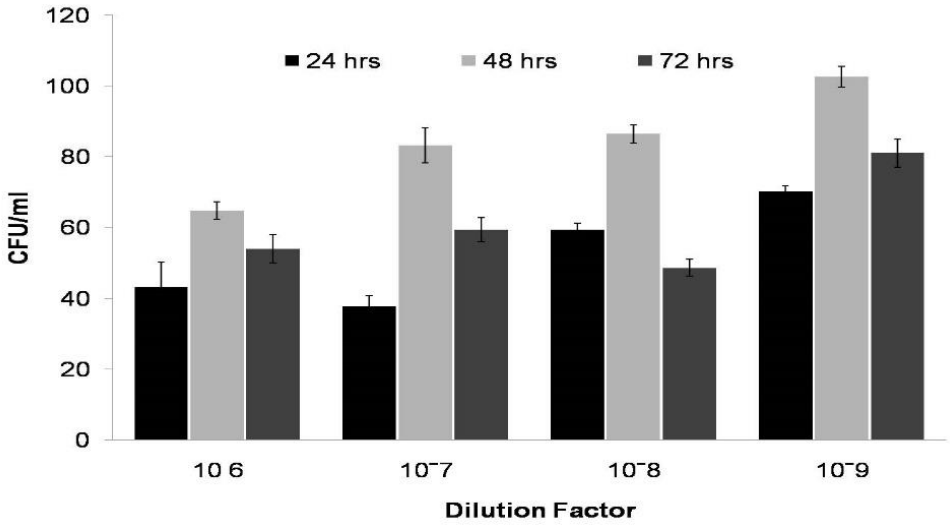

Figure 2. Microbial growth activity.

2.1.6. Comparison study.

The whole wheat bread containing and excluding lactobacillus strains were compared with each other (*Lactobacillus acidophilus* and *Lactobacillus rhamnosus*). Whole wheat bread with and without the *Lactobacillus* was baked and weighed. Nutrition estimation was done on protein, carbohydrates, and lipids. The values were then juxtaposed with the type1 and type2 whole wheat bread. The types above are bread with the *lactobacillus strains* (TYPE 1–WITH LAB) and without the *Lactobacillus* strains (TYPE 2–WITHOUT LAB), respectively shown in (figure 3) [1]. The nutrition test was evaluated for carbohydrates, proteins, and lipids by adding 2 ml of reagent and 1ml of the sample. The color changes were then observed, and the OD values were noted with the help of a spectrophotometer (figure 4).

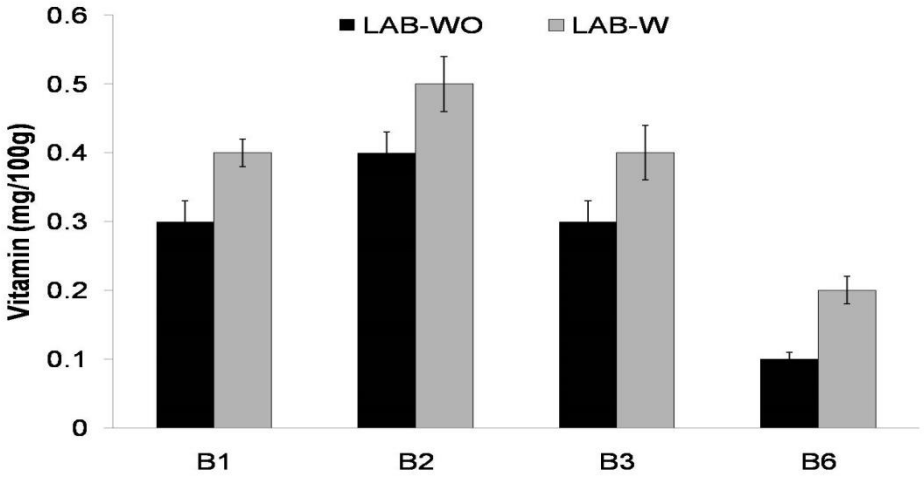

Figure 3. Comparison of VITAMIN B.

