„Dunărea de Jos” University of Galați

Doctoral School of Fundamental Sciences and Engineering

PHD THESIS

SUMMARY

Modern approaches in lactic acid bacteria starter cultures’ obtainment to be used in bakery, with technological and functional impact

PhD student,

Bogdan PĂCULARU-BURADA

Scientific coordinator,

Prof.univ.dr.eng. Gabriela-Elena BAHRIM

Series I 1. BIOTEHNOLOGIES No. 15
GALAȚI
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Introduction

The lactic acid bacteria are studied and used to obtain fermented products with specific technological, functional, and sensorial characteristics, for the diversification, preservation, and food safety's assurance of food products and food ingredients. The metabolic properties of the lactic acid bacteria starter cultures', correlated with the intrinsic and extrinsic parameters of the fermentation processes influence the fermented products' quality and the metabiotic potential (prebiotics, probiotics, postbiotics, and paraprobiotics) (Ranadheera *et al.*, 2022; Singhal *et al.*, 2021).

The postbiotic compounds biosynthesized by lactic acid bacteria are intensively studied in the scientific literature for their technological and functional properties, among postbiotics being included any organic and water-soluble compound resulting from a fermentation process, as well as cellular components originated from lactic acid bacteria (Shafipour Yordshahi *et al.*, 2020). Therefore, organic acids (short-chain fatty acids), exopolysaccharides, vitamins, and bioactive peptides are considered postbiotics when they exert proven beneficial effects for some diseases' treatment and consumers' health (Arora *et al.*, 2021).

Numerous scientific studies highlight the pseudocereals' and legumes' capacity (amaranth, quinoa, chia, buckwheat, millets, chickpea, beans, soybean), unexploited enough in food industry, to partially or totally substitute the conventional wheat flour in bakery products, the main benefits being associated to the lack of gluten, an enriched and balanced amount of proteins, amino acids, unsaturated fats, and minerals (Yaver and Bilgiçli, 2019). By lactic acid fermentation and sourdoughs production the nutritional and sensorial properties of the gluten-free raw materials are improved, the biochemical modifications of the fermentation substrates induced by the selected lactic acid bacteria strains contribute, as well, to the obtainment of fermented products with enhanced functionality (Garrido-Galand *et al.*, 2021).

Furthermore, the diversification of gluten-free bread products supplemented with sourdoughs, obtained by a controlled or spontaneous fermentation of some unconventional raw materials, respectively by using unconventional artisanal cultures for bakery (milk kefir grains and water kefir grains), leads to shelf-life's extension, improved textural properties and consumers' health improvements (Comasio *et al.*, 2019; Ito and Arai, 2021).

Taking into consideration the variety of valuable microbial strains which can be isolated and selected based on specific biotechnological properties, depending on the desired objectives, there are nowadays multiple ways for the biovalorification of lactic acid bacteria and unconventional flours, as fermentation substrates, to obtain value-added fermented products, promoting the modern principles of circular economy, improvement of life's quality and environmental protection.

The doctoral thesis entitled ”***Modern approaches in lactic acid bacteria starter cultures' obtainment to be used in bakery, with technological and functional impact***” aimed at isolating some performant lactic acid bacteria strains and select them, along with the study of some artisanal cultures (kefir grains) with specific technological and functional properties, for further utilization in the obtainment of gluten-free sourdoughs and unconventional bakery products, with extended microbiological stability, rich in postbiotic compounds with superior functionality.

The research carried out during the doctoral studies aimed the following scientific objectives:

- Isolation of some lactic acid bacteria strains originated from agri-food matrices and diverse ecosystems, evaluation of their capacity to ferment gluten-free substrates based on chickpea, quinoa, and buckwheat flours, by analyzing the postbiotics' antimicrobial spectrum, in different pH and temperature conditions, against baking industry's spoilage microorganisms (*Aspergillus niger*, *Aspergillus flavus*, *Penicillium* spp., and *Bacillus* spp), as well as the study of the probiotic character and phylogenetic analysis for the performant lactic acid bacteria strains.
- Identification of the most important biotechnological parameters to obtain a gluten-free sourdough with enhanced functionality, by mathematical modelling and statistical analysis.
- Utilization of artisanal cultures (kefir grains) in the gluten-free sourdoughs fermentation process, using chickpea, quinoa, buckwheat, and okara, and a comparative analysis of the sourdoughs' properties, obtained by a mixed fermentation with multiple microorganisms from the water and milk kefir grains' consortia, in order to select the most competitive artisanal starters for utilization in gluten-free sourdoughs production.
- Obtaining some gluten-free bakery products supplemented with sourdoughs and evaluation of the modifications that occur, regarding the technological and functional properties, when flours are substituted with sourdoughs in the bread making recipe.

The doctoral thesis is structured in two sections, as following:

I. DOCUMENTARY STUDY, entitled “***Modern approaches about lactic acid bacteria implications in sourdough obtainment for applications in bakery***” presents, in one chapter, structured in five subchapters, recent researches in the field of sourdoughs' obtainment and use, highlighting the nutritional composition of the unconventional raw materials such as pseudocereals and legumes. There are systematized, also, the metabolic characteristics of lactic acid bacteria starter cultures' and the methods which can improve the strains' capacity to biosynthesize postbiotic compounds, with positive impact on the bakery products supplemented with sourdoughs.

II. EXPERIMENTAL STUDY presents the results of the original investigations realized during the doctoral studies, being structured in four chapters, respectively:

Chapter 2 entitled "***Isolation, characterization, and selection of some lactic acid bacteria strains to be used in unconventional technologies in bakery***" presents the isolation techniques of the lactic acid bacteria from different natural sources, their metabolic diversity, and the effect of individual strains' cultivation, evaluated by principal component analysis, on the acidification capacity, antimicrobial potential of the fermented cell-free supernatants after pH value adjustment or thermal treatment. Furthermore, it was carried out a comparative study on the exopolysaccharides' extraction methods, biosynthesized by selected lactic acid bacteria strains, on the biopolymers' yield and their antioxidant properties, the probiotic character of the selected lactic acid bacteria and the phylogenetic characterization being also evaluated.

"***Optimization of the fermentation conditions to obtain a gluten-free sourdough fermented with selected lactic acid bacteria strains***" presented in **Chapter 3** aimed at identifying the biotechnological parameters with significant impact on a lactic acid fermented gluten-free sourdough, using the Plackett-Burman and Response Surface Methodology mathematical modelling techniques, that allowed the study of the interactions between the independent variables (dough yield, type and volume of inoculum used, amount of okara added or the temperature and time of the fermentation process), on the responses (total titratable acidity, antimicrobial activity against indicator microorganisms *A. niger*, *A. flavus*, *Penicillium* spp., and *Bacillus* spp.). After the mathematical model's validation, the resulting sourdough was characterized by enhanced technological and functional properties, and the HPLC analysis emphasized, by a qualitative analysis, the modifications induced by lactic acid fermentation regarding the concentrations of short-chain fatty acids and bioactive compounds.

The artisanal cultures, respectively the water and milk kefir grains were used and selected for some gluten-free sourdoughs production, the results presented in **Chapter 4**, entitled "***Obtaining some gluten-free sourdoughs by fermentation with kefir grains***", demonstrate these artisanal starter cultures' capacity, made of multiple consortia of beneficial microorganisms (bacteria and yeasts), to be used in order to fulfill the expected biotechnological objectives. The modifications of the microbial consortia, determined by the cultivation on the unconventional gluten-free substrates, were emphasized by scanning electron microscopy (SEM) techniques, and the quantitative analysis of the postbiotic compounds by HPLC confirmed the functionality of the obtained fermented products, correlated to the antifungal effect against the indicator mould species tested respectively *Aspergillus niger*, *Aspergillus flavus*, *Penicillium* spp.

The impact of chickpea, quinoa, and buckwheat flours' substitution with sourdoughs obtained by lactic acid fermentation with a selected co-culture comprising the selected lactic acid bacteria strains, respectively by fermentation with a selected multiple culture of kefir grains, was presented in **Chapter 5** entitled "***Testing the functionality of sourdough in unconventional bakery products***", in which it was demonstrated the biopreservative effect of the formulated sourdoughs, as well as their contribution to a controlled release of bioactive compounds with antioxidant activity and postbiotic effect after *in vitro* digestion**.**

Each chapter of the experimental study is divided in the following subchapters: *Introduction*, which presents the current trends in the studied field and the research objectives; *Materials and methods*, where the raw materials, reagents, investigation methods, experimental data analysis and interpretation are presented; *Results and discussion*, summarizes the obtained results by comparison with other similar data from the scientific literature; *Partial conclusions* and *References*.

Chapter 6, ***General conclusions***, presents the final conclusions resulting from the carried out experimental studies, which aimed at elaborating some gluten-free bakery products supplemented with sourdoughs, characterized by superior technological and functional properties, determined by the utilization of chickpea, quinoa, buckwheat, and okara flours along with the metabolic effect of some selected lactic acid bacteria strains belonging to *Lactobacillus* spp., respectively by the consortia's involvement made of bacteria and yeasts strains within the selected water kefir grains' microbiota.

The doctoral thesis consists of 213 pages, including 40 figures and 31 tables. The documentary study represents 12% and the experimental part 88%.

Finally, the ***original contributions*** of the PhD thesis are presented, which contribute to the enrichment of the fundamental and practical concepts in the studied field, the research perspectives, as well as the dissemination of the obtained results are summarized. As such, the research results were used to elaborate and publish **5 articles**, **3 articles** published in ISI journals (*Microorganisms*, *Applied Sciences*, *LWT – Food Science and Technology*) and **2 articles** published in international databases indexed journals (*The Annals of the University Dunarea de Jos of Galati, Fascicle VI – Food Technology* and *Innovative Romanian Food Biotechnology*), **2 book chapters** accepted for publication at international publishing houses, and **17 communications** at national and international representative scientific events in Biotechnology.

The research activities included in the PhD thesis were carried out by the modern research infrastructure from the *Integrated Center of Research, Expertise and*

Technological Transfer (BioAliment-TehnIA) (www.bioaliment.ugal.ro), within the Faculty of Food Science and Engineering, "Dunărea de Jos" University of Galați.

During the doctoral studies, the PhD student was involved in **3 research projects** and entrepreneurial skills' development, with convergent objectives to the PhD thesis, as follows:

- ✓ **COST ACTION 18101/**2019-2023 - *Sourdough biotechnology network towards novel, healthier and sustainable food and bioprocesses* (SOURDOMICS https://sourdomics.com/en/).
- ✓ **SMIS 123847/**2019-2020 - Academic excellence and entrepreneurial values – scholarships system to ensure opportunities for the training and development of entrepreneurial skills for PhD students and postdocs *(ANTREPRENORDOC*; http://www.antreprenordoc.ugal.ro/ro/),
- ✓ **PCE 159**/2021-2023 - *Novel transition from probiotics to metabiotics - New emerging concepts for food functionalization as a health promoting strategy* (*BIOTICS+; https://www.biotics.ugal.ro/index.php/en/*).

The doctoral thesis was realized under the scientific coordination of Prof.dr.eng. Gabriela-Elena BAHRIM, as PhD supervisor, and the committee members: Prof.dr.eng. Iuliana BANU, Associate prof.dr.biol. Vasilica BARBU, Associate prof.dr.eng. Mihaela Aida VASILE and Associate prof.dr.eng. Luminița Anca GEORGESCU.

Chapter 3. Optimization of the fermentation conditions to obtain a gluten-free sourdough fermented with selected lactic acid bacteria strains

3.1. Introduction

The spontaneous fermentation of the flours from cereals', pseudocereals', and legumes' processing, or flours made of food by-products can contribute to the diversification of the sourdoughs regarding the technological and bioactive properties. These fermented products can be used in the bread making to offer specific sensorial features, the technological, nutritional, and functional improvements induced by the fermentation process being also considered (Arora *et al.*, 2021; Gobbetti *et al.*, 2020; Novotni *et al.*, 2021). Starter cultures made of selected lactic acid bacteria strains were used to obtain sourdoughs with properties that contribute to the shelf-life extension of the bakery products, being also able to improve the textural, nutritive, and functional characteristics (Diowksz *et al.*, 2020; Sakandar *et al.*, 2019).

The technological and functional properties of the sourdoughs depend on multiple factors, the most important being represented by the metabolic particularities of the lactic acid bacteria strains used in the fermentation process, respectively by the parameters of the fermentation process (Bartkiene *et al.*, 2019; Menezes *et al.*, 2020) and the subsequent treatments applied for the fermented products (high pressure treatment, ohmic heating, ultrasonication, exposure to UV radiations) in order to enhance the effect determined by the post- or paraprobiotic compounds (Moradi *et al.*, 2020; Vallejo-Cordoba *et al.*, 2020).

The optimization studies based on diverse techniques for the experimental design and mathematical modelling (Plackett-Burman Design – PBD; Response Surface Methodology – RSM) are frequently used in the researches from the food industry and/or biotechnology (Ma and Wang, 2016).

The scientific objectives in this stage of the study aimed at identifying the influence of some biotechnological parameters on the fermentation process conducted in controlled conditions with a semi-solid fermentation system, using some gluten-free flours and selected lactic acid bacteria strains. By the experimental design, mathematical modelling and statistical analysis using the PBD and RSM techniques, the objective was to obtain a gluten-free sourdough with enhanced bioactive properties.

