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Evaluation of Sensory and Physicochemical Characteristics of Vitamin B_{12} Enriched Whole-Meal Sourdough Bread Fermented with *Propionibacterium freudenreichii*

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Abstract: The sustainable production of vegan or vegetarian food rich in vitamin B_{12} is a challenge. *Propionibacterium freudenreichii* fermentation has been identified as an effective method for the enhancement of vitamin B_{12} content in foods. However, limited studies have been conducted on the co-fermentation of *P. freudenreichii* with other bacteria. This study investigated the co-fermentation of *P. freudenreichii* with Lactic acid Bacteria (LAB) and its effects on the sensory characteristics of whole-meal sourdough bread (WMSB) in comparison to WMSB produced with LAB alone. The effects of *P. freudenreichii* co-fermentation on WMSB vitamin B_{12} content were also evaluated. Results indicated that *P. freudenreichii* co-fermentation with LAB significantly reduced ($p < 0.05$) crumb hardness when compared with WMSB produced with only LAB (4532 ± 176 g and 5313 ± 846 g respectively). A significantly higher adhesiveness ($p < 0.05$) was also observed due to the presence of *P. freudenreichii*. Triangle testing showed that perceptible differences were observed between the two WMSB types investigated in this study ($p < 0.05$). Qualitative data from focus group testing indicated that WMSB produced with co-fermentation has a more homogeneous texture. However, improvement in aroma, texture, and taste was possible in both bread types. The co-fermentation of *P. freudenreichii* with Lactic acid Bacteria (LAB) was successful in producing bread with enriched vitamin B_{12} levels (ranging from 0.89 to 1.44 µg 100 g^{-1}). Overall, the co-fermentation of *P. freudenreichii* presents an opportunity to improve the nutritional value of WMSB.

Keywords: *Propionibacterium freudenreichii*; vitamin B_{12}; fermented food; consumer acceptance

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1. Introduction

Vitamin B_{12} (cobalamin) is an essential micronutrient that must be sourced from the human diet. It is required for the formation of red blood cells, DNA synthesis, and plays a crucial role in the development of the human nervous system [1]. Vitamin B_{12} deficiencies can lead to various complications, with common associated health issues including cardiovascular disease. Elevated levels of homocysteine, which is linked to heart disease and stroke, can result from vitamin B_{12} deficiency [2]. The recommended dietary intake of vitamin B_{12} for men and women aged 14 and over is 2.4 µg/day, whereas for the elderly and women during pregnancy, this requirement increases to 2.6–2.8 µg/day [3]. Impaired cognitive function is also associated with Alzheimer's, dementia, and overall cognitive decline. Therefore, it has been suggested that the elderly consume supplemented or fortified foods to ensure adequate absorption in the gastrointestinal tract [4]. Initially, pernicious anemia was considered the primary disease associated with vitamin B_{12} deficiencies, and it was thought to be caused solely by the inability to properly absorb the vitamin through our dietary intake. However, subsequent studies have revealed other factors that affect the vitamin B_{12} absorption, including digestive disorders, insufficient stomach acid, the autoimmune destruction of gastric parietal cells, and intrinsic factor [1]. Vitamin B_{12} deficiency has been associated with the dysregulation of interleukin-6 (IL-6) levels and alterations in folic acid status [5,6]. Vitamin B_{12} deficiencies are experienced by 15% of the global population [3], especially children, women of reproductive age, and the elderly. The elderly are most at risk due to a high prevalence of atrophic gastritis [4]. Atrophic gastritis can result in a decreased ability to absorb protein bound vitamin B_{12} and bacterial overgrowth in the stomach. Typically, people obtain vitamin B_{12} from animal-derived foods [1]. However, the sustainability concerns surrounding vitamin B_{12} arise from the environmental impacts associated with industrial-scale animal farming, which include deforestation, water pollution, greenhouse gas emissions, and biodiversity loss [7]. Nonetheless, there are sustainable alternatives available. These alternatives involve cultivating vitamin B_{12}-producing bacteria through fermentation processes and consuming plant-based foods fortified with vitamin B_{12}. These options provide environmentally friendly means of obtaining vitamin B_{12}.

Bread, which is made from a combination of flour, water, and other ingredients, has been a staple of the human diet for thirty thousand years and has been adapted in many forms by different cultural groups [8]. Sourdough bread is distinct from other varieties because it is created by naturally fermenting a mixture of flour and water using naturally occurring yeasts and bacteria instead of commercial yeast. The natural fermentation process that sourdough bread undergoes gives it an exceptional taste and texture [9]. In 2020, the sourdough bread market reached a value of USD 3.4 billion globally, and it is estimated to grow at a CAGR of 5.1% from 2021 to 2028 [10]. The global need for sourdough bread is on the rise as it is recognized as a nutritious source of carbohydrates, crucial nutrients, dietary fiber, and phytochemicals. Moreover, sourdough baked products have a prolonged shelf life due to the production of antifungal compounds, bacteriocins, and higher acidity, making them superior to items fermented solely by yeast [10]. A higher sensory quality is achieved by increasing elasticity and the concentration of volatiles, as well as a greater nutritional value due to reducing antinutritional compounds like phytase enzymes [11]. The fermentation process used to create sourdough involves preparing a starter containing flour, water, and lactic acid bacteria. This process heavily influences the nutritional composition, texture, and sensory qualities of the final product. By choosing to use whole-meal flour for both the starter and breadmaking, the bacteria have more nutrients to grow and feed on, which increases the efficiency of fermentation and the resulting byproducts [12,13]. Additionally, whole-meal sourdough bread, made from whole grains with retained bran and germ layers, offers a higher nutritional content and a distinct flavor profile compared to refined wheat dough bread. Considering that sourdough bread enriched with certain bacterial strains can result in improved sensory characteristics, and the recognition that sourdough bread fortified with vitamin B_{12} offers nutritional advantages, our study aims to explore the feasibility of developing a sourdough bread that not only provides nutritional benefits but also delivers enhanced sensory qualities.

