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Isolation, Characterisation and Vitamin B12 Production Optimization of *P. freudenreichii* from Turkish Traditional Kars Gravyer

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Abstract

In this study, nine different *Propionibacterium freudenreichii* strains were isolated from Kars Gravyer produced by traditional methods in Turkey and identified by sequencing the 16S–23S intergenic region using species-specific primers. The isolated strains were examined in vitro for the presence of the β-galactosidase enzyme, autoaggregation ability, sensitivity against eight selected antibiotics and survivability under harsh conditions in order to determine their potential probiotic properties. After probiotic potentials were evaluated, an experimental design was made to optimize the production of vitamin B12 in a 3 L glass bioreactor *P. freudenreichii* NUV774. While all strains showed similar resistance (92–98%) to gastric juice (0.3% pepsin, pH 3.0), they showed resistance to intestinal fluid (0.1% pancreatin, 0.3% bile salt, pH 8.0) between 60% and 92%. It was determined that the viability after 3 and 6 h of incubation in 0.5% and 1% bile salt differed between strains. All isolates exhibited resistance to ciprofloxacin, ampicillin, and trimethoprim–sulphamethoxazole; however, most were sensitive to ofloxacin. Overall, *P. freudenreichii* strains showed resistance to the gastrointestinal tract, tolerance to pH 3.0, and high tolerance to bile salts. As a result of optimization, maximum vitamin B12 production was found to be 156.8 mg/L. The optimum operating conditions were calculated as temperature = 36.9 °C, aeration = 2.430 vvm, and agitation = 159.120 rpm. Hence, *P. freudenreichii*, as future probiotic strain candidates, will offer an alternative source to *Lactobacillus*, *Bifidobacterium* and some *Bacillus* spp. In addition, this study denoted that the alteration of the production of active vitamin B12 by *P. freudenreichii* occurs in a strain-dependent manner.

Keywords: dairy propionibacteria; *Propionibacterium freudenreichii*; vitamin B12; optimization; probiotic properties

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1. Introduction

Dairy propionibacteria strains play a substantial role in the dairy industry, especially in the production of Swiss-type cheeses, together with a mixture of homofermentative streptococci and lactobacilli [1]. The dairy species consist of *P.acidipropionici*, *P. freudenreichii*,

P. jensenii and *P. thoenii* [2]. There are nearly 40 types of cheese produced traditionally in Turkey, including many cheeses such as Basket Cheese, Izmir Tulum Cheese, Kars Gravyer Cheese, and Afyon Cheese, which have the same hole structure as Swiss cheeses [3]. The propionibacteria are responsible for the eye formation and characteristic aroma during the ripening process of such cheeses [4]. Therefore, propionic acid bacteria are of great technological and economic importance.

P. freudenreichii, which is one of the most notable dairy propionibacterium species, kept its former taxonomic classification. Orla Jensen and von Freudenreich first described this bacterium, which was isolated from samples of Emmental cheese in 1906 [5,6]. *P. freudenreichii* is a member of the Actinobacteria phylum with a high GC content of approximately 57–70% of its genome. This strain is aerotolerant, non-motile, non-spore forming, mesophilic, Gram-positive, and has low nutritional needs in order to survive in harsh conditions. In terms of morphology, it is a pleomorphic rod-shaped microorganism with a tendency to aggregate, forming clusters resembling Chinese characters [7]. Although 30 °C is the ideal temperature for growth, they grow at 15–40 °C with a pH of 5.1–8.5 [8]. Some *P. freudenreichii* strains promote weight gain and improved intestinal health. Besides, the organism has been associated with the industrial production of vitamin B12 and strain-dependent probiotic and anti-inflammatory properties because of the production of several beneficial metabolites [9]. Propionibacteria species stimulated the growth of Bifidobacteria and Lactobacilli [10]. *P. freudenreichii* is a bacterium with the ability to de novo synthesize the active form of B12 [11]. However, the ability of B12 production among different strains of *P. freudenreichii* differs considerably [12].

Probiotics are defined by the Food and Agriculture Organization (FAO) and the World Health Organization (WHO) as "living microbes that provide health benefits to the host when taken into the body in sufficient amounts" [13]. However, in order for a microorganism to be used in food supplements as a probiotic, it must be "generally recognized as safe" (GRAS) as defined by the US Food and Drug Administration (FDA) [14]. *P. freudenreichii* is also among the microorganisms considered GRAS, and some studies have reported that it does not have any adverse effects for human consumption [15]. Moreover, this microorganism has been identified in fecal samples from a discrete cohort of preterm, breastfed infants, suggesting that it may be a component of the healthy human gut microbiota [16].

Vitamin B12 is a water-soluble molecule with a red crystalline structure that is essential for the metabolism of many organisms [17]. Vitamin B12 is involved in the activity of methionine synthase and R-methylmalonyl-CoA mutase enzymes in human metabolism, and approximately 1 mg per day is required. It is a crucial coenzyme, particularly in human tissue metabolism, and a lack of it can lead to a number of illnesses, including neurological difficulties and megaloblastic anemia [18,19]. Therefore, more than 10 tons of Vitamin B12 are produced annually commercially using different types of bacteria. Vitamin B12 synthesis is mostly carried out by the microorganisms Propionibacterium freudenreichii and Pseudomonas denitrificans in industrial applications [20]. However, in contrast to *P. denitrificans*, *P. freudenreichii* is a generally-recognized-as-safe (GRAS) microorganism and can grow on a wide variety of different inexpensive carbon and nitrogen sources [21]. Vitamin B12 is mostly obtained through fermentation and is produced by many pharmaceutical companies to meet demand around the world [22].

In Turkey, there are many types of homemade cheese produced by spontaneous fermentation and traditional methods without using a specific starter culture [23]. In this paper, we isolated nine different *P. freudenreichii* strains from Kars Gravyer, one of the traditional home-made Turkish cheeses. First, the 16S–23S intergenic region of the isolated bacteria was amplified using species-specific primers and identified by sequencing.

Subsequently, these nine strains were tested for resistance to gastric juice (0.3% pepsin, pH 3.0), intestinal juice (0.1% pancreatin, 0.3% bile salt, pH 8.0), low pH (2.0 and 3.0) and bile salt (0.5% and 1.0%), sensitivity to 8 different antibiotics, presence of the β-galactosidase enzyme and autoaggregation ability. The *P. freudenreichii* strains capacity to produce active Vitamin B12 was revealed by High-Pressure Liquid Chromatography (HPLC). The aim of this study is to determine the potential probiotic properties of the *P. freudenreichii* strain, which is of great importance in biotechnology processes and is increasingly used nowadays, and to reveal the production of Vitamin B12 in active form. It is believed that the results of the study; will lead to the widespread use of Propionic acid bacteria in the industry and will make positive contributions to microbial Vitamin B12 production processes.

2. Materials and Methods

2.1. Isolation of P. freudenreichii from Kars Gravyer

Samples of Kars Gravyer (n = 3) made with traditional methods and completed their ripening period were obtained from a local cheesemaker in Kars, Turkey. Before being analyzed, he samples were kept at 4 °C until delivery to the Microbiology Laboratory of the Nuvita Biosearch Center (NBC) (Alyors Inc., Istanbul, Turkey) and immediately processed. 10 g of Kars Gravyer samples were transferred into sterile bags under aseptic conditions and suspended in 90 mL of sterile saline solution (0.85% NaCl). After homogenization (paddle blender, ISOLAB Laborgeräte GmbH, Eschau, Germany), serial dilutions were prepared from the homogenate using saline solution. Aliquots of these dilutions were plated on yeast extract-sodium lactate (YEL) agar and incubated for 7–10 days at 30 °C under anaerobic conditions. The YEL medium consisted of 1% yeast extract (Neogen®, Lansing, MI, USA), 1% sodium lactate (Sigma-Aldrich®, St. Louis, MO, USA), 1% peptone from casein (Neogen®), 0.025% KH_2PO_4 (Sigma-Aldrich®), 0.0005% $MnSO_4 \cdot 4H_2O$ (Sigma-Aldrich®), 1.5% agar (Liofilchem®, Waltham, MA, USA), and distilled water. The medium was sterilized at 121 °C for 15 min after the final pH was adjusted to 7.0 ± 0.2. For identification, all the isolates that met the basic traits of *P. freudenreichii*—namely, nonspore formation, Gram positivity, and catalase positivity—were taken into consideration. The isolates were routinely propagated in YEL broth under anaerobic conditions and stored at −80 °C in liquid medium (YEL broth) supplemented with 50% (vol/vol) glycerol as a cryoprotectant.