3.2. Materials and methods

The investigation methods were:

- optimization of the fermentation process to obtain a sourdough by using mathematical modelling and statistical analysis techniques (Plackett-Burman and Response Surface Methodology);
- evaluation of the acidification capacity by the titrimetric method;

- evaluation of the antifungal and antibacterial properties using the inhibition ratio against indicator strains;
- evaluation of the postbiotics' content from the optimized sourdough using HPLC methods.

3.3. Results and discussion

3.3.1. Optimization of the fermentation process to obtain a sourdough with improved bioactive properties

Preliminary selection of the biotechnological parameters with impact on the fermentation process

Considering these results, six independent variables were selected for the optimization of the fermentation process by RSM technique, respectively: dough yield, lactic acid bacteria strain *Lactiplantibacillus plantarum* MIUG BL 21 and *Lactiplantibacillus pentosus* MIUG BL 24 inoculum volume, okara's addition, fermentation temperature and time (Păcularu-Burada *et al.*, 2021).

Optimization of the fermentation process by response surface methodology (RSM)

The previously selected factors, respectively **A** – Dough yield, **B** – Volume of inoculum of *Lpb. plantarum* MIUG BL 21 (% v/w), **C** – Volume of inoculum of *Lpb. pentosus* MIUG BL 24 (% v/w), **D** – Okara's addition (% g/g), **E** – Fermentation time (hours), and **F** – Fermentation temperature (°C), were included in the Response Surface Methodology (RSM).

The mathematical model obtained after RSM analysis was validated by a simultaneous evaluation of all the analyzed responses, the results being presented in **Table 3.5**.

Table 3.5. Validation of the mathematical model after RSM analysis

Responses	Predicted value	Experimental value
TTA, mL NaOH 0.10 N	33.12	40.21±2.16
Antifungal activity against *A. niger* I,%	70.20	71.42±1.00
Antifungal activity against *A. flavus* I,%	94.30	95.00±0.50
Antifungal activity against *Penicillium spp.* I,%	94.70	95.71±0.70
Antibacterial activity against *Bacillus* spp. I,%	100.00	100.00±0.00
Composite desirability	0.94	-

By mathematical modelling and statistical analysis, it was identified an optimal combination of factors (independent variables) with impact on the fermented product's properties, as such: dough yield – 475; volume of inoculum of *Lpb. plantarum* MIUG BL 21 – 2.90% (v/w); volume of inoculum of *Lpb. pentosus* MIUG BL 24 – 5% (v/w); freeze-dried okara addition – 16.90% (g/g); fermentation temperature – 31.40°C, and fermentation time – 66.10 hours.

The optimized variant of the studied biotechnological parameters leads to a bioprocess characterized by a composite desirability of 94%, which confirms the statistical validation of the obtained experimental results.

3.3.2. Analysis of the postbiotic compounds from the sourdough obtained in optimized biotechnological conditions

The gluten-free sourdough obtained in optimized biotechnological conditions was subjected to the reverse phase chromatographic analysis (RP-HPLC) to highlight the content of postbiotic compounds, organic acids, phenolic acids and flavonoids with bioactive properties. The results are presented in **Figure 3.5**, **Figure 3.6** and **Figure 3.7**.

Sourdough fermentation following the established optimized conditions determined the increment of lactic and propionic acid, corresponding to the peaks 3 and 6, after 66 hours of fermentation at 31.40°C, compared to the control sample (unfermented substrate). It can be concluded that the lactic acid bacteria strains have a heterofermentative metabolism that can contribute to improve the technological and functional properties of the fermented products (Moon *et al.*, 2018).

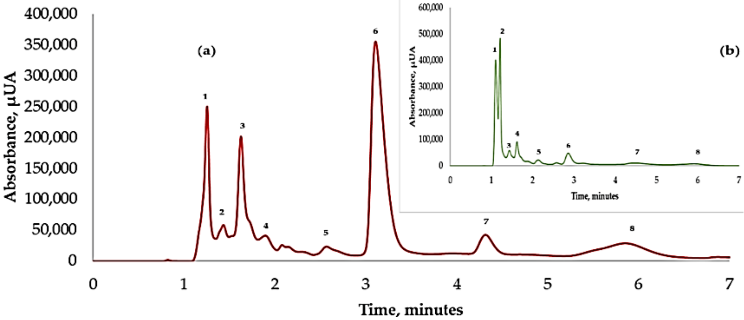

Figure 3.5. HPLC chromatograms for the separation of organic acids (λ = 210 nm) from the sourdough obtained by the optimized process (a) and control sample (unfermented medium) (b), using an injection volume of 20 µL, from a sample concentration of 400 mg/mL. Peaks: 3- lactic acid; 5- acetic acid; 6- propionic acid; 1, 2, 4, 7, 8 – unidentified compounds

Moreover, the sourdough's composition, obtained in optimized conditions, regarding the phenolic acids and flavonoids was also analyzed. For these compounds as well, to highlight the modifications determined by the controlled fermentation with selected lactic acid bacteria strains, it was analyzed simultaneously a control sample (unfermented medium). The experimental data presented in **Figure 3.6** and **Figure 3.7** certify the benefits of the lactic acid fermentation upon the modification of the bioactives' composition from the fermented product. As such, from the control sample there were separated 27 bioactive compounds, the concentration of gallic acid being higher, compared with quercetin. From the fermented sample there were eluted more than 10 polyphenols, only some of them had superior concentrations, determined by the intensity of the separated peaks. The concentration of vanillic acid is higher in the fermented sample, compared to the control sample, with a maximum intensity determined at the wavelength of 280 nm. On the opposite, the increment of ferulic acid content from the fermented product, using the lactic acid bacteria strains, was determined by analyzing the chromatograms obtained at the wavelength of 320 nm.

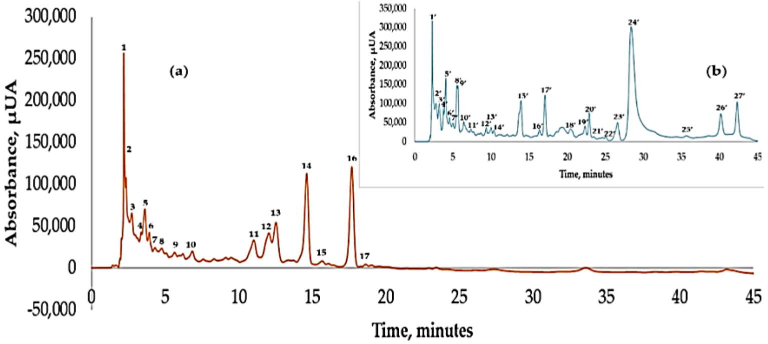

Figure 3.6. HPLC chromatograms for the separation of polyphenols (λ = 280 nm) from the sourdough obtained by the optimized process (a) and control sample (b), for an injection volume of 10 µL, from a sample concentration of 400 mg/mL. Peaks: 9- caffeic acid; 10- vanillic acid; 12- ferulic acid; 1-8, 11, 13-17 – unidentified compounds; 8′- chlorogenic acid; 9′- caffeic acid; 10′- vanillic acid; 23′- quercetin; 24′- gallic acid; 26′-epicatechin; 1'-7', 11'-22', 25', 27' – unidentified compounds

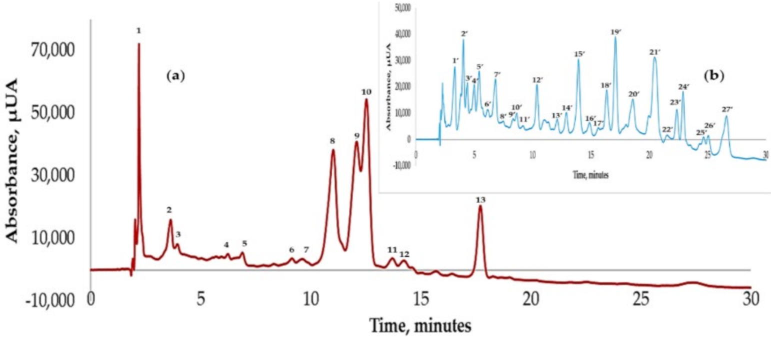

Figure 3.7. HPLC chromatograms for the separation of polyphenols (λ = 320 nm) from the sourdough obtained by the optimized process (a) and control sample (b), for an injection volume of 10 µL, from a sample concentration of 400 mg/mL. Peaks: 4- vanillic acid; 9- ferulic acid; 1-3, 5-8, 10-13 – unidentified compounds; 5′- chlorogenic acid; 7′- vanillic acid; 13′- ferulic acid; 23′- quercetin, 24′- gallic acid; 1'-4', 6', 8'-12', 14'-22', 25'-27' – unidentified compounds

It can be concluded that the optimized fermentation process to produce gluten-free sourdough, using as starters the selected strains *Lpb. plantarum* MIUG BL 21 and *Lpb. pentosus* MIUG BL 24, in co-culture, determined beneficial modifications on the chromatographic profiles of the bioactive compounds, with major impact on the improvement of the fermented product's functionality.

3.4. Partial conclusions

The most important biotechnological parameters with impact on the obtainment of a gluten-free sourdough, unconventional, realized by the controlled fermentation of the semi-solid medium, were analyzed by the statistical analysis and mathematical modelling using PBD and RSM techniques.

By the formulation of the fermentation medium based on a sterile fermentation substrate with gluten-free flours of chickpea, quinoa, and buckwheat, supplemented with okara (soybean's pulp residue resulted after processing), fermentation in controlled conditions with selected cultures of lactic acid bacteria (three lactobacilli strains and one strain of *Leuconostoc mesenteroides* spp. *mesenteroides*), experimental design, mathematical modelling and statistical analysis, it was elaborated an optimized bioprocess to obtain a gluten-free sourdough with enhanced bioactive properties.

The main independent variables (biotechnological parameters) with influence on the fermented product's quality were: dough yield, inoculum's composition and dimension, okara's addition, fermentation time and temperature. Consequently, the sourdough with superior bioactive properties is obtained by the following optimized fermentation conditions: dough yield – 475; amount of okara added – 16.90% (g/g); volume of inoculum of *Lpb. plantarum* MIUG BL 21 – 2.90% (v/w); volume of inoculum of *Lpb. pentosus* MIUG BL 24 – 5% (v/w); fermentation temperature – 31.40°C; fermentation time – 66.10 hours.

The beneficial effects on the biochemical transformations of the compounds from the fermentation substrate's composition, induced by the selected starter cultures (*Lpb. plantarum* MIUG BL 21 and *Lpb. pentosus* MIUG BL 24) cultivated in co-culture, are certified by the presence of postbiotic compounds in the fermented product, specifically the presence of organic acids, phenolic acids, flavonoids and their derivatives, with positive effect on the bioactive properties (antimicrobial activity, antioxidant activity etc.).

The obtained results confirm the antimicrobial properties of the resulted sourdough, made by following the optimized fermentation process, create the premises for this fermented product to be efficiently used as a bioingredient in bakery products and feed, to ensure the extended microbiological stability and food safety (prevention of mycotoxins' production during mold growth) for this kind of products.

Chapter 4. Obtaining some gluten-free sourdoughs by fermentation with kefir grains

4.1. Introduction

Kefir beverages are included among the probiotic functional products that offer multiple benefits for the consumers' health. The most important positive effects associated with a regular consumption of these functional products are related to the *in vivo* benefits, antimutagenic, anti-inflammatory, antimicrobial, antioxidant etc. (Du *et al.*, 2021; Zeng *et al.*, 2022). Kefir grains are artisanal starter cultures that reunite wild consortia of microorganisms made of lactic acid bacteria, acetic acid bacteria, yeast strains and bacteriophages, that have a complex metabolic activity, able to produce after fermentation and bioconversion processes bioactive compounds (postbiotics), organic acids, amino acids and bioactive peptides, vitamins, minerals and exopolysaccharides with technological and functional properties (Pihurov *et al.*, 2021).

This chapter aimed at testing different freeze-dried water or milk kefir grains, to be used as multiple inoculum to obtain, in controlled fermentation conditions, some gluten-free sourdoughs. The adaptation capacity of the microbial consortium from the kefir grains and its biochemical and functional properties were evaluated after the controlled fermentation of a substrate made of a mixture of flours from chickpea, quinoa, buckwheat, and okara.

The obtained sourdoughs were analyzed to assess the inhibition potential against some saprophytic molds in baking industry, *Aspergillus niger*, *Aspergillus flavus*, *Penicillium* spp. The scavenging capacity of the DPPH free radical, respectively the modifications caused by the controlled fermentation with kefir grains upon some postbiotic compounds within the fermented medium's composition (organic acids and polyphenolic compounds) were also determined. Furthermore, a selection of the most performant kefir grains was carried out, in order to ferment the formulated substrate, by successive multiplications in fermentation systems with liquid (LSF) or semi-solid medium, using as inoculum 0,20% (w/w) freeze-dried kefir grains or 10% (w/w) fermented product. This approach aimed at reducing the amount of the starter culture used and the simplification of the fermentation process to facilitate the transfer to the industrial conditions.

