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RESEARCH ARTICLE

METABOLIC FUNCTIONALITY OF THE SYNERGISTICALLY AMELIORATED CONSORTIUM BASED ON WILD KEFIR GRAINS AND SELECTED MICROORGANISMS FOR BOVINE COLOSTRUM FERMENTATION

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Abstract: The aim of the study was the investigation of the fermentative behaviour of multiple starter cultures with wild kefir grains and selected microorganisms. The enhanced consortium was used for the bovine colostrum fermentation in order to obtain the reproducibility of the fermentation process in terms of the technological and functional properties of the fermented product, i.e. acidity, antioxidant and antimicrobial activities. Also, the scaling up technology for the production of fermented colostrum was performed in order to analyse the technological and functional properties of the consortium used as starter culture. Different fermented samples were obtained and analysed considering the pH, titratable acidity, antimicrobial activity (agar well-diffusion assay) and antioxidant potential (DPPH method). Fermented products with different characteristics were observed taking into consideration the variation of the biotechnological conditions or during the scale-up of the fermentation process. In order to offer a stable technological solution ready to be used in large scale manufacturing processes a technical variant which demonstrated reproducibility was proposed. The fermented product obtained by successive batches of fermentation showed almost 79% of acidity, 30% of the antioxidant potential, and the antimicrobial activity remained constant in comparison with the laboratory inoculum, which was considered 100%.

Keywords: kefir grains, synergistically ameliorated consortium, bovine colostrum, fermentation, scaling up technology

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Introduction

Kefir is an ancient fermented beverage produced by the multiple actions of the lactic acid bacteria (LAB), yeasts, and acetic acid bacteria that are associated in symbiosis in milk kefir grains, as an artisanal consortium of cells that form a complex matrix with kefiran and other polysaccharides (de Oliveira Leite *et al*., 2013; Tan *et al*., 2020). This complex consortium of microorganisms produces a distinctive fermented product with unique functional properties (Farnworth *et al*., 2003; Gaware *et al*., 2011; Guzel-Seydim *et al*., 2011). Due to the complex microbiota present in the kefir starter and for functional benefits associated with the consumption of this milk fermented beverage, kefir was designated as a natural probiotic product (de Oliveira Leite *et al*., 2013; Farag *et al*., 2020). Recent studies demonstrated that kefir and its bioactive components have essential healthy properties such as antimicrobial, antihypertensive, antioxidative, anticytotoxic, hypocholesterolemic, anticarcinogenic and immunomodulatory activity and also improve the lactose digestion (Cotârleţ *et al*., 2020; Farag *et al*., 2020).

Milk kefir grains can be preserved as freeze-dried or wet, but constant washing reduces their fermentation potential because some cells could be removed from the polysaccharides matrix surface (de Oliveira Leite *et al*., 2013). It was mentioned that freeze-dried kefir grains maintain their activity for 12–18 months (Garrote *et al.*, 2010). This offer a valuable perspective for the kefir grains preservation in order to be used as a starter culture.

Colostrum is the first secretion of the mammary gland being rich in proteins, fat, lactose, vitamins, minerals, and antimicrobial substance (i.e. immunoglobulins, lactoferrin, lactoperoxidase, lysozyme and cytokines (Silva *et al.*, 2019). The possibility to ferment the bovine colostrum with the kefir grains was previously demonstrated (Cotârleţ *et al*., 2019; Cotârleţ *et al*., 2020). Due to the consumer's preference and its high perishability, the colostrum is still under-utilized. Recently, the bovine colostrum was used for food supplementation, nutraceuticals, or in medicinal therapies (Bartkiene, *et al*., 2018; Silva *et al.*, 2019). In recent years, only a few studies mention the production of dairy foods with different bovine colostrum concentrations (Ayar *et al*., 2016; Nazir *et al*. 2018).

