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RESEARCH ARTICLE

The development of novel probiotic fermented plant milk alternative from flaxseed oil cake using *Lactobacillus rhamnosus* GG acting as a preservative agent against pathogenic bacteria during short-term refrigerated storage

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ABSTRACT

This study is aimed at investigating the suitability of flaxseed oil cake plant milk alternative (FOCM) to produce an innovative probiotic drink using *Lactobacillus rhamnosus* GG (LGG). Three variants of beverage were prepared: without supplementation, supplemented with 1% of glucose and supplemented with 1% of fructose. The viability of LGG, levels of polyphenolics, flavonoids and antioxidant activity were analysed. Moreover, color, viscosity and titratable acidity measurements were performed. Fermented beverages were artificially contaminated with pathogenic bacteria, and preservative effect of LGG was evaluated. The fermentation improved antioxidant activity, polyphenolics and flavonoids content, whereas viscosity of the samples decreased. During storage at 6°C for 48 hours the viability of LGG in all the samples was over the recommended probiotic minimum level ($>10^6$ CFU/mL). Supplementation with sugars significantly enhanced acidification of FOCM. Acidification was linked with preservative effect of LGG against pathogenic microflora. A significant reduction of Gram-negative pathogens was observed, whereas moderate activity was noted against Gram-positive bacteria. Due to the functional and biochemical characteristics conferred to the fermented beverages, the use of LGG showed adequate potential for industrial application. There is a potential for these products to be used where non-dairy alternatives for probiotic consumption are desired.

Keywords: *Lactobacillus rhamnosus* GG; fermentation; probiotic; flaxseed oil cake; plant based milk alternative

INTRODUCTION

Probiotic bacteria and their health effects are a focus of international food research. Probiotics are defined as: "living microorganisms that, when administered in adequate amounts, are beneficial to host's health, though a positive action on intestinal microbiota" (FAO/WHO, 2006). Therapeutic benefits of probiotics can vary according to their specific strains (Williams and Hekmat, 2017). Incorporation of selected strains of the genera *Bifidobacterium* and *Lactobacillus* in dairy as well as non-dairy products is extensively studied worldwide (Kandylis et al., 2016; Williams and Hekmat, 2017; Szparaga et al., 2019). The consumption of appreciable amounts of fermented products confers health benefits primarily by balancing intestinal microflora (Ranadheera et al., 2017). This is mainly due to a large number of active and living microorganisms in fermented foods, with the minimum counts of viable lactic acid bacteria (LAB) at least 10^6 CFU/mL (Colony Forming Units). This level has been deemed the therapeutic minimum (Szparaga et al., 2019). The efficacy of added probiotic bacteria depends on inoculum level and their viability must be maintained throughout storage of the product's shelf life (Alegre et al., 2011; Vesterlund et al., 2012). As the public demand for potential beneficial health effects

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of probiotic has increased, many products have been proposed as carrier foods for probiotics to improve the health and nutrition of consumers (Jia et al., 2016). Most probiotics are available in form of fermented dairy products (such as yogurt and kefir), as well as dietary supplements (Łopusiewicz et al., 2019; Łopusiewicz et al., 2020a). However, consumer's demand for dairy alternatives increased due to health (lactose intolerance, allergies, cholesterol) issues, and their awareness about the effects of their dietary choices on environment (Bernat et al., 2014; Kandylis et al., 2016; Łopusiewicz et al., 2019). Therefore, a need has arisen to offer consumers an alternative to fermented dairy products by exploring new non-dairy matrices as probiotics carriers (Kehinde et al., 2020; Nazhand et al., 2020). Plant-based products are perceived as healthy foods because they are rich in antioxidants, vitamins, dietary fiber, and minerals. Additionally, they do not contain any dairy ingredients such as allergens, lactose and cholesterol that might prevent their use by certain segments of the population (Łopusiewicz et al., 2019; Szparaga et al., 2019; Łopusiewicz et al., 2020a). Plant milk substitutes are water-soluble colloidal extracts, suspensions, or emulsions consisting of dissolved and disintegrated plant material that resemble animal milk in appearance, color and texture (Bernat et al., 2014; Atalar, 2019; Kehinde et al., 2020). Currently, consumers are interested in novel "milk" plant-based products, thus industries are forced to explore different raw materials, as well as apply novel processing technologies with the aim to develop new functional products (Łopusiewicz et al., 2019; Szparaga et al., 2019; Kehinde et al. 2020). Plant-based dairy analogues are one approach, and the development of such products has attracted rising interest. However, the majority of literature concerns the feasibility of producing fermented soybean milk, which is related to the fact, that manufacture of high quality plant-based beverages containing probiotics poses a serious challenge (Bernat et al., 2014; Szparaga et al., 2019).

