Article

Statistical Approach to Potentially Enhance the Postbiotication of Gluten-Free Sourdough

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Featured Application: This optimized gluten-free sourdough is suitable for utilization as a functional ingredient in gluten-free bakery recipes. The sourdough's production following a controlled fermentation process can increase the predictability of the postbiotic properties of the fermented product. Postbiotics are healthy alternatives to obtain foods with enhanced antimicrobial and technological features.

Abstract: Fermented products are permanently under the attention of scientists and consumers, both due to nutritional importance and health promoting effects. The fermented functional foods contribute to a more balanced diet and increase the immune responses (among many other health effects) with positive implications for quality of life. In this sense, improving the sourdough's fermentation to boost the biotic (postbiotic and paraprobiotic) properties of the sourdough-based products has positive impacts on the nutritional and functional properties of the final baked products. These enhanced sourdoughs can be obtained in controlled fermentation conditions and used as sourdough bread improvers or novel bioingredients. In this context, our work aimed to optimize, using statistical tools, a gluten-free sourdough based on chickpea, quinoa, and buckwheat fermentation with selected lactic acid bacteria (LAB) to enhance its postbiotic properties. The most important biotechnological parameters were selected by Plackett–Burman Design (PBD) and then Response Surface Methodology (RSM) was applied to evaluate the interactions between the selected factors to maximize the gluten-free sourdough's properties. As a result, the optimized fermented sourdough had antimicrobial activity with inhibition ratios between 71 and 100% against the *Aspergillus niger*, *Aspergillus flavus*, *Penicillium* spp. molds and against the *Bacillus* spp endospore-forming Gram-positive rods. The optimized variant showed a total titratable acidity (TTA) of 40.2 mL NaOH 0.1N. Finally, the high-performance liquid chromatography (HPLC) analysis highlighted a heterofermentative profile for the organic acids from the optimized sourdough. Among flavonoids and polyphenols, the level of caffeic and vanillic acids increased after lactic acid fermentation. The comparison between the optimized sourdough and the control evidenced significant differences in the metabolite profiles, thus highlighting its potential postbiotication effect.

Keywords: gluten-free flours; selected lactic acid bacteria; solid state fermentation; sourdough; design of the experiments; optimization; postbiotics

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1. Introduction

The spontaneous fermentation of flours from cereals, pseudocereals, legumes or flours from their byproducts results in a sourdough—a fermented bread dough typically characterized by acidic taste and aroma, which can be used further to manufacture differentiated bakery products with economic and nutritional added-value [1–3]. Selected lactic acid bacteria (LAB) starters were employed in the sourdough's fermentation to maximize the

sourdough bread's shelf-life or to enhance the nutritional and functional value [3–5]. The utilization of the metabiotics (including postbiotics and paraprobiotics) to enhance the functional properties of the fermented products represent novel conceptual approaches applied in the food and feed industry that contribute to a healthier lifestyle [6]. Dietary fibers, known also as prebiotics, support the proliferation of probiotics in the colon [7]. Postbiotic compounds (metabolites and/or microbial cells fractions) are usually produced by microorganisms during fermentation, and they have valuable functional and health promoting effects. Additionally, paraprobiotics (“ghost probiotics”) are the result of probiotic cells’ disruption by different treatments and the exploitation of these products [8,9]. The functional and technological features of the sourdoughs depend on various factors, the most important being the metabolic properties of the LAB strains and the fermentation parameters [3,10,11] as well as the treatments applied to the fermented products in order to promote postbiotic and paraprobiotic compounds, e.g., high pressure treatments, ohmic heating, ultrasonication or UV light exposure [12,13]. Freeze-drying or spray-drying were successfully used in sourdough technology [14,15] in order to improve its bioavailability behavior and the reported results concluded that sourdough breads are digested faster compared to leavened baked goods [16], and, moreover, sourdough can contribute towards the reduction in the use of salt in baked goods due to its sour taste [17,18]. The antidiabetic, hypocholesterolemic, antimicrobial and antioxidative effects of postbiotics and paraprobiotics from LAB strains were highlighted in the literature, and this is a research topic that is still being intensively studied [3,9,19]. All these strategies supporting sustainability are characterized by a responsible utilization of the available resources to fulfill consumers’ expectations and to ensure their well-being. The products manufactured by socially responsible companies that have implemented sustainable policies are frequently chosen by Romanian consumers [20,21].

Chickpea, quinoa and buckwheat are gluten-free raw materials characterized mainly by their complex amino acids composition with a low allergenicity and anticarcinogenic effects. Besides their protein content, quinoa and buckwheat are consumed worldwide due to their contents of fatty acids, vitamins, minerals and antioxidant compounds, molecules that support diverse health benefits [3,22–25]. The nutritional and functional value of these gluten-free seeds can be enhanced by malting, cooking or fermentation [26,27]. Beverages, pasta [28,29], bread and sourdough were successfully made using the above-mentioned pseudocereals and legumes [30–32].

Okara is the main byproduct from the soymilk industry, rich in proteins, fibers and antioxidants. The solid-state fermentation (SSF) of okara with *Kluyveromyces marxianus* [33,34] or *Saccharomyces cerevisiae* strains increased the content of soluble dietary fibers and crude fats and decreased the level of antinutritional factors [35]. Moreover, the *Lactobacillus plantarum* strain increased the antioxidant activity of the fermented okara [36]. Symbiotic products that prevent diabetes and cardiovascular diseases based on okara and *Lactobacillus casei* were also studied and correlated [37–39].

Experimental designs using the Plackett–Burman Design (PBD) and Response Surface Methodology (RSM) were widely used in research in the biotechnology and food industries [40]. For example, recipes for a sweet bread product [41], and for gluten-free noodles were optimized by RSM [42]. The rheological and textural implications of the replacement of salt or wheat flour with wheat sourdough and pseudocereal flours were determined as well by RSM [43,44].

The aims of this work were to establish the influence of the biotechnological parameters on the fermentation of selected gluten-free flours in controlled solid-state fermentation with selected LAB strains, using the statistical tools (PBD and RSM) for the mathematical modelling and analysis. The optimized parameters were selected with the objective of enhancing the antimicrobial and technological characteristics, from the perspective of the development of assisted bioprocesses which are able to retain the desired postbiotics and to enhance their functional potential.

2. Materials and Methods

2.1. Materials, Microorganisms and Culture Conditions

The LAB strains used in this study were previously selected based on specific functional properties, respectively, the acidification capacity, the production of exopolysaccharides and the antimicrobial spectra at different pH values and temperatures [45]. Specifically, four LAB strains as part of the Microorganisms Collection of Dunărea de Jos University of Galati (acronym MIUG), corresponding to three *Lactobacillus* spp. strains (coded MIUG BL 21, MIUG BL 24 and MIUG BL 38) and one *Leuconostoc* spp. strain (coded MIUG BL 40) were tested. The presumptive identification at species level was carried out by Biolog Microbial Identification System (Biolog Inc., Hayward, CA, USA) using 94-well Gen III MicroPlates for aerobes. The manufacturer's instructions were carefully followed, as previously described [46–48]. The LAB strains were presumptively identified as follows: MIUG BL 21 being *Lactobacillus paracasei* ssp. *paracasei*; MIUG BL 24 being *Lactobacillus parabuchneri*; MIUG BL 38 being *Lactobacillus brevis* and MIUG BL 40 being *Leuconostoc mesenteroides* ssp. *mesenteroides*.

The stock cultures of LAB were reactivated in de Man, Rogosa and Sharpe (MRS) broth. After 48 h of incubation at 37 °C (Binder BF4000, Tuttlingen, Germany) under aerobiosis, each inoculum was diluted in MRS broth to reach an optical density at the wavelength of 600 nm (OD_{600nm}) of 2.0, using a spectrophotometer (Libra S22 UV-VIS, Biochrom, Cambridge, UK) [49]. The cell viable counts of LAB in each inoculum was also determined using saline (0.9%, w/v) serial dilutions inoculated in MRS agar plates [50,51]. For the established optical density (2.0 at $\lambda = 600$ nm) and incubation conditions (48 h, 37 °C, aerobiosis), an inoculum size of ca. 1×10^7 colony-forming units per milliliter (CFU/mL) was obtained for all LAB strains. The mold strains used in this study as the indicator microorganisms for the antifungal effect were isolated and purified as reported in our previous work [45]. Prior to their use, the stock cultures of *Aspergillus niger*, *Aspergillus flavus* and *Penicillium* spp. strains were reactivated on Potato Glucose agar (PGA) slants at 25 °C (Pol-Eko Aparatura, Poland) for 96 h under aerobiosis. The indicator microorganism used for the antibacterial activity was a *Bacillus* spp. strain previously isolated from quinoa seeds, as reported elsewhere [45]. This bacterial strain was routinely reactivated from stock cultures by cultivation overnight on Nutrient broth at 37 °C under aerobiosis.