Taking into account these premises, the present study reports the obtainment of functional fermented products based on bovine colostrum fermented with a synergistically ameliorated

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consortium of bacteria and yeasts. The proposed consortium comprised freeze-dried milk kefir grains culture enhanced with a selected yeast strain (*Candida lipolytica*) and FreshQ4®, Chr. Hansen commercial culture improved the characteristics of the fermented product and assured the reproducibility of the scaled-up biotechnological process.

Materials and methods

Microorganisms

The artisanal starter culture (ASC) was obtained by processing the fresh milk kefir grains, through successive passages in sterile milk (3.5% fat) previously autoclaved at 105°C, for 10 min, using an autoclave (Sanyo MLS-3020U, Groningen, The Netherlands) and then freeze-dried by a freeze-dryer (Martin Christ Alpha 1-4 LD, Osterode am Harz, Germany) (the process that is being patented). Initially, the stock kefir grains were preserved in a 40% glycerol (w/v) solution at -70°C in ultra-freezer (Angelantoni, Cimacolle, Italy). The freeze-dried artisanal starter culture was preserved in a glass container hermetically closed for 6 months at 4°C.

Yeast culture, *Candida lipolytica* MIUG D67, was preserved in 40% glycerol at -70°C and it was reactivated by cultivation on Yeast extract glucose chloramphenicol agar (YGC) for 72 h, at 30°C in an incubator (Binder BF4000, Tuttlingen, Germany). The inoculum was obtained by transferring the fresh yeast biomass into sterile 0.9% NaCl solution and obtaining cells suspension, which was counted with the Thoma cytometer. The yeast strain *C. lipolytica* MIUG D67 and the indicator microorganisms for the antimicrobial activity assay, *Aspergillus niger* MIUG M5 and *Bacillus subtilis* MIUG B1, were provided from the Microorganisms Collection of the Bioaliment Research Platform (acronym MIUG) of Faculty of Food Science and Engineering of ”Dunărea de Jos” University of Galați, Romania.

The FreshQ4® commercial probiotic culture was purchased from Chr. Hansen, Denmark.

All the chemicals, reagents, and commercial culture media were purchased from Sigma-Aldrich (Steinheim, Germany).

Fermented samples obtainment using different inoculums

Firstly, 8% (w/v) bovine colostrum (Axyar, Belgium) was autoclaved at 105°C for 10 min. Then, four samples were prepared as following:

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Sample 1 – 100 mL of sterile colostrum was inoculated with 0.125% (w/v) freeze-dried ASC and then incubated for 96 h, at 30°C, under the stationary conditions. This sample was considered as a control sample.

Sample 2 – 100 mL of sterile colostrum was inoculated with 3.8 mL of *C. lipolytica* MIUG D67 suspension, with a concentration of 2.62 x 10^8 CFU/mL and incubated on a rotary shaker (Lab Companion SI-300, GMI, Minneapolis, MN, USA) for 48 h, at 30°C and 150 rpm, then 0.125% (w/v) of freeze-dried ASC was added and incubated further for 48 h, at 30°C, under stationary conditions.

Sample 3 – 100 mL of sterile colostrum was inoculated with 3.8 mL of *C. lipolytica* MIUG D67 suspension, with a concentration of 2.62 x 10^8 CFU/mL and incubated on a rotary shaker for 48 h, at 30°C and 150 rpm. Afterward, 0.125% (w/v) freeze-dried ASC and 0.2% (w/v) Fresh Q4® culture were added and incubated further for 48 h, at 30°C, under the stationary conditions.

Sample 4 – 100 mL of sterile colostrum was inoculated with 3.8 mL of *C. lipolytica* MIUG D67 suspension, with a concentration of 2.62 x 10^8 CFU /mL, 0.125% (w/v) freeze-dried ASC and 0.2% (w/v) Fresh Q4® culture and incubated for 96 h, at 30°C, under the stationary regime.