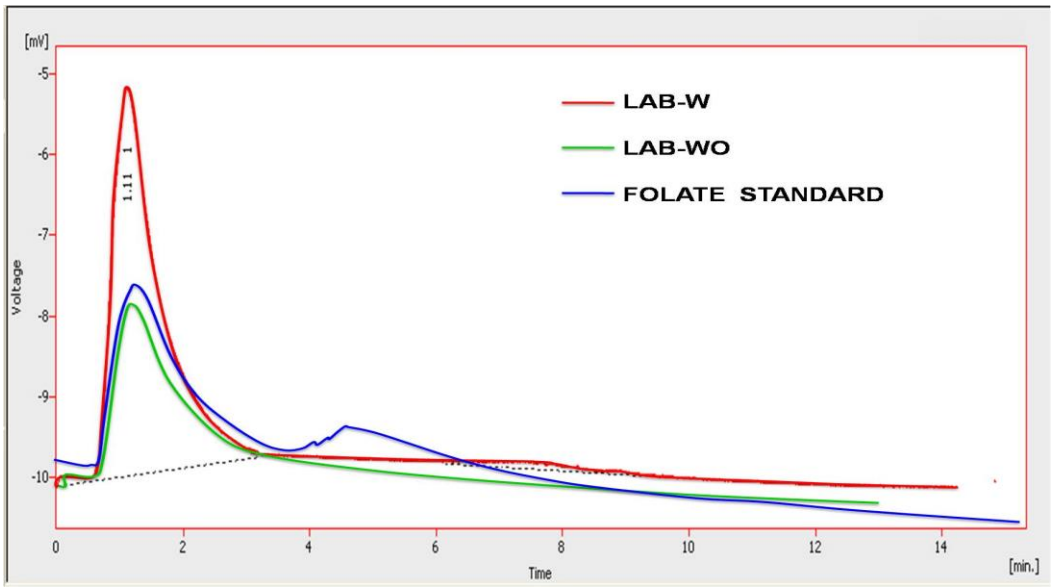

Figure 4. The folic acid curve for without lactic acid bacteria

2.1.7. Antioxidant assay.

A highly essential antioxidant property is possessed by the selenium present in wheat. The antioxidant assay was accordingly performed using FRAP–(ferrous reducing antioxidant power) to estimate the antioxidant level. Phosphate buffer, potassium ferricyanide, trichloro acetic acid, ferric chloride solution, and distilled water were some of the chemical reagents

made used. Quantities consisting of 100 µl and 200 µl from the sample whole wheat bread made with and without *Lactobacillus* species (figure 5) were taken in test tubes. 1.25 ml of phosphate buffer and 1.25 ml of potassium ferricyanide were added and blended in each of the test tubes, respectively, in [16]. A hot air oven was employed to place the tubes at 30ºc for about 30 minutes. After the incubation period, the test tubes were cooled, and 1.25 ml of trichloro-acetic acid was added and blended. The mixture was then transferred to centrifuge tubes and centrifuged for approximately 10 minutes at 3000 rpm. A volume of 1.25 ml was meticulously transferred from the supernatant into test tubes upon centrifugation. The distilled water of 1.25 ml volume was added and blended to that. Ultimately, 0.5 ml of freshly prepared ferric chloride was added to the tubes, and the color change was monitored to detect the antioxidant activity. The color change indicated antioxidant activity from yellow to sea blue. A spectrophotometer was employed to measure the absorbance at 695 nm. Ascorbic acid was used as the standard to compare antioxidant levels(figure 6).

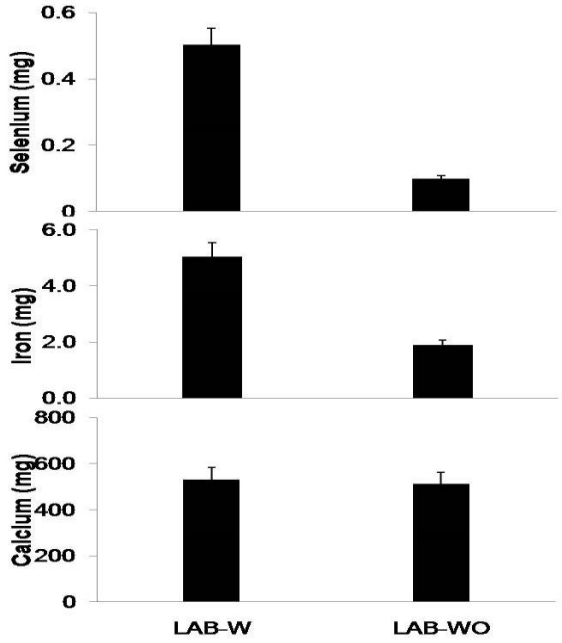

Figure 5. Whole WHEAT BREAD – with and without lactic acid bacteria.