Whole seeds of chickpea, white quinoa, buckwheat, and non-genetically modified soybeans were purchased from Solaris Plant S.R.L, Bucharest, Romania. The seeds were finely ground with a coffee mill for approximately 5–7 min (DeLonghi, Bucharest, Romania), and the respective flours were further used for the experiments in this study. The soybeans were previously soaked in tap water for 12 h at room temperature. Afterwards, soymilk was obtained from the soaked beans and water 1:4 (w/v) using a blender (Bosch, Cluj, Romania) for 5 min [33]. Finally, the solid residue from the previous soymilk's preparation, called okara, was collected, freeze-dried (Martin Christ, Osterode am Harz, Germany) and stored at 4 °C in dark airtight jars.

For the formulation of the fermentation medium, the chickpea, quinoa, and buckwheat flours were mixed in equal proportions (1:1:1, w/w) together with the amount of okara powder ranging between 0–16.9 g% (w/w) according to the PBD or RSM runs and sterilized at 121 °C for 15 min (Panasonic MLS-3871L, Bucharest, Romania). The distilled water was sterilized separately from the dry ingredients. The fermentation substrates were made by mixing the sterilized ingredients under aseptic conditions in a laminar-flow chamber (SafeFast, Faster, Cornaredo, Italy). The dough yield (DY) was calculated using Equation (1). Accordingly, softer (higher water content) sourdoughs have higher DY's [3,52]. The sourdough's ingredients and solid-state fermentation (SSF) conditions (LAB strain inoculum, fermentation time and temperature) were designed according to the statistical software (see Sections 3.1.1 and 3.1.2). After the fermentation, the resulting sourdough samples

were freeze-dried and stored in sterile containers at 4 °C. All the chemicals, reagents, and commercial culture media were purchased from Sigma-Aldrich (Steinheim, Germany).

$$\text{Dough Yield} = \frac{\text{dough weight (g)}}{\text{flour weight (g)}} \times 100 \quad (1)$$

2.2. Analysis of the Properties of Sourdoughs

2.2.1. Total Titratable Acidity

For the determination of the total titratable acidity (TTA), 10 g of freeze-dried sourdough was mixed with 90 mL of distilled water. TTA was evaluated using an automatic titrator (TitroLine Easy, Schott Instruments, Mainz, Germany) and the results were expressed as the volume of NaOH 0.1N (mL) required to reach the pH value of 8.5 [53,54].

2.2.2. Antifungal Properties of Sourdoughs

The PGA slants of *Aspergillus niger*, *Aspergillus flavus* and *Penicillium* spp. strains were used to collect the spores in a saline solution supplemented with 0.1% (v/v) Tween 80 (Sigma-Aldrich, Steinheim, Germany). Serial dilutions were made and the concentration of spores was established at 10^4 spores/mL using a Thoma counting chamber (Paul Marienfeld GmbH & Co. KG, Lauda-Königshofen, Germany) [55,56].

Petri dishes with 2% (w/v) of each freeze-dried sourdough sample were sterilized by UV for 30 min in a laminar-flow chamber [57]. Then, 20 mL of sterilized and melted (ca. 45–50 °C) PGA was poured into the dishes. After the homogenization of the mixture and solidification of the culture medium, a volume of 10 µL of fungal spores suspension (10^4 spores/mL) was used for the inoculation on the surface of the culture medium [58]. The mycelium growth was measured after 96 h of incubation at 25 °C under aerobiosis. The antifungal activity was expressed as inhibition ratio using Equation (2):

$$I = \frac{Ac - At}{Ac} \times 100 \quad (2)$$

where I is the growth inhibition percentage, Ac is the diameter (in mm) of the mycelial growth for the control sample (PGA without freeze-dried sourdough) and At is the diameter (in mm) of the mycelial growth on the culture medium supplemented with 2% (w/v) of the tested sourdough sample from PBD or RSM [59].

2.2.3. Antibacterial Properties of Sourdoughs

The antibacterial activity of the prepared sourdoughs was determined following the experimental protocol described by Cotârleş et al. [60] with slight modifications. The overnight culture of *Bacillus* spp. strain was diluted in Nutrient Broth, to reach a target optical density of 0.05 (OD_{600nm}) using a spectrophotometer [61]. Afterwards, the bacterial suspension (10 µL) was used for the inoculation in the Petri dishes with sterilized and melted (ca. 45–50 °C) Nutrient Agar. After 24 h of incubation at 37 °C under aerobiosis, the diameter (in mm) of the bacterial colonies was measured, and the inhibition ratio was calculated using Equation (2), where Ac is the diameter (in mm) of the bacterial growth for the control sample and At is the diameter (in mm) of the bacterial growth on the culture medium supplemented with 2% (w/v) of the tested sourdough samples.

2.3. Optimization of Sourdough Fermentation Parameters

2.3.1. Plackett–Burman Design (PBD)

In the current study, a preliminary selection of the fermentation conditions (independent variables or factors) to produce sourdoughs was assessed through PBD. The mathematical modelling was produced using Equation (3):

$$Y = \beta_0 + \sum \beta_i X_i \quad (3)$$

where Y represents the response—i.e., the independent variables or observations, which were the TTA, expressed as mL NaOH 0.1N, and antifungal and antibacterial activities, expressed as inhibition ratios: β_0 is the model intercept; β_i is the linear coefficient; and X_i is the level of the independent variables [62,63].

The analysis of the mathematical models was undertaken after collecting results from 12 independent runs taking into consideration all the factors and levels listed in Table 1.

Table 1. Plackett–Burman Design (PBD) independent variables (or factors).

Codification of the Independent Variables/Factors	Minimum Value, −1	Maximum Value, +1
A, Dough Yield (DY)	250.00	700.00
B, Inoculum of <i>L. paracasei</i> ssp. <i>paracasei</i> MIUG BL 21 (% v/w)	0.00	4.00
C, Inoculum of <i>L. brevis</i> MIUG BL 38 (% v/w)	0.00	4.00
D, Inoculum of <i>L. parabuchneri</i> MIUG BL 24 (% v/w)	0.00	4.00
E, Inoculum of <i>Leu. mesenteroides</i> ssp. <i>mesenteroides</i> MIUG BL 40 (% v/w)	0.00	4.00
F, Okara (% w/w)	0.00	10.00
G, Fermentation temperature (°C)	25.00	45.00
H, Fermentation time (h)	24.00	96.00

2.3.2. Response Surface Methodology (RSM)

The factors that determined the significant predictable postbiotic effects on each of the studied responses were selected after PBD analysis. Moreover, it is important to highlight the significant interaction effects between the most important factors listed in Table 2.

Table 2. Response Surface Methodology (RSM) factors.

Independent Variable/Factors	Minimum Value, −1	Central Value, 0	Maximum Value, +1	Axial Points	
				− α	+ α
A, Dough Yield (DY)	250.00	475.00	700.00	−60.14	1010.14
B, Inoculum of <i>L. paracasei</i> ssp. <i>paracasei</i> MIUG BL 21 (% v/w)	0.00	2.00	4.00	−2.75	6.75
C, Inoculum of <i>L. parabuchneri</i> MIUG BL 24 (% v/w)	0.00	2.00	4.00	−2.75	6.75
D, Okara (% w/w)	0.00	5.00	10.00	−6.89	16.89
E, Fermentation temperature (°C)	25.00	35.00	45.00	11.21	58.78
F, Fermentation time (h)	24.00	60.00	96.00	−25.62	145.62

The interaction effects were identified by a Central Composite Design (CCD) by RSM. With those 6 independent variables (A to F) and the corresponding levels previously described (Table 2), a two-level factorial design, half-fractional, CCD with 53 runs was employed, following the second order polynomial Equation (4):

$$Y = \beta_0 + \sum_{i=1}^k \beta_i X_i + \sum_{i=1}^k \beta_{ii} X_i^2 + \sum_{i=1}^k \beta_{ij} X_i X_j \quad (4)$$

where Y represents the response—i.e., TTA expressed as mL NaOH 0.1N, and antifungal and antibacterial activities, expressed as inhibition ratios; β_0 is the constant of the model; β_i , β_{ii} , and β_{ij} are the coefficients for the linear, quadratic and interaction effects; X_i and X_j are independent variables, and k is the number of the studied factors [64].