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Laboratory stage

In an Erlenmeyer flask, a volume of 100 mL fermentation media based on bovine colostrum 8% (w/v), autoclaved and cooled down was inoculated with 3.8 mL of *Candida lipolytica* MIUG D67 suspension (2.62 x 10^8 CFU/mL), and incubated for 48 h, at 30°C and 150 rpm. Then, 0.125% (w/v) freeze-dried ASC and 0.2% (w/v) FreshQ4® were added and further incubated for 48 h at 30°C, under stationary conditions (sample 3). The obtained fermented product was considered a laboratory inoculum that was used for the fermentation of the next batches.

Micro pilot fermentation stages

In this stage the primary and secondary starter cultures were obtained. 8% autoclaved and cooled bovine colostrum was inoculated with 10% starter culture (laboratory inoculum). Thus, in the Erlenmeyer flask, 10 mL of laboratory inoculum was added in 100 mL of the fermentation medium and incubated at 30ºC, for 72 h, in the stationary conditions (primary starter culture). Then, in 3 L laboratory bioreactor, Applikon BioBundle (USA), the primary starter culture (100

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mL) was further inoculated in 1 L sterile medium, following the biotechnological parameters previously described (secondary starter - production culture). Afterward, in 16 L pilot bioreactor pilot, Bioengineering (Switzerland), 1 L of production starter culture was inoculated in 10 L of the fermentation medium and incubated at 30ºC, for 72 h, in stationary regime. After that, the fermented product was freeze-dried and stored at 4°C. The flowchart for the scaling up technology was showed in Figure 1.

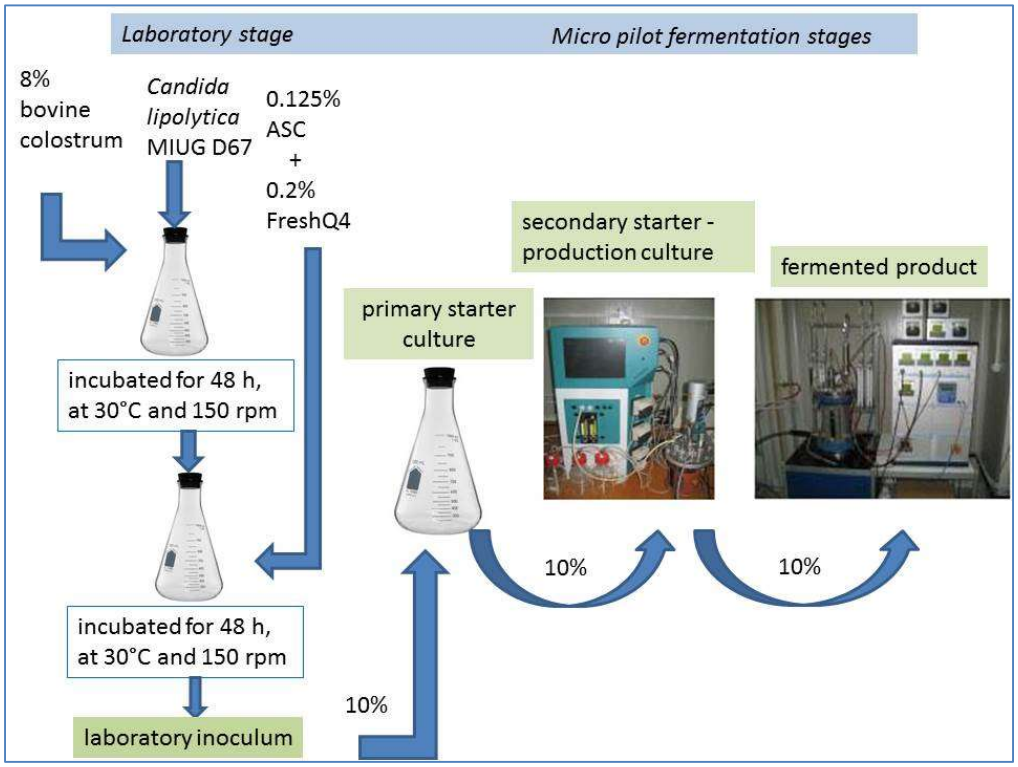

Fig.1. Schematic process diagram for scaling up the technology

pH and acidity

The pH of the fermented samples was measured with the MP 2000 pH meter (Mettler Toledo, Switzerland). The total titratable acidity assay (TTA) was performed in accordance with Cotârleţ *et al*., 2019. The TTA was expressed as Thörner degrees (ºTh).