4.2. Materials and methods

The investigation methods were focused on:

- the selection of the performant artisanal culture by successive cultivation in fermentation systems with liquid or semi-solid medium, to obtain a gluten-free sourdough with improved bioactive properties;
- the microstructural analysis of the starter cultures and fermented products by scanning electron microscopy (SEM);
- the evaluation of the pH and total titratable acidity;

- the evaluation of the antifungal properties based on the inhibition ratio against the indicator strains;
- the evaluation of the antioxidant properties by DPPH and ABTS methods;
- the evaluation of the postbiotics' composition from the sourdoughs by HPLC methods.

4.2.2. Fermentation processes in successive batches

The fermentations were conducted in three different batches, each of them with a specific purpose. The obtained sourdough samples, fermentation batches, and inoculum used for fermentations are described in **Table 4.1**.

Table 4.1. Codification and obtainment method for the sourdoughs made by successive fermentations batches

Fermentation batch	Experimental variant	Inoculum	Sourdough sample obtained
1	V1	WKG1 (0.20% w/w)	A
	V1	MKG (0.20% w/w)	B
	V2	WKG1 (0.20% w/w)	C
	V3		D
	V2	MKG (0.20% w/w)	E
	V3		F
	V2	WKG2 (0.20% w/w)	G
	V3		H
2	V2	WKG2 (0.20% w/w)	S1-S4
		S4 (10% w/w)	S5-S8
		S8 (10% w/w)	S9
		S9 (10% w/w)	S10
3	V2	WKG2 (0.20% w/w)	S1L
		S1L (10% w/w)	S2L
		S2L (10% w/w)	LSF
		WKG2 (0.20% w/w)	S1F
		S1F (10% w/w)	S2F
		S2F (10% w/w)	SSF

In the first fermentation batch a selection of the freeze-dried artisanal starter cultures of WKG and MKG was made, taking into account different preliminary treatments applied on the fermentation substrate (V1-V3).

The second fermentation batch aimed at evaluating the stability of the microbial consortia from the freeze-dried GKA2, by successive fermentations in LSF system, considering the properties of the resulting fermented products (S1-S10).

In the third fermentation batch there were realized two successive multiplications followed by incubation in aerobiosis at 25°C, for 48 hours, in LSF system, in order to confirm the effectiveness of the obtained fermented product. Additionally, in this fermentation batch it was evaluated the impact of okara supplementation on the sourdoughs' functionality, obtained by fermentation in SSF system, following the same biotechnological conditions (Păcularu-Burada *et al.*, 2021).

The sourdoughs obtained from all the fermentation batches, after 48 hours of fermentation in aerobiosis, at 25°C, were freeze-dried and stored at 4°C, in sterile, opaque, and airtight containers until analysis.

4.3. Results and discussion

4.3.1. Scanning electron microscopy analysis of the freeze-dried artisanal starter cultures and sourdoughs

The morphological studies of the freeze-dried kefir grains' microsystems, respectively of the gluten-free sourdoughs, realized by the scanning electron microscopy (SEM) techniques, it was highlighted that the utilization of inulin as a cryoprotectant agent in the freeze-drying process of WKG was beneficial for the complex microbial consortium. As such, it was observed that some specific structures were formed after freeze-drying (**Figure 4.4a**), that facilitated the natural embedding of the microorganisms. Inside these structures can be observed spherical bacterial cells (cocci) or rod-shaped ones (bacilli), as it is shown in **Figure 4.4b** and **Figure 4.4c**.

The stabilization of MKG by freeze-drying with inulin determined the microorganisms from the consortium to adhere, in a first stage, to the polymers naturally formed in the consortium's association (kefiran), the cells being associated also with inulin, located at the surface of the freeze-dried matrix (**Figure 4.4d**). The SEM micrographs show the yeast cells' dominance, and cylindrical and spherical shaped bacterial cells, in different development stages (**Figure 4.4e**,**f**). During the fermentation process qualitative and quantitative modifications of the microbiota occurred, which were confirmed by SEM analysis. Consequently, in the fermented product made with the freeze-dried culture of WKG2, the synergistic relationships between lactic acid bacteria and yeast strains were highlighted, these observations can be explained by the adhesion of the bacterial cells on the surface of yeast cells (**Figure 4.5a**-**c**). Therefore, when the freeze-dried MKG was used as inoculum, it was observed a reduction of the microbial population, effect that could be attributed to the microbiome's inadaptations following the fermentation conditions to obtain sourdough, using gluten-free flours and okara as fermentation substrates (**Figure 4.5d**-**f**).

Similar results were reported by other authors, that observed qualitative and quantitative variations regarding the MKG and WKG microbiota, correlated with the geographical origin of the artisanal cultures, fermentation conditions (aerobiosis or anaerobiosis, temperature, time), or determined by the fermentation substrate's composition (nutritional compounds, growth factors, inhibitory compounds, pH, a_w). It was demonstrated that the fermentation medium's supplementation with inulin or fruits can induce a selection of the microorganisms from the WKG consortium, the lactobacilli and yeast being dominant (Pendón *et al.*, 2021). Wang *et al.* (2018) observed the morphological modification of lactic acid bacteria strains (*Lactobacillus* spp.) depending on the biotechnological conditions (intrinsic, extrinsic, and biological). In the previous study, the abundance of long and short lactobacilli, was observed in the same time at the surface and inside of the freeze-dried fermented products' matrixes, that demonstrate the adaptability of the lactic acid bacteria strains from the WKG on the formulated fermentation substrate, as well as the suitable fermentation conditions used.

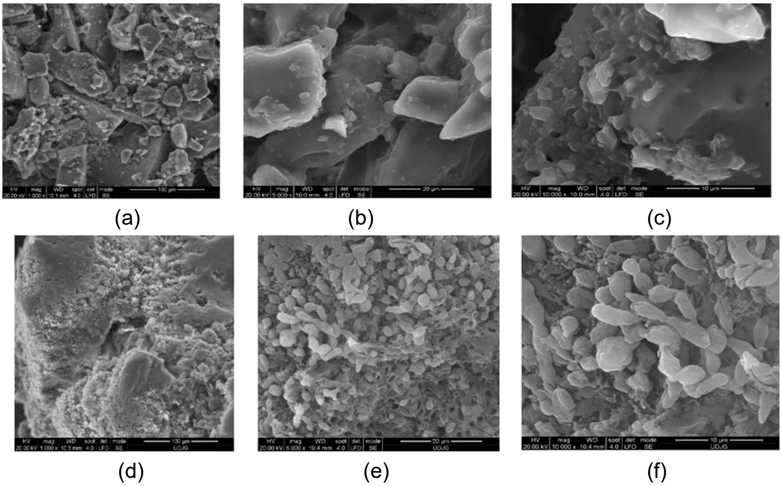

Figure 4.4. SEM micrographs of the kefir grains WKG2 (a-c) and MKG (d-f), freeze-dried with 20% (w/v) inulin solution

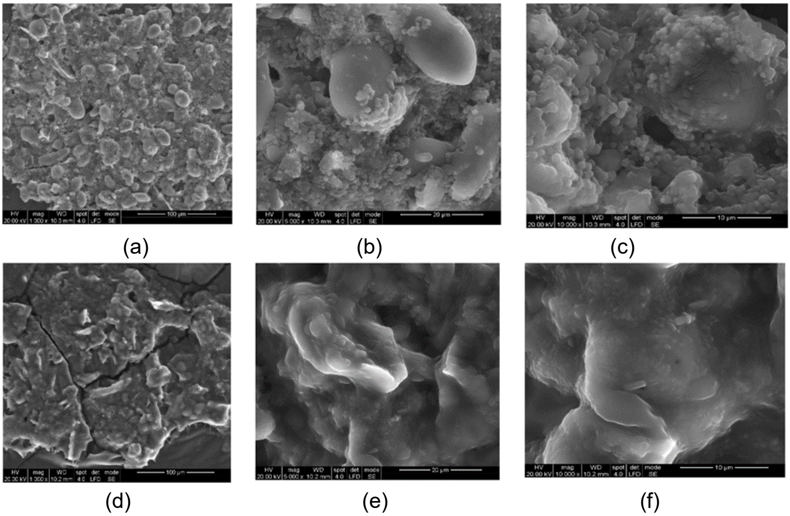

Figure 4.5. SEM micrographs of the sourdough fermented with WKG2 (V2), sample G (a-c) and sourdough fermented with MKG (V1), sample B (d-f), from the first fermentation batch

Spherical or lemon shaped yeast cells were observed by SEM analysis of the MKG artisanal cultures, being associated to *Saccharomyces* spp., *Pichia* spp., *Kluyveromyces* spp., respectively cylindrical or spherical shaped bacteria belong to *Lactobacillus* spp., *Lactococcus* spp., *Pediococcus* spp. or *Bacillus* spp. All these microorganisms are included in the complex microbiome of MKG, as literature describes (Bengoa *et al.*, 2019; Zeng *et al.*, 2022). The experimental data obtained in this study are comparable with other scientific studies reported by Garofalo *et al.* (2015), that highlighted by SEM analysis the differences between the kefir grains' microbiota, specifically the microorganisms' diversity and the ratio between the bacteria and yeast strains, such differences being influenced by numerous factors related to the ecosystem and the utilization conditions in various fermentation processes.

For the biotechnological variants to obtain sourdough by the fermentation of the gluten-free flours (chickpea, quinoa, and buckwheat) supplemented with okara, the most adaptable starters were the freeze-dried cultures with WKG1 and WKG2, these ones being recommended for further utilization.

4.3.2. Variation of pH and total titratable acidity for the sourdoughs obtained by fermentation with freeze-dried kefir grains

The pH and total titratable acidity (TTA) values are important parameters, correlated to the microbiological stability of foods, the beneficial effects being determined by the antimicrobial role of organic acids (lactic acid, acetic acid, propionic acid, medium-chain polyunsaturated fatty acids etc.). The pH values determined for the gluten-free sourdoughs obtained in the first fermentation batch (V1-V3), that used the freeze-dried starters with WKG1, WKG2, and MKG, ranged between 4.55-6.35 (**Table 4.2**), the pH value of the fermented product being affected by starter culture used, respectively by the preliminary treatments applied on the fermentation substrate.

In the second fermentation batch, the freeze-dried culture of WKG2 was used to obtain ten sourdough variants by fermentation in LSF system, with 0.20% (w/w) starter culture for the first three samples (S1-S3), respectively 10% (w/w) fermented medium from sample S4 to inoculate the subsequent fermentation substrates. The pH values for the samples obtained in the second fermentation batch varied significantly between 6.40-6.52, when the sourdoughs fermented by WKG2 freeze-dried culture were analyzed, respectively those inoculated with an aliquot of previously fermented sourdough from sample S4. Using 10% (w/w) inoculum resulting from a previous fermentation facilitated microbiota's stabilization originated from the freeze-dried WKG2, by successive cultivation in the formulated fermentation substrate. The obtained experimental results highlighted that the fermentation substrate's inoculation with a previously made sourdough can be applied for two or three subsequent fermentations.

Analyzing the sourdoughs from the third fermentation batch, obtained after two successive multiplications, it was concluded that the metabolic activity of the microorganisms from the WKG2 consortium as well as the characteristics of the resulting fermented product, associated to the pH and TTA values depend on the fermentation medium's composition and water activity. As such, it was determined a TTA value of 9.10 mL NaOH 0.10 N, with a pH value of 6.00 for the sourdough obtained by fermentation in the LSF system, whereas for the SSF fermented sourdough, a lower pH value (pH=5.83) was determined, along with 18.20 mL NaOH 0.10 N used for titration (TTA), as shown in **Table 4.2**, fact that demonstrates the effectiveness of a concentrated fermentation substrate's utilization, with a reduced amount of water.

MKG utilization to obtain sourdough was studied by Mantzourani *et al.* (2014), being reported a pH value of 4.00 and 12 mL of NaOH 0.10 N (TTA). These values were determined after 48 hours of fermentation in aerobiosis, at 30°C, using as inoculum 30% (w/w) MKG. Multiple lactic acid bacteria strains (*Leuconostoc citreum*, *Weissella cibaria*, *Lactobacillus amylovorus*) were isolated and studied due to their technological and functional properties in order to obtain sourdoughs with superior characteristics. Thus, starting from the pH values of 6.10-6.40, a decreasing was observed during the fermentation process, until pH=4.00 was reached, correlated with a TTA value greater than 10 mL NaOH 0.10 N (Belz *et al.*, 2019; Müller *et al.*, 2021). Furthermore, it was demonstrated that the fermentation time and temperature, as well as the nutrients from the substrate can affect the acidification capacity of some spontaneously fermented sourdough based on wheat and corn flour (Adebo *et al.*, 2021; Syrokou *et al.*, 2020).

The results reported in this subchapter for the pH and TTA are comparable with the data reported by Drakula *et al.* (2021), respectively a pH value with variations around 4.00 and the best TTA values for TTA ranging between 15.41-18.97, determined for some sourdoughs made of yellow pea flour fermented with *Lactobacillus* spp. strains.