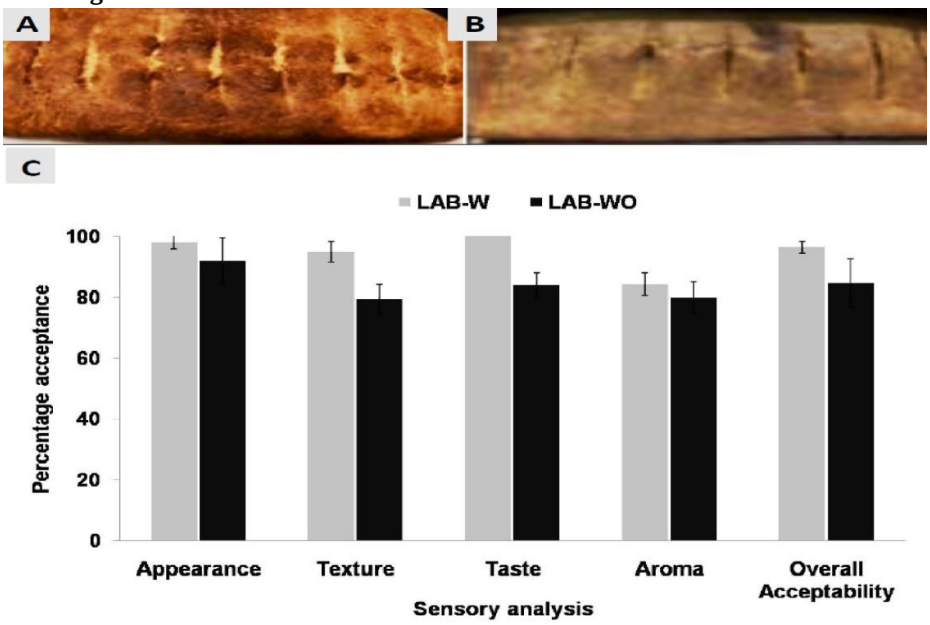

Figure 6. Sensory analysis of nutritionally enhanced bread with non-*Lactobacillus bacterial* bread.

2.1.8. Vitamin and mineral analysis.

Chromatography techniques and spectroscopy methods were implemented to analyze the vitamins and minerals. For vitamins, the HPLC method was followed. The standard, buffer, and stock solutions were prepared, and the concentration was found by observing the peaks within the standard.

3. Results and Discussion

3.1. Results.

The protein and carbohydrate levels were calculated for the 24, 48, and 72-hour fermented samples. The absorbance was discovered and multiplied with the standard concentration of protein and carbohydrates to find their concentrations in fermented samples. The protein and carbohydrate levels were higher in 48 hours of fermented wheat flour when compared with 24 and 72 hours of fermented wheat flour. DNSA test affirmed the presence of carbohydrates through the change of color from yellow to deep brown. Burette test confirmed the presence of protein through the color change from blue to purple, whereas the curdy white appearance acquired through the vanillin test asserted the presence of lipids. The protein & the carbohydrate concentration were evaluated in the wheat fermented for 48 hours. Microbes were analyzed through microbial activity grown at each hour of fermentation [8]. The wheat flour, fermented for 48 hours, showed extensive colony formation compared with the wheat fermented for 24 and 72 hours. Counting the colonies was done by multiplying the number of colonies present in each grid box with the total number of grid boxes on the Petri plate. It was multiplied, the colonies were counted, and comparison tests were performed for the type 1 and 2 whole wheat bread. The whole wheat bread made with lactobacillus strains (*Lactobacillus acidophilus* and *Lactobacillus rhamnosus*) was Type 1, and the bread made without the strain was type 2; their nutrients were also compared [27,28]. The nutrients were estimated by comparing both types. Type-1 bread had a high protein and carbohydrate content, whereas the lipid–fat content was higher on the bread made without adding lactobacillus strains (type2). Tests were conducted to prove a rise in folate and selenium levels as wheat is considerably rich in vitamins and minerals. The standard curve for folic acid – vitamin B9 was utilized to compare folic acid with and without LAB. 1.86 was the standard peak obtained here, then used for comparison. In the curve of folic acid without the lab, the peak was compared with the standard curve and analyzed. An approximated 72.6% was found to be the percentage of the area and 78.2%, the percentage of height. The retention time was found to be 1.100. In the curve of the folic acid with lab (*Lactobacillus acidophilus* and *Lactobacillus rhamnosus*), the peak was compared with the standard curve and analyzed. The percentage of the area was an estimated 78% and 84.5%, the percentage of height. The retention time was found to be 1.110. It was found that the one with the *Lactobacillus* spp. had a larger percentage of area and height while comparing both peaks from the graph and also possessed a higher retention time. This proved that the folic acid - vitamin B9 content was higher on the one containing lactobacillus spp. It was also enriched with folate content, an essential vitamin. It was then tested for the mineral selenium. They were increased in with lab with a quantity of 0.5022mg and found to be decreased in without lab without a quantity of 0.099mg. Increased selenium levels were found in the wheat bread made with lactobacillus spp. proving antioxidant activity. The presence of antioxidant properties was tested using FRAP assay. Compared with the standard