Antimicrobial activity

The antimicrobial activity of the fermented products was tested against *Bacillus subtilis* MIUG B1 and *Aspergillus niger* MIUG M5 strains, that frequently cause food spoilage, by using the

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agar well diffusion assay (Macaluso *et al*., 2016; Cotârleţ *et al*., 2019). The diameter of the inhibition zone was measured and expressed in mm.

Antioxidant activity

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) (Fluka Chemie, Buchs, Switzerland) radical scavenging capacity of the samples was performed according to Cotârleţ *et al*., 2019, and expressed as mM Trolox Equivalent (TE)/mL.

Statistical analysis

Analysis of variance (ANOVA) followed by Tukey test was performed using Minitab 19, version 1.1. (Minitab LLC, USA). Differences between samples were considered significant for p values < 0.05.

Results and discussion

Testing the fermentative behaviour of the synergistically ameliorated consortium

In the present study, four samples were obtained by fermenting the bovine colostrum with different starter culture inoculums. Firstly, the technological properties studied for estimating the fermentative capacity of the starter cultures were pH and total titratable acidity. The relationship between the titratable acidity and pH was not fully elucidated yet, even it is known that pH decreases with the increases of the acidity (Barukčić *et al.,* 2017). This tendency is confirmed by the results reported in this work (Table 1).

Table 1. Acidification characteristics of the fermented samples

Sample	pH	Total titratable acidity, °Th
1	4.18 ± 0.00^{d}	112.50 ± 0.50^{a}
2	5.58 ± 0.07^{a}	50.00 ± 0.50^{d}
3	4.56 ± 0.08^{c}	100.00 ± 0.87^{b}
4	5.08 ± 0.01^{b}	87.50 ± 0.50^{c}

The values are means for three replicates ± standard deviation; means, for the same column, that do not share a letter are significantly different, using Tukey test for $p<0.05$

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The fermented sample that presented the lowest pH value (4.18) and the highest acidity (112.50 ºTh) was sample 1 (control). Comparable values were achieved for sample 3, where the bovine colostrum was fermented with an enhanced consortium of selected yeast and commercial lactic acid bacteria (LAB). Our previous studies reported higher pH and acidity values for the fermented colostrum with fresh kefir grains enhanced with selected yeast and commercial LAB (Cotârleţ *et al*., 2019; Cotârleţ *et al*., 2020). The fermented product obtained with a consortium based on fresh kefir grains (2.5%, w/v) and *Candida lipolytica* MIUG D67 indicated higher acidity (200°Th), after 48 h of fermentation. Cotârleţ *et al*. (2020) highlighted that using the fresh kefir grains fermented products with higher values of the acidity were achieved (168°Th). Unfortunately, the use of the fresh kefir grains is not considered a scale-up solution because the multiplication and preservation of the inoculum are disadvantageous techniques for industrial use.

The results from the antioxidant and antimicrobial activities of the fermented samples are shown in Table 2. Sample 3 registered the highest antioxidant activity (1.89 mM TE/mL) compared to sample 1 (0.64 mM TE/mL). Therefore, sample 3 presented comparable values of the acidity and antimicrobial potential compared to the control (sample 1), but the antioxidant potential was almost 3 times higher. Sample 3 was fermented with *Candida lipolytica* MIUG D67 for 48 h and then 0.125% freeze-dried ASC, respectively 0.2% FreshQ4® were added and fermented further for 48 h, at 30°C. This yeast strain was previously selected based on the proteolytic and lipolytic activities (Cotârleţ *et al*., 2019).