Table 4.2. Gluten-free sourdoughs' characteristics obtained by fermentation with freeze-dried kefir grains (WKG and MKG) of the unconventional substrates (chickpea, quinoa, buckwheat, and okara)

Sourdough sample	Starter culture	Experimental variant	Sourdough's characteristics					
			pH	TTA, mL NaOH 0.10 N	Antifungal activity, I%			Antioxidant activity (DPPH), I%
					A. niger	*A. flavus*	*Penicillium* spp.	
Fermentation batch 1[*]								
A	WKG1	V1	5.18±0.00^{d}	30.00±0.60^{abc}	44.51±3.60^{c}	56.36±1.13^{b}	57.82±5.81^{b}	16.29±1.07^{c}
B	MKG		4.55±0.01^{f}	42.40±0.30^{a}	5.49±0.64^{d}	14.09±2.37^{ef}	14.18±2.18^{d}	17.58±0.20^{c}
C	WKG1	V2	6.24±0.07^{b}	22.90±0.08^{cd}	61.19±3.66^{b}	34.10±3.50^{cd}	29.54±0.45^{c}	18.67±0.50^{bc}
D		V3	5.74±0.02^{c}	23.60±0.20^{bcd}	100.00±0.00^{a}	94.55±5.45^{a}	100.00±0.00^{a}	27.49±1.06^{a}
E	MKG	V2	4.93±0.02^{e}	38.00±0.20^{ab}	n.d.	5.45±0.90^{f}	n.d.	18.19±1.05^{c}
F		V3	4.81±0.01^{e}	41.60±0.58^{a}	n.d.	22.70±3.82^{de}	12.18±4.18^{d}	21.92±0.81^{b}
G	WKG2	V2	6.69±0.13^{a}	10.90±0.15^{de}	100.00±0.00^{a}	30.45±5.90^{cd}	100.00±0.00^{a}	27.53±0.61^{a}
H		V3	6.35±0.01^{b}	12.33±0.00^{e}	64.97±2.71^{b}	40.00±3.94^{c}	n.d.	28.63±2.70^{a}
Fermentation batch 2[*]								
S1	WKG2	V2	6.48±0.00^{abc}	8.80±0.15^{ab}	100.00±0.00^{a}	34.68±1.73^{a}	76.04±3.88^{b}	19.79±3.06^{ab}
S2			6.47±0.00^{bc}	9.00±0.12^{ab}	100.00±0.00^{a}	30.83±1.17^{ab}	67.71±5.67^{b}	22.22±0.60^{ab}
S3			6.50±0.00^{ab}	8.00±0.40^{ab}	100.00±0.00^{a}	25.08±1.26^{bc}	40.22±3.83^{b}	18.13±1.67^{b}
S5			6.47±0.00^{bc}	9.20±0.70^{ab}	100.00±0.00^{a}	28.12±0.55^{bc}	100.00±0.00^{a}	24.38±1.10^{a}
S6			6.44±0.01^{cd}	9.60±0.13^{ab}	100.00±0.00^{a}	35.54±1.42^{a}	100.00±0.00^{a}	24.24±3.88^{a}
S7			6.40±0.00^{d}	10.40±0.02^{a}	100.00±0.00^{a}	36.67±1.37^{a}	100.00±0.00^{a}	19.24±1.00^{ab}
S8			6.52±0.01^{a}	7.90±0.70^{ab}	100.00±0.00^{a}	34.41±1.46^{a}	100.00±0.00^{a}	18.10±1.54^{b}
S9			6.52±0.00^{ab}	7.00±0.40^{b}	100.00±0.00^{a}	24.17±2.64^{c}	100.00±0.00^{a}	17.63±1.23^{b}
S10			6.50±0.03^{ab}	8.00±0.15^{ab}	n.d.	13.54±1.30^{d}	18.34±3.10^{b}	21.34±0.47^{ab}
Fermentation batch 3[**]								
LSF	WKG2	V2	6.00±0.01^{a}	9.10±0.10^{b}	100.00±0.00^{a}	100.00±0.00^{a}	100.00±0.00^{a}	17.85±1.99^{b}
SSF			5.83±0.03^{b}	18.20±0.30^{a}	100.00±0.00^{a}	100.00±0.00^{a}	100.00±0.00^{a}	25.91±2.00^{a}

[*] LSF fermentation system, [**] LSF or SSF fermentation system. Average values for 3 replicates ± standard deviation. Different letters denote significant differences in a column, considering the samples from the same fermentation batch; n.d. not determined

4.3.3. Antifungal activity of the gluten-free sourdoughs fermented with kefir grains

The results for the antifungal activity against *A. niger*, *A. flavus* and *Penicillium* spp, determined by the gluten-free sourdoughs fermented with kefir grains varied depending on the indicator mold strain used. Therefore, the involvement of the inoculation system with successive fermentations influenced the inhibitory potential of the fermented products, by improving the antifungal activity after the microbial composition's stabilization on the fermentation substrate used.

Varying the type of starter by the fermentation approach used for the first fermentation batch, in LSF system, there were determined inhibition ratios between 5.45-94.55 against *A. flavus* indicator strain, induced by the obtained fermented medium (**Table 4.2**).

The main criterion for the performant sourdoughs' selection, including the freeze-dried starter cultures with WKG and MKG for the fermentation, was represented by the antifungal properties determined by the resulting fermented products, taking into account also the pH and TTA values, respectively the antioxidant properties and sensorial features. Consequently, for further studies the sourdough sample G was selected, due to this sample's quality attributes associated with the improvement of the technological and functional properties.

The utilization of the freeze-dried fermented sample as antifungal agent (2% w/v) from the sourdough samples S1-S9 totally inhibited the *A. niger* strain, a similar effect being observed for the *Penicillium* spp. Strain when the sourdough samples S5-S9 were used. Values with variations between 35.54-36.67% were calculated for the inhibition of *A. flavus* when the freeze-dried fermented product from the sample S6 and S7 was used.

The two successive multiplications from the third fermentation batch, in LSF and SSF systems, using the WKG2 starter culture, allowed an improved biosynthesis of the antifungal compounds, which inhibited the indicator mold strains' development (**Figure 4.6**). Other similar studies from the literature emphasized that the strains belonging to *Lactobacillus* spp. and *Pediococcus* spp., isolated from the MKG microbiota were able to inhibit differently *Penicillium* spp. or *Aspergillus* spp. strains, the antagonistic effect being correlated with the biosynthesized organic acids' spectrum (Farag *et al.*, 2020; Georgalaki *et al.*, 2021). It should be mentioned that the yeast strains are part of the kefir grains' microbiota, these microorganisms contribute to the antifungal properties of the sourdoughs on the studied mold strains, due to some enzymatic transformations of the compounds from the fermentation substrate. It was demonstrated that the fermented media using *Saccharomyces boulardii* strains had an inhibitory effect upon *Aspergillus* spp. growth (Goktas *et al.*, 2021; Purutoğlu *et al.*, 2020).

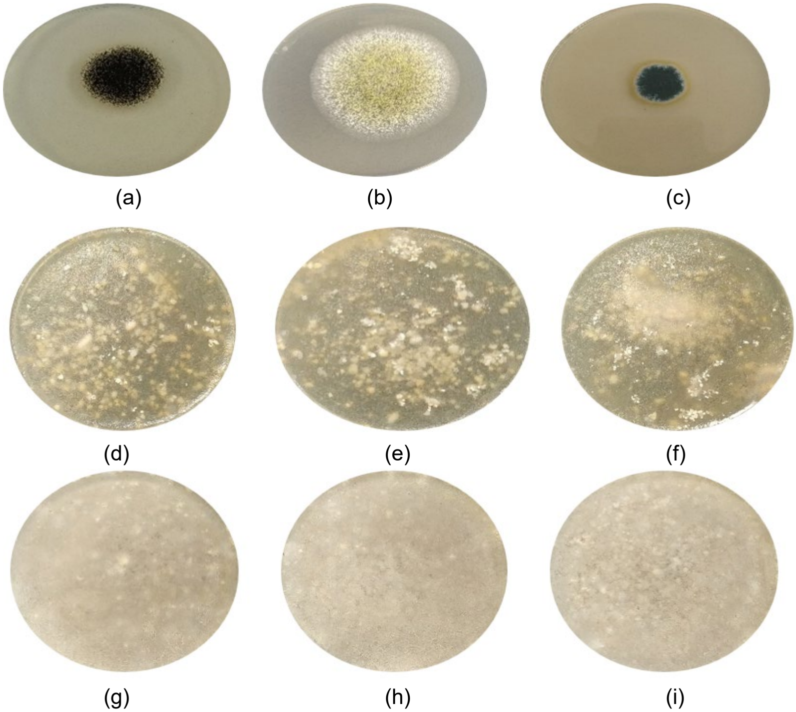

Figure 4.6. Antifungal activity of the sourdoughs obtained in the third fermentation batch with WKG2 starter culture, on the indicator mold strains: *A. niger*, control (a), LSF sourdough (d) and SSF sourdough (g); *A. flavus*, control (b), LSF sourdough (e) and SSF sourdough (h); *Penicillium* spp., control (c), LSF sourdough (f) and SSF sourdough (i)

4.3.4. Antioxidant activity of the gluten-free sourdoughs fermented with kefir grains

The antioxidant properties of the sourdoughs fermented by kefir grains, evaluated by DPPH method, varied depending on the fermentation system used (LSF or SSF), on the starter cultures used, respectively on the preliminary treatments applied on the substrates before inoculation. The best DPPH free radical scavenging ratios, higher than 27%, were determined by the sourdoughs obtained in the first fermentation batch (LSF), fermented by the starter cultures WKG1 and WKG2 (**Table 4.2**). Using as inoculum the previously fermented medium (10% w/w) for the inoculation of some new fermentation substrates, contributed to the improvement of the antioxidant properties. Consequently, maximum values for DPPH inhibition, respectively 24.38% and 24.24%, were determined when the samples coded S5 and S6 were analyzed, from the second fermentation batch in LSF system.

The experimental results demonstrate, as well, the fact that the antioxidant properties of the fermented products were minimized when more than two successive multiplications were carried out using 10% (w/w) inoculum from the sample coded S4. The sourdough obtained in the third fermentation batch , in LSF system, determined a DPPH inhibition of 17.85%, when two successive multiplications were made. It was concluded that the supplementation of the fermentation medium with 16.89% (w/v) and sourdough's consistency determined significant differences on the DPPH scavenging properties.

The sourdough obtained by fermentation in SSF system (third fermentation batch), using WKG2 as starter culture, incubated for 48 hours at 25°C in aerobiosis, determined a DPPH inhibition ratio of 25.91% (**Table 4.2**).

Some scientific studies were focused on the sustainable biovalorification of soy whey, the starter cultures made of WKG being used for the fermentation of this by-product. These fermented beverages were characterized by an improved antioxidant potential, with DPPH inhibition capacity ranging between 40-80% (Azi *et al.*, 2020a; Azi *et al.*, 2020b). Such experimental data emphasize the importance of the microbial strains from the WKG consortium, as well as the fermentation's spectrum variability, correlated to the chemical composition of the fermentation substrate and the metabolic properties of the starter cultures. A superior antioxidant activity can be determined by the bioactive peptides, phenolic acids or flavonoids from the fermentation substrate's composition or by the compounds formed after the biochemical activity of the microorganisms involved in the fermentation process (Gunenc *et al.*, 2017; Tu *et al.*, 2019).

4.3.5. Postbiotic compounds biosynthesized in the sourdough through the activity of the microorganisms from the kefir grains microbiota

The sourdough samples from the third fermentation batch, in LSF and SSF system, were analyzed by HPLC in order to separate, identify and quantify some postbiotic compounds with impact on the antimicrobial and antioxidant activity. There were highlighted significant variations ($p<0.05$) of the organic acids' content from the sourdough samples realized in LSF and SSF fermentation systems, in the third fermentation batch, after two successive fermentations and incubation in aerobiosis at 25°C for 48 hours, using the freeze-dried WKG2 as starter.

The sourdough obtained in SSF system, using the fermentation medium supplemented with 16.89% (w/v) okara, the concentration of organic acids was higher compared to those determined for the LSF made sourdough. Among the organic acids quantified by HPLC analysis from the SSF sourdough, the highest concentration was attributed to lactic acid (2357.56 mM/kg dry weight), followed by acetic acid (783.87 mM/kg dry weight), citric acid (208.65 mM/kg dry weight), propionic acid (169.70 mM/kg dry weight) and butyric acid (8.97 mM/kg dry weight). The fermentation quotient's values, presented in **Table 4.3**, varied between 3.01-3.82, without significant differences among the fermented samples obtained in LSF or SSF system. A fermentation quotient between 1.50-4.00 can enhance the sensorial and textural features of the bakery products with sourdoughs (Galli *et al.*, 2019).

The experimental data from this study for the fermentation quotient are comparable with the data reported in other scientific articles from the literature for some sourdoughs made with semolina or millets (Gaglio *et al.*, 2021; Wang *et al.*, 2019). Three sourdoughs with a stable microbiological composition, originated from different artisanal bakeries, were characterized by concentrations of acetic acid between 39-99 mM/kg sourdough, respectively concentrations of lactic acid between 53-85 mM/kg sourdough. It was demonstrated that these organic acids from the fermented products can ensure the microbiological stability, which impacts the shelf-life extension of the sourdough breads, when the acids are in undissociated forms in the final products (Debonne *et al.*, 2020).