Propionibacterium freudenreichii (*P. freudenreichii*) is a gram-positive probiotic bacterium that has been studied for its potential health benefits, including improving digestive health by supporting the growth of beneficial gut bacteria, supporting the immune system, reducing inflammation, reducing the risk of colon cancer, and enhancing vitamin B_{12} status [14,15]. Research has found that it can suppress *Helicobacter pylori* activity, which can ameliorate the infection of atrophic gastritis [16]. Because of its considerable potentials such as its antifungal and antimicrobial characteristics, as well as its knack for improving flavor and aroma attributes, *P. freudenreichii* has been widely utilized as a ripening culture in the production of Swiss-style cheeses and has been found to provide advantages in bread making [17,18]. *P. freudenreichii* is also regarded as the most optimal bacterium for industrial-scale production of vitamin B_{12} because of its capability to synthesize propionic acid (PAB) in the presence of lactic acid. Co-fermentation of Lactic Acid Bacteria (LAB) and *P. freudenreichii* has been shown to be effective in producing vitamin B_{12} [19], and has the potential to improve the shelf-life of sourdough bread via acid production. Moreover, the co-fermentation of LAB and *P. freudenreichii* can elicit the synergistic effects of exopolysaccharide and acid production, resulting in improved antifungal and anti-stalling properties, and texture-building abilities in wheat bread [20]. Our study introduced an innovative approach by utilizing whole-meal sourdough bread instead of wheatmeal in the enrichment of vitamin B_{12}. This choice provides a favorable environment for *P. freudenreichii* to thrive and produce vitamin B_{12}, aided by the additional nutrients and substrates present in the bran and germ.

The objective of this study was to determine the feasibility of using *P. freudenreichii* for sourdough fermentation to create a vitamin B_{12}-fortified bread, and to assess if consumers would find it agreeable based on sensory criteria. The assessment primarily targeted the texture, pH, and acidity of *P. freudenreichii* sourdough when compared to traditional LAB sourdough. The expectation was that *P. freudenreichii* sourdough would demonstrate superior texture quality and lower levels of pH and titratable acidity than the control group. Several sensory and instrumental analyses were administered to evaluate and analyze these factors, as well as additional variables.

2. Materials and Methods

2.1. Sourdough Bread Making

Sourdough starter (SD) was prepared in a beaker by combining 1.2 g of *Lactobacillus lactis* (Cheese Culture, Mad Millie, Auckland, New Zealand) with whole meal flour (Edmonds, Auckland, New Zealand) and tap water in a ratio of (1.2:1, *w*/*v*), and then allowed to naturally ferment at 37 °C for 24 h in a Sanyo incubator (MIR-153, Osaka, Japan). The *P. freudenreichii* sourdough starter (PSD) was prepared by mixing 0.36 g of *P. freudenreichii* (Propionic Culture 0.5u, The Urban Cheese Company, Christchurch, New Zealand) with 1.2 g of *Lactobacillus lactis* (Cheese Culture, Mad Millie, Auckland, New Zealand) in a small amount of water for pre-activation and then combined with 240 g whole meal flour and 204 g tap water to make a batter. The mixture was fermented at 37 °C for 24 h in the incubator.

The fermented sourdough starter (SD or PSD) was weighed 400 g and was mixed with 240 g whole meal flour, 8 g sugar (Chelsea, Auckland, New Zealand), 7.6 g canola oil (Simply, Auckland, New Zealand), 6 g table salt (Pams, Auckland, New Zealand) and 146 g tap water. Bread doughs were mixed in a mixer equipped with flat beater (Delta Food Mixer, Auckland, New Zealand) for 1 min at speed 1 and then for 4 min at speed 4, altogether 5 min followed by resting for 1 h in a 37 °C incubator. Bread was placed into a pre-heated oven (Moffat oven E32M, Christchurch, New Zealand) and baked at 180 °C for 20 min to make sourdough bread (SDB) and *P. freudenreichii* sourdough bread (PSDB). After being taken out of the pans, the bread was allowed to cool on a rack until it reached room temperature. It was then refrigerated to assist in conducting instrumental and sensory evaluations, while additional samples were frozen in the freezer for mineral analysis.