In other studies it was demonstrated that the fermented products obtained with a consortium based on fresh kefir grains and *Candida lipolytica* MIUG D67 have an increased antioxidant activity, respectively 2.69-3.15 mM TE/mL, after 48 h of fermentation, at 30°C with ameliorated consortium (Cotârleţ *et al*., 2019; Cotârleţ *et al*., 2020).

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Table 2. Antioxidant and antibacterial activities of the fermented bovine colostrum samples

Sample	Antioxidant activity, mM TE/mL	Antibacterial activity, mm *(B. subtilis)*
1	0.64 ± 0.08^{b}	3.50 ± 0.17^{a}
2	1.54 ± 0.33^{a}	1.00 ± 0.10^{c}
3	1.89 ± 0.31^{a}	3.00 ± 0.10^{b}
4	1.55 ± 0.44^{a}	n.d.

The values are means for three replicates ± standard deviation; n.d. – not determined; means, for the same column, that do not share a letter are significantly different, using Tukey test for $p<0.05$

Fermented samples 1 and 3 demonstrated the highest antibacterial activity (3.5-3.0 mm), which proved that ameliorated consortium produced the antimicrobial compounds by bovine colostrum biotransformation (bioconversion and fermentation), such as organic acids (lactic acid, acetic acid, and propionic acid), fatty acids, bioactive peptides and exopolysaccharides (de Oliveira Leite *et al*., 2013; Moradi *et al*., 2020).

The broadest antibacterial spectra against eight food pathogens and spoilage microorganisms (*Bacillus cereus, Staphylococcus aureus, Listeria monocytogenes, Enterococcus faecalis, Escherichia coli, Salmonella enteritidis, Pseudomonas aeruginosa* and *Cronobacter* spp.) were obtained after at least 48 h of fermentation using four types of kefir (Kim *et al*., 2016).

A total inhibition of *Bacillus cereus* ATCC 33019 and *Bacillus subtilis* ATCC 6633 was reported for the peptides extracted from sheep milk fermented with kefir grains (de Lima *et al*., 2018).

At a concentration of 200 µL of the coconut milk kefir showed an inhibition zone of 6 mm for *Aspergillus niger* (Lakshmi *et al*., 2017). In our study, 100 µL of fermented products inhibited sporulation of *Aspergillus niger.* Ismaiel *et al.* (2011) studied the influence of various concentrations of fermented kefir filtrate on *Aspergillus flavus* AH3 strain's growth, demonstrated that the sporulation was inhibited.

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The fermented products, resulting from each batch were characterized in terms of pH and TTA, as Table 3 shows.

Table 3. The fermentation dynamic during the scaling-up technology

Sample	pH	Total titratable acidity, ºTh
Laboratory inoculum	3.94±0.01[a]	146.82±2.58[a]
Primary starter culture	3.89±0.02[a]	140.72±4.00[a]
Secondary starter culture	3.90±0.02[a]	118.25±2.82[b]
Fermented product	3.91±0.00[a]	115.87±0.53[b]

The values are means for two replicates ± standard deviation; means, for the same column, that do not share a letter are significantly different, using Tukey test for $p<0.05$

The data presented in Table 3 highlighted that all the fermented products shown that the acidification power decreased during fermentation stages. Instead, the fermented product (the laboratory inoculum) had the highest value for total titratable acidity (146.82ºTh). The decreasing trend was correlated with the number of the batches. Taking into account that for primary, secondary and production steps of the fermentation a concentration of 10% from a previously fermented product was used as inoculum, the scaled-up process was quite expected, probably based on a reduced inoculum concentration and also of a selected adaptation of some strains which act in synergism.