Using the freeze-dried culture of WKG2 in controlled bioprocesses seemed to be efficient to obtain sourdoughs rich in postbiotics (organic acids and polyphenols). Zongo *et al.* (2020) studied the metabolic activity of a WKG culture to obtain a water kefir supplemented with palm sap. In the fermented beverage multiple organic acids were identified (acetic, citric, lactic, pyruvic, propionic, and succinic acid) that contribute to the food safety, sensorial and functional properties. The HPLC analysis was carried out also for the bioactive compounds' separation from the gluten-free sourdoughs and unfermented substrates. The gallic acid content significantly increased ($p<0.05$) from 302.62 µg/g dry weight or 512.62 µg/g dry weight in the unfermented samples, up to 1384 µg/g dry weight, respectively 1446.50 µg/g dry weight in the sourdoughs obtained in LSF or SSF fermentation systems. Moreover, the epicatechin content from the LSF and SSF sourdoughs was improved, its maximum amounts ranged between 18238-23798 µg/g dry weight (**Table 4.4**).

Bioactives' quantification from the analyzed fermented samples (**Table 4.4)**, emphasized the impact of the optimized fermentation parameters from this study, on the bioactive compounds' enrichment. Consequently, in the sourdough obtained by fermentation in SSF system, using WKG2 starter culture, the antioxidant potential was improved, whereas, for the sourdough obtained in the LSF system, a modification of the bioactive compounds' spectrum was observed.

Table 4.3. Organic acids' composition and fermentation quotient for the gluten-free sourdoughs (LSF or SSF fermentation system, V2, with freeze-dried WGK2 starter culture) and control samples (unfermented substrates) from the third fermentation batch

Sample	**Organic acids (mM/kg dry weight)**					**Fermentation quotient**
	Lactic acid	Acetic acid	Citric acid	Propionic acid	Butyric acid	
Fermented product in LSF system	1510.77±2.40^{b}	592.21±3.60^{b}	83.54±0.75^{c}	48.49±0.49^{b}	3.47±0.00^{b}	3.82±0.02^{c}
Control LSF (unfermented substrate)	42.14±1.20^{d}	2.54±0.00^{d}	194.52±0.94^{b}	n.d.	n.d.	24.83±0.71^{b}
Fermented product in SSF system	2357.56±0.80^{a}	783.87±1.20^{a}	208.65±0.37^{a}	169.70±0.49^{a}	8.97±0.41^{a}	3.01±0.00^{c}
Control SSF (unfermented substrate)	523.70±0.00^{c}	15.29±0.00^{c}	73.66±0.19^{d}	21.34±0.00^{c}	n.d.	51.39±0.00^{a}

Average values for 3 replicates ± standard deviation. Different letters denote significant differences in a column; n.d. not determined

Table 4.4. Polyphenols' concentration from the gluten-free sourdoughs, obtained by fermentation in LSF or SSF system (V2) using the freeze-dried WKG2 culture, and the control samples (unfermented substrates) from the third fermentation batch

Bioactive compound, μg/g dry weight	**Concentrations determined at the wavelength of:**							
	280 nm		**320 nm**		**280 nm**		**320 nm**	
	Sample							
	Fermented product in LSF system	**Control LSF**	**Fermented product in LSF system**	**Control LSF**	**Fermented product in SSF system**	**Control SSF**	**Fermented product in SSF system**	**Control SSF**
Gallic acid	1384.00± 29.00^{aA}	512.62± 16.17^{bB}	2848.00± 56.10^{aA}	1153.37± 99.64^{bB}	1446.50± 94.30^{aA}	303.62± 0.88^{bB}	1525.92± 12.65^{aB}	669.20± 39.70^{bC}
Epicatechin	18238.00± 20.09^{aB}	2172.40± 35.50^{bC}	n.d.	11615.54± 27.17^{aA}	23798.00± 48.00^{aA}	17731.00± 36.00^{bB}	12425.00± 41.00^{aA}	10659.30± 19.20^{bB}
Caffeic acid	n.d.	n.d.	n.d.	n.d.	100.35± 4.18^{aA}	n.d.	66.16± 4.42^{aA}	n.d.
Myricetin	n.d.	n.d.	n.d.	n.d.	96.38± 1.24^{bB}	599.20± 9.53^{aA}	n.d.	1937.17± 70.86^{aA}
Hesperidin	n.d.	n.d.	n.d.	n.d.	927.30± 28.80^{aA}	97.33± 4.30^{bB}	589.30± 41.90^{bB}	2155.90± 54.10^{aA}
Quercetin 3-β-D-glucoside	n.d.	n.d.	n.d.	n.d.	138.56± 5.91^{bB}	496.70± 4.45^{aA}	n.d.	476.48± 2.86^{aA}
Apigenin	1.46± 0.20^{bB}	2.47± 0.06^{aA}	0.34± 0.00^{bC}	0.59± 0.05^{aB}	1.36± 0.03^{bB}	2.04± 0.01^{aA}	n.d.	0.76± 0.01^{aA}

Bioactive compound, µg/g dry weight	**Concentrations determined at the wavelength of:**							
	280 nm		**320 nm**		**280 nm**		**320 nm**	
	Sample							
	Fermented product in LSF system	**Control LSF**	**Fermented product in LSF system**	**Control LSF**	**Fermented product in SSF system**	**Control SSF**	**Fermented product in SSF system**	**Control SSF**
Isorhamnetin	103.35± 5.24^{aA}	97.96± 2.00^{aA}	0.65± 0.04^{aB}	n.d.	52.70± 0.51^{bC}	79.72± 0.43^{aB}	n.d.	2.34± 0.21^{aA}
Quercetin	n.d.	75.82± 0.06^{aA}	n.d.	n.d.	n.d.	n.d.	n.d.	72.88± 0.21^{aA}
Vanillic acid	n.d.	n.d.	n.d.	80.85± 0.46^{aA}	n.d.	n.d.	n.d.	n.d.
Kaempferol	n.d.	24.94± 1.57^{aA}	n.d.	3.49±0.04^{aA}	n.d.	n.d.	n.d.	n.d.

Average values for 3 replicates ± standard deviation. Different lowercase letters denote significant differences between sourdough and control at the same wavelength, whereas different uppercase letters denote significant differences between the samples analyzed at the same wavelength; n.d. not determined

The caffeic acid content determined in a sorghum-based sourdough increased from 45.80 µg/g to 183.40 µg/g, after fermentation with a *Weissella confusa* strain, result that is in accordance with the experimental data obtained for the sourdough obtained in SSF system, reported in this chapter of the doctoral thesis. A similar behavior was observed for the isorhamnetin content from the LSF sourdough, whereas the amount of quercetin derivatives decreased in the sorghum-based sourdough made by Wang *et al.* (2020). By fermentation in SSF system of the soybean flour with a *Lactobacillus casei* strain, the enrichment of gallic acid content was observed when the fermented product was analyzed (Li *et al.*, 2020), conclusion that is similar to the results of this study for the gluten-free sourdoughs fermented by the WKG2 culture, when the process was carried out in LSF or SSF fermentation system.

In the SSF sourdough β-glucosides and aglycones originated from okara's composition were used as nutritional compounds by the microorganisms from the WKG artisanal culture (Tu *et al.*, 2019). The hydroxybenzoic and hydroxycinnamic acids from a fruit-based product, fermented with *Pediococcus pentosaceus* or *Lactobacillus plantarum* strains had antifungal properties, the effect depends on the concentration (Omedi *et al.*, 2019). The diversity and concentrations of the bioactive compounds from the analyzed fermented samples are affected by multiple factors, the most important being represented by the type and concentration of the starter culture, fermentation substrate's composition, fermentation system, and multiple intrinsic, extrinsic, and biological factors that interfere, especially for the products with commercial sterility.

4.4. Partial conclusions

The potential of the artisanal cultures (milk and water kefir grains) to be used as starter cultures in the fermentation process of some gluten-free flours (chickpea, quinoa, buckwheat, and okara) to obtain sourdough with enhanced bioactive properties was followed. It was evaluated and demonstrated the adaptation capacity of these natural consortia of microorganisms, as well as the stability of the biochemical and functional properties, by using successive fermentations divided in batches and inoculum's propagation, to simplify and scale-up the fermentation process for industrial purposes.

The functionality and stability of kefir grains by freeze-drying with 20% (w/v) inulin solution, as a cryoprotectant agent, was demonstrated.

Flow charts aiming at improving the postbiotics' composition in the obtained fermented products made with chickpea, quinoa, buckwheat, and okara flour, varying the inoculum's type and concentration, the fermentation substrate's composition, the preliminary treatments applied or the fermentation system used, were designed, which had a major impact on the antifungal and antioxidant properties.

The best fermentation performances were obtained by using the freeze-dried WKG2, characterized by a superior adaptation capacity to the tested biotechnological conditions, being also able to metabolize the chemical compounds within the formulated fermentation substrate in order to improve the postbiotic composition (organic acids, phenolic acids, and flavonoids) of the fermented product, to obtain a gluten-free sourdough with superior technological and functional characteristics. Consequently, the gluten-free sourdoughs obtained in this study, by an innovative utilization of the artisanal starter cultures, can contribute to the diversification of the gluten-free products with extended microbiological stability.

This study offers innovative perspectives on the identification of novel uses of WKG in fermentation processes involving plant-based substrates. It was emphasized, also, the versatility of the microorganisms from the WKG consortium, that facilitates the diversification of foods, food ingredients or feed, with improved functionality.

Chapter 5. Testing the functionality of sourdough in unconventional bakery products

5.1. Introduction

Gluten is an important proteinaceous component often found in the conventional raw materials used in bread making. Its presence in bread offers technological benefits and increases consumers' acceptance for the obtained final products, due to the gluten's capacity to develop a stable matrix after dough's kneading, this process allowing also the embedding of air and CO_2 produced during fermentation (Alizadehbahaabadi *et al.*, 2021).

Considering that numerous people choose to eliminate the gluten from their diet, it is essential to have a scientific basis to support the consumers, in order to develop gluten-free products with improved quality and functionality (Miranda-Ramos *et al.*, 2020). The gluten-free bakery products' diversification is the main objective of the scientists from this field, to fulfil the consumers' requirements about the nutritional quality of foods, but also to enhance the health promoting effects determined by functional bakery products' consumption (Ibidapo *et al.*, 2020). In the scientific literature, there are numerous methods to obtain gluten-free bakery products with improved functionality, the fermentation with selected lactic acid bacteria and yeast strains, or their consortia, respectively the supplementation with different ingredients (whey, onion skins' extracts, pseudocereals and legumes flours or brans) being the most common approaches to achieve the established objectives (Gobbetti *et al.*, 2019; Ito and Arai, 2021; Roman *et al.*, 2019).

Most of the gluten-free loaves are made by rice flour, but some scientific studies emphasize the beneficial health effects determined by the consumption of gluten-free products obtained using unconventional raw materials such as millets, quinoa, sorghum, teff, buckwheat, or chickpea. The health effects are associated to the lower glycemic index, improved resistant starch content, and superior protein digestibility (Chakraborty *et al.*, 2020; Romão *et al.*, 2021; Sahagún *et al.*, 2020). Simultaneously, the pseudocereals and legumes are characterized by a high level of proteins, fats, dietary fibers, minerals, and bioactive compounds with anti-inflammatory, antiviral, anticarcinogenic, or antidiabetic effects, being also efficient in cardiovascular or Alzheimer diseases' prevention (Brites *et al.*, 2022).

Controlled fermentations of the raw materials, involving artisanal consortia of microorganisms, or by using selected lactic acid and yeast strains, to obtain sourdoughs can improve the final products' characteristics (Bender and Schönlechner, 2020). The utilization of composite flour mixtures comprising legumes, pseudocereals, and cereals, is recommended to obtain a balanced nutritional composition, the sprouting and soaking contributing to the functionality enhancement due to specific enzymes' activity. Thus, the ratio between ingredients should be considered when bread production is intended, because some sensorial features may be negatively affected (Xu *et al.*, 2020).

Sourdough addition in bread can extend the products' shelf-life due to the bioingredient's postbiotic compounds with antimicrobial effect, which were biosynthesized by the microorganisms, that inhibit molds' growth, on one side. On the other side, it was demonstrated that sourdough's utilization contributes to the salt reduction in bread recipes (Debonne *et al.*, 2020; Voinea *et al.*, 2020).

In this chapter, the impact of the gluten-free flours' mixture, made of chickpea, quinoa, and buckwheat, substitution with sourdoughs using concentrations of 10% or 25% (w/w), obtained by fermentation with selected lactic acid bacteria strains belonging to *Lactobacillus* spp. (Păcularu-Burada *et al.*, 2020; Păcularu-Burada *et al.*, 2021), respectively by fermentation with a freeze-dried culture of water kefir grains. Some technological and functional properties were evaluated, as follows: microbiological stability, total titratable acidity and pH, texture, *in vitro* digestibility and bioactive compounds' release, respectively the sensorial properties of the experimental gluten-free breads obtained, in order to recommend some of them to be produced at industrial scale.

5.2. Materials and methods

The investigations on the gluten-free breads were focused on:

- pH and total titratable acidity assessment;
- texture profile analysis;
- evaluation of the microbiological stability in storage conditions;
- evaluation of the bioactive compounds' behavior during *in vitro* digestion (total polyphenolic content assessed by Folin Ciocâlteu method; total flavonoids content determined by aluminium chloride assay; antioxidant properties evaluation using DPPH and ABTS methods; starch digestion by DNS method);
- sensorial characteristics' evaluation using a 9 points hedonic scale.