(ascorbic), the antioxidant activity was observed higher on the sample made from *Lactobacillus* spp. [16]. The change of color from yellow to blue indicated the presence of antioxidant activity. The wheat bread made with and without using *Lactobacillus* strains was tested and evaluated for its characteristics. Qualified 7 evaluators were employed to carry out the sensory analyses. Attributes such as appearance, texture, taste, aroma, and overall acceptability were evaluated out of 100. The evaluation results proved that whole wheat bread made with lactobacillus spp. possessed better attributes when compared with the bread made without *Lactobacillus* spp. The wheat bread with *Lactobacillus* strain was also preserved for a longer time, resisting contamination, while the bread made without *Lactobacillus* species got infested with fungi within 3-4 days.

3.2. Discussion.

In the current study, *Lactobacillus* strains and *Saccharomyces cerevisiae* were used to ferment wheat to manufacture bread. It was observed that the protein, carbohydrate, and lipid levels had elevated, and also, a soft texture and aroma were present due to the enhancement of essential vitamins and minerals. It possesses several health benefits. Various researchers conducted similar studies in which the results were observed and juxtaposed with the present study. One such researcher proved that "*S. cerevisiae* consumes lactic acid in a dough environment, consequently slowing down the resultant acidification of the lactic acid production by LAB, in turn allowing extended growth of LAB in a sourdough consortium [9]". This study yielded similar results. The lactic acid bacteria – *Lactobacillus* was consumed by yeast (*Saccharomyces cerevisiae*), reducing the sourness that arises when combining it with flour. The taste, texture, and nutrition were noted to have enhanced due to the interaction between lactic acid bacteria and yeast, attracting customers for its taste and possessing enriched nutrients such as protein, carbohydrates, and lipids. It was then compared with the samples fermented for 24, 48, and 72 hours. "MRS agar plates positioned on top of dough plates displayed serial dilutions on a 24 hours culture. Upon completion of growth, the colonies in the appropriate dilutions (10–50 colonies in 10 µl) were photographed and evaluated" [29]. These colonies were also counted in a similar manner. The colonies obtained in 48 hours were more extensive compared to 24 and 72 hours. Moreover, the nutrient levels (proteins, carbohydrates, lipids) were higher in 48 hours. The usage of the *Lactobacillus rhamnosus* and *Lactobacillus acidophilus* provided several health benefits and were helpful in the enrichment of vitamins, minerals, and numerous essential nutrients. Investigations were carried out to add other well-known probiotic strains viz *L. rhamnosus* to bread products. In a similar study, utilizing *Lactobacillus rhamnosus* displayed more satisfying results due to its prominence. The *Lactobacillus rhamnosus* maintains its viability in the gastrointestinal tract and possesses powerful adhesive properties. Consequently, it aids in strengthening the overall immune function of the human body. It is possible to treat urinary tract infections with the help of these strains, and it is also proven to be highly beneficial for the liver. It protects the body against alcohol-induced liver injuries. It has also been proven to reduce gastrointestinal tract disorders in human beings. It protects the body from pathogenic diseases by fighting fungi and viruses. "*Lactobacillus* strains such as *L. rhamnosus* are associated with the reduction of atopic eczema, otherwise known as dermatitis" [30,31]. This study utilizes *Lactobacillus rhamnosus*, which is popularly known for preventing the development of eczema and being beneficial to the skin. It possesses anti-obesity and anti-inflammatory properties and has also proved to be helpful during the stages of pregnancy. Selenium was found in abundance, a principal mineral