Regarding the antioxidant activity, it can be concluded that the initial fermented product (laboratory inoculum) registered the highest antioxidant potential (1.78 mM TE/mL), decreasing as the fermentation batches were performed (Table 4). This fact could be explained by the reduced dimension of the inoculum and the type of the microbiota which could influence the process and the fermented product characteristics.

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Yilmaz-Ersan *et al*. (2018) determined an antioxidant potential, for fermented kefirs from cow and ewe milk, between 2.10 – 6.25 mg TE/100 mL.

Table 4. The functional properties of the fermented samples

Sample	Antioxidant activity, mM TE/mL	Antibacterial activity against *Bacillus subtilis*, mm
Laboratory inoculum	1.78±0.02[a]	4.2±0.14[a]
Primary starter culture	1.02±0.18[b]	2.2±0.07[b]
Secondary starter culture	0.73±0.16[b]	2.5±0.07[b]
Fermented product	0.53±0.02[b]	4.2±0.07[a]

The values are means for two replicates ± standard deviation; means, for the same column, that do not share a letter are significantly different, using Tukey test for $p<0.05$

The data presented in Table 4 and Figure 2 highlighted that the yeast strain, which was involved in the first stage of colostrum fermentation, was able to produce bioactive peptides with antimicrobial and antioxidant properties. Afterward, the activity of the selected yeast decreased in competition with the wild microorganisms from the artisanal culture, much more adapted to the substrate and to the consortium.

a)

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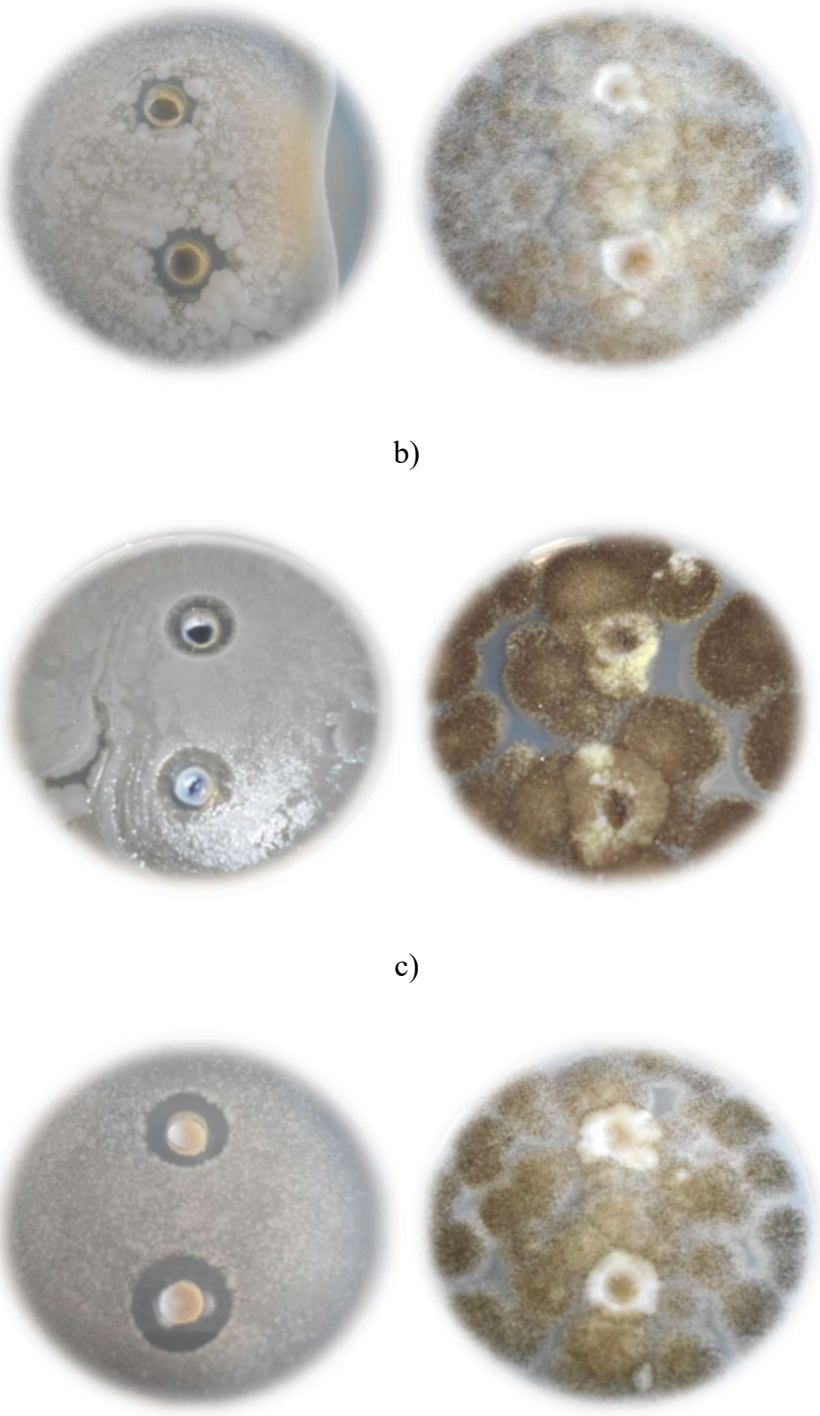