5.2.2. Obtainment of gluten-free bread

The recipes for the experimental gluten-free breads were made taking into account similar scientific studies from the literature, numerous preliminary tests, respectively manufacturer's instructions for the bread made with commercial premix (Aguiar *et al.*, 2020; Khemiri *et al.*, 2020; Sahin *et al.*, 2020; Santos *et al.*, 2020; Santos *et al.*, 2021).

The sourdough used for the experimental samples coded P2 and P3 was obtained after a fermentation process with selected lactic acid bacteria strains, as it was previously described (Păcularu-Burada *et al.*, 2020; Păcularu-Burada *et al.*, 2021). The sourdough used for the experimental samples coded P4 and P5 was made by fermentation in semi-solid medium using as inoculum for fermentation the freeze-dried culture of water kefir grains, previously selected (WKG2), obtained in optimized conditions (Păcularu-Burada *et al.*, 2022).

Bread doughs were realized by making a homogenous mixture using the recipe's ingredients and a bread making machine (Studio Casa, Bucharest, Romania) that ensured the kneading and proofing of the obtained dough. Afterwards, the dough was divided into individual pieces of 60 g and the baking was carried out at 200°C, for 60 minutes (Puerta *et al.*, 2021), using an air-circulating electric oven (Albatros, Smart Electro-Distribution, Bucharest, Romania).

5.3. Results and discussion

5.3.1. Technological and functional properties of the bread supplemented with gluten-free sourdough obtained in unconventional fermentation conditions

Acidification capacity

The gluten-free bread samples, supplemented with sourdoughs obtained and used in the experimental variants previously described, had pH values between 5.25 and 5.91, results being influenced by the substitution level of the flours with sourdoughs, as well as by the fermented products' characteristics, correlated to the starter cultures' type used in the fermentation process (**Figure 5.6**).

For some experimental gluten-free breads supplemented or not with sourdoughs based on chia, quinoa, and hemp, there were determined pH values between 4.67-5.49, whereas the TTA values varied between 0.99-2.68 mL NaOH 0.10 N (Jagelaviciute and Cizeikiene, 2021). For the studied breads the pH values varied between 4.70-5.60, and TTA between 3.00-4.70 depending on the substitution level and obtainment method.

The results of this study for the total titratable acidity (TTA) and pH are comparable with those reported by Martins *et al.* (2022). The previously mentioned authors observed that the substitution of rice flour with unfermented acorn flour in various concentrations (0%, 23%, 35% w/w) determined a pH decrease from 6.09 to 5.77. Rice flour substitution with acorn flour sourdough in a gluten-free bread recipe, using the same substitution levels, lead to pH values between 4.89-4.98.

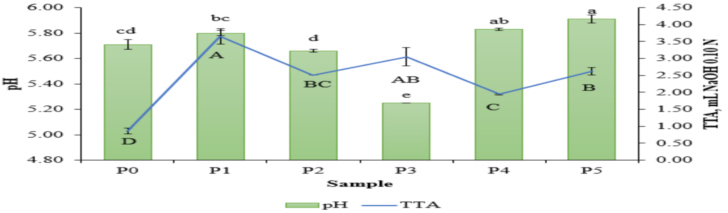

Figure 5.6. pH and total titratable acidity (TTA) variations for the gluten-free bread samples (average vales for 3 replicates ± standard deviation; different lowercase letters denote significant differences for the pH values; uppercase letters denote significant differences for the TTA)

Texture profile analysis for the bread supplemented with gluten-free sourdough

The lowest value for hardness, respectively 1.30 N (**Table 5.2**) was determined for the ready-to-eat gluten-free bread purchased from the market (P6), result that can be associated to the product's composition, based mainly on rice flour and different types of starch. The proteins from the chickpea, quinoa, and buckwheat flours, used for the obtainment of samples (P1-P5) contributed to the development of more dense structures which affected the experimental data for the hardness, ranging between 1.84-2.33 N. The flours' substitution from the bread making recipe with 10% (w/w) sourdough fermented with selected lactic acid bacteria strains (*Lactiplantibacillus plantarum* MIUG BL 21 and *Lactiplantibacillus pentosus* MIUG BL 24) or with the freeze-dried culture of water kefir grains (WKG2), sample P2 and sample P4, respectively, determined hardness values comparable with those determined for the sample P6 (commercial gluten-free bread).

Similar studies conducted by other authors highlighted that the chickpea flour addition contributed, on one side, to the hardness increase, whereas the lactic acid fermentation can decrease the values for this parameter (Guardado-Félix *et al.*, 2020; Xiao *et al.*, 2016). Baker's yeast utilization along with the sourdoughs affect the water and oil absorbtion, the amount of sourdough employed, the temperature and time for bread dough proofing can also modify the final product's hardness (Caponio *et al.*, 2022; Naji-Tabasi *et al.*, 2022).

Table 5.2. Experimental values for the analyzed textural parameters assessed for the gluten-free breads

Sample code	Textural features evaluated			
	Hardness, N	Cohesiveness, -	Springiness, mm	Chewiness, mJ
P0	2.33±0.79^{a}	0.42±0.05^{cd}	4.03±0.09^{ab}	3.83±1.79^{a}
P1	2.12±0.39^{a}	0.52±0.08^{ab}	3.93±0.25^{ab}	4.21±1.34^{a}
P2	2.07±0.54^{ab}	0.49±0.09^{abc}	4.12±0.10^{a}	3.98±2.05^{a}
P3	2.22±0.66^{a}	0.40±0.08^{cd}	3.44±0.39^{c}	3.20±1.53^{a}
P4	1.84±0.60^{ab}	0.46±0.07^{bc}	3.73±0.45^{abc}	3.49±1.60^{a}
P5	2.33±0.36^{a}	0.37±0.02^{d}	3.66±0.30^{bc}	3.06±0.69^{a}
P6	1.30±0.25^{b}	0.57±0.04^{a}	4.06±0.09^{a}	2.83±0.73^{a}

Different letters in one column denote significant differences for the same parameter

The bakery products' chewiness is influenced mainly by the dough proofing conditions, respectively its time and temperature, during this step of the manufacturing process enzymatic reactions occur, the starch content being modified, the pores' structure is developing, which will influence the subsequent textural features (Naji-Tabasi *et al.*, 2022).

Liang *et al.* (2022) observed that the chewiness decreases along with an increased concentration of sourdough in the bread recipe, the sourdough being previously fermented with selected lactic acid bacteria strains. Thus, involving different starter cultures for sourdough's fermentation did not modify significantly the chewiness, result that is in accordance with the experimental data determined for the sourdough gluten-free breads made with chickpea, quinoa, and buckwheat flours.

Microbiological stability of gluten-free breads

The antifungal properties of the gluten-free bread samples obtained were improved by flours' substitution with sourdoughs. As such, the storage time was extended with 2-4 days, depending on the amount of sourdough added, respectively on the type of starter culture used for sourdough's fermentation (**Figure 5.7**).

The experimental results can be compared with other data from the scientific literature. Consequently, the most performant *Lactobacillus plantarum* strains, used for sourdough fermentation and whole wheat sourdough bread making, determined a storage time extension with 3 days, whereas other strains of *Lactobacillus* spp. included in the same study accelerated the bread's contamination with molds, compared to the control sample (Sun *et al.*, 2020).

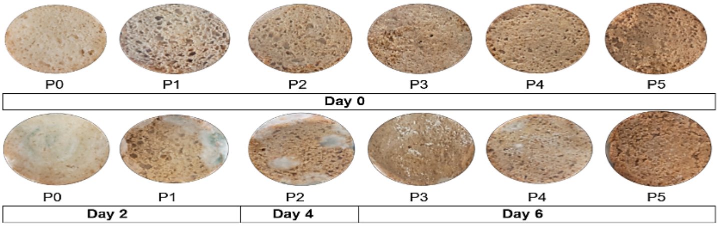

Figure 5.7. Gluten-free bread samples' contamination progress during storage at 25°C

The combination between two sourdoughs (24% w/w total sourdough) fermented by *Lactobacillus amylovorus* or *Weissella cibaria* strains, determined a bread's shelf-life extension up to six days, afterwards being observed the first signs of contamination (Belz *et al.*, 2019).

Release of bioactive compounds during in vitro digestion

The total polyphenolic content of the gluten-free bread samples was influenced by the recipe's ingredients. As such, at the beginning of the *in vitro* digestion protocol (oral phase, t_0) for the sample P0, obtained with commercial premix for gluten-free bread, it was assessed a total polyphenolic content of 136.95 mg

GAE/100 g dry weight, whereas for the sample made with gluten-free flours of chickpea, quinoa, and buckwheat it was calculated a total polyphenolic content of 177.15 mg GAE/100 g dry weight. It was observed that the gluten-free flours' substitution from the recipes with sourdoughs fermented with selected lactic acid bacteria strains determined a decrease of the total polyphenols, respectively 100.77 mg GAE/100 g dry weight, for sample P2 and 106.62 mg GAE/100 g dry weight for sample P3. A different behavior was observed for the gluten-free flours' substitution with sourdoughs fermented in semi-solid system (SSF) with the freeze-dried starter of water kefir grains (WKG2), a substitution level of 25% (w/w) for sample P5, enriched the total polyphenolic content (314.98 mg GAE/100 g dry weight). The lactic acid fermentation of wheat bran with selected strains of *Enterococcus faecalis* determined an improvement of total polyphenolic content up to 153 mg GAE/100 g dry weight (Mao *et al.*, 2020).

The combination of some lactic acid bacteria strains (*Pediococcus* spp.) and baker's yeast (*Saccharomyces cerevisiae*) determined an improvement of the polyphenolic content, especially flavonoids, when a gluten-free sourdough based on sorghum flour, the fermentation process using both strains being beneficial upon the functional properties (Olojede *et al.*, 2020a). Wang *et al.* (2019) concluded that the dough fermentation with selected yeast strains can improve the total polyphenolic content. The impact of sourdough addition as well as its fermentation time on some functional properties was studied by Caponio *et al.*, 2022. These authors determined a total polyphenolic content variation between 15.60-99.80 mg GAE/100 g for the gluten-free breads, such results being comparable with the data obtained in this chapter of the PhD thesis. A major release of the flavonoids from the analyzed gluten-free breads was observed, in simulated salivary fluid, after 2 minutes of simulated *in vitro* digestion. This result can be influenced by the complexes formed between the flavonoids and starch, which are unstabilized by α-amylase's presence, thus increasing the total flavonoid content.

It was emphasized the beneficial effect of the selected starter cultures, used to obtain the sourdough, on the total flavonoid content. The flavonoids' functionality depends on the starter culture's type involved in the sourdough's obtainment bioprocess, respectively on the sourdough's concentration used for the gluten-free bread production.

Liang *et al.* (2022) observed, also, a different level of total flavonoid content, depending on the starter culture with lactic acid bacteria strains used for sourdough's fermentation and sourdough bread production (30% w/w). The calculated values varied between 24.40-27.80 mg rutin equivalents/ 100 g, being comparable with the experimental data obtained in this PhD thesis. The fermentation temperature for the sourdough affects as well the total flavonoid content (Yakubu *et al.*, 2022).

A comparative analysis of the antioxidant activity by both DPPH and ABTS method, emphasized the differences between the scavenging activity of the analyzed free radicals. Therefore, at the beginning of the simulated *in vitro* digestion protocol, sample P0, made with commercial flour premix for gluten-free bread was characterized by an antioxidant value (DPPH) of 12.55 mM TE/100 g dry weight, after the gastrointestinal digestion, a lower value, of 4.70 mM TE/100 g dry weight being determined. Similarly, for sample P5, made with 25% (w/w) sourdough obtained in the same fermentation conditions, the antioxidant activity by DPPH method was reduced from 4.01 to 2.26 mM TE/100 g dry weight. The recipe gluten-free flours' substitution by 10% (w/w) sourdough, obtained after fermentation with selected lactic acid bacteria strains (sample P2) positively affected the antioxidant activity (DPPH), at the end of the simulated *in vitro* digestion protocol being determined a concentration of 5.20 mM TE/100 g dry weight.

On the other side, for the gluten-free bread samples P0-P3 were not determined positive results for the antioxidant activity by ABTS method after the *in vitro* digestion, in simulated salivary fluid or after gastric digestion. The control sample with chickpea, quinoa, and buckwheat flours (P1) determined a release of the compounds with inhibitory activity against ABTS, after 80 minutes of intestinal digestion, being calculated a maximum value of 4.35 mM TE/100 g dry weight, whereas, after the same treatment, it was determined a maximum value for the antioxidant activity by ABTS (1.75 mM TE/100 g dry weight) for sample P2, supplemented with 10% (w/w) sourdough fermented by selected lactic acid bacteria strains. A higher flours' substitution level using the same type of sourdough, respectively 25% (w/w), determined enhanced antioxidant activities (ABTS), respectively 2.90 mM TE/100 g dry weight, after 120 minutes of simulated gastrointestinal digestion.