2.2. Determination of Vitamin B_{12} Content

Vitamin B_{12} content before and after 3-day fermentation in PSDB was extracted following the method of the Association of Official Agricultural Chemists (AOAC 2014.02) as described by Giménez [21]. The dough (PSD) was allowed to ferment at room temperature for 3 days covered with a bowl. During the first two hours, the PSD was periodically subjected to the stretch and fold motion every 30 min. Over time, the PSD rose and developed bubbles. Once the dough had risen and become puffy, it was shaped after its third day of fermentation. The work surface was lightly dusted with flour, and the dough was placed on top of it. The sides of the dough were gathered towards the center and formed into a ball. The dough was then given a 20-min rest before being baked. The PSDB making methods were mentioned in Section 2.1. To obtain the vitamin B_{12} extract, PSDB samples were subjected to 0.4 M sodium acetate buffer (pH 4) along with 1 mL of a 1% sodium cyanide solution at a temperature of 100 °C for a duration of 30 min. Immunoaffinity columns (EASI-EXTRACT® VITAMIN B_{12} (LGE), R-Biopharm, P88, Darmstadt, Germany) were used for purifying and concentrating extracts. The high-performance liquid chromatography (HPLC) with C18 column (150 × 3.0 mm (ACE, Scot, UK) was used to analyze vitamin B_{12}

in cyanocobalamin for PSDB on Day 0 and Day 3, and UV detection at 361 nm. Calibrating the chromatographic system with a vitamin B_{12} working standard solution (2, 10, 20, 40, 60,100 ng. mL^{-1}) The flow rate was set at 0.25 mL/min, with an injection volume of 100 µL. The identification of vitamin B_{12} was confirmed by comparing the retention time and UV spectrum of the bread sample test solution with that of the vitamin B_{12} stock standard (100 µg. mL^{-1}).

2.3. Determination of Sourdough pH and Total Titratable Acidity (TTA)

The pH and acidity of both bread samples (SDB and PSDB) were tested using an automated potentiometric titration system (Metrohm 702 SM Titrino, Herisau, Switzerland) as previously described by Jekle et al. [22]. Samples were loaded in triplicate into the automated titration system for the determination of titratable acidity. A bread sample weighing 10 g was mixed with 90 mL of distilled water and then titrated with a 0.1 mol L^{-1} NaOH solution until a pH of 8.5 was reached. The volume of NaOH solution (mL) required in two separate measurements was combined to obtain the TTA.

2.4. Texture Analysis

Texture analysis of bread samples (SDB and PSDB) was conducted using a TA.XT plus Texture Analyzer from Stable Micro Systems equipped with a P/25 probe (25 mm) and 5 kg was calibration of force [23]. Three 2 × 2 cm cubes of each bread type were measured. Samples were placed directly under the probe to be pressed. In contact with the sample, the probe measured forces of insertion and withdrawal. The assessment of this characteristic involved the utilization of the following TA setting parameters: pre-test speed: 1.0 mm. s^{-1}, test speed: 1.7 mm. s^{-1}, post-test speed: 10.0 mm. s^{-1}, strain: 40%, trigger type: auto—5 g, data acquisition rate: 250 pps. Hardness quantifies the initial force used to deform the food object (g) and adhesiveness is the rate at which the bread comes away from the probe and is represented by the work of adhesion (g. s).

2.5. Loaf specific Volume Evaluation

Breads were weighted after cooking and the loaf specific volume was measured using the seed displacement method [24]. The volume was calculated according to Equation (1).

$$V_0 = \frac{V_1}{M_1} \tag{1}$$

V_0 = Loaf specific volume (m^3. kg^{-1}), V_1 = Sample volume (m^3), and M_1= sample weight (kg).

2.6. Color Evaluation

The lightness (L*), red (+a*) and yellow (+b*) colorimetric indices of bread crumb was measure using a Chroma Meter CR-400 Minolta (Konica Minolta, Inc., Tokyo, Japan). Triplicate 2 × 2 cm cube samples of both bread types (SDB and PSDB) were measured triplicate.

2.7. Bread Moisture Content Evaluation

Both SDB and PSDB samples were broken into small pieces, weighed (W_0), placed on a small cylinder metal tray, and kept in a 150 °C oven overnight. After drying, samples were weighted (W_1) and calculated the moisture content (%, db) by Equation (2).

$$Moisture\ content(\%, db) = \frac{W_0 - W_1}{W_0} \times 100 \tag{2}$$

W_0 = Sample weight before drying (g), and W_1 = Sample weight after drying (g).

2.8. Mineral Compositon Anaysis

Mineral composition of both bread types (SDB and PSDB) was analyzed. Samples were placed in the freezer upon receipt and then freeze dried for a period of at least 48 h at 0.5 mbar (chamber pressure). Temperature setting were at 25 °C under vacuum. (Model E.D. 5.3, Cuddon Ltd., Blenheim, New Zealand) After freeze-drying, 0.5 g samples were digested at 220 °C in a microwave digestor (CEM Corporation, Matthews, NC, USA) using 5 mL nitric acid and 2.5 mL hydrogen peroxide to dissolve the minerals [25]. Digested samples were then analyzed by calibrated Inductively Coupled Plasma Optical Emission Spectrophotometer (ICP-OES).

2.9. Sensory Evaluation

All human related sensory tests were approved by the Lincoln University Human Ethics Committee (Approval No: 2020-35). All participants involved in studies were over the age of 18 and claimed to be non-allergic to bread ingredients. Before participating in any testing all participants were required to sign a consent form.

2.9.1. Triangle Test

The triangle test is used as a discriminative method to gauge an overall difference between two products [26]. During this test, a total of 15 panelists were presented with three samples each, which were allocated with 3-digit random numbers. Among the three samples presented, two were identical and one was different. One *P. freudenreichii* sourdough bread (PSDB) sample and two sourdough bread (SDB) samples were given to panelists in a random manner. Panelists were instructed to taste samples from left to right, and then indicated the sample they thought was different.