for pregnant individuals in preventing NTDs (neural tube defects) in their infants. One of the authors has stated that "the addition of folic acid to flour has averted a significant number of NTDs in infants. Research trials have proven that the ability to reduce the incidence of NTDs increased by 72% when supplementing the pregnant individual with folic acid" [32]. Folic acid was enhanced in the current study and would help prevent NTDs through regular intake. The distinctive behaviors of the microorganisms present in the two discrete lactic fermentation processes were studied. The quantity of selenium added and its concentration in the enriched sample were evaluated. In a similar manner, it was deduced that the addition of lactobacillus species enhanced the concentration of selenium. Wheat flour is generally preferred as it possesses high selenium content. When fermented and fortified with the aid of lactobacillus species, it heightens the mineral content even more, procuring high selenium levels, which could prove essential. The bread and wheat flour containing elevated selenium content was recorded [33]. A similar method was followed in the current study. Using yeast and lactobacillus species to make bread and whole wheat flour proliferated the selenium content. Comparisons were executed, including and excluding lactobacillus species. The bread made using lactobacillus species and fermented wheat flour improved the selenium concentration. The selenium was almost double the quantity when comparing the bread made with lactobacillus species to the bread made without the *Lactobacillus* spp. [34-36]. The wheat dough prepared in the presence of LLactobacillus exuded more characteristic changes than the dough made without the *Lactobacillus* spp. A researcher proved that "the usage of sourdoughs improved the overall characteristics of bread. Furthermore, utilizing a specific LAB strain while manufacturing sourdough bread may delay the onset of firmness and staling" [37,38]. Another observation made through this study was that the usage of lactobacillus species (*Lactobacillus acidophilus* and *Lactobacillus rhamnosus*) prevented staling. The bread made with lactobacillus species was found to have not decayed even after 10 days. Fungal infections were not noticeable, and it even gave off a fresh aroma and taste for a long time, while the bread was made without the *Lactobacillus* spp. decayed too soon and gave off an unpleasant odor. A separate author has stated that lactobacilli are associated with food production because of their preservative action owing to acidification and enhancement of flavor, texture, and nutrition [39-41]. The current study also observed the texture and flavor to have heightened. The bread made in the presence of LLactobacillus possessed more flavors and enriched nutrients.

4. Conclusions

In conclusion, whole wheat bread made from lactobacillus strains (*Lactobacillus acidophilus* and *Lactobacillus rhamnosus*) resulted in high levels of proteins and carbohydrates. The fermented wheat bread was enriched in proteins and carbohydrates when coupled with the *Lactobacillus* spp. It was observed that the vitamin levels were high, and the folate vitamin was enhanced, an essential vitamin in the human diet. The minerals calcium, selenium, and iron also increased. Selenium is a rare mineral that possesses several health benefits and antioxidant properties. The whole wheat bread was noted to have been enriched with essential vitamins and minerals that have proved highly beneficial to humankind. The slice of bread carries numerous vital nutrients which would be sufficient on a daily intake basis. Deficiency diseases could be prevented by regularly taking these foods, and due to their antioxidant property, the fight against the aging process could also seem less challenging. The risk of cancer and cardiovascular diseases could also be eradicated. The body's immunity can

be boosted, and the RBC count will increase, which would help enhance hemoglobin levels. It helps to maintain the immune and digestive systems and also aids in burning calories. Unlike normal bread, this whole wheat bread is made from the bacterial probiotic *Lactobacillus* spp. contains no preservatives and has been known to stay preserved for a longer time without stalling. This whole wheat bread made with Lactobacillus will be successful in helping the human population as it makes frequently consumed food more nutritious without demanding the consumers to change their food habits.

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Conflicts of Interest

The authors declare no conflict of interest.

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