2.4. The Composition of Organic Acids, Flavonoids and Polyphenolic Compounds in Sourdoughs by RP-HPLC-DAD

Organic acids, flavonoids and polyphenolic compounds were separated and partially identified by Reversed Phase (RP) High-Performance Liquid Chromatography system coupled with a Diode Array Detector (DAD). The organic acids in the unfermented (control) and the optimized fermented sourdough obtained by RSM were separated and partially identified using an HPLC system (Perkin Elmer, Flexar, Waltham, MA, USA) equipped with a solvent degasser, column oven, binary pump system and a DAD detector (Perkin Elmer, Flexar, Waltham, MA, USA). A Hamilton PRP X300 column (250 × 4.1 mm, 7 µm particle size, Hamilton, Bonaduz, Switzerland) was used for the separation of the organic acids using a 20 mM H₂SO₄ acidified water as the mobile phase, under isocratic elution conditions, at 60 °C, and an optimal flow-rate of 1.5 mL/min. The total run time for the separation was 45 min. The post-run time of 20 min was used to re-equilibrate the column. The detection of the organic acids was made in the ultra-violet-C range at a wavelength of 210 nm ($\lambda = 210$ nm) after 15 min and integrated using the Chromera software, v. 3.3 [65].

The flavonoids and polyphenolic compounds from the optimized sourdough were separated by a Surveyor HPLC system (Finnigan Surveyor LC, Thermo Scientific, Waltham, MA, USA) equipped with autosampler, quaternary pump, photodiode-array (PDA) and UV-VIS detectors. A Synergi 4u Fusion-RP-80Å column (150 × 4.6 mm, 4 µm particle size; Phenomenex, Torrance, CA, USA) was used for the separation following the methodology described by Montiel-Sánchez et al. (2020) [66] with minor modifications. Briefly, a binary elution system composed by 100% (v/v) methanol (solvent A) and 3% (v/v) formic acid (solvent B) was used. The separation was carried out at 30 °C for a total run time of 55 min using the following elution pattern with a linear gradient for the separation and further identification of these above-mentioned bioactive compounds: starting from 91% B within 0–20 min; decreasing at 65% B within 20 min (from 20 to 40 min); and increasing at 91% B (from 40–55 min). The post-run time of 20 min was used to re-equilibrate the column. The separation was made at an optimal flow-rate of 1 mL/min. The compounds of interest were simultaneously detected in the UV-B and UV-A ranges at the wavelengths of 280 and 320 nm, respectively. The detection and integration were made resorting to the Xcalibur software, v. 2.1.

Prior to the HPLC separations, 2 g of freeze-dried sourdough samples, respectively, the sample fermented under optimized conditions and the unfermented sample (control) were resuspended in 5 mL of 20 mM H₂SO₄ acidified water or 5 mL 70% (v/v) methanol for the organic acids, flavonoids and polyphenols separation, respectively. The mixtures were dissolved by sonication for 45 min, using an ultrasonic bath (MRC, Holon, Israel). Before the HPLC injection, the supernatants of the sample mixtures were collected after centrifugation at 6000 rpm and 4 °C for 10 min (Hettich Universal 320R, Tuttlingen, Germany) and filtrated through 0.22 µm syringe filters (Merck, Darmstadt, Germany) [67].

The organic acids, flavonoids and polyphenols of the analyzed samples were identified after their separation by comparing the retention times to external standards. All external standards were purchased from Sigma-Aldrich (Steinheim, Germany).

2.5. Statistical Analysis

Plackett–Burman Design (PBD) was used to determine the most important variables for the functional properties of the sourdough. As previously mentioned, 8 independent variables were evaluated: A—dough yield; B—inoculum of *L. paracasei* ssp. *paracasei* MIUG BL 21 strain (% v/w); C—inoculum of *L. brevis* MIUG BL 38 strain (% v/w); D—inoculum of *L. parabuchneri* MIUG BL 24 strain (% v/w); E—inoculum of *Leu. mesenteroides* ssp. *mesenteroides* MIUG BL 40 strain (% v/w); F—okara (% w/w); G—fermentation temperature (°C); and H—fermentation time (h). The effect of the above-mentioned factors was tested on the following responses: TTA (mL NaOH 0.1N); antifungal activity against *Aspergillus niger*, *A. flavus*, and *Penicillium* spp. and antibacterial activity against *Bacillus* spp. (I, %, Equation (2)). Among all those factors, 6 of them had a significant impact on the

studied responses, namely: *A*—dough yield (Equation (1)); *B*—inoculum of *L. paracasei* ssp. *paracasei* MIUG BL 21 strain (% *v/w*); *C*—inoculum of *L. parabuchneri* MIUG BL 24 strain (% *v/w*); *D*—okara (% *w/w*); *E*—fermentation temperature (°C); and *F*—fermentation time (h). These six factors were further included in a half-fractional Central Composite Design (CCD) using the RSM analysis with 53 runs (32 cube points, 9 center points in cube and 12 axial points). The negative values for the axial points ($-\alpha$) were considered 0. Minitab 19 (v. 1.1, LLC, College, PA, USA) was used for the design of experiments and one-way analysis of variance (ANOVA) was evaluated considering a confidence interval of 95%. The fitting of the models was evaluated based on the regression coefficient (R^2), lack of fit, and *p*-value < 0.05. Two parallel experimental runs were performed, and the reported results are the average values of duplicate measurements for each analysis.

3. Results and Discussion

3.1. Sourdough Fermentation Process Optimization

3.1.1. Selection of the Most Important Fermentation Parameters by Plackett–Burman Design (PBD)

Following the combinations of factors at different levels (Table 1) obtained by PBD, the variations between the experimental results for the studied responses were observed, as it is shown in Table 3. The acidification capacity expressed as TTA varied between 5.90 to 38.90 mL NaOH 0.1N. As expected, the lowest TTA value was determined for the uninoculated dough incubated for 24 h at 25 °C. The most acidified sample was obtained with a mixed inoculum containing the LAB strains of *L. paracasei* ssp. *paracasei* MIUG BL 21, *L. brevis* MIUG BL 38 and *Leu. mesenteroides* ssp. *mesenteroides* MIUG BL 40 after the fermentation of a medium containing 10% (*w/w*) okara powder. For some samples, a mixed inoculum did not determine a high level of TTA, most likely due to the competitive and/or antagonistic effects between the studied LAB strains. Similar results were reported by Siepmann et al. [68], who reported differences between the TTA values when various strains of *Lactobacillus* spp. and *Pediococcus pentosaceus* were co-inoculated as starter cultures for the wheat-based sourdoughs. For some LAB strains, a fermentation temperature of 35 °C caused TTA values below 4.0 mL NaOH 0.1N, whereas a mixed inoculum composed by the four studied LAB strains caused a significant increase in TTA values to 13.0 mL after the sourdough's fermentation at 28 °C. The reported results underline the importance of the fermentation temperatures on the microbial and enzymatic activities of pseudocereal and legume flours.

Table 3. Experimental results of the total titratable acidity (TTA) and inhibition ratio (*I*, %, defined by the Equation (2)) obtained by Plackett–Burman Design (PBD) analysis. The meaning of the factors *A* to *H* is given in Table 1.