2.9.2. Preference Consumer Test

A 9-point hedonic consumer test was conducted with a semi-trained panel of 20 panelists [25]. Panelists were trained on sight using two commercial sourdough loaves that varied in the amount of sourdough. Freya's German Whole Meal Sourdough, which contained 3% of rye sourdough, and Gluten Freedom Sweet Potato Sourdough, which contained 25% organic sourdough. The parameters we were testing for were appearance, aroma, taste, and texture. In the training stage, panelists were asked to take these factors into consideration while assessing the commercial sourdoughs. Bread samples were thawed and stored overnight in individual containers. Once panelists were ready, they were taken into the sensory testing room where individually they were given two numbered samples: 459 (SDB) and 672 (PSDB). Panelists were instructed to taste each sample and rate the appearance, aroma, taste, texture, and overall preference on a 9-point hedonic scale provided.

2.9.3. Focus Group Study

Focus group evaluations were run to explore the product development and understand consumer opinion regarding a product [27]. Groups consisted of six panelists respectively, who were recruited based on the criteria of having a bread-consuming habit by asking their bread consuming frequency, concerns with vitamin B_{12} enrichment food intake, and experience in sensory analysis. They ranged in age from 18 to 25 years old. Throughout the discussion, a trained moderator facilitated and monitored group interactions. The moderator explained sourdough bread's nutritional qualities and eventually its potential health benefits. The focus group was performed in a previously prepared meeting room on campus and lasted 1 h. Participants sat in a round table format so that participants could make their own decisions without interruption by potential positional leadership. Panelists were given the two commercial breads, Freya's German Whole Meal Sourdough Toast Bread; Gluten Freedom Sweet Potato Sourdough, to gain an idea of sourdough currently in the market. The most frequently used descriptions for the bread were considered significant and then selected as a reference for sourdough bread samples. Panelists then tasted the

SDB and PSDB, respectively. Panelists' descriptions were collected in terms of appearance, aroma, taste, and texture, and wording that received more than half of the votes were considered significant descriptions.

2.10. Statistical Analysis

The data obtained from all determinations were analyzed using a *t*-test. Statistic significant difference ($p < 0.05$) between samples were calculated using Mini Tab 19 software (Minitab LLC, PA, USA) and Excel 2016 were used to manage and visual data (Microsoft Co., Redmond, WA, USA).

3. Results

3.1. Determination of Vitamin B_{12} Concentration

A two-sample t-test was performed to compare the vitamin B_{12} concentration of PSDB on Day 0 and Day 3. There was a significant difference in PSDB vitamin B_{12} concentration between Day 0 (0.89 ± 0.17) and Day 3 (1.44 ± 0.07) ($p < 0.05$). In the PSDB, relatively lower level of vitamin B_{12} (0.77–1.01 µg.100 g^{-1}) was detected on day 0, while after 3 days fermentation, PSDB were confirmed to contain 1.39–1.49 µg.100 g^{-1} vitamin B_{12} as expected (Figure 1).

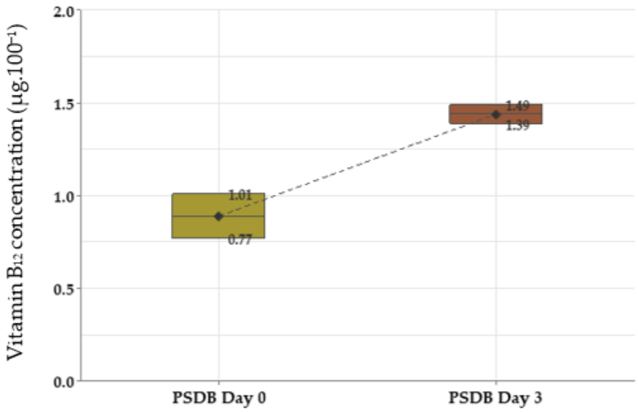

Figure 1. Boxplot of variations in Vitamin B_{12} Levels Throughout a 3-Day Fermentation Period.

3.2. Bread Instrumental Analysis and Detection of Mineral Content

It can be seen in Figure 2 that the difference of appearance and inner structure of SDB and PSDB. Although the difference is not ideal, it remains within the acceptable range.

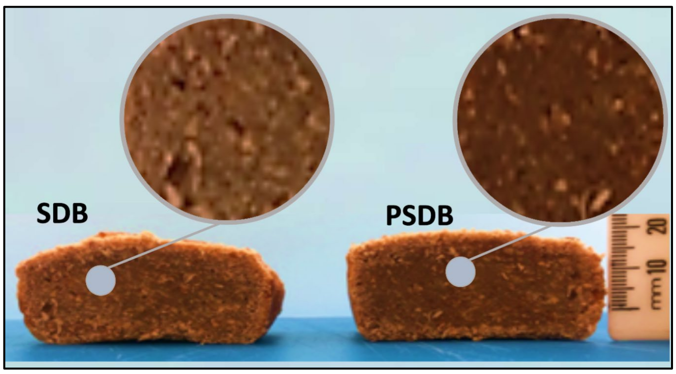

Figure 2. The Cross-Sectional Imaging of Sourdough Bread (SDB) and *P. freudenreichii* Sourdough Bread (PSDB).