2.2. Identification of Isolates

2.2.1. Phenotypic Identification

The isolates grown for 7–10 days of incubation on the YEL medium surface were submitted to Gram staining, tested for catalase production, and pigmentation. For catalase analysis isolates, one of the colonies that grew after the end of incubation was placed on a slide, and one drop of 3% H_2O_2 solution was added onto the colony. Test results were evaluated according to bubble formation in the samples. The isolates were placed in an atmosphere containing 5% CO_2 for 10 days, and at the end of the period, the colonies' formation of cream, orange, and brown pigments was evaluated [24]. Gram staining analysis was carried out after catalase and pigmentation tests, and the isolates stained in accordance with the protocol were examined under a microscope [25,26].

2.2.2. Genotypic Characterization by RAPD-PCR

For the typing of *P. freudenreichii* strains using primer M13 (5′-GAGGGTGGCGGTTC-3′), randomly amplified polymorphic DNA (RAPD) polymerase chain reaction (PCR) analysis was performed [27]. Genomic DNA was extracted with the PureLink™ Genomic DNA Mini Kit (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions.

The reaction mixture (final volume 25 µL) consisted of 10 µL sterile dH_2O, 12.5 µL Q5® High-Fidelity 2X Master Mix (New England Biolabs, Ipswich, MA, USA), 1.5 µL 10 mM M13 primer, and 1 µL of the DNA template. RAPD-PCR was performed using T100™ Thermal Cycler (Bio-Rad, Hercules, CA, USA) with the following program: Initial denaturation for 30 s at 98 °C, 40 cycles at 98 °C for 10 s, 59 °C for 20 s, and 72 °C for 20 s; then final extension of 72 °C for 2 min. The PCR products were loaded on a 1% (*w*/*v*) agarose gel with 1× Tris Acetate EDTA (TAE) buffer; 6× DNA Loading Dye (Thermo Scientific™, Waltham, MA, USA) was used as a DNA molecular weight marker. The gel electrophoresis was performed at 90 V for one hour, and the DNA bands were detected by visualizing under UV illumination with ethidium bromide in a Gel Doc EZ Imager (BIO-RAD Gel Documentation System, Hercules, CA, USA).

2.2.3. 16S–23S rRNA Gene Amplification and Sequencing

For amplifying the 16S–23S intergenic region to identify *P. freudenreichii*, species-specific PfrI (5′-AGGAGCCTTTTCGCCATC-3′) and PfrII (5′-TAGCTTGTCACACAAA-ACTC-3′) primers were used [28]. The PCR reaction was performed in a final 25 µL reaction volume containing 1 µL DNA template, 12.5 µL Q5® High-Fidelity 2X Master Mix (New England Biolabs, Ipswich, MA, USA), 1.25 µL 10 mM primers PfrI and PfrII, and 9 µL sterile dH_2O. The amplification program consisted of an initial denaturing step at 98 °C for 1 min followed by 25 cycles of denaturation at 98 °C for 10 s, annealing at 52 °C for 30 s and elongation at 72 °C for 1 min and one cycle of final extension at 72 °C for 2 min, performed in a thermal cycler (Bio-Rad, Hercules, CA, USA). Amplified PCR products were run on a gel to check the band patterns, and PCR products were sequenced at Medsantek, İstanbul, Turkey. The obtained nucleotide sequences were analyzed with the NCBI database using the BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi, access date: 23 January 2025) algorithm with a similarity criterion of 97–100% and submitted to GenBank.

2.3. Investigation of the Probiotic Potential of P. freudenreichii

2.3.1. Preparation of Bacterial Cell Suspensions

Briefly, strains were grown in YEL media for 48 h and centrifuged at 4400× *g* for 10 min at 4 °C (Allegra X-30R, Beckman Coulter, Brea, CA, USA). Then the supernatant and cell pellet were separated. The harvested cell pellets were washed twice with sterile saline solution (0.85% NaCl, *w*/*v*) by centrifugation, and then the cell density was adjusted to 1.0 (~10^8 CFU/mL) at OD600 nm.

2.3.2. Resistance to Simulated Gastric and Intestinal Fluids

The resistance of simulated gastric and intestinal fluids assay of *P. freudenreichii* strains was performed according to the methods described by Hacioglu and Kunduhoglu [29] with slight modifications. Simulated gastric juice was prepared by suspending 0.3% pepsin (Sigma-Aldrich, St. Louis, MO, USA) in YEL medium adjusted to pH 3.0 with 1 M HCl. The bacterial cell suspensions were inoculated at an inoculum size of 1% into the gastric juice. After the mixing, the initial bacterial count was determined by colony count and incubated at 30 °C for two hours with shaking. Following the incubation period, the final count of bacteria was determined. The gastric juice containing bacterial cells was separated by centrifugation at 4400× *g* for 10 min, and after the removal of gastric juice, the pellet was used for the intestinal fluid test. Simulated intestinal fluid was prepared by dissolving bile salt (0.3%) and pancreatin (0.1%) in sterile saline solution adjusted to pH 8.0 with 1 M NaOH. This fluid was resuspended in the remaining pellet, which was washed with saline solution by centrifugation at 4400× *g* for 10 min and incubated at 30 °C for two hours with shaking. The viable cell population was determined by colony counts before and after

incubation on YEL agar plates. The survival percentage was calculated by comparing cell counts before and after digestion for each strain.

2.3.3. Tolerance to Low pH and Bile Salt

To determine the acid tolerance of strains, the bacterial cell suspensions whose density was adjusted to 1.0 (~10^8 CFU/mL) at OD600 nm were added at an inoculum size of 1% to YEL medium adjusted to pH 2.0, 3.0, and 7.0 (control). Low pH study was conducted at pH 2.0 and pH 3.0. The broths were then incubated at 30 °C for six hours anaerobically, and aliquots of the samples were taken after 0, 3, and 6 h of incubation. These samples were serially diluted in sterile saline solution (0.85% NaCl), and the number of surviving cells was determined on YEL agar medium [30,31]. For the bile salt tolerance assay, YEL broth containing a final concentration of 0, 0.5, and 1.0% (*w*/*v*) of bile salt (Oxgall, Oxoid, UK) was inoculated with bacterial cell suspension at an inoculum size of 1%. The broths were incubated at 30 °C for six hours and cell viability was determined by serial dilution and plating onto YEL agar after 0, 3, and 6 h of incubation [32].

2.3.4. Antibiotic Susceptibility

Antibiotic susceptibility was determined by the disc diffusion method. The procedure was adapted from [33]. Nine strains identified as *P. freudenreichii* were tested for resistance to 8 antibiotic discs produced by Oxoid Ltd., Cheshire, UK. These were: ampicillin (20 µg), belonging to β-lactam; ciprofloxacin (5 µg), levofloxacin (5 µg), and ofloxacin (5 µg), belonging to fluoroquinolones; erythromycin (15 µg), belonging to macrolides; trimethoprim (1.25 µg)/sulfamethoxazole (23.75 µg), belonging to sulfanomides; vancomycin (30 µg), belonging to non-β-lactam cell wall; and rifampicin (15 µg), belonging to rifamycins. Each strain was inoculated in YEL broth and incubated for 48 h at 30 °C. The fresh YEL broth culture of *P. freudenreichii* strains (the final concentration was 10^7 CFU/mL; 100 µL) was poured and spread onto YEL agar plates. Then, antibiotic discs were placed on the surface of YEL agar plates and incubated at 30 °C anaerobically. The inhibition zone diameters were measured after incubation, and each isolate was classified as sensitive (S), intermediate (I), or resistant (R) according to the inhibition zone diameters in agreement with the CLSI tables [34].