Run	Factors								Responses				
	<i>A</i>	<i>B</i>	<i>C</i>	<i>D</i>	<i>E</i>	<i>F</i>	<i>G</i>	<i>H</i>	TTA, mL NaOH 0.1N	<i>I</i> , % <i>A. niger</i> ¹	<i>I</i> , % <i>A. flavus</i>	<i>I</i> , % <i>Penicillium</i> spp. ¹	<i>I</i> , % <i>Bacillus</i> spp. ¹
1	250.00	4.00	4.00	0.00	4.00	0.00	25.00	24.00	11.23	21.27	56.36	6.90	2.23
2	700.00	0.00	0.00	0.00	4.00	10.00	45.00	24.00	11.00	−0.81	43.18	−1.55	−9.48
3	700.00	0.00	4.00	0.00	0.00	0.00	45.00	96.00	10.67	41.64	45.45	8.90	−34.50
4	250.00	4.00	0.00	0.00	0.00	10.00	45.00	96.00	13.73	31.26	57.73	29.83	−36.07
5	700.00	4.00	0.00	4.00	4.00	0.00	45.00	24.00	14.77	−8.88	51.82	16.21	2.69
6	250.00	4.00	4.00	4.00	0.00	10.00	45.00	24.00	10.23	−6.43	51.36	55.00	−16.42
7	700.00	4.00	0.00	4.00	0.00	0.00	25.00	96.00	27.10	−7.97	51.82	21.55	16.60
8	700.00	0.00	4.00	4.00	0.00	10.00	25.00	24.00	27.87	−32.96	29.09	−2.41	−11.59
9	250.00	0.00	4.00	4.00	4.00	0.00	45.00	96.00	11.60	10.03	50.00	23.45	−5.46
10	250.00	0.00	0.00	4.00	4.00	10.00	25.00	96.00	28.77	10.76	43.18	24.83	25.55
11	250.00	0.00	0.00	0.00	0.00	0.00	25.00	24.00	5.90	13.55	15.00	−21.03	25.27
12	700.00	4.00	4.00	0.00	4.00	10.00	25.00	96.00	38.90	−6.72	50.91	26.90	65.22

¹ Negative values resulting from the calculation of the inhibition ratio. These values have statistical relevance for the mathematical modelling.

Another key-variable for the TTA response was represented by the fermentation time (Table 3). Our results suggested that an extension of the incubation time up to 96 h could result in higher levels of TTA, depending on the LAB strains used as inoculum. In a work from Comasio et al. [54], the TTA values above 10.0 mL NaOH 0.1N were obtained with some selected *Lactobacillus* spp. strains, after 72 h of fermentation at 30 °C of a wheat-based sourdough. Furthermore, the amount and type of flours and other ingredients used in the sourdough recipes for bakery products, as well as the number and type of LAB strains employed during the fermentation and the consistency of the sourdoughs (i.e., the DY) may cause significant changes upon the acidification potential. Accordingly, different types of cereal flours have different buffer capacities, and different LAB strains in the same or different dough mixtures lead to different types of organic acids and respective yields of production [53,69–71].

The high antimicrobial activities of the fermented products (Table 3) were determined against *Aspergillus niger* and *Aspergillus flavus* as indicator fungal strains, after 96 h of incubation at 45 °C and after 24 h of fermentation at 25 °C, respectively. The data showed in Table 3 indicate that some LAB strains used for the fermentation of sourdough were not able to inhibit the fungal or bacterial growth, namely of *Penicillium* spp. or *Bacillus* spp. strains, respectively. However, the antimicrobial compounds produced during the SSF process of the gluten-free flours formulated media by LAB strains used in this study possessed different inhibitory potentials against the indicator strains of molds and bacterium (Table 3).

According to the ANOVA results displayed in Tables S1–S5, the type of LAB strains used as the inoculum, the addition of okara in the fermentation medium, as well as the fermentation temperature and time led to distinct impacts on the analyzed responses. Particularly, the factors A (dough yield), D (inoculum of *L. parabuchneri* MIUG BL 24 strain, % v/w), F (okara, % w/w), G (fermentation temperature, °C) and H (fermentation time, h) had significant impacts ($p < 0.05$) on the TTA. The mathematical model generated on the TTA was also significant ($p = 0.014$) with a regression coefficient (R^2) of 0.9827 (Table S1).

The growth of the fungal indicator strains used in this study revealed different behaviors, depending on the strain. *Aspergillus niger*'s growth was significantly inhibited ($p < 0.05$) by the variation of the factors A (dough yield), D (*L. parabuchneri* MIUG BL 24 strain inoculum, % v/w), and H (fermentation time, h) involved in the sourdoughs' fermentation processes, as showed in Table S2.

Moreover, the analysis of variance (ANOVA) from Tables S3 and S4 unfolded the influence of the factor B (*L. paracasei* ssp. *paracasei* MIUG BL 21 strain inoculum, % v/w) on the inhibitory potential of the sourdoughs against *Aspergillus flavus* and *Penicillium* spp. strains.

The temperature (factor G) through the SSF greatly influenced the sourdoughs' anti-fungal activity against *Aspergillus flavus*. In contrast, the same factor G had no significant effect against *Penicillium* spp. Moreover, the *L. parabuchneri* MIUG BL 24 strain inoculum (factor D), along with the fermentation time (factor H), contributed significantly to attain a sourdough with an inhibitory effect against *Penicillium* spp. The antibacterial activity of the sourdoughs against *Bacillus* spp. was strongly influenced ($p < 0.05$) by the factor G (fermentation temperature, °C). This statistical model was significant ($p = 0.026$) but its regression coefficient was only 66.66% ($R^2 = 0.6666$) (Table S5).

Taking into consideration that the fermentation temperature had a significant impact on the antibacterial activity of the sourdoughs against the *Bacillus* spp. strain, and simultaneously proved to be an important factor regarding other generated models (viz., TTA and growth inhibition of *Aspergillus flavus*), the antibacterial activity of the sourdoughs was, therefore, included in the further studies by RSM.

Overall, by analyzing all the models with the responses of interest using the PBD, factor E (*Leu. mesenteroides* ssp. *mesenteroides* MIUG BL 40 inoculum) previously identified as an important exopolysaccharide producing strain [45], it has not been statistically significant for the responses studied in this work. Thus, six factors were proved to be significant on the sourdough properties, chiefly dough yield, *L. paracasei* ssp. *paracasei* MIUG BL 21 and

L. parabuchneri MIUG BL 24 strains' inoculum concentration, okara amount, fermentation temperature and time—thus being selected for the further optimization studies by RSM.

3.1.2. Optimization of Gluten-Free Sourdough's Solid-State Fermentation (SSF) by Response Surface Methodology (RSM)

The factors *A*, dough yield; *B*, *L. paracasei* ssp. *paracasei* MIUG BL 21 strain inoculum (% *v/w*); *C*, *L. parabuchneri* MIUG BL 24 strain inoculum (% *v/w*); *D*, okara (% *w/w*); *E*, fermentation temperature (°C); and *F*, fermentation time (h) (Table 2) previously selected (see Section 3.1.1) were used for the RSM analysis, the results for each response being listed in Table 4.

(1) Acidification Capacity (TTA)

Regarding the acidification capacity of the studied LAB strains in the sourdoughs, it can be observed from Table 4 that the highest values for the TTA were achieved at 35 °C after 60 h of fermentation with different inoculum types and concentrations. Analyzing the results (Table 4) in the sourdough samples with the lowest consistency (i.e., highest DY of 700), an increase in TTA was observed with increasing temperature and okara concentration, when the sourdough was inoculated uniquely with the *L. parabuchneri* MIUG BL 24 strain. Moreover, the *L. paracasei* ssp. *paracasei* MIUG BL 21 strain produced higher amounts of organic acids when the fermentation time ranged between 60 h and 96 h. A synergistic effect on the TTA values was detected when the studied lactobacilli strains were used together and, simultaneously, when the fermentation medium was supplemented with 10% (*w/w*) okara powder and 96 h of fermentation was applied.

The used indicator molds and the bacterial strains (i.e., *Aspergillus niger*, *Aspergillus flavus*, *Penicillium* spp. and *Bacillus* spp., respectively), were inhibited in different manners by the obtained samples of the sourdough formulated based on the optimization design and principles. The negative inhibition ratios were calculated. Thus, these values helped for a better discrimination of the results towards an optimized sourdough formulation.