b)

c)

d)

Fig. 2. Antimicrobial activity of the fermented samples obtained during the scaling-up experiment (duplicates), a) laboratory inoculum, b) primary starter culture, c) secondary starter culture, d) fermented product, against *B. subtilis* (left) şi *A. niger* (right)

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Regarding the antimicrobial activity, it can be pointed out that the strongest antibacterial spectrum was obtained against *B. subtilis* MIUG B1 for all fermented products. Diameters of the inhibition zone between 2.2 - 4.2 mm were measured. The best results were obtained when the initial fermented product and the fermented product obtained in the production stage were used (Table 4). The antimicrobial spectrum for the fermented product obtained in the production stage could also be explained by the fact that after successive batches, the starter microorganisms were able to form an optimized consortium with functional properties. Instead, regarding the antifungal activity against *A. niger* MIUG M5, it was observed that almost all the fermented samples inhibited the sporulation (Figure 2).

Chifiriuc *et al*. (2011) reported an inhibition zone of 30 mm, against *B. subtilis,* when 24-48 h fermented kefir was used. The precise mechanism of the microbial growth inhibition is still unidentified. The LAB from wild kefir grains, especially lactobacilli, could produce a variety of antimicrobial compounds, that are able to reduce the food pathogens (*Bacillus subtilis*, *Fusarium graminearum, Aspergillus flavus* (Prado *et al*., 2015; Bartkiene *et al*., 2018), to extend the shelf-life of the fermented foods, or to treat and prevent the gastro-intestinal. The growth inhibition of the aerobic species (*Bacillus* spp.) could be explained by oxidative phosphorylation. Moreover, in high concentration, acetaldehyde, diacetyl, ethyl and amino acids possess antimicrobial activity against yeasts and molds and demonstrated antioxidant potential (Chifiriuc *et al*., 2011; Deeseenthum, *et al*., 2018).

Conclusions

This study presents some possibilities to enhance the technological and functional of the inoculum for the bovine colostrum fermentation by associating the artisanal and selected starter cultures. This strategy improves the characteristics of the fermented products. The study highlights a new approach for scaling-up the technology in order to obtain fermented colostrum with an enhanced artisanal culture by using as inoculum 10% of the previously fermented product. Besides, the functionality of the enhanced synergistically ameliorated consortium, the reproducibility of the fermentation (fermentation acidity, antioxidant activity and antimicrobial activity) was demonstrated. The results offer an innovative perspective to extend the fermentation of the bovine colostrum at pilot or industrial levels in order to be used in food industry and nutraceuticals.

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