Sourdough bread with the addition of *P. freudenreichii* (PSDB) had a darker color and a more homogenized inner structure compared with bread without *P. freudenreichii* (SDB) (Table 1). PSDB has a significantly lower specific volume ($p = 0.007$) than SDB, as well as a softer texture with increased adhesiveness ($p = 0.006$). SDB had the initial pH and TTA value of 5.80 and 0.54 mL (respectively), whereas *P. freudenreichii* sourdough bread (PSDB) had a pH of 5.7 and a TTA of 0.51 mL. There are negligible variations in acidity and pH value between SDB and PSDB.

Table 1. Comparative Instrumental Analysis of Sourdough Bread (SDB) and *P. freudenreichii* Sourdough Bread (PSDB).

Sample	Specific Volume ($m^3.kg^{-1}$)	Moisture Content (%)	Hardness (g)	Adhesiveness (g.s)	pH	Acidity (mL)	Color		
							L *	a *	b *
SDB	1.34 ± 0.00 [a]	44.6 ± 0.6	5313 ± 846 [a]	-119 ± 63.0 [a]	5.76 ± 0.00	0.54 ± 0.00	39.0 ± 0.60	4.2 ± 0.10	9.0 ± 0.20
PSDB	1.27 ± 0.00	48.0 ± 6.6	4532 ±176	-338 ± 279	5.72 ± 0.10	0.52 ± 0.00	40.8 ± 3.50	4.5 ± 0.30	9.3 ± 0.90

* All values are means ± standard deviations (n = 3). Values in the same column followed by [a] are significantly higher according to *t*-tests ($p < 0.05$).

Among two types of sourdough bread investigated in this study, the mineral content was found no significant difference ($p > 0.05$) in Table 2. As expected, a similar mineral profile was observed in both SDB and PSDB. The supplement of *P. freudenreichii* appeared to have no effect on the sourdough bread minerals constituents.

Table 2. Mineral profile of sourdough bread (SDB) and *P. freudenreichii* sourdough bread (PSDB).

Sample mg.kg^{-1}	Al	Ca	Cd	Cu	Fe	K
SDB	2.9 ± 0.60	307.6 ± 30.0	0.0235 ± 0.0	3.38 ± 0.20	32.2 ± 6.10	2638 ± 89.0
PSDB	2.7 ± 0.10	318.17 ± 2.80	0.0246 ± 0.0	3.38 ± 0.06	30.8 ± 3.00	2729 ± 20.0
	Mg	**Mn**	**Na**	**P**	**S**	**Zn**
SDB	723.3 ± 634	26.3 ± 1.50	3206 ± 342	2198 ± 123	1107 ± 31.0	19.2 ± 3.70
PSDB	688 ± 37.0	27.0 ± 0.80	3501 ± 304	2195 ± 23	1149 ± 34.0	18.3 ± 0.60

All values are means ± standard deviations (n = 3).

3.3. Sensory Characteristic Analysis

The results of the triangle test indicate that there is a noticeable difference between the two product samples being compared. Out of the 15 panelists, 9 were able to correctly identify the odd sample, indicating that there exists a perceivable difference between SDB and PSDB. This suggests that the sensory characteristics of the two samples are different enough for some individuals to perceive a noticeable difference.

To further investigate the sensory attributes responsible for this difference, 9-Point Hedonic Scale Analysis of Consumer Acceptability was conducted, and the results are presented in Figure 3. SDB and PSDB were assessed as similar in flavor, appearance and overall preference. However, the addition of *P. freudenreichii* to bread formulas mitigated the bread aroma, whereas improving the texture of sourdough bread.

Focus group session was conducted to collect consumer opinions about the vitamin B_{12}-enhanced sourdough bread mainly regarding the possible impact on bread sensory profile. The presence of *P. freudenreichii* in bread formula strongly affected bread texture description. Both SDB and PSDB exhibit a dense surface texture that emits a yeasty and palatable aroma (Figure 4). However, SDB had a drier and grainier appearance accompanied by a whole-meal scent and a comparatively bland flavor profile. In contrast, PSDB exudes a sour fragrance and presents an overtly homogenous texture, characterized by a salty and sour taste sensation.