The most acidified sourdough sample—obtained with the co-culture of both selected lactobacilli strains determined a total inhibition of *Aspergillus niger*, *Penicillium* spp. and *Bacillus* spp., whereas only a growth inhibition ratio of 75.9% was attained for *Aspergillus flavus*. Another promising result was found when the sourdough was fermented with a co-culture of 2% (*v/w*) of each *L. paracasei* ssp. *paracasei* and *L. parabuchneri* strain's inoculum at 35 °C for 60 h. The combination of these parameters along with the use of 16.9% (*w/w*) of okara powder provided a sourdough with the capacity to fully inhibit the growth of *Aspergillus flavus*, *Penicillium* spp. and *Bacillus* spp.

Each mold and spoilage bacteria strains used as the indicators in this study were fully inhibited by specific sourdoughs prepared under certain combinations of factors obtained by the RSM model. In fact, in this work it was essential to identify the most important fermentation factors, and their most favorable amounts and combinations in order to maximize the sourdough's overall antimicrobial properties and its content of the studied compounds with potential bioactive and/or technological functionalities, viz. organic acids, flavonoids and polyphenolic compounds [3,72,73].

Table 4. Experimental results of the total titratable acidity (TTA) and inhibition ratio (*I*, %, defined by the Equation (2)) obtained by Response Surface Methodology (RSM). The meaning of the selected factors *A* to *F* is given in Table 2.

Run	Factors						Responses				
	<i>A</i>	<i>B</i>	<i>C</i>	<i>D</i>	<i>E</i>	<i>F</i>	TTA, mL NaOH 0.1N	Antifungal Activity against <i>A. niger</i> I, % ¹	Antifungal Activity against <i>A. flavus</i> I, % ¹	Antifungal Activity against <i>Penicillium</i> spp. I, % ¹	Antibacterial Activity against <i>Bacillus</i> spp. I, % ¹
1	250.00	4.00	0.00	0.00	45.00	24.00	9.30	−35.00	24.09	42.50	18.00
2	250.00	0.00	4.00	0.00	45.00	24.00	10.50	−57.00	4.09	27.50	30.00
3	250.00	4.00	4.00	0.00	45.00	96.00	20.00	−34.38	11.14	28.75	27.50
4	475.00	2.00	2.00	5.00	35.00	145.62	22.00	15.00	56.82	67.50	78.00
5	475.00	2.00	2.00	5.00	35.00	60.00	26.00	40.00	81.82	100.00	100.00
6	475.00	2.00	6.75	5.00	35.00	60.00	30.00	100.00	75.91	100.00	100.00
7	250.00	4.00	4.00	10.00	45.00	24.00	23.00	40.00	61.82	62.50	42.00
8	250.00	4.00	4.00	10.00	25.00	96.00	24.00	70.00	66.36	62.50	100.00
9	475.00	2.00	2.00	5.00	35.00	60.00	24.00	40.00	63.18	66.25	20.00
10	−60.14	2.00	2.00	5.00	35.00	60.00	0.00	0.00	0.00	0.00	0.00
11	700.00	0.00	0.00	0.00	25.00	96.00	2.00	−14.00	43.64	2.00	−55.00
12	700.00	0.00	4.00	0.00	25.00	24.00	9.00	32.00	35.00	20.00	36.00
13	475.00	2.00	2.00	16.89	35.00	60.00	28.05	60.00	100.00	100.00	100.00
14	700.00	0.00	0.00	10.00	25.00	24.00	9.00	−36.00	11.82	−32.79	−61.00
15	700.00	4.00	4.00	10.00	45.00	96.00	29.00	0.00	31.06	50.00	63.00
16	700.00	4.00	0.00	0.00	25.00	24.00	10.00	30.00	45.91	40.00	73.00
17	700.00	4.00	4.00	0.00	25.00	96.00	22.00	55.00	49.09	41.25	100.00
18	250.00	0.00	0.00	10.00	25.00	96.00	8.00	30.00	20.91	−28.45	−7.00
19	700.00	4.00	4.00	0.00	45.00	24.00	20.00	8.00	50.00	68.75	54.17
20	475.00	2.00	2.00	5.00	35.00	60.00	24.00	56.00	60.45	65.00	83.33
21	475.00	2.00	2.00	−6.89	35.00	60.00	23.00	40.00	62.27	77.50	95.83
22	250.00	0.00	0.00	10.00	45.00	24.00	10.00	−35.00	−9.09	−23.32	−24.17
23	700.00	4.00	0.00	0.00	45.00	96.00	14.00	18.50	47.73	56.25	55.00
24	475.00	2.00	2.00	5.00	35.00	60.00	15.00	2.00	100.00	38.75	72.00
25	250.00	4.00	0.00	10.00	45.00	96.00	9.00	−9.00	−18.64	27.50	14.00
26	1010.14	2.00	2.00	5.00	35.00	60.00	15.00	15.00	46.82	38.92	100.00
27	250.00	0.00	4.00	10.00	45.00	96.00	20.00	−30.00	0.00	33.75	83.00
28	250.00	0.00	4.00	0.00	25.00	96.00	19.00	58.00	59.55	70.00	100.00
29	475.00	2.00	2.00	5.00	35.00	60.00	26.00	43.00	50.00	68.75	90.00
30	250.00	4.00	4.00	0.00	25.00	24.00	9.00	25.00	32.73	38.75	38.33
31	700.00	0.00	4.00	10.00	45.00	24.00	21.00	20.00	45.15	41.25	47.50

Table 4. Cont.

Run	Factors						Responses				
	A	B	C	D	E	F	TTA, mL NaOH 0.1N	Antifungal Activity against <i>A. niger</i> I, % ¹	Antifungal Activity against <i>A. flavus</i> I, % ¹	Antifungal Activity against <i>Penicillium</i> spp. I, % ¹	Antibacterial Activity against <i>Bacillus</i> spp. I, % ¹
32	475.00	2.00	2.00	5.00	35.00	60.00	24.00	45.00	55.91	61.25	81.67
33	700.00	4.00	0.00	10.00	25.00	96.00	25.00	20.00	58.64	61.25	100.00
34	250.00	4.00	0.00	10.00	25.00	24.00	10.00	10.00	38.18	33.75	−10.00
35	475.00	2.00	2.00	5.00	35.00	−25.62	9.00	3.00	37.73	41.25	60.00
36	250.00	4.00	0.00	0.00	25.00	96.00	19.00	75.00	76.82	70.00	100.00
37	250.00	0.00	0.00	0.00	45.00	96.00	7.00	−20.00	31.82	−21.38	−16.00
38	475.00	2.00	2.75	5.00	35.00	60.00	15.00	36.00	56.82	52.62	63.33
39	475.00	2.00	2.00	5.00	35.00	60.00	25.00	88.00	62.73	67.50	100.00
40	475.00	2.75	2.00	5.00	35.00	60.00	23.00	40.00	62.27	35.75	90.00
41	250.00	0.00	4.00	10.00	25.00	24.00	10.00	−2.00	27.27	43.75	50.83
42	475.00	6.75	2.00	5.00	35.00	60.00	27.00	44.00	69.55	87.50	100.00
43	700.00	0.00	4.00	0.00	45.00	96.00	20.00	20.00	40.91	40.00	54.17
44	475.00	2.00	2.00	5.00	35.00	60.00	25.00	50.00	50.45	81.25	91.67
45	700.00	4.00	0.00	10.00	45.00	24.00	10.00	−36.00	11.36	31.25	59.17
46	700.00	0.00	0.00	0.00	45.00	24.00	7.00	−11.00	−6.82	−21.56	−40.00
47	700.00	0.00	4.00	10.00	25.00	96.00	25.00	15.00	72.73	55.00	100.00
48	250.00	0.00	0.00	0.00	25.00	24.00	7.00	−21.00	15.91	−35.93	−17.00
49	475.00	2.00	2.00	5.00	58.78	60.00	11.00	2.50	0.00	36.25	58.00
50	475.00	2.00	2.00	5.00	35.00	60.00	25.00	60.00	61.82	67.50	100.00
51	475.00	2.00	2.00	5.00	11.21	60.00	5.00	7.00	38.64	43.75	60.00
52	700.00	0.00	0.00	10.00	45.00	96.00	10.00	−35.00	−11.36	−31.67	−60.00
53	700.00	4.00	4.00	10.00	25.00	24.00	12.00	32.00	25.00	25.00	34.00

¹ Negative values resulting from the calculation of the inhibition ratio. These values have statistical relevance for the mathematical modelling.