The experimental data reported by other researchers regarding the antioxidant activity by DPPH method, showed that, the compositional characteristics of the analyzed sample affect the obtained results. Consequently, for a rye-based bread it was calculated a concentration of 0.30 mM TE/100 g dry weight (Lachowicz *et al.*, 2020). Other scientific studies demonstrated the importance of fermentation time and strains used for the starter cultures to obtain products with DPPH scavenging activity (Colosimo *et al.*, 2020; Liang *et al.*, 2022). Adding some broccoli leaf powder in a gluten-free bread recipe improved the antioxidant activity from 27 to 95 μM TE/100 g (Krupa-Kozak *et al.*, 2021), these reported values being below those obtained in the experimental study conducted in this chapter of the PhD thesis.

In vitro digestion of starch

The identification of different starch fractions, such as rapidly and slowly digestible starch, respectively resistant starch, as well as the occurring quantitative modifications determined by the substitution of gluten-free flours with different concentrations of sourdoughs was followed in order to intensify the functional character of the obtained gluten-free breads. Consequently, based on the results presented in **Table 5.7**, differences on the starch fractions' content were observed for the analyzed samples.

The study carried out by Bottani *et al.* (2018) highlight the ability of some selected lactic acid bacteria strains belonging to *Leuconostoc* spp. and *Lactobacillus* spp. to enrich the resistant starch content from some bread samples, values lower than 10% being determined for this starch fraction. The raw materials used for bread making directly affect the content of rapidly digestible, slowly digestible, and resistant starch (Di Cairano *et al*., 2020), in other cases the ratio between these fractions can be modified after diverse treatments, such as sprouting and ultrasonication or thermal treatment and fermentation, as the studies of You *et al.* (2016) and Lu *et al.* (2018) suggest. The resistant starch determined for a baking product made with wheat flour supplemented with purple potato flour varied between 34.90-42.90% depending on the amount of flour added in bread's dough (Liu *et al*., 2019).

Table 5.7. Starch fractions from the gluten-free bread samples after *in vitro* digestion

Sample	**Starch fraction, %**		
	Rapidly digestible starch	**Slowly digestible starch**	**Resistant starch**
P0	15.37±0.12^{cC}	52.70±0.32^{aD}	31.93±0.39^{bA}
P1	23.23±1.24^{bA}	69.62±1.43^{aA}	7.15±0.21^{cE}
P2	17.73±0.23 ^{cB}	52.50±0.16^{aD}	29.77±0.39^{bA}
P3	22.67±0.11^{bA}	66.63±1.71^{aB}	10.69±1.81^{cD}
P4	19.61 ±0.99^{bB}	61.00±0.79^{aC}	19.39±0.22^{bC}
P5	18.12±0.63^{cB}	54.35±0.33^{aD}	27.53±0.52^{bB}

[1] *Avergage values for 3 replicates ± standard deviation. Different lowercase letters denote significant differences in a row, whereas different uppercase letters denote significant differences in a column*

5.3.2. Sensorial properties of gluten-free breads supplemented with sourdough

The experimental gluten-free bread samples were subjected to a sensorial evaluation by an unspecialized group of panelists, the average scores for each attribute being presented in **Figure 5.8**.

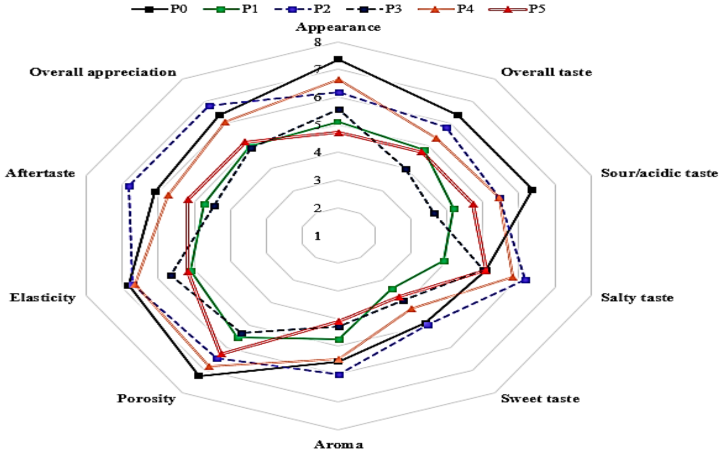

Figure 5.8. Average values of the sensorial features evaluated for the gluten-free breads

Using a similar approach, different types of gluten-free breads were made with quinoa, germinated quinoa, or with quinoa sourdough. The sensory analysis of these bread products emphasized the panelists' preference for the gluten-free sourdough quinoa bread. The highest scores were attributed to aroma, elasticity, and acidic taste, whereas the salty and sour tastes were depreciated (Franco *et al.*, 2021).

The experimental results obtained after the sensorial analysis, reported in this chapter, are confirmed by the study realized by Yeşil and Levent (2022), which concluded that the pseudocereals' flours (amaranth, buckwheat, quinoa) used for gluten-free bread production, and the microorganisms involved in the fermentation process (spontaneous microbiota or baker's yeast) may have a major impact on the textural and sensorial of the final products.

5.4. Partial conclusions

It was studied the impact of sourdough addition, using concentrations of 10% and 25% (w/w), obtained with different starter cultures to make some unconventional gluten-free bakery products employing chickpea, quinoa, and buckwheat flours. The effects on the functional, textural, and sensorial properties were studied by comparison with some control samples made of commercial gluten-free flours or chickpea, quinoa, and buckwheat flours' mixture without sourdough addition.

The experimental results emphasize an improvement of the microbiological stability in storage conditions, by a shelf-life extension with two up to four days, determined by the starter culture used for sourdough fermentation, the amount of flour substituted with sourdough contributed also to this result. The microbiological stability improvement of food products, by the extension of the necessary time for spoilage molds to develop, is associated to the sourdough's postbiotic compounds with antifungal potential.

Textural profile's evaluation for the obtained bread samples, including also another commercial, ready-to-eat, gluten-free bread sample (P6), highlighted the modification of some textural characteristics such as hardness, cohesiveness, and springiness, determined by gluten-free flours' substitution with sourdoughs.

After the simulated *in vitro* digestion of the experimental gluten-free bread samples (P0-P5), a different behavior was observed for the starch and bioactive compounds with antioxidant properties, such outcomes being determined by the recipe's ingredients used for bread making and by the starter cultures used for the fermentation processes to obtain sourdoughs. The flour mixture made of chickpea, quinoa, and buckwheat improved the polyphenolic content, including flavonoids, the sourdough addition contributed to a controlled release of these compounds with antioxidant activity during the simulated digestion protocol. The results after the *in vitro* digestion are influenced by the substitution level of the gluten-free flours with sourdoughs, the utilization of the sourdough made by fermentation with the water kefir grains starter contributed to an improved release of the functional compounds from the obtained bakery products.

The conducted studies have innovative character and show novel perspectives for the gluten-free raw materials' valorization to obtain some unconventional bakery products with superior functionality. Based on the quality criteria of the obtained gluten-free bakery products, considering the functional and sensorial properties, as well as the extension at a larger scale of the manufacturing process for these bakery products with chickpea, quinoa, and buckwheat flours, adding 10% (w/w) sourdough obtained by fermentation in controlled, optimized, conditions, it is considered to be a cost-efficient process with prospects to be scaled-up, promoting a healthy diet and the improvement of life quality.

Chapter 6. General conclusions

The doctoral thesis aiming at studying some efficient biotechnological variants to obtain sourdoughs by fermentation of some gluten-free flours comprising chickpea, quinoa, and buckwheat, using selected starter cultures, including microbial consortia from artisanal cultures (water and milk kefir grains), in order to obtain some bioingredients with improved technological and functional properties for bakery applications. Based on the experimental results presented and the partial conclusions detailed at the end of each chapter, there can be summarized the following general conclusions:

- The sourdough production methods for bakery applications are intensively studied in order to understand, improve, and optimize the employed biotechnological processes, taking into account the available unconventional raw materials that can be used involving the metabolic potential of some performant microorganisms, to improve the nutritional value and the technological and functional properties of the resulting fermented products, valuable bioingredients with multiple uses, extended also towards other food products, nutraceuticals, or feed production.
- Research specialists and manufacturers permanently diversificate the bakery products' types, obtained by "clean" technologies, designed for all the consumers or for some of them with special personalized diets (gluten- or lactose-free diet, vegan/vegetarian diet, hyperproteic diet). These novel food products must be characterized by enhanced nutritional, sensorial, and functional properties with a restricted addition of preservatives and ingredients to assure the shelf-life and food safety.
- Taking into consideration the metabolic diversity and functional adaptability of lactic acid bacteria, there were isolated and preliminary studied 60 strains with different origins, their selection being carried out based on some metabolic performance criteria and statistical relevance, as following: acidification properties, antimicrobial properties against molds and spoilage bacteria strains (spoilage microorganisms affecting the bakery products' food safety), capacity to biosynthesize some extracellular enzymes with impact in bakery, and exopolysaccharides, to be further used for gluten-free sourdough fermentation

process. Four performant strains were selected, coded MIUG BL 21, MIUG BL 24, MIUG BL 38, belonging to *Lactobacillus* spp., and MIUG BL 40, belonging to *Leuconostoc* spp. These strains are included in the Collection of Microorganisms from the Bioaliment Platform (acronym MIUG) (https://www.unicer.ugal.ro/index.php/ro/uc-acreditate-institutional), this Collection being affiliated to the pan-European research infrastructure *The Microbial Resource Research Infrastructure* (MIRRI) (https://www.mirri.org/).

- The probiotic properties of the selected lactic acid bacteria strains were studied, being demonstrated that the strain MIUG BL 21 is characterized by a superior probiotic potential due to the cells' survival after cultivation in the medium with low pH values, respectively supplemented with bile salts. Furthermore, this strain was resistant to a large spectrum of antibiotics, survival after *in vitro* gastrointestinal digestion, antipathogenic, and anticarcinogenic effect.
- Three of the performant strains were phylogenetically identified being taxonomically classified as *Lactiplantibacillus plantarum* (MIUG BL 21), *Lactiplantibacillus pentosus* (MIUG BL 24) and *Lacticaseibacillus rhamnosus* (MIUG BL 38).
- The exopolysaccharides' biosynthesis capacity was evaluated for the strains *Lactobacillus* spp. (MIUG BL 39) and *Leuconostoc* spp. (MIUG BL 40), being highlighted the inoculum's impact, and the extraction techniques used on the separated exopolysaccharides' yield, respectively on the antioxidant properties of these biopolymeric compounds.
- It was formulated and optimized the fermentation medium's composition made of gluten-free flours of chickpea, quinoa, buckwheat, and okara, and the fermentation conditions to obtain a sourdough, by using the experimental design, mathematical modelling, and statistical analysis tools, such as Plackett-Burman (PBD) and Response Surface Methodology (RSM). The selected lactic acid bacteria strains involved in the fermentation processes, *Lactiplantibacillus plantarum* MIUG BL 21 and *Lactiplantibacillus pentosus* MIUG BL 24, demonstrated metabolic performances and contributed to the bioactives' composition enrichment (organic acids and polyphenols) for the obtained fermented products. The technological and functional properties of the resulted sourdough can be correlated, by establishing for each targeted response the relevant parameters (independent variables), the intercorrelated effect of them, and the numerical values that lead to optimized processes, specifically designed for the desired objective, respectively the intensification of the antioxidant or antimicrobial properties (antifungal, antibacterial).
- It was demonstrated that postbiotics, the metabolites resulting after bioprocessing or released from the microbial cells used as starters, improve the technological and functional properties of the obtained gluten-free sourdough, being recommended as a valuable bioingredient with multiple applications in food, nutraceuticals, feed production etc., with impact on the life's quality.
- The fermentation substrate's formulation involved the utilization of the by-product named okara, made of soybeans pulp after processing, this ingredient

seemed to be beneficial for the lactic acid bacteria strains' nutritional requirements, being as well a promoter for the bioactives' production by fermentation and bioconversion processes.

- The possibility to use artisanal cultures made of lactic acid bacteria, acetic acid bacteria, and yeast strains that form the milk or water kefir grains' microbiota, as starter cultures to ferment the gluten-free flours substrates made of chickpea, quinoa, buckwheat and okara, to obtain sourdough was studied. For the process simplification and inoculum's stabilization, the kefir grains were freeze-dried with inulin solution (20% w/v) as a cryoprotectant agent.
- There were designed multiple technological variants (flow charts) to obtain sourdoughs, by varying the type and volume of inoculum, by carrying out the process in successive fermentation batches using fermentation systems with liquid (LSF) or semi-solid (SSF) medium, being emphasized the impact of the fermentation substrate's preliminary treatments on the technological and functional properties of the fermented product.
- The comparative study of the artisanal cultures, by cultivation on the unconventional substrates, allowed the identification of the novel possibilities to use performant water kefir grains to obtain gluten-free sourdoughs based on chickpea, quinoa, buckwheat, and okara. The microbiota's complexity of these natural microbial consortia, considering their microbiological composition (type and ratio among microorganisms) and the metabolic functionality (biochemical and physiological properties), depends on these cultures' origin which influence simultaneously the adaptability and functionality in particular fermentation conditions. Involving microbial consortia with synergistic activity, which are in symbiosis, in performant bioprocesses, offers numerous benefits on the bioactive properties of the resulting sourdoughs.
- The substitution of the gluten-free flour mixture made of chickpea, quinoa, and buckwheat with sourdoughs fermented by the freeze-dried culture of water kefir grains in various concentrations (10% and 25% w/w) determined beneficial modifications on the technological and functional properties of the gluten-free breads supplemented with this bioingredient. The benefits were demonstrated by *in vitro* digestion studies considering all the gastrointestinal tract's stages.
- Depending on the substitution level a shelf-life extension was observed, being also determined beneficial modifications of some textural characteristics and a controlled release of the functional compounds, respectively flavonoids, phenolic acids, and other compounds with antioxidant properties, the resistant starch with prebiotic effect being as well determined. After the sensorial analysis, the consumers preferred the bakery products supplemented with 10% (w/w) sourdough.
- The proposed solutions contribute to the bakery products' diversification, allowing the scaling-up of the processes based on the final products' cost-efficiency and quality principles, promoting also the "clean-label" concept.
- The reported results reinforce the innovative character of the studied and demonstrated concepts of the PhD thesis on both scientific and practical basis, that

are in accordance entirely with the targeted scientific objectives, enrich the knowledge in the field and open up new perspectives to understand and manage the biotechnological processes with applications in sourdoughs' production, with positive impact on life's quality, by a superior resources' valorization and by applying the circular economy principles.