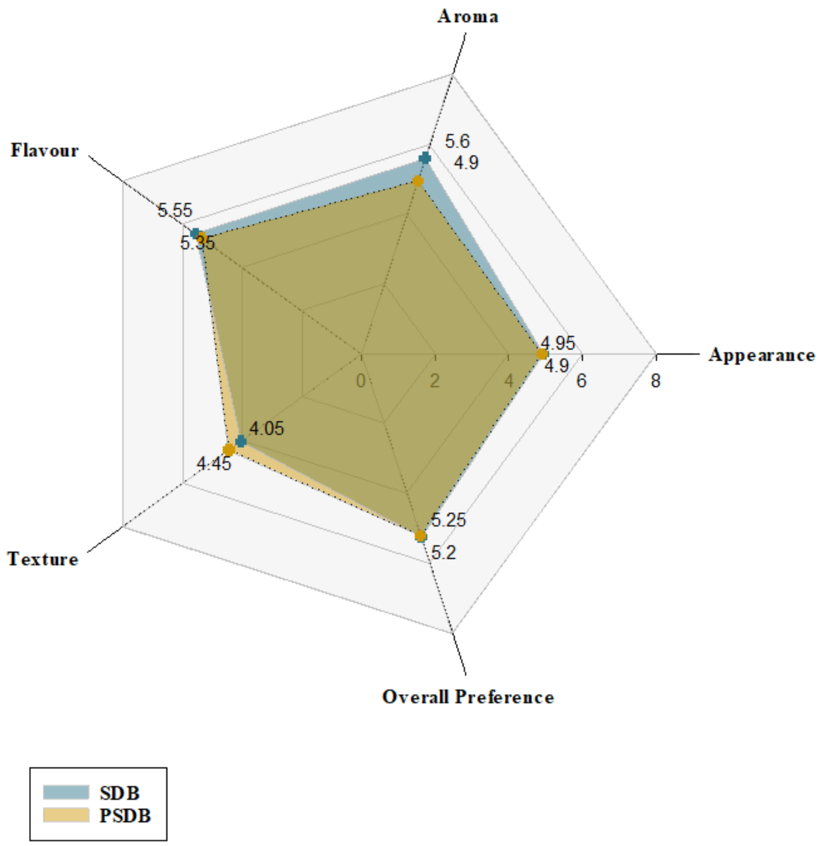

Figure 3. 9-Point Hedonic Scale Analysis of Consumer Acceptability for Sourdough Bread (SDB) and *P. freudenreichii* Sourdough Bread (PSDB).

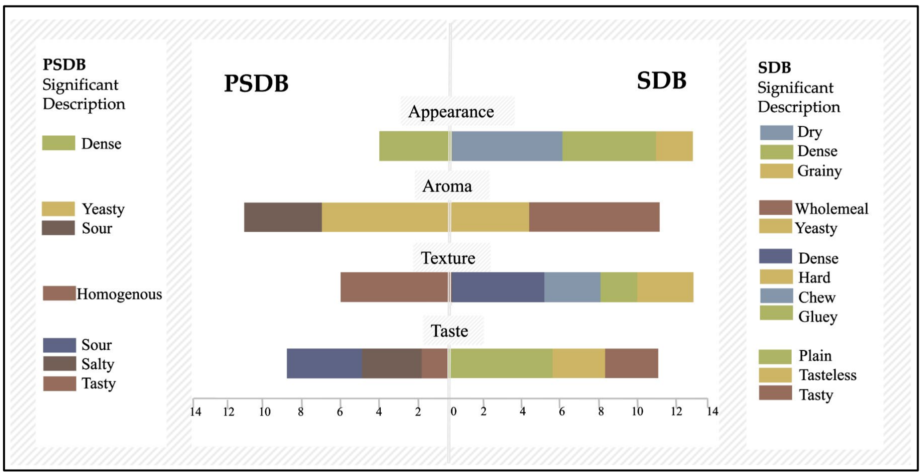

Figure 4. Classification of Bread Characteristics into Four Categories (Appearance, Aroma, Texture, and Taste) Based on Focus Group Study. Analysis of Significant Attributes (n > 3) Affecting Results.

4. Discussion

4.1. Detection of Vitamin B_{12}

The incorporation of *P. freudenreichii* in bread dough has led to a notable enhancement in its nutritional composition. In this study, the concentration of vitamin B_{12} increased almost twofold following a 3-day fermentation period (Figure 1). Previous research has demonstrated the ability of *P. freudenreichii* to produce vitamin B_{12} with high efficiency and noteworthy results [28–31]. Piao and others demonstrated that *P. freudenreichii* can produce vitamin B_{12}, and the introduction of certain cob genes, as well as successful multigene expression, can significantly increase its production. Piao and others' findings show a 1.7- to 1.9-fold increase in vitamin B_{12} production when cobA, cbiLF, or cbiEGH genes were introduced than the microorganism without any clone genes, and a 2.2-fold increase when a combination of endogenous and exogenous genes was expressed [32]. These findings suggest that *P. freudenreichii* possesses the enzymatic machinery necessary to biosynthesize vitamin B_{12}. The capacity for vitamin B_{12} production has been shown to be particularly effective in the context of sourdough bread fermentation, where the presence of *P. freudenreichii* can lead to a significant increase in vitamin B_{12} content. Xie and others revealed a noteworthy enhancement in the vitamin B_{12} content through the fermentation process of wheat bran using *P. freudenreichii* and LAB. The levels of vitamin B_{12} witnessed a substantial rise from 40 ng/g dw on Day 0 to 332 ng/g dw on Day 3 [19]. Moreover, the vitamin B_{12} content in fermented foods varies depending on the type of food and production method. Kimchi (1.78 μg.100 g^{-1}) and kefir (1.06 μg.100 g^{-1}) tend to contain higher levels of vitamin B_{12}, while miso (0.15–0.25 μg.100 g^{-1}), sauerkraut (0.5 μg.100 g^{-1}), and tempeh (0.06–0.11μg.100 g^{-1}) generally contain lower amounts [33–36].

The results of this study provide evidence to suggest that *P. freudenreichii* may be a promising candidate for enhancing the nutritional quality of fermented foods, without significant effects on their mineral composition (Table 2). This highlights the potential of *P. freudenreichii* as a tool for improving the health benefits of food products that undergo fermentation processes. Further investigation is required to fully elucidate the mechanisms underlying *P. freudenreichii*-mediated vitamin B_{12} biosynthesis, as well as to optimize the use of this bacterium in food production for maximum nutritional benefit. Consequently, the PSDB was hypothesized to be regarded as a potential candidate for a healthy food option, provided that its texture and sensory properties remain unaffected. Therefore, instrumental and sensory analyses were carried out to evaluate PSDB characteristics in comparison to SDB.