Regarding the TTA, the impact of each independent variable on its value can be determined by the mathematical model expressed in Equation (5) and obtained via RSM:

$$TTA = -46.500 + 0.051A + 1.270B - 1.690C - 0.050D + 2.338E + 0.305F - 0.032B^2 - 0.143C^2 - 0.002D^2 - 0.031E^2 - 0.001F^2 + 0.001AB + 0.001AC + 0.002AE - 0.170BC - 0.040BD - 0.021BE + 0.014BF + 0.059CD + 0.074CE + 0.019CF - 0.001DE - 0.004EF \quad (5)$$

The significant interaction effects on TTA ($p < 0.05$) were identified, on the one hand, between variables C and E (*L. parabuchneri* MIUG BL 24 strain's inoculum (% v/w), and the fermentation temperature, °C), as it is shown in Table S6 for the ANOVA results. The TTA values increased along with the increment of the inoculum volume of *L. parabuchneri* MIUG BL 24 strain. The maximum TTA values could be reached when the inoculum volumes above 6.74 (% v/w) of *L. parabuchneri* MIUG BL 24 strain were used simultaneously with temperatures ranging between 35.90 and 47.92 °C (Figure 1a,b). On the other hand, a significant interaction effect was identified between variables E and F (fermentation temperature, °C and fermentation time, h, respectively) for the TTA. A prolonged fermentation time determined a high level of TTA, whereas a minimized response was observed at a fermentation temperature below 21 °C or above 45 °C (Figure 1c,d).

Figure 1. Surface (left) and contour (right) plots for the two-way interactions between the significant fermentation factors on the total titratable acidity (TTA, mL NaOH 0.1N). Surface (a) and contour (b) plots for the interaction between the factors C and E, and (c) surface and (d) contour plots for the interaction between the factors E and F. The mean of the factors A to F is given in Table 2.

As displayed in Table S6 the regression coefficient (R^2) for the mathematical model of TTA was 88%, showing that the model fits very well with the experimental values and is in agreement with the P-value obtained for the lack of fit ($p > 0.05$).

(2) Inhibitory Effects

Regarding the microbial growth inhibition studies, the *Aspergillus niger* strain's growth was significantly influenced ($p < 0.05$) only by the linear effect of the sourdough's fermenta-

tion variables B , C and E (Table 2) by their quadratic effects A^2 , E^2 , F^2 , respectively. Table S6 highlighted the good fit of the above-mentioned model to the experimental results, proven by the P-value for the lack of fit (0.25) and, concurrently, by the regression coefficient higher than 70% ($R^2 = 0.72$). The regression Equation (6) obtained for the inhibition against *Aspergillus niger* strain by RSM was as follows:

$$I, \% = -174.000 + 0.127A + 20.300B + 5.200C + 1.350D + 5.710E + 2.333F - 1.012B^2 + 0.137C^2 - 0.105D^2 - 0.106E^2 - 0.008F^2 - 0.006AB + 0.012AC - 0.007AD + 0.004AE - 0.590BC + 0.140BD - 0.242BE - 0.006BF + 0.475CD - 0.097CE - 0.062CF + 0.079DE - 0.020DF - 0.019EF \quad (6)$$

Furthermore, the mathematical model corresponding to the inhibitory effect of the formulated sourdoughs against *Aspergillus flavus* strain was expressed by the Equation (7):

$$I, \% = -143.200 + 0.143A + 10.900B - 2.640C + 0.930D + 6.830E + 1.628F - 0.592B^2 - 0.572C^2 + 0.013D^2 - 0.106E^2 - 0.004F^2 - 0.004AB + 0.004AC + 0.001AE - 1.131BC + 0.009BD + 0.028BE - 0.044BF + 0.700CD + 0.186CE - 0.030CF - 0.034DE - 0.026DF - 0.023EF \quad (7)$$

A significant interaction effect on the antifungal activity of the formulated sourdoughs against *Aspergillus flavus* strain was perceived between the factors' fermentation time and temperature. It can be concluded, from the surface (Figure 2a) and contour (Figure 2b) plots, that a fermentation temperature higher than 44 °C would minimize the antifungal effects of the gluten-free fermented products against *Aspergillus flavus* strain, which is obviously related to the effect of the temperature on the LAB's activity and growth-rates.

Figure 2. Surface (a) and contour (b) plots for the two-way interactions between the significant fermentation variables E and F on the antifungal activity (inhibition ratio, %) against *Aspergillus flavus*. The mean of the factors A to F is given in Table 2.

Regarding the inhibitory effect of the formulated sourdoughs against *Penicillium* spp. and *Bacillus* spp. indicator strains, the inhibition effects induced by varying the factors B and C (Table 2) in their 2-way interaction were shown in Figures 3 and 4. For the antifungal activity against *Penicillium* spp. indicator strain the calculated regression Equation (8) was:

$$I, \% = -193.200 + 0.200A + 20.250B + 16.750C + 0.520D + 6.400E + 1.517F - 1.261B^2 - 0.612C^2 - 0.010D^2 - 0.089E^2 - 0.005F^2 - 0.005AC + 0.001AD + 0.001AE - 3.979BC - 0.029BD + 0.069BE - 0.020BF + 0.416CD + 0.073CE - 0.020CF - 0.002DE - 0.010DF - 0.021EF \quad (8)$$

Consequently, a full inhibition against *Penicillium* spp. indicator strain was determined by the sourdoughs inoculated with a co-culture containing above 5% (v/w) of *L. paracasei* ssp. *paracasei* MIUG BL 21 strain and up to 0.20% (v/w) of *L. parabuchneri* MIUG BL 24 strain. Similarly, the total inhibition is likely to be reached when a maximum of 1.10% (v/w), of *L. paracasei* ssp. *paracasei* MIUG BL 21 strain together with no more than 6.68% (v/w) of *L. parabuchneri* MIUG BL 24 strain inoculum was employed in the biotechnological process of the sourdough's SSF fermentation (Figure 3a,b).

Furthermore, *Bacillus* spp. strain growth was totally inhibited by two formulated gluten-free sourdough samples: (i) the sample fermented by the co-culture containing at least 3.76% (v/w) of *L. parabuchneri* MIUG BL 24 strain and maximum 2.35% (v/w) of *L. paracasei* ssp. *paracasei* MIUG BL 21 strain, or (ii) the sourdough sample fermented with a minimum volume of 4.39% (v/w) of *L. paracasei* ssp. *paracasei* MIUG BL 21 strain combined with maximum 2.21% (v/w) of *L. parabuchneri* MIUG BL 24 strain (Figure 4a,b).

Figure 3. Surface (a) and contour (b) plots for the two-way interactions between the significant fermentation variables B and C, on the antifungal activity (inhibition ratio, %) against *Penicillium* spp. The mean of the factors A to F is given in Table 2.

Figure 4. Surface (a) and contour (b) plots for the two-way interactions between the significant fermentation variables B and C, on the antibacterial activity (inhibition ratio, %) against *Bacillus* spp. The mean of the factors A to F is given in Table 2.

Resorting to the obtained experimental data, the following Equation (9) was attained for the antibacterial activity of the sourdoughs against *Bacillus* spp. strain as follows:

$$I, \% = -202.000 + 0.163A + 16.400B + 22.600C - 3.290D + 7.550E + 2.061F - 0.800B^2 - 1.390C^2 - 0.108D^2 - 0.096E^2 - 0.006F^2 + 0.027AB + 0.001AD + 0.001AE - 5.720BC - 0.315BD - 0.203BE + 0.032BF + 0.585CD - 0.064CE + 0.070CF + 0.069DE + 0.014DF - 0.031EF // \quad (9)$$

It can be concluded that each selected starter culture has a different fermentation behavior when composed of a co-culture of these LAB strains (Figure 4). Additionally, the inoculum size possesses a strong influence on the fermented product's composition and properties.