2.3.5. Autoaggregation Assay

Autoaggregation viabilities were performed as described by Rocha-Ramírez et al. [35] with some modifications. The suspension of bacterial cells (10^8 CFU/mL) was prepared with sterile saline solution (0.85% NaCl), shaken in a vortex for 10 s, and incubated without agitation at 30 °C. The bacterial suspensions were then monitored at different times (1, 2, 3, 4, and 5 h), and each time, aliquots were taken from the supernatant, and the absorbance was measured at 600 nm with a UV-VIS spectrophotometer (UV-1900, Shimadzu, Kyoto, Japan).

The autoaggregation percentage was calculated as:

$$Autoaggregation\ (\%) = (1 - At\ /\ A0) \times 100 \quad (1)$$

where A_0 is the initial absorbance, and A_t is the final absorbance at 1, 2, 3, 4, and 5 h of incubation.

2.3.6. β-Galactosidase Activity

β-galactosidase activity of *P. freudenreichii* strains was determined using the plate assay method Angmo et al. [36]. 48-h-grown *P. freudenreichii* cultures were streaked on modified YEL agar plates containing 10 µL of IPTG (iso-propylthio β-D galactopyranoside) and 60 µL of X-gal (5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside) per 100 mL of YEL agar.

The inoculated plates were then incubated at 30 °C anaerobically. The appearance of the blue-colored colonies on YEL agar indicated the presence of β-galactosidase activity.

2.4. A-Optimal Experimental Design and Optimization

An *A*-optimal experimental design is an appropriate selection to conduct an experiment due to the individual variances of the regression coefficients. Moreover, the aim is to minimize the trace of the moment matrix [37,38]. Due to this awareness, an *A*-optimal design was chosen for each experimental run. The specified design factors and their levels are presented in Table 1.

Table 1. The specified three design factors and their levels.

Notation	Design Factor	Unit	Low-Level Uncoded/Uncoded	High-Level Uncoded/Uncoded
x_1	Temperature	°C	25.00/−1	37.00/+1
x_2	Aeration	vvm	0.00/−1	4.00/+1
x_3	Agitation	rpm	50.00/−1	250.00/+1

When using the least squares method, the estimated response function of the production of vitamin B12 is calculated as follows:

$$\hat{y}_1(\mathbf{x}) = \mathbf{p}\left[(\mathbf{X}_A)'(\mathbf{X}_A)\right]^{-1}(\mathbf{X}_A)'\mathbf{c} \text{ and } \mathbf{c} = \begin{pmatrix} y_{1,1} & y_{1,2} & \dots & y_{1,20} \end{pmatrix}' \quad (2)$$

where $\mathbf{X}_A$ is an *A*-optimal design model matrix. Also, $\mathbf{p}$ and $\mathbf{c}$ are parameters and response variable vectors, respectively. The next step is the optimization phase. For this phase, the desirability-focused optimization model is presented to obtain the optimum operating conditions of the design factor when maximizing the production of vitamin B12. The aim of the model is to maximize the desirability when satisfying the boundary requirements of the specified three design factors. The model is presented as follows:

$$\begin{aligned} &\text{maximize } d(\hat{y}_1(\mathbf{x})) \\ &\text{subject to } -1 \le \mathbf{x} \le +1 \text{ (Boundary requirements)} \\ &\text{where } d(\hat{y}_1(\mathbf{x})) = \begin{cases} 0, \ \hat{y}_1(\mathbf{x}) < l \\ \left(\frac{\hat{y}_1(\mathbf{x})-l}{t-l}\right), \ l \le \hat{y}_1(\mathbf{x}) \le t \\ 1, \ \hat{y}_1(\mathbf{x}) > l \end{cases} \text{ and } \mathbf{x} = \begin{pmatrix} x_1 & x_2 & x_3 \end{pmatrix}' \\ &\text{Find : Optimum operating conditions of the design factors} \\ &\qquad \text{and the estimated response value of B12} \end{aligned} \quad (3)$$

$d(\hat{y}_1(\mathbf{x}))$ is the desirability function associated with the response variable. Also, l and t are the lower and target values of the response, respectively. In this study, Design Expert (ver. 12.0.3.0 64-bit Minneapolis, MN, USA) was employed when conducting the data analysis and ANOVA, and plotting the 3D graphs. In addition, MATLAB (ver R2014a 64-bit, Natick, MA, USA) was utilized to acquire the optimum operating conditions of the specified three design factors for the B12 production.

2.5. Fermentation Process and Determination of Vitamin B12 with HPLC

In the vitamin B12 production preliminary studies conducted on the Erlenmeyer scale, the most promising strain was NUV774. To determine the vitamin B12 production abilities of *P. freudenreichii* NUV774 strain, the method proposed by Liu et al. [20] was modified. In this study, YEL broth was used as a pre-culture medium, while modified medium containing 54.3 g/L glucose, 30 g/L yeast extract, 2.7 g/L KH_2PO_4, and 0.005 g/L $CoCl_2 \cdot 6H_2O$ was used as fermentation medium. The pH value of the media was adjusted to a value between 6.8 and 7.0 by adding 3 M NaOH solution before autoclaving.

500 µL of the cryopreserved cells of *P. freudenreichii* strains were transferred to 150 mL of modified YEL medium and grown for 24 h at 30 °C anaerobically. Subsequently, fermentation processes was conducted in a 3 L total volume glass bioreactor (Minifors 2, Infors, Bottmingen, Switzerland) containing 1.5 L of fermentation medium, inoculated with 10% (*v*/*v*) of the pre-cultures and incubated at 30 °C for 168 h. Anaerobic fermentation was carried out for 72 h, and at the end of this period, 100 mM DMBI (5,6-dimethylbenzimidazole, Sigma-Aldrich, USA) was added to the media. After this point, fermentation continued for 96 h under agitation and aerobic conditions. In the optimization study, aeration was provided after aerobic conditions were achieved. Aeration was not supplied for the first 72 h.

At the end of the period, 20 mL of fermentation broth was taken and transferred into sterile falcon tubes. After centrifugation at 4000× *g* for 15 min, the supernatant was removed, and the pellet was homogenized with 2 mL of 8% (*w*/*v*) $NaNO_2$ and 2 mL of glacial acetic acid. Then, this mixture was kept in a boiling water bath for 30 min, and at the end of the period, the supernatant was passed through a filter with a 0.22 µm pore size and used in HPLC analysis.

The final sample was resolved on a reverse-phase hypersil BDS C-18 column (4.6 × 250 mm, 5 µm, Scharlau, Hamburg, Germany) using a Shimadzu LC-2050C HPLC (Kyoto, Japan) with a PDA detector and operating at 30 °C and monitored at 361 nm. The mobile phase consisted of acetonitril and water (80:20 [*v*/*v*]) at a flow rate of 1.0 mL/min. The methylcobalamin standard was from Glentham Life Sciences (Corsham, UK). The analysis methods used to determine the probiotic potential and vitamin B12 production ability of *P. freudenreichii* are shown in Figure 1.