4.2. Textural Properties

The texture of bread is an essential aspect that influences its overall quality. In the case of PSDB, lower crumb hardness and higher adhesiveness are observed (Table 1), indicating a softer and juicier crumb that gives a more homogeneous bite compared to SDB (Figure 4). Results from focus groups and 9-pointed hedonic consumer tests support PSDB's homogeneous texture and the high score of textural quality (Figures 3 and 4).

The soft texture of PSDB can be attributed to its moisture distribution, as the moisture levels decrease as crumb rigidity increases. One hypothesis to explain the observed differences in bread texture and moisture content could involve the distribution of moisture within the bread, as well as the rate of stalling. It has been suggested that the presence of *P. freudenreichii* in bread may confer an anti-stalling effect, attributed to the production of exopolysaccharides during the co-fermentation of lactic acid bacteria and propionic acid bacteria [19,37]. This may slow down the staling process and help the bread to retain more moisture over time. However, it is possible that some degree of stalling occurred during the preparation of bread samples for sensory testing, as they were thawed and stored overnight in individual containers. Previous studies have reported a decrease in crumb rigidity over time with storage and storage with crust has been shown to result in increased firmness [38]. Thus, any differences in moisture distribution within the bread may have been amplified following the storage period, leading to perceived differences in crumb softness.

Recent studies demonstrated that the co-fermentation of Lactic Acid Bacteria (LAB) and Propionic Acid Bacteria (PAB) in synergy with exopolysaccharides can provide a natural alternative to the use of propionic acid as a preservative [19]. Propionic acid is a naturally occurring food-grade organic acid commonly utilized as a preservative by the baking industry to prevent microbial overgrowth. However, its use has been associated with negative perceptions among consumers, who consider it unnatural and believe that it can adversely affect the rheology and sensory characteristics of dough. This process enhances the textural quality of bread while extending its shelf life and providing organic acids, including propionic acid, that inhibits fungal growth [39]. Meanwhile, our study also revealed the efficacy of co-fermentation with LAB and PAB as an alternative to conventional preservatives in the bakery industry. These findings suggest that co-fermentation with LAB and PAB can offer a natural and effective means to prevent microbial overgrowth in bread without compromising its quality, serving as an attractive option for consumers who seek natural and minimally processed products. Further research is needed to fully elucidate the mechanisms underlying the anti-stalling effect of *P. freudenreichii*, and to determine the optimal conditions for its use in bread production to maximize its beneficial properties.

The distinct textural variances observed between SDB and PSDB may also be attributed to the variation in the number of exopolysaccharides (EPS) generated during the fermentation. Studies have shown that *P. freudenreichii* is capable of producing higher levels of EPS compared to other lactic acid bacteria commonly used in sourdough fermentation [40,41]. The LAB and PAB co-fermentation in PSDB has also shown synergistic effects, leading to improved functionality [19]. The blending of EPS and propionic acid by-products produced during co-fermentation has the potential to enhance the textural quality of bread. Previous studies have demonstrated that the EPS produced via co-fermentation exhibits anti-stalling effects, which are similar to a commercial bread improver [20,42,43]. Likewise, EPS has also been shown to increase the water-holding capacity of the dough and lead to a more uniform crumb structure, which can improve the overall quality of bread [44–46]. Therefore, it is plausible that the production of EPS by *P. freudenreichii* in the PSDB may have contributed to its more homogeneous texture compared to SDB.

4.3. Sensory Analysis

Sensory testing is a widely used method to assess consumer acceptance [47]. Consumers are highly sensitive to the texture, flavor, and aroma of food products [48,49]. Manufacturers identify the specific sensory attributes to make educated choices regarding the improvement of their products and advertising strategies [50–52]. The results of the triangle test demonstrate that there is a significant difference between the two bread samples, as evidenced by the ability of 9 out of 15 panelists to accurately distinguish the odd sample. The outcome of the triangle test can be influenced by various factors, such as the intensity of the sensory attributes being evaluated and the familiarity of the panelists with the product [26,53]. Additionally, human perception of differences in food products is well-established and influenced by several factors, including individual taste perception and prior exposure to the food product [54,55]. Considering the limitations of the triangle test, we proceeded to conduct a 9-point hedonic consumer test to discern the discriminability of the samples with regard to various sensory attributes.

The 9-point hedonic consumer test conducted on SDB and PSDB samples in this study revealed notable differences in aroma and texture (Figure 3). The texture differences can be attributed to the factors mentioned above and there exist several variables that can influence the aroma profile, such as the presence of diverse microorganisms, co-fermentation process, and individual variability in taste and preference. Hu and others [56] have established that various strains of lactic acid bacteria and yeast can generate different aroma compounds in bread. Thus, the incorporation of *P. freudenreichii* could have contributed to the observed differences in aroma. It was illustrated from the focus group session in Figure 4 that the presence of *P. freudenreichii* in bread formula affected its sensory attributes, especially texture, aroma and taste. The dense texture of both SDB and PSDB can be attributed to the presence of lactic acid bacteria in the sourdough fermentation process, which can improve the gluten network and create a firmer and more elastic crumb structure [57–59]. The different aroma and taste profiles observed between the two samples could be explained by the production of various volatile compounds during the fermentation process [60]. For example, organic acids like acetic acid and lactic acid can contribute to a sour flavor profile in bread [61,62]. Acetic acid has a pungent and vinegar-like aroma, while lactic acid has a milder and creamier aroma [63]. Meanwhile, diacetyl, produced by lactic acid bacteria during fermentation, has a buttery or creamy aroma and flavor [64]. The concentration of diacetyl in sourdough bread can vary depending on the fermentation conditions and the microorganisms present in the sourdough starter [65], which can greatly influence the overall sensory profile of the bread.