Different culture medium constituents, fermentation temperatures and incubation times or inoculum sizes were screened via Plackett–Burman Design (PBD), Response Surface Methodology (RSM) or Box–Behnken Design (BBD) to identify the most suitable conditions for the specific enzyme biosynthesis [63,74]. The statistical studies for the

process optimization are very common in food science and other biotechnological fields, to decrease the number of time-consuming experimental runs, costs and technical resources. In particular, various factors were screened by PBD and RSM in order to establish those with significant effects on the TTA levels of a sourdough fermented with *L. paracasei* ssp. *paracasei* [75]. A stable matrix able to ensure the *L. curvatus* viability during storage was optimized by RSM, being successfully used in the sourdough technology [76]. Moreover, the variation of the dough yield between 175–225 was statistically studied to conclude that this factor positively affects the textural characteristics of a sourdough cake [77]. Sourdough bread's physicochemical and textural properties (viz. TTA, porosity, chewiness and hardness) were optimized after the RSM analysis by Abedfar et al. (2019) [78]. The regression coefficients reported in the above-mentioned study varied between 43.4–97.9% and 29.5–95.2% for R^2 and adjusted R^2 (R^2_{adj}), respectively. In our RSM analyses, values in the same range were attained (viz. R^2 71.4–87.7% and R^2_{adj} 40.4–74.4%).

The antifungal effect of LAB metabolites has been intensively studied by several researchers to conclude that the fungal growth is related to multiple biotechnological factors. The most important factors have been claimed to be the type of exogenous LAB starter strains used for fermentation and their initial concentration. *Aspergillus flavus*, *Aspergillus niger* and *Aspergillus tubingensis* indicator strains were successfully inhibited by *Lactobacillus* spp. and *Leuc. mesenteroides* strains studied by Ouidir et al. [67] and Çakır et al. [79]. In addition, the fungal contamination of bread by *Penicillium* spp. strains was delayed by 3 days when a whole wheat sourdough inoculated with selected strains of *L. plantarum* was used [56]. The same effect was observed when 30% (*w/w*) of a commercial sourdough was used [80] or essential oils from aromatic plants [81], respectively, when a mixture of eugenol-citral was added to the wheat bread doughs [82].

A delayed bacterial spoilage determined by *Bacillus subtilis*, *B. cereus* and *B. licheniformis* was observed after the in vitro assays using *Lactobacillus* spp. strains for the sourdough bread making [79,83]. Finally, the study conducted by Pereira et al. [84] led to the conclusion that bread roty spoilage can be effectively delayed by up to 3 days when the moisture content and pH value are controlled. The scientific works mentioned above reinforce the LAB strains' effects that contribute to sustainability and innovation in the production of food and feed that positively affect quality of life [85]. Moreover, sustainable approaches were reported by some authors regarding the utilization of the microorganisms from the sourdough microbiota as starters for other fermentation processes, results with positive impact on the economic efficiency and diversification of the products [86,87]. Thus, the stabilized sourdoughs could be used as artisanal starters for the fermentation processes associated with the agrifood byproducts bio-valorization through multiple bioprocessing techniques (fermentations, bioconversions) that support the circular economy and the environmental protection [88,89]. Finally, the postbiotics produced by LAB have multiple implications in the food and feed preservation and safety assurance. The in vivo effects of the fermented products as a valuable source of biotics (prebiotics, postbiotics) are intensively studied regarding the health effects on both humans and animals, considering also the microbiome complexity and functionality [90].

In the current study, the RSM model was validated by the multi-response optimization tool, considering all the analyzed response variables (see Table 2). Based on the mathematical modelling studies, the following optimal combination of independent variables was established: 475.0 dough yield; co-inoculation of 2.9% (*v/w*) of *L. paracasei* ssp. *paracasei* MIUG BL 21 strain and 5.0% (*v/w*) of *L. parabuchneri* MIUG BL 24 strain; 16.9% (*w/w*) of okara; 31.4 °C fermentation temperature; and 66.1 h of fermentation time. The results for the considered responses under the optimized conditions are listed in Table 5. This optimized sourdough variant was experimentally produced, and the parameters analyzed. These experimental results excellently fit with the predicted ones (Table 5), as proved by the composite desirability (94%)—which allowed the validation of the statistically optimized model [91]. Such value for the composite desirability is comparable to that of 98% obtained in a study for the formulation of gluten-free noodles [42].

Table 5. Predicted versus experimental results for the optimized sourdough. Inhibition ratios obtained according to Equation (2).

Response	Predicted Value	Experimental Value
TTA, mL NaOH 0.1N	33.12	40.21 ± 2.16
Antifungal activity against <i>Aspergillus niger</i> I, %	70.20	71.42 ± 1.00
Antifungal activity against <i>Aspergillus flavus</i> I, %	94.30	95.00 ± 0.50
Antifungal activity against <i>Penicillium</i> spp. I, %	94.70	95.71 ± 0.70
Antibacterial activity against <i>Bacillus</i> spp. I, %	100.00	100.00 ± 0.00
Composite desirability	0.94	

Our results for the antimicrobial properties and TTA (Table 5) are in agreement to those reported in the literature. Particularly, inhibition ratios between 20 and 60% against *Aspergillus* spp. and *Penicillium* spp. indicator strains were determined for a chickpea sourdough [28], whereas a TTA value of 43.4 was obtained for a hemp sourdough fermented for 24 h at 30 °C [92].

The satisfactory results, determined by the optimized gluten-free formulation from our work, suggest that this fermented product, obtained in controlled SSF fermentation conditions involving selected strains of LAB, has valuable properties with impact on enhancing the antimicrobial and technological properties of the gluten-free sourdough bread that will be assessed in the future.

3.2. The Organic Acids, Flavonoids and Polyphenolic Compounds Bioactive Composition of the Optimized Sourdough

Under the optimal fermentation conditions, the resulting gluten-free sourdough was subjected to chromatographic analysis, by RP-HPLC-DAD, to investigate its profile of organic acids, flavonoids and polyphenolic compounds, as depicted in Figures 5–7. Following the established optimized variant, the LAB strains involved in the fermentation of the complex substrate made of chickpea, quinoa, buckwheat and okara increased the amount of lactic (peak 3) and propionic acids (peak 6), after 66 h of fermentation at 31.4 °C (Figure 5a), in comparison to the unfermented sample (Figure 5b). On the other hand, based on the peak's intensity, the optimized fermented sample showed a profile where a slight increase of the amount could be observed for the non-identified peaks 7 and 8 in Figure 5a, when compared to the same peaks in Figure 5b. Taking into consideration the organic acids that eluted from the fermented sourdough (Figure 5a), it can be stated that the mixed inoculum of the selected LAB starter used for fermentation displayed a heterofermentative metabolism that could increase the antimicrobial and technological features of baked goods [93].

A dairy product fermented by *L. rhamnosus* showed antimicrobial properties due to the acetic, lactic, citric, propionic and succinic acids synthesized in the fermented medium [94]. Additionally, malic, tartaric and fumaric acids were identified in a wheat-soybean sourdough fermented with different mixed starters of *Lactobacillus* spp. [95]. It is well-known that the amount and type of organic acids found after the fermentation of different matrices depend largely on the inoculum strain(s) and the composition of the fermentation substrate [72,96]. The relatively high level of organic acids eluted as peaks 1 and 2 in the unfermented sourdough (Figure 5b) proves that it originated from the flours, as it was reported in some works [97,98]. Indeed, the accumulation of organic acids in the pseudocereal and legume seeds is a stress adaptation to inadequate environmental conditions [99].

Figure 5. RP-HPLC-DAD ($\lambda = 210$ nm) chromatograms for the organic acids in the optimized sourdough (a) and unfermented optimized sample (b), according to the elution pattern and chromatographic conditions described in Section 2.4. The injection volume (IV) was 20 μ L and the concentration of the sample was 400 mg/mL. Peak's identification: 3—lactic acid; 5—acetic acid; 6—propionic acid.

Figure 6. RP-HPLC-DAD ($\lambda = 280$ nm) chromatograms for the flavonoids and polyphenols in the optimized sourdough (a), and the unfermented optimized dough (b), according to the elution pattern and the chromatographic conditions described in Section 2.4. The injection volume (IV) was 10 μ L and the concentration of the sample was 400 mg/mL. Peak's identification: 9—caffeic acid; 10—vanillic acid; 12—ferulic acid; 8'—chlorogenic acid; 9'—caffeic acid; 10'—vanillic acid; 23'—quercetin; 24'—gallic acid; 26'—epicatechin.