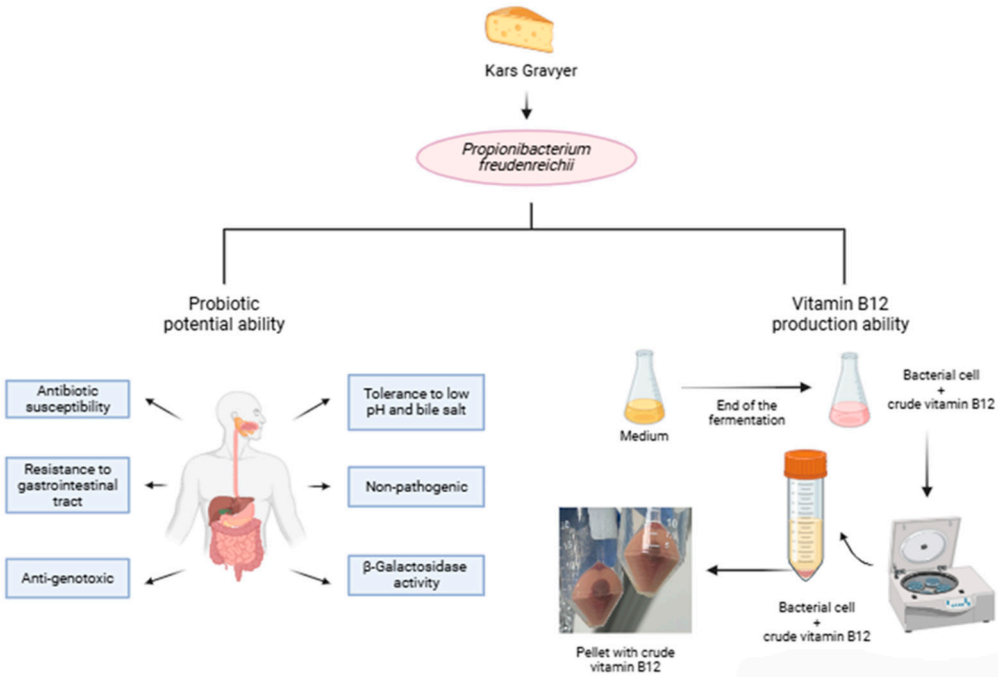


Figure 1. Graphical abstract illustrating the analysis performed within the scope of this study.

2.6. Statistical Analysis

All experiments were carried out at least in duplicate, and the results were expressed as mean values ± standard error (SE). Data obtained for each test were analyzed by means of a one-way ANOVA test. Tukey's post hoc test was applied for comparisons of means; differences were considered significant at $p < 0.05$. Statistical analyses were carried out using the statistical package IBM SPSS version 29.0.2.0.

3. Results and Discussion

In this research, *P. freudenreichii* strains were isolated from Kars Gravyer samples produced by traditional methods in Turkey, and some of their probiotic properties were determined. Nine *P. freudenreichii* strains were isolated from traditional Turkish Kars Gravyer samples. All of the isolates were Gram-positive, catalase-positive, capable of pigmentation, and had an irregular, short rod shape under the microscope. The 16S–23S sequences of the genotypically identified strains in this study were deposited in GenBank (Table 2). The *P. freudenreichii* strain has been found in a variety of traditional cheese samples, according to numerous previous studies [39–42].

Table 2. GenBank accession number of *Propionibacterium freudenreichii* strains.

Strain Number	Identification	GenBank Accession Number
NUV 766	*Propionibacterium freudenreichii*	PP410368
NUV 767	*Propionibacterium freudenreichii*	OR766040
NUV 768	*Propionibacterium freudenreichii*	OR781470
NUV 769	*Propionibacterium freudenreichii*	OR781471
NUV 770	*Propionibacterium freudenreichii*	OR781472
NUV 771	*Propionibacterium freudenreichii*	OR781473
NUV 772	*Propionibacterium freudenreichii*	OR781474
NUV 773	*Propionibacterium freudenreichii*	OR781475
NUV 774	*Propionibacterium freudenreichii*	OR781476

3.1. Resistance to Simulated Gastric and Intestinal Fluids

A microorganism needs to be able to survive in the gastrointestinal tract in order to be classified as a probiotic. The effect of simulated gastric and intestinal juices on the viability of nine *P. freudenreichii* strains is presented in Table 3. After 2 h of incubation in gastric fluid with a pH of 3.0, the viability of all strains decreased compared to the initial number, while their resistance ranged from 92% to 98% when compared to the viability in the control medium. The resistance of all isolated *P. freudenreichii* strains to the gastric fluid was found to be higher [42] or similar [39] compared to previous studies on the same species. However, the viability of the strains that survived in the gastric fluid decreased after being transferred to the intestinal fluid and incubated here (Table 3). The data show that the strains' resistance to artificial intestinal fluid ranged from 60% to 92% ($p < 0.05$). While the resistance of the nine isolated *P. freudenreichii* strains to the gastric juice was relatively similar ($p > 0.05$), strain-specific resistance was observed in the simulated intestinal fluid. *P. freudenreichii* tentatively survive and sustain metabolic activity in the gastrointestinal tract. Furthermore, it bring about an increase in the number of intestinal bifidobacteria and also causes an alleviation in toxin-producing Bacteroides species [43]. In a study examining the durability of commercial probiotic products in the gastrointestinal system, it was determined that the viability of bacteria in commercial products named Enterogermina, Yovis, and VSL3 remained stable in the gastric juice for up to 120 min [44]. On the other hand, bacterial viability in other probiotic products decreased during the 30–120 min incubation period. Thus, in vitro survival tests showed that it is possible for *P. freudenreichii* strains to survive the harsh conditions of the human digestive system.

3.2. Tolerance to Low pH and Bile Salt

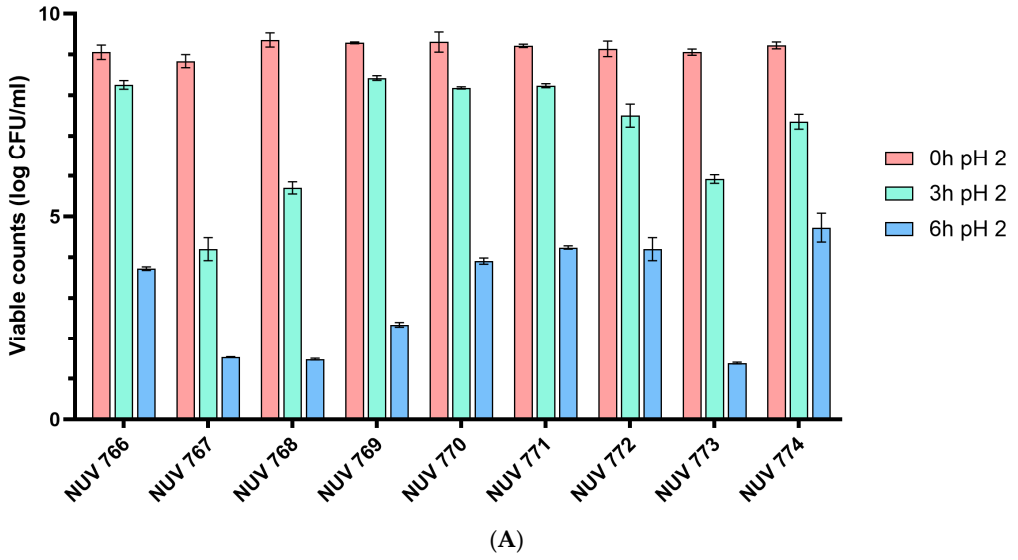
Resistance to low pH and bile salts is of great importance in predicting the survival and growth of potential probiotic strains in the harsh conditions [32]. In healthy individuals, the pH in the gastrointestinal system ranges from 2 to 6.7 [45]. In this study, we investigated the survivability of *P. freudenreichii* strains in highly acidic environments, specifically at pH 2 and pH 3. Following incubation at pH 2.0 for 3 and 6 h, the viability of the strains

decreased compared to the initial count (0 h), with each strain exhibiting this decrease at a different rate (Figure 2A). The NUV 769 strain exhibited higher viability than the other strains in the 3-h incubation period, and the NUV 774 strain exhibited higher viability in the 6-h incubation period. At pH 3, the viability of all strains was found to be close to the initial amount during a six hours incubation period ($p > 0.05$) (Figure 2B). Thus, all strains had a high tolerance to pH 3.0. In a similar study, dairy propionibacteria showed lower viability in simulated gastric juices at pH 2.0 than in simulated gastric juices with pH 3.0 or pH 4.0. Also, similar to the result we found, they found that approximately 77% of the strains showed similar levels of viability at pH 3.0 [42]. In the work of Campaniello et al. [46], the four different species' dairy propionibacteria showed tolerance to low pH values, and none of them significantly declined after 3 h at pH 2.5. They also examined the resistance of the strains to 0.3% bile salt. Propionibacteria survived in acidic environments and in the presence of bile salts [46].

Table 3. Survival of *P. freudenreichii strains* in simulated gastric and intestinal fluids.