Chapter 7. Original contributions and research perspectives

The original contributions of the doctoral thesis derive from the following aspects:

- There was isolated and analyzed considering metabolic and morphological aspects, a large number of lactic acid bacteria strains (60 strains) from the specific microbiota of some cereals', legumes', pseudocereals' flours, respectively from dairy fermented products' microbiota, or from other natural ecosystems. For the performant strains' selection based on specific biotechnological and functional properties, it was carried out a simultaneous analysis of multiple metabolic variables using the Principal Component Analysis. Four strains belonging to *Lactobacillus* spp. (*Lactiplantibacillus plantarum* MIUG BL 21, *Lactiplantibacillus pentosus* MIUG BL 24, *Lacticaseibacillus rhamnosus* MIUG BL 38) and *Leuconostoc* spp. (*Leuconostoc mesenteroides* spp. *mesenteroides* MIUG BL 40) were selected and stored in the MIUG Collection of Microorganisms, with national and international relevance.
- It was proposed the utilization of gluten-free flours from legumes (chickpea), pseudocereals (quinoa and buckwheat), and the freeze-dried by-product from soymilk's production (okara) as fermentation substrates, these raw materials being insufficiently exploited in biotechnological processes to obtain value-added fermented products.
- There were proposed new flow charts for sourdough's obtainment biotechnology, based on metabolic and physiological efficiency of the starter cultures used, correlated to the technological and functional properties of the resulted fermented products, as well as for the processes' simplification and economic efficiency to be used for practical application at industrial level.
- The efficiency of the artisanal starter cultures was demonstrated, respectively water kefir grains, to be used as starters for the fermentation of the substrate made of gluten-free flours (chickpea, quinoa, buckwheat) and okara, with numerous benefits upon the bioactive properties of the fermented product, recommended as a valuable bioingredient with antimicrobial and antioxidant effect. These exclusively carried out studies will be disseminated in a patent registration.
- Establishing a gluten-free bread making recipe using chickpea, quinoa, and buckwheat and studying the impact of sourdoughs, obtained by fermentation with selected lactic acid bacteria starter cultures and artisanal cultures (kefir grains), on the final products' functionality regarding targeted bioactive compounds' behavior during *in vitro* digestion are, also, arguments that support the originality of the PhD thesis. Based on the developed skills during the doctoral studies, being aware of the scientific evolution of the studied field, the research perspectives will be focused

on the sustainable biovalorification of the available but insufficient exploited raw materials and by-products, respectively on the probiotic microorganisms' study (lactic acid bacteria and yeasts) and their efficient control to biosynthesize metabiotics (prebiotics, probiotics, postbiotics, and paraprobiotics).

Chapter 8. Dissemination of research results

The dissemination of the research results carried out during the doctoral studies, was made in the following scientific papers published in relevant journals from the field or presented at prestigious international and national conferences, as such:

A. Articles published in ISI indexed journals

1. **Păcularu-Burada, Bogdan**, Ceoromila (Cantaragiu), Alina-Mihaela, Vasile, Mihaela Aida, Bahrim, Gabriela-Elena, 2022. *Novel insights into different kefir grains usefulness as valuable multiple starter cultures to achieve bioactive gluten-free sourdoughs.* LWT – Food Science and Technology, 165, 113670. **Impact factor: 4.952.**
2. **Păcularu-Burada, Bogdan**, Turturică, Mihaela, Rocha, João Miguel, Bahrim, Gabriela-Elena, 2021. *Statistical Approach to Potentially Enhance the Postbiotication of Gluten-Free Sourdough.* Applied Sciences, 11(11), 5306**. Impact factor: 2.679.**
3. **Păcularu-Burada, Bogdan**, Georgescu, Luminița Anca, Vasile, Mihaela Aida, Rocha, João Miguel, Bahrim, Gabriela-Elena, 2020. *Selection of wild lactic acid bacteria strains as promoters of postbiotics in gluten-free sourdoughs.* Microorganisms, 8(5), 643. **Impact factor: 4.167.**

B. Articles published in journal indexed in international databases

1. **Păcularu-Burada, Bogdan**, Bahrim, Gabriela-Elena, 2021. *Extraction and antioxidant activity assessment of postbiotic exopolysaccharides produced by selected lactic acid bacteria.* Innovative Romanian Food Biotechnology, 20 (journal indexed in international databases, https://www.gup.ugal.ro/ugaljournals/index.php/IFRB/about).
2. **Păcularu-Burada, Bogdan**, Georgescu, Luminița Anca, Bahrim, Gabriela-Elena, 2020. *Current approaches in sourdough production with valuable characteristics for technological and functional applications.* The Annals of the University Dunarea de Jos of Galati. Fascicle VI – Food Technology, 44(1), 132-148 (journal indexed in Web of Science Core Collection, Clarivate Analytics Master Journal List, Emerging Sources Citation Index).

C. Book chapters

1. Constantin, Oana Emilia, **Păcularu-Burada, Bogdan**, Bahrim, Gabriela-Elena, 2022. Yeast strains from sourdough as potential clean-label starters for fermentation processes. In: Ceresino, Elaine, Juodeikiene, Grazina, Miescher, Susanne, Rocha, João Miguel (Eds.) *Sourdough microbiota and starter cultures for industry*. Gewerbestrasse, Switzerland: Springer Nature – accepted for publication.
2. **Păcularu-Burada, Bogdan**, Pihurov, Marina, Cotârleț, Mihaela, Enachi, Elena, Bahrim, Gabriela-Elena, 2022. Introduction to sourdough enzymology. In: Vaquero, Marco Garcia, Rocha, João Miguel (Eds.) *Sourdough Innovations: Novel Uses of Metabolites, Enzymes and Microbiota from Sourdough Processing*. Boca Raton, Florida, USA: CRC Press – accepted for publication.

D. Papers presented at international scientific events

1. **Păcularu-Burada, Bogdan**, Bahrim, Gabriela-Elena, Vasile, Mihaela Aida, 2022. *New Strategy to Develop Metabiotic Sourdoughs for Gluten-Free Bread Functionalization*, 8th World Congress on New Technologies – NewTech'22, 03-05th of August 2022, Prague, Czech Republic.
2. Pihurov, Marina, **Păcularu-Burada, Bogdan**, Cotârleț, Mihaela, Grigore-Gurgu, Leontina, Bahrim, Gabriela-Elena, 2022. *Study of newly lactobacilli strains to be used as delivery systems for matabiotics*, Congress on food quality and safety, health and nutrition – NUTRICON 2022, 08-10th of June 2022, Ohrid, Republic of North Macedonia.
3. **Păcularu-Burada, Bogdan**, Vasile, Mihaela Aida, Cotârleț, Mihaela, Pihurov, Marina, Bahrim, Gabriela-Elena, 2021. *Study on the newly lactic acid bacteria strains for their probiotic behaviour*, 10th Edition of Euroaliment Symposium, 07-08th of October 2021, Galați, Romania.
4. Pihurov, Marina, **Păcularu-Burada, Bogdan**, Vasile, Mihaela Aida, Cotârleț, Mihaela, Bahrim, Gabriela-Elena, 2021. *Increasing the usage of agro-food byproducts through Lactobacillus spp. fermentation by using the Plackett-Burman Design*, 10th Edition of Euroaliment Symposium, 07-08th of October 2021, Galați, Romania.
5. Vasile, Mihaela Aida, **Păcularu-Burada, Bogdan**, Pihurov, Marina, Cotârleț, Mihaela, Bahrim, Gabriela-Elena, 2021. *Biotechnological parameters with impact on bovine colostrum tribiotication by fermentation with selected lactic acid bacteria*, 10th Edition of Euroaliment Symposium, 07-08th of October 2021, Galați, Romania.

6. Bahrim, Gabriela-Elena, Pihurov, Marina, **Păcularu-Burada, Bogdan**, Râpeanu, Gabriela, Stănciuc, Nicoleta, 2021. *Biotics+: From concepts to applications for generation of innovative tailored functional food*, 10th Edition of Euroaliment Symposium, 07-08th of October 2021, Galați, Romania.
7. Pihurov, Marina, **Păcularu-Burada, Bogdan**, Vasile, Mihaela Aida, Cotârleț, Mihaela, Bahrim, Gabriela-Elena, 2021. *Lactic acid bacteria isolated from the artisanal cultures as postbiotics promoters by fermentation of unconventional substrates*, 11th Edition of Probiotics, Prebiotics & New Foods, Nutraceuticals & Botanicals for Nutrition & Human and Microbiota Health, 12-14th of September 2021, Rome, Italy.
8. Vasile, Mihaela Aida, **Păcularu-Burada, Bogdan**, Pihurov, Marina, Cotârleț, Mihaela, Bahrim, Gabriela-Elena, 2021. *Postbiotic composition of the bovine colostrum improved by lactic acid fermentation in optimized conditions*, 11th Edition of Probiotics, Prebiotics & New Foods, Nutraceuticals & Botanicals for Nutrition & Human and Microbiota Health, 12-14th of September 2021, Rome, Italy.
9. Cotârleț, Mihaela, Pihurov, Marina, **Păcularu-Burada, Bogdan**, Vasile, Mihaela Aida, Bahrim, Gabriela-Elena, 2021. *Enhancement of the postbiotics production by lactic acid bacteria on agri-food residual substrates*, 11th Edition of Probiotics, Prebiotics & New Foods, Nutraceuticals & Botanicals for Nutrition & Human and Microbiota Health, 12-14th of September 2021, Rome, Italy.
10. **Păcularu-Burada, Bogdan,** Georgescu, Luminița Anca, Bahrim, Gabriela-Elena, 2020. *Postbiotic Exopolysaccharides Biosynthesized by Selected Lactic Acid Bacteria Strains for Gluten-Free Sourdough*, European Biotechnology Congress 2020, 24-26th of September 2020, Prague, Czech Republic.
11. **Păcularu-Burada, Bogdan**, Cotârleț, Mihaela, Vasile, Mihaela Aida, Bahrim, Gabriela-Elena, 2019. *Assessment of lactic acid bacteria enzymatic activities on chromogenic substrates for the functional food products obtainment*, Modern Biotechnological Advances for Human Health – BAHH, 28th-31st of May 2019, Bucharest, Romania.

E. Papers presented at national scientific events

1. **Păcularu-Burada, Bogdan**, Bahrim, Gabriela-Elena, 2022. *Effects of gluten-free breads supplementation with sourdoughs involving unconventional starters*, The Tenth Edition of Scientific Conference of Doctoral Schools (SCDS-UDJG 2022), 9-10th of June, Galați, Romania.

2. **Păcularu-Burada, Bogdan**, Bahrim, Gabriela-Elena, 2021. *Selection of lactic acid bacteria strains with probiotic characteristics*, The Nineth Edition of Scientific Conference of Doctoral Schools (SCDS-UDJG 2021), 10-11th of June, Galați, Romania.
3. **Păcularu-Burada, Bogdan**, Bahrim, Gabriela-Elena, 2021. *Postbiotic Exopolysaccharides Obtained by Controlled Fermentation Processes with Selected Lactic Acid Bacteria: Extraction and Characterization*, Conference on European Integration – Realities and perspectives, 16th Edition – Danubius University of Galați, 2021, 14-15th of May, Galați, Romania.
4. **Păcularu-Burada, Bogdan**, Bahrim, Gabriela-Elena, 2021. *Sourdough – a Functional Remedy in COVID-19 Pandemic Context*, Conference on European Integration – Realities and perspectives, 16th Edition – Danubius University of Galați, 2021, 14-15th of May, Galați, Romania.
5. **Păcularu-Burada, Bogdan**, Georgescu, Luminița Anca, Bahrim, Gabriela-Elena, 2020. *Exopolysaccharides biosynthesized by newly lactic acid bacteria strains with impact on gluten-free sourdough biotechnology*, The Eighth Edition of Scientific Conference of Doctoral Schools (SCDS-UDJG 2020), 18-19th of June, Galați, Romania.
6. **Păcularu-Burada, Bogdan**, Georgescu, Luminița Anca, Bahrim, Gabriela-Elena, 2019. *Sourdough Formulation with Improved Antimicrobial Properties*, The Seventh Edition of Scientific Conference of Doctoral Schools (SCDS-UDJG 2019), 13-14th of June, Galați, Romania.