The composition of the optimized gluten-free sourdough was also investigated by RP-HPLC-DAD and compared to the unfermented sourdough, to observe the differences in the composition of the most important bioactive compounds. The lactic acid fermentation process clearly modified the concentration of the flavonoids and polyphenols determined at wavelengths of 280 nm (Figure 6) and 320 nm (Figure 7), respectively. In the uninoculated sourdough sample, 27 bioactive compounds were separated (Figures 6b and 7b), showing a higher diversity of these compounds when compared to the fermented sourdough (Figures 6a and 7a). The gallic acid content was higher than quercetin in the unfermented sample (Figures 6b and 7b). In contrast, more than 10 flavonoids and polyphenols can be observed in the chromatograms presented in Figures 6a and 7a. In the fermented optimized sourdough, only some bioactive compounds with the highest peaks' intensity could be separated from the baseline. The peaks' intensities corresponding to caffeic and vanillic acids were higher in the control sample, their maximum absorbances being identified at 280 nm. On the other hand, ferulic acid was eluted at its maximum intensity in the fermented sourdough when the wavelength of 320 nm was used. The high number of

peaks eluted between 4–19 min (Figure 6a), corresponding to ferulic, gallic, cinnamic acid or quercetin derivatives, that were the effect of the metabolic activities of the selected LAB used as starter cultures, were preliminarily identified by comparing the retention times to those reported in the literature [100,101]. Similarly, in the unfermented sample (Figure 6b), the *p*-coumaric acid (peak 11'), vanillic (peak 12') and caffeic acid (peak 16') derivatives were identified [102,103]. The acidic conditions of the fermented medium and other microbial enzymes may significantly affect the composition of flavonoids and polyphenols in the final sourdough, thus resulting in structural changes. Another reason for this difference between the uninoculated and fermented sourdoughs can be related to the ratio between the bounded and free phenolic compounds and flavonoids, the first ones being more stable. The concentration of the phenolic acids in some gluten-free cereals was studied by Zeng et al. [104] using HPLC methods, to conclude that the content of bounded phenolic acids was higher than the free phenolics.

Figure 7. RP-HPLC-DAD ($\lambda = 320$ nm) chromatograms for the flavonoids and polyphenols in the optimized sourdough (a), and the unfermented optimized dough (b), according to the elution pattern and the chromatographic conditions described in Section 2.4. The injection volume (IV) was 10 μ L and the concentration of the sample was 400 mg/mL. Peak's identification: 4—vanillic acid; 9—ferulic acid; 5'—chlorogenic acid; 7'—vanillic acid; 13'—ferulic acid; 23'—quercetin, 24'—gallic acid.

Flavonoids (quercetin, catechin and kaempferol), tannins (proanthocyanidins), phenolic acids (caffeic, chlorogenic, ferulic, gallic, vanillic) and their derivatives were identified in buckwheat [105,106], quinoa, and chickpea seeds [107–109]. The intensity of each compound is different based on the wavelength used for the HPLC data acquisition [110], our results being supported by these above-mentioned works.

The antioxidant properties of pseudocereals and legumes are related to the applied processing methods [111,112]. Consequently, it was highlighted by Han et al. [113] that the levels of saponins and antinutritional factors depend on the milling process of quinoa. Furthermore, the antioxidant properties of buckwheat were modified after ultrasound treatment [114] whereas the importance of the HPLC elution solvent (acetonitrile) for the extraction of isoflavones from okara was stated by Nile et al. [115].

Jiang et al. [116] and Zhang et al. [117] affirmed that the molecular complexes between bioactive compounds and starch or proteins from flours can be formed in food systems, and their stability and properties are affected mainly by the pH, temperature and nutritional composition of the matrix [118,119]. The above-mentioned conclusion would probably be the reason why the bioactive composition of our samples was different, along with the fact that the fermentation substrate was obtained by combining several flours, with different biologically active compound profiles.

Finally, it is obvious that different chromatographic profiles of the fermented optimized sourdough and control sample (unfermented sourdough) were obtained in this work, such changes being justified by the specific metabolic activity of the selected LAB strains co-culture (*L. paracasei* ssp. *paracasei* MIUG BL 21 strain and *L. parabuchneri* MIUG BL 24 strain), in correlation with intrinsic and extrinsic factors' variation, under the optimized fermentation conditions established by RSM. Some researchers found that *Lactobacillus* spp. strains can synthesize enzymes (oxidases, esterases, decarboxylases and dehydrogenases) able to destabilize specific complexes and affect the individual structures of the polyphenolic compounds [120,121].

These results confirm that, by RSM, the interactions between the previously selected independent variables were established in order to determine the optimal conditions to achieve a gluten-free sourdough with enhanced antimicrobial properties against spoilage molds and bacteria, correlated to the organic acids content, phenolic content and functional properties.

4. Conclusions

The most important biotechnological parameters for an unconventional gluten-free sourdough obtained by a controlled solid-state fermentation process were analyzed through the mathematical modelling and statistical analysis support offered by Plackett–Burman Design and Response Surface Methodology tools.

Starting from a complex sterile substrate composition based on chickpea, quinoa, and buckwheat flours supplemented with okara (the solid residue of soymilk preparation), using as starter cultures the previously selected strains (three lactobacilli and a *Leu. mesenteroides* ssp. *mesenteroides*, respectively) and by varying the fermentation conditions according to the established design of the experiments, the most important parameters which impacted the fermented product's characteristics were determined. It was demonstrated that, following the designed biotechnological conditions, the variables with significant effects on the sourdough preparation are: the dough yield, *L. paracasei* ssp. *paracasei* MIUG BL 21 and *L. parabuchneri* MIUG BL 24 strains' inoculum type and size, the amount of okara, the temperature and the time of fermentation, respectively. As such, the proposed fermentation parameters for the optimal sourdough's production established by RSM analysis are: the dough yield 475.0, 16.9% (w/w) okara, 2.9% (v/w) *L. paracasei* ssp. *paracasei* MIUG BL 21 strain inoculum, 5.0% (v/w) *L. parabuchneri* MIUG BL 24 strain inoculum, 66.1 h of fermentation at 31.4 °C.

The synergistic effect of the starter co-culture of *L. paracasei* ssp. *paracasei* MIUG BL 21 and *L. parabuchneri* MIUG BL 24 strains act in the controlled fermentation conditions towards the conversion of the flour's constituents into the desired postbiotics, mainly organic acids, flavonoids, polyphenols and their derivatives, with a great impact on the antimicrobial properties. The bioactive content and the antimicrobial properties of the optimized sourdough are valuable advantages to recommend this product as a bioingredient for food or feed formulations in order to improve their microbiological stability.

Supplementary Materials: The following are available online at <https://www.mdpi.com/article/10.3390/app11115306/s1>, Table S1: ANOVA for the Total Titratable Acidity (TTA, mL NaOH 0.1N) from the PBD. The meaning of the factors A to H is given in Table 1; Table S2: ANOVA for the antifungal activity of the sourdoughs, measured as the inhibition effect (I, %, Equation (2)), against *Aspergillus niger* from the PBD. The meaning of the factors A to H is given in Table 1; Table S3: ANOVA for the sourdoughs' antifungal activity, measured as the inhibition effect (I, %, Equation (2)), against *Aspergillus flavus* from the PBD. The meaning of the factors A to H is given in Table 1; Table S4: ANOVA for the sourdoughs' antifungal activity, measured as the inhibition effect (I, %, Equation (2)), against *Penicillium* spp. from the PBD. The meaning of the factors A to H is given in Table 1; Table S5: ANOVA for the sourdoughs' antibacterial activity, measured as the inhibition effect (I, %, Equation (2)), against *Bacillus* spp. from the PBD. The meaning of the factors A to H is given in Table 1; Table S6: ANOVA for the Total Titratable Acidity (TTA, mL NaOH 0.1N), antifungal activity of the sourdoughs against *Aspergillus niger*, *Aspergillus flavus*

and *Penicillium* spp., and antibacterial activity against *Bacillus* spp., measured as inhibition effect (I , %, Equation (2)) from RSM. The meaning of the factors A to F is given in Table 2.

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