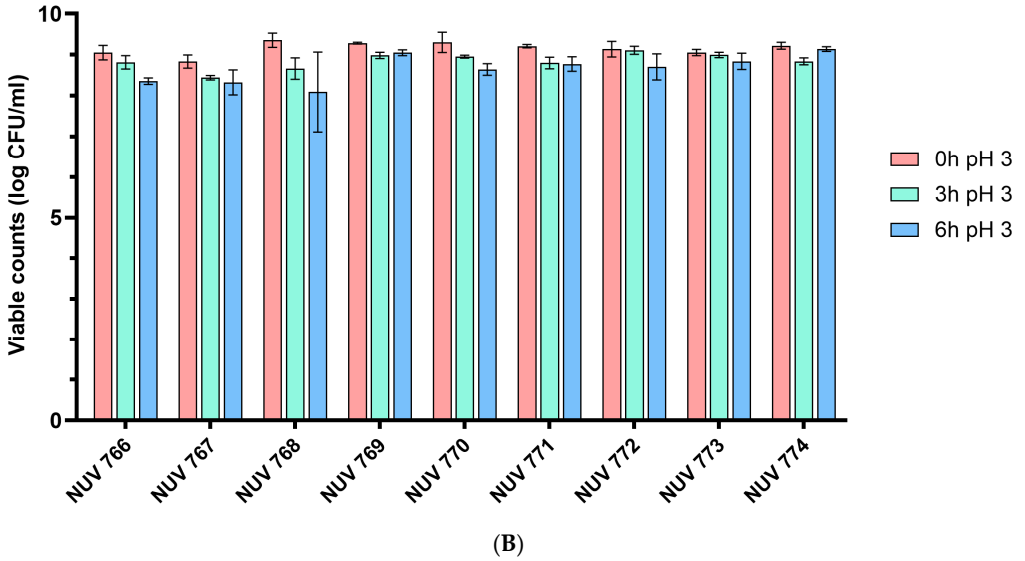
Strain Number	Initial Counts (log cfu/mL)	Survival After 2 h in the Gastric Fluid		Survival After a Consecutive 2 h in the Simulated Intestinal Fluid	
		(log cfu/mL)	%	(log cfu/mL)	%
NUV 766	9.10 ± 0.04	9.03 ± 0.04	98	7.21 ± 0.01	76 ^{CD}
NUV 767	8.65 ± 0.09	8.41 ± 0.03	97	5.96 ± 0.06	67 ^{AB}
NUV 768	9.07 ± 0.06	8.42 ± 0.05	92	5.60 ± 0.11	60 ^{A}
NUV 769	9.25 ± 0.04	9.12 ± 0.07	98	7.91 ± 0.13	84 ^{DE}
NUV 770	9.35 ± 0.07	9.13 ± 0.08	97	7.06 ± 0.08	75 ^{BC}
NUV 771	9.30 ± 0.03	9.12 ± 0.04	97	8.24 ± 0.01	86 ^{E}
NUV 772	9.30 ± 0.06	9.04 ± 0.06	97	6.77 ± 0.10	71 ^{BC}
NUV 773	9.22 ± 0.05	9.04 ± 0.01	96	8.09 ± 0.02	85 ^{E}
NUV 774	9.28 ± 0.06	9.13 ± 0.05	98	8.62 ± 0.07	92 ^{E}

Values are expressed in mean ± standard error ($n = 3$). $^{A–E}$ Different letters in the same column indicate significant differences among samples according to Tukey's post hoc test ($p < 0.05$).



(**A**)

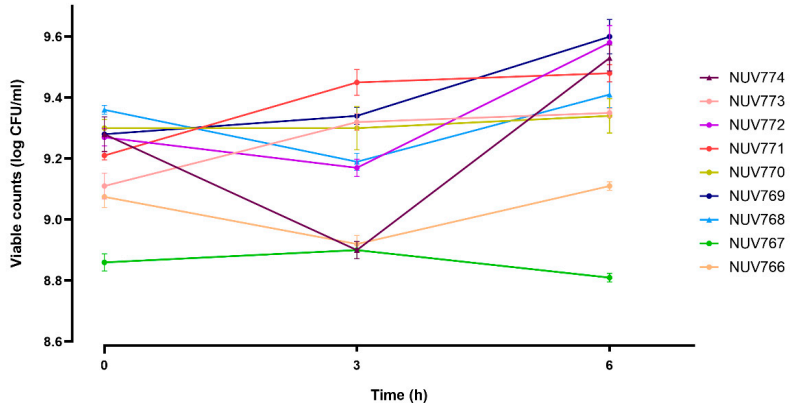
Figure 2. *Cont.*



(**B**)

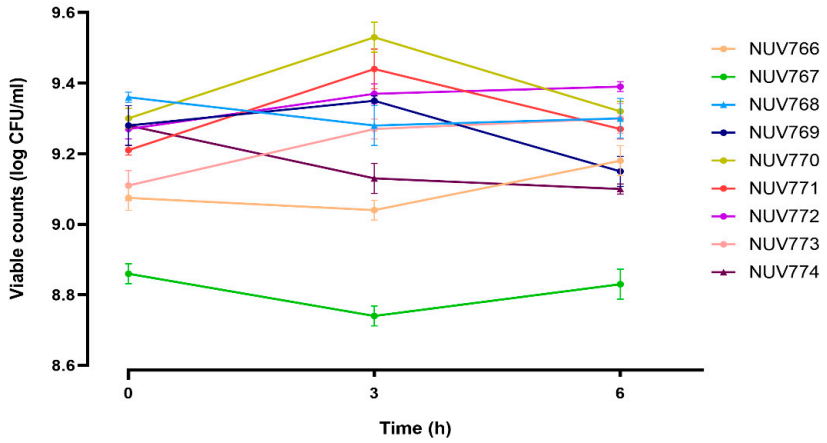
Figure 2. Viability of the strains expressed as log cfu/mL after 0, 3 and 6 h incubation at pH 2 (**A**) and pH 3 (**B**).

After 3 and 6 h of incubation in 0.5% and 1% bile salt, it was determined that the viability of some strains decreased and some increased compared to the control media. The presence of bile salt promoted the growth of some strains and caused the viability of others to decrease. Looking at the results of this analysis, it was seen that the resistance of the strains to different concentrations of bile salt at different times is strain-specific (Figure 3). Darilmaz and Beyatli [3] investigated the resistance of 15 *P. freudenreichii* strains they isolated to three different concentrations of bile salt (0.06, 0.15 and 0.30%) and stated that the viability of each strain was different depending on the concentration. Ibrahim et al. [47] reported minimal loss of viability for some dairy propionibacteria strains exposed to 1% bile salts for 48 h, while significant decreases were observed for other species. The most important considerations in probiotic selection are pH tolerance and bile salt resistance. The survival of the isolated *P. freudenreichii* species in low pH and bile salts environments was considered important in terms of probiotic properties. Low pH and bile salt tolerance studies results are promising when compared with the of different dairy propionibacterium strains.

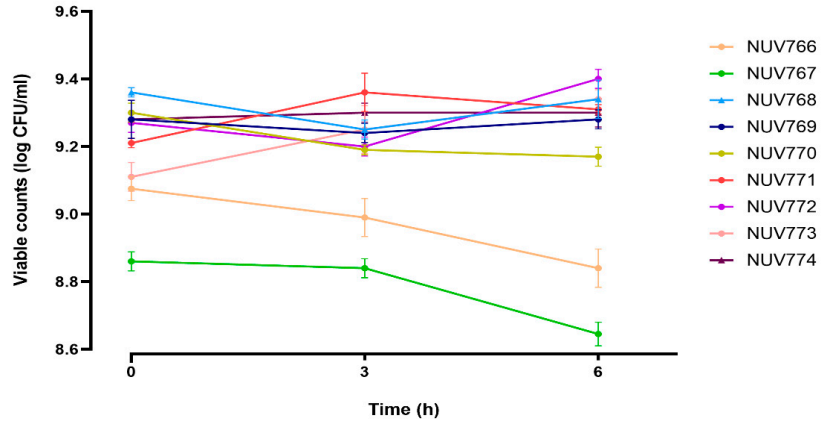


(**a**)

Figure 3. *Cont.*



(**b**)



(**c**)

Figure 3. Survival rate of *P. freudenreichii* strains at different bile salt concentrations. (**a**) Control media. (**b**) The tolerance of strains to 0.5% bile salt. (**c**) The tolerance of strains to 1.0% bile salt.