P. freudenreichii is known to produce various volatile compounds during fermentation that can affect the aroma and flavor of bread, including short-chain fatty acids such as acetic acid, propionic acid, and butyric acid, as well as other compounds like diacetyl, acetoin, and 2,3-pentanedione [18,66–68]. The production of these volatile compounds can be influenced by various factors, such as the concentration of *P. freudenreichii* in the bread dough, the fermentation time and temperature, and the composition of the bread dough [69]. Different strains of *P. freudenreichii* can also produce varying concentrations and types of volatile compounds, which can lead to different sensory attributes in the final product [18,70,71]. For instance, Wang et al. [66] found that adding *P. freudenreichii* to bread dough resulted in an increase in acetic and propionic acid concentrations, as well as an increase in the intensity of sour and cheesy aroma notes in the final bread product. However, the results indicated that when *P. freudenreichii* was added, there were no significant variations observed in the pH value (pH 5.72) compared to SDB (pH 5.76), as presented in Table 1. Additionally, the acidity test revealed no significant distinction between SDB (0.54 mL) and PSDB (0.52 mL). Surprisingly, despite having similar acidity and pH value, the PSDB had a distinctly sour aroma and taste compared to SDB, as noted by the focus group in Figure 4. In the study conducted by Amerine et al. [72], the participants were able to detect differences of 0.05 pH units among four different types of acids. Even when the titratable acidity was adjusted by increments of 0.02, 0.03, 0.04, and 0.05 mL, the panel members were still able to rank the samples based on their level of sourness. This observation suggests that it is plausible for consumers to discern slight variations in sourness between SDB and PSDB, despite the absence of statistically significant differences in pH value and acidity. This finding could be considered as one of the potential explanations for the ability of consumers to perceive these subtle differences.

One potential explanation for the sourness disparity could be the variation in flavor compounds or volatile organic compounds (VOCs) found in the PSDB and SDB [73]. These compounds could contribute to the perception of sourness and impact the overall flavor characteristics. It is conceivable that the inclusion of *P. freudenreichii* or other ingredients in the PSDB formulation led to the production of specific flavor compounds that intensified the sour taste and aroma [68]. Moreover, sensory perception is a subjective experience that can be influenced by individual variations in taste sensitivity. Even if the pH and acidity levels are identical, individuals may interpret and describe flavors in distinct ways due to their unique taste preferences and sensitivities. Another study found that *P. freudenreichii* can produce diacetyl and acetoin during fermentation, which can contribute to a buttery flavor and aroma in bread [74]. However, consumers did not like the aroma of PSDB as revealed in Figure 3. They reported that it had a more yeasty and stronger sour aroma than SDB, as shown in the focus group results in Figure 4. The intense and stronger sour aroma of PSDB, as identified during the focus group session, might have been overwhelming or unpleasant for certain consumers, potentially resulting in a lower score or rating. The preference for aroma or texture of the SDB or PSDB may vary among individuals. Some panelists may prefer the aroma of the SDB due to personal preference, while others may prefer the texture of the PSDB, which could be a result of the presence of exopolysaccharides and propionic acid. Consequently, the more desirable aroma profile of SDB for the majority of consumers might have contributed to its higher score. Conversely, the specific strains and concentrations of *P. freudenreichii* employed during fermentation, along with the fermentation conditions, can all influence the ultimate sensory characteristics of the bread. Given the multifaceted nature of sensory evaluation, susceptible to influence from diverse factors, and acknowledging the intricate nuances in aroma gradations, additional research may be necessary to attain a holistic comprehension of the fundamental causes involved [75–77]. Overall, the presence of *P. freudenreichii* in bread formula can significantly influence the sensory attributes of the bread, particularly its aroma and flavor profile. Other factors, such as individual differences in taste and preference, as well as variations in the specific strains and concentrations of microorganisms used in fermentation, could also have contributed to the observed differences in aroma grade between the two bread samples.

5. Conclusions

In the research conducted, it was found that introducing *P. freudenreichii* to the sourdough fermentation process resulted in a rise in vitamin B_{12} levels and better texture compared to a control sample that did not contain *P. freudenreichii*. Although the overall preference was alike in both samples, sensory variations were noted in the aroma, texture, and perceived salty taste in the *P. freudenreichii* sourdough. The enhanced structural composition of the sourdough was due to the cooperative effects of exopolysaccharides and propionic acid, which offered anti-microbial and anti-stalling properties, greater water absorption, and improved texture. No significant differences were observed in mineral factors, pH and acidity in both samples. Furthermore, additional changes to the recipe and method are necessary to increase consumer appeal by enhancing loaf volume and crumb structure. By investigating the cultivation of vitamin B_{12}-producing bacteria through fermentation, this study aims to advance sustainability by mitigating the environmental repercussions linked to conventional animal-based vitamin B_{12} sources.

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