3.3. Antibiotic Susceptibility

Since the antibiotic resistance gene is a transferable gene between microorganisms in the gastrointestinal tract, probiotic microorganisms should not carry antibiotic resistance genes. A few antibiotics are naturally resistant to *Propionibacterium* strains isolated from dairy products, and this resistance does not appear to be plasmid-encoded [48]. Therefore, we wanted to determine the antibiotic susceptibility of potential probiotic *P. freudenreichii* strains by using eight different antibiotics commonly used in Medicine [49]. The examined *Propionibacterium freudenreichii* strains were resistant to ampicillin, which inhibits cell wall synthesis, ciprofloxacin, which has a bactericidal effect by inhibiting DNA synthesis, as well as trimethoprim–sulphamethoxazole, as shown in (Table 4). On the other hand, all strains were sensitive to the tested antibiotics vancomycin, erythromycin, levofloxacin, and rifampicin. While 56% of the strains were sensitive to ofloxacin, a fluoroquinolone antibiotic, 44% were found to be intermediately sensitive as shown in (Table 4). Yuksekdag et al. [24] found in their study that most *P. freudenreichii* strains (69%) were resistant to ampicillin. Darilmaz et al. [23] reported that one of the two *P. freudenreichii* strains they isolated from Kars Gravyer was resistant to ampicillin, and the other was sensitive. Results from studies on the antibiotic susceptibility of the different *P. freudenreichii* strains showed that susceptibility was strain-specific. Therefore, it must be proven genotypically whether strains with possible probiotic qualities have a transferable resistance gene [50,51].

Table 4. Antibiotic resistance of *P. freudenreichii* strains.

Strain Number	Antibiotic Resistance *							
	VA	CIP	AMP	E	LEV	OFX	RA	SXT
NUV 766	S	R	R	S	S	S	S	R
NUV 767	S	R	R	S	S	S	S	R
NUV 768	S	R	R	S	S	S	S	R
NUV 769	S	R	R	S	S	S	S	R
NUV 770	S	R	R	S	S	I	S	R
NUV 771	S	R	R	S	S	I	S	R
NUV 772	S	R	R	S	S	I	S	R
NUV 773	S	R	R	S	S	S	S	R
NUV 774	S	R	R	S	S	I	S	R

AMP = ampicillin, CIP = ciprofloxacin, VA = vancomycin, E = erythromycin, LEV = levofloxacin, OFX = ofloxacin, RA = rifampicin, SXT = trimethoprim–sulphamethoxazole; * R = Resistant, S = Sensitive and I = Intermediate resistance.

3.4. Auto-Aggregation

Auto-aggregation of bacteria is necessary for adhesion to epithelial cells and thus for the colonization of the gastrointestinal tract. Autoaggregation appears to be the first step in the adhesion process, allowing bacteria to form a barrier and preventing undesired bacteria from adhering [45]. Auto-aggregation is one of the characteristics that provides potential advantages for microorganisms in colonizing the intestinal tract [52,53]. All the strains tested in this study exhibited some degree of auto-aggregation (Table 5) from the first time (from 1.30% to 5.85%), which continually increased with time. After 24 h of incubation, the highest aggregation activity was recorded in NUV 767, while the lowest activity was found in NUV 768. Not much information was found about the autoaggregation ability of *P. freudenreichii* strains in the literature. Krausova et al. [54] isolated strains belonging to *Lactobacillus* and *Bifidobacterium* species in their study and found their autoaggregation abilities between 21.7% and 69.7%. This proved that autoaggregation ability can be found in various ranges depending on the bacterial genera and strain. The auto-aggregation ability of *P. freudenreichii* strains will be important evaluation parameter in the selection of potential probiotic strains.

Table 5. Autoaggregation (%) during 24 h and β-galactosidase activity of *P. freudenreichii* strains.

Strain Number	Auto-Aggregation (%)						β-Galactosidase Activity
	1 h	2 h	3 h	4 h	5 h	24 h	
NUV 766	2.17 ± 0.10^{D}	6.91 ± 0.06^{B}	7.04 ± 0.01^{C}	11.01 ± 0.11^{A}	11.40 ± 0.10^{B}	59.77 ± 0.03^{B}	+
NUV 767	1.30 ± 0.13^{E}	3.11 ± 0.07^{G}	4.41 ± 0.12^{F}	6.61 ± 0.02^{E}	8.52 ± 0.04^{G}	60.17 ± 0.13^{A}	-
NUV 768	1.87 ± 0.11^{D}	5.23 ± 0.12^{D}	6.51 ± 0.04^{D}	8.97 ± 0.05^{C}	9.17 ± 0.01^{F}	34.73 ± 0.24^{I}	+
NUV 769	1.53 ± 0.04^{DE}	3.27 ± 0.26^{FG}	7.35 ± 0.01^{C}	8.98 ± 0.16^{C}	10.92 ± 0.06^{C}	55.42 ± 0.17^{E}	+
NUV 770	1.32 ± 0.07^{E}	4.39 ± 0.01^{E}	7.89 ± 0.19^{B}	10.96 ± 0.21^{A}	12.83 ± 0.08^{A}	42.38 ± 0.07^{H}	+
NUV 771	1.56 ± 0.16^{DE}	3.62 ± 0.03^{F}	5.38 ± 0.03^{E}	8.50 ± 0.07^{D}	8.90 ± 0.02^{F}	51.44 ± 0.34^{F}	+
NUV 772	4.73 ± 0.10^{B}	7.14 ± 0.08^{B}	7.35 ± 0.01^{C}	9.35 ± 0.14^{C}	9.77 ± 0.12^{E}	46.03 ± 0.26^{G}	-
NUV 773	5.85 ± 0.03^{A}	9.23 ± 0.11^{A}	9.72 ± 0.11^{A}	10.02 ± 0.02^{B}	10.52 ± 0.02^{D}	58.54 ± 0.15^{D}	-
NUV 774	3.07 ± 0.00^{C}	5.94 ± 0.21^{C}	6.62 ± 0.07^{D}	8.34 ± 0.08^{D}	8.92 ± 0.10^{F}	59.14 ± 0.11^{C}	+

Values are expressed in mean ± standard error ($n = 3$). $^{A–I}$ Different letters in the same column indicate significant differences among samples according to Tukey's post hoc test ($p < 0.05$).

3.5. β-Galactosidase Activity

The β-galactosidase enzyme is widely used in the dairy industry and hydrolyzes lactose, the main carbohydrate in milk, into glucose and galactose, which can be absorbed across the intestinal epithelium [55]. In most cases, lactose intolerance—a digestive insufficiency—is caused by low activity of the β-galactosidase enzyme [56]. In this context,

among all the beneficial effects of probiotics, their effect on regulating lactose intolerance is important. Since the presence of this enzyme is considered an advantageous feature for probiotic bacteria, the β-galactosidase activity of the isolated strains was tested. About 67% of the strains showed green color colonies on X-Gal plates, signifying their potent ability to produce the enzyme β-galactosidase (Table 4). β-galactosidase activity analysis of *P. freudenreichii* strains using X-gal plates has not been found in the literature, but Vinderola and Reinheimer [57] reported that a high value of β- galactosidase enzyme in commercial strains of *L. delbrueckii* subsp. *bulgaricus* isolated from cheese. An important dairy propionibacterium, *A. acidipropionioci* has been found to have high β-galactosidase activity. It has been determined that it produces some oligosaccharides thanks to its high β-galactosidase activity [58]. Also, it is thought that *P. freudenreichii* strains can produce some prebiotics owing to their β-galactosidase activities.

3.6. Fermentation Optimization Results

In fermentation optimization, 20 bioreactor experiments were carried out with the NUV774 strain, which was selected considering preliminary Erlenmeyer studies and probiotic potential. Table 6 also shows the *A*-optimal design points. Table 6 contains the ten required *A*-optimal design points, the five lack-of-fit design points, and five replicated design points. Indeed, the ten required design points estimate the model coefficients. However, additional A-optimal model points enhance the A-optimality criterion and the precision of the estimates. Therefore, additional lack-of-fit and replicated A-optimal design points are included in the study to enhance the quality of precision. Moreover, vitamin B12 (mg/L) production is the response variable. Notice that the *A*-optimal design points are randomly constructed in order to eliminate an experimental bias. The *A*-optimal design points are obtained in 923.400 s.

Table 6. *A*-optimal design points and the collected data.

	The Production of Vitamin B12			
Run	x_1 **(°C)**	x_2 **(vvm)**	x_3 **(rpm)**	**Response** y_1 **(mg/L)**
1	37.000	4.000	50.000	94.230
2	33.520	4.000	141.346	129.560
3	25.000	0.000	50.000	36.210
4	37.000	0.000	250.000	85.250
5	25.000	4.000	132.529	57.300
6	30.361	0.000	160.760	75.330
7	25.000	4.000	250.000	71.280
8	30.356	2.214	50.000	98.250
9	32.046	4.000	250.000	66.900
10	37.000	2.214	160.731	156.850
11	25.000	1.160	141.447	46.740
12	37.000	0.000	50.000	39.120
13	37.000	4.000	50.000	68.260
14	29.091	2.639	181.798	82.900
15	25.000	1.648	250.000	55.380
16	37.000	0.000	50.000	58.980
17	37.000	0.000	250.000	61.540
18	33.513	1.827	250.000	110.450
19	25.000	0.000	50.000	31.640
20	30.356	2.214	50.000	96.980

The optimization step is significant to maximize the production of B12 (mg/L) when obtaining the optimum operating conditions of the specified three design factors. The reduced cubic model was obtained for the response, and it was calculated as follows:

$$\hat{y}_1(\mathbf{x}) = 109.04 + 49.16x_1 + 12.21x_2 - 28.46x_2^2 - 16.53x_3^2 + 9.96x_1^2x_3 - 34.85x_1x_2^2 \quad (4)$$

In (4), the variance inflation factors (VIFs) are checked, and there is no severity of multicollinearity issues in the regression analysis. Table 7 shows the ANOVA results and summary of statistics for the B12 production response when the number of experimental runs (n) is twenty. In Table 7, the R^2 (the coefficient of determination), R^2_{adj} (the adjusted coefficient of determination), R^2_{pre} (the absolute prediction coefficient of determination) values are obtained at 0.8450, 0.7734, and 0.6602, respectively. The difference between R^2_{adj} and R^2_{pre} is 0.1132. Thus, an agreement between R^2_{adj} and R^2_{pre} are observed for the model fit. Furthermore, the model fit is appropriately associated with the summary of statistical findings. Then, an adequate signal was computed at 14.7575; so, the model might navigate the A-optimal design region. Next, the F-value and p-values were calculated at 11.81 and 0.0001, respectively. Hence, the model is found to be significant. Moreover, the significant model terms were found to be x_1, x_2, x_2^2, and $x_1x_2^2$. Note that x_3^2 and $x_1^2x_3$ were obtained to be non-significant in the model, and it is included the reduced model to support hierarchy. Furthermore, the lack of fit was found to be non-significant for the reduced model.

Table 7. Summary of the statistical analysis and ANOVA for the response variable.

Summary of Statistics for the Response						
R^2	0.8450	R^2_{adj}	0.7734			
Adequate precision	14.7575					
ANOVA for the reduced model						
Source	SS	DF	MS	F-value	p-value	Explanation
Model	16222.44	6	2703.74	11.81	0.0001	Significant
x_1	6554.27	1	6554.27	28.63	0.0001	Significant
x_2	1904.38	1	1904.38	8.32	0.0128	Significant
x_2^2	2949.32	1	2949.32	12.88	0.0033	Significant
x_3^2	977.02	1	977.02	4.27	0.0594	Non-significant
$x_1^2x_3$	883.48	1	883.48	3.86	0.0712	Non-significant
$x_1x_2^2$	2426.86	1	2426.86	10.6	0.0063	Significant
Residual	2976.42	13	228.96			
Lack of Fit	2149.65	8	268.71	1.63	0.3074	Non-significant
Pure Error	826.76	5	165.35			
Total	19198.86	19				

Note: SS = Sum of squares, DF = Degrees of freedom, and MS = Mean square.

For the results of the optimization phase, desirability was obtained at 1.000, denoting a completely desirable response value of the vitamin B12 production. The optimum operating conditions were calculated as follows: x_1 = 36.900 °C, x_2 = 2.430 vvm, and x_3 = 159.120 rpm. Moreover, the estimated response value was obtained at 157.835 mg/L. The two experimental runs were carried out for the validation study with the optimum operating conditions found from the introduced model. The vitamin B12 productions were obtained to be 149.230 mg/L and 154.780 mg/L. In the experimental study, the deviations

can be observed when replicating using the same conditions several times. Furthermore, the validation results are consistent with the optimization results. Notice that the tenth run in Table 6 is x_1 = 37.000 °C, x_2 = 2.214 vvm, and x_3 = 160.731 rpm, and the response variable is 156.850 mg/L. The conditions of the tenth run in Table 6 are pretty similar to the optimization results. If we rerun the tenth run in Table 6 several times, we may obtain less than 156.850 mg/L for the vitamin B12 production. Based on the findings, model verification is vital, along with a couple of experiments, to validate the optimization results.

The 3D graphs are presented in Figures 4 and 5. The temperature level is around the high level for the optimum operating condition. On the other hand, the aeration level could be around the middle level for the optimum condition. Moreover, the agitation level could be around the middle level for the optimum condition. These 3D graphs visually verified the optimization results.

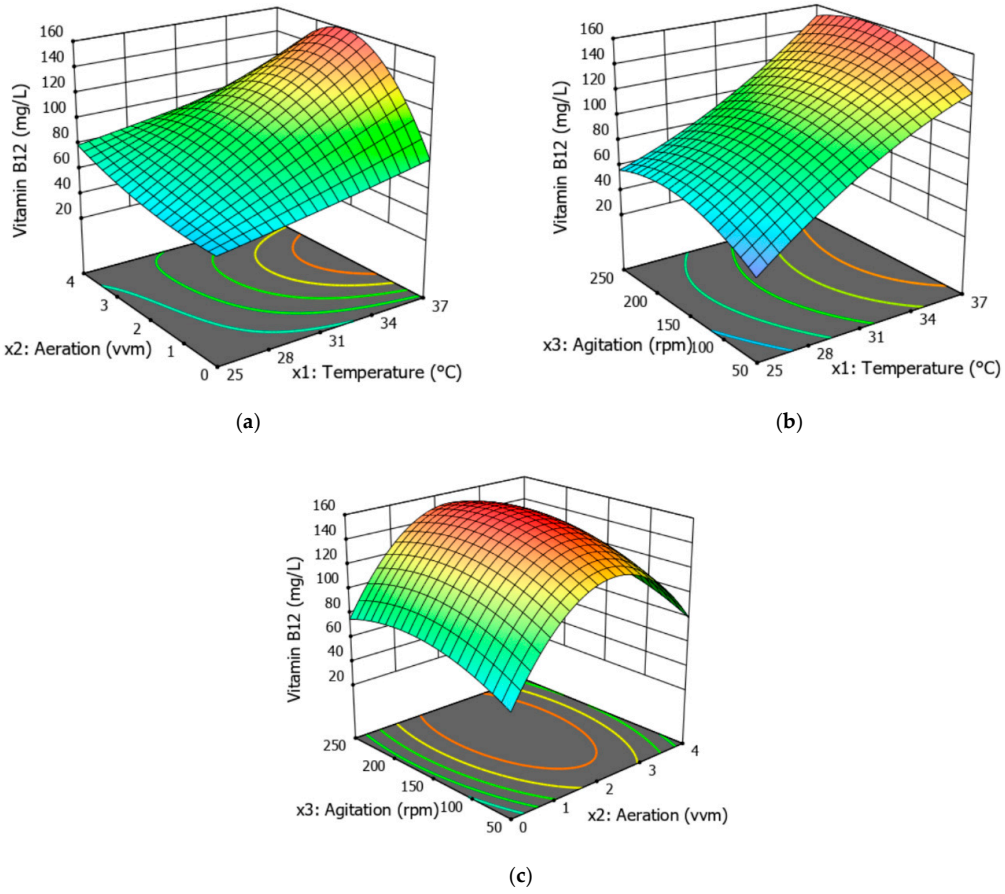


Figure 4. 3D plots for (**a**) x_1 and x_2 design factors and associated with the vitamin B12 production response, (**b**) x_1 and x_3 design factors and associated with the vitamin B12 production response, and (**c**) x_2 and x_3 design factors and associated with the vitamin B12 production response.

Enrichment of food materials through fermentation with selected starter cultures is an efficient way to provide micronutrients to consumers utilization of vitamin B12 as a dietary supplement has become increasingly significant due to the rise in the vegan and vegetarian demographic. For this reason, the use of producers such as non-genetically modified wild-type strains of *P. freudenreichii* is of interest, especially in markets where consumer awareness is high [59,60]. The amounts of vitamin B12 synthesized by the isolated *P. freudenreichii* strains were determined using a calibration curve obtained using methylcobalamin standards of varying concentrations. The results showed that all isolated strains were capable of producing vitamin B12, but the production amounts were strain-specific. Details of the chromatogram for NUV774 and methylcobalamin standard for Vitamin B12

analysis performed on HPLC are provided in the Supplementary Materials. As a result of the study conducted by Liu et al. [20] for the optimization of vitamin B12 production by *P. freudenreichii* CICC10019, it was determined that the amount of B12 produced by the strain was 8.32 mg/L. Chamlagain et al. [61] found *P. freudenreichii* with a maximum Vitamin B12 yield of 2.5 µg/mL in whey permeate supplemented with DMBI. Chamlagain et al. [62] investigated vitamin B12 production in a solubilized protein extract of biotreated wheat bran containing *P. freudenreichii*. 24-h supplementation of $CoCl_2$ with the biotreated extract resulted in a 5.4-fold increase in B12 production (maximum 49 ± 3.5 ng/mL). This suggests that Co is one of the main factors affecting B12 production, which is confirmed by the literature. Calinoiu et al. [63] used wheat bran and oat bran in a Vitamin B12 production environment with *P. freudenreichii* DSM 20271. In mediums without any supplementation, *P. freudenreichii* DSM 20271 obtained approximately 120 and 56 µg B12/kg of fresh product, respectively. Research on the vitamin B12 generation by the *P. freudenreichii* strain indicates that variables like strain, fermentation medium, and fermentation time influence the quantity of vitamin B12 produced [64,65]. This indicates that the vitamin B12 yield from this strain can be enhanced and its production can be scaled up without genetic modification. The key here is believed to be the richness of the nutrient medium and the optimization of bioprocess operating conditions.

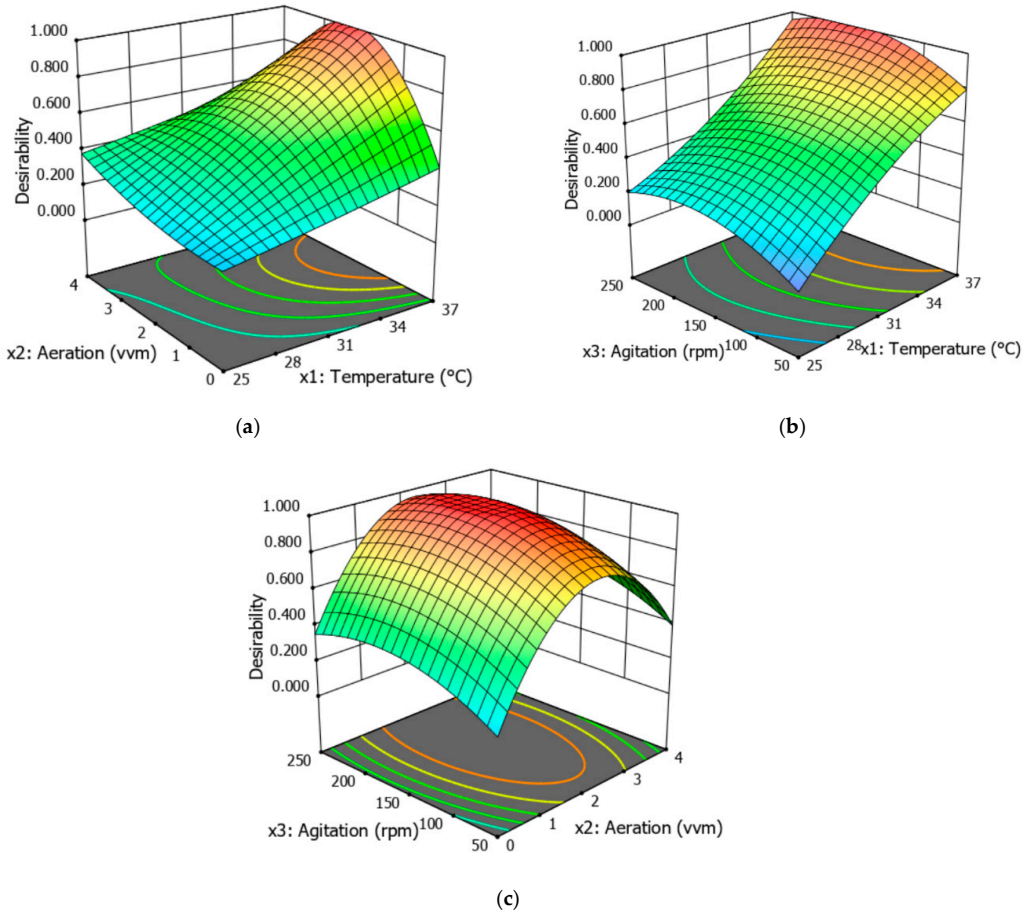


Figure 5. 3D plots for (**a**) x_1 and x_2 design factors and associated with the desirability function, (**b**) x_1 and x_3 design factors and associated with the desirability function, and (**c**) x_2 and T_3 design factors and associated with the desirability function.

4. Conclusions

In conclusion, the probiotic characteristics and vitamin B12 production ability of *P. freudenreichii* strains isolated from Kars Gravyer samples were determined. After the

identification of nine *P. freudenreichii* strains, their resistance to antibiotics, β-galactosidase activities, autoaggregation percentages, and resistance to harsh conditions were tested to determine probiotic characterization. While the resistance of the strains to the gastric environment was similar, their sensitivity to simulated intestinal fluid differed. The viability of propionibacteria was affected by pH 2.0, almost all of the tested strains survived well at pH 3.0. Specifically, at the end of both 3 h and 6 h incubation periods, NUV771 and NUV774 strains showed a higher tolerance to pH 2.0 than other strains, while the tolerance of the strains to pH 3.0 was found to be similar. Bile salt added at two different concentrations stimulated the growth of some bacteria. Antibiotic resistance, beta-galactosidase activities, and autoaggregation abilities of the strains varied on a strain basis. Among the isolated strains, NUV771 and NUV774 stand out with their probiotic potential, while NUV774 stands out with its vitamin B12 production potential as a result of preliminary vitamin B12 production experiments. As a result of optimization, the yield values obtained in preliminary experiments were exceeded by 50%. When the data were examined, it once again revealed the importance of microbial process development and optimization. Study findings clearly reveal that some *P. freudenreichii* strains showed high capacity of probiotic characteristics and will provide an alternative source to Lactobacillus and Bifidobacterium for future probiotic development. On the other hand, their ability to produce vitamin B12 makes these strains isolated from domestic sources commercially valuable. The studies will continue toward the industrial-scale production and commercialization of the the *P. freudenreichii* NUV774 strain.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/fermentation11120704/s1, Figure S1: Chromatography images of methylcobalamin standard and NUV774.

Author Contributions: A.E.K. and E.D. conceived the study; A.E.K., Z.B.A. and A.Ö. analyzed the data, and wrote the first draft of the manuscript. E.D. supervised and investigated the experiment and revised the manuscript. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflicts of interest.

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