A high survivability of the probiotic microorganisms in food products during their specified shelf life, and the potential antimicrobial action of the probiotics against contaminating pathogens during production process and shelf life are desirable (Sireswar et al., 2017; Minervini et al., 2020). With regard to plant-based beverages, stable product development is a challenge, as the use of chemical preservatives is usually avoided as it does not match with the "fresh" image. Moreover, chemical additives, commonly used in food products to inhibit microbial growth, improving quality and extending shelf-life, are negatively perceived by consumers (Alegre et al., 2011; Castellano et al., 2017; Ricci et al., 2019). While the majority of consumers prefer consumption of minimally processed, low, or synthetic preservatives free food, even with the most recent technological advancements, issues related to food safety and security still remain to be completely resolved (Sireswar et al., 2019). In order to ensure food safety while trying to match consumer expectations, the research of natural preservatives as alternatives to chemicals represents an interesting avenue. In this context various natural additives such as essential oils, enzymes, peptides, organic acids, chitosan, bacteriocins, and bacteriophages have been considered (Kwiatkowski et al., 2019; Ricci et al., 2019). Control of pathogenic microflora in fermented products is important because these pathogens and/or their toxins, may have an adverse effect, causing food poisoning and diarrhea (Mpofu et al., 2016; Bartkiene et al., 2020; Minervini et al., 2020). Despite the presence of modern security safety systems, the number of cases of foodborne illnesses and poisoning that are caused also by antibiotic-resistant bacteria seems to be increasing worldwide (Sireswar et al., 2017; Kwiatkowski et al., 2019; Ricci et al., 2019). Under these circumstances, the application of food suitable LAB cultures as protective agents during fermentation, processing, and storage of food products offers an interesting strategy for biopreservation (Mpofu et al., 2016; Castellano et al., 2017; Sireswar et al., 2017;Ricci et al., 2019; Minervini et al., 2020). Among microorganisms, many LAB have the GRAS (Generally Recognized as Safe) status, and ability to produce antimicrobial compounds, such as organic acids (lactic, acetic, propionic, succinic, etc.), diacetyl, bacteriocins, hydrogen peroxide, as well other metabolites (Makras et al., 2006; Zhang et al., 2011; Castellano et al., 2017; Iglesias et al., 2018). LAB can also act as microbial barriers against undesirable microorganisms through competitive exclusion of pathogen binding in gastrointestinal tract and modulation of host's immune system (Servin, 2004; Fayol-Messaoudi et al., 2005; Sun et al., 2010). Moreover, LAB play an essential role in formation of unique flavor, aroma and texture characteristics of food products (Castellano et al., 2017; Łopusiewicz et al., 2019, 2020a). However, it is necessary to point out, that the efficacy of each LAB strain as probiotic and/or protective culture may differ, and needs standardization for different food systems (Sireswar et al., 2017, 2019).

Plant processing generates a large number of by-products rich in bioactive compounds and nutrients, that may be successfully fermented and metabolized (Łopusiewicz et al., 2019, 2020a; Ricci et al., 2019; Szparaga et al., 2019). It was reported that plant milks may be good matrices for maintenance high survivability of probiotics (Bernat et al., 2014; Szparaga et al., 2019; Kehinde et al., 2020). Flaxseed oil cake (FOC) is underestimated cheap by-product of flaxseed (*Linum usitatissimum* L.) oil pressing, and is a source of many bioactive substances such as proteins, fiber and lignans. In previous

works, it was proved, that FOC is a safe, suitable material to develop non-dairy kefir-like, yogurt-like and Camembert-like fermented products characterized by high bioactivity and microorganisms viability during refrigerated storage (Łopusiewicz et al., 2019, 2020a, 2020b).

No studies have been published on the use of flaxseed oil cake to produce milk-like extract used in the development of probiotic LGG-fermented drink. Also, despite well-characterized probiotic properties of LGG, there are limited scientific reports regarding its use in plant milks fermentation processes. Moreover, a little information is available on the survival and growth of pathogens in non-dairy products containing probiotics. Thus, the aim of the presented study was to produce LGG-fermented plant milk alternative based on flaxseed oil cake, with/and without addition of sugars (glucose and fructose), and evaluation of microbiological and physicochemical properties during short-term refrigerated storage.

MATERIALS AND METHODS

Materials and chemicals

Flaxseed oil cake (FOC) obtained via cold press technique was kindly donated by ACS Sp. z o.o. (Bydgoszcz, Poland). The proximate composition of FOC was as follows: solids content 80.50%, protein content 41.97%, carbohydrates content 27.99%, fiber content 6.29%, fat content 6.11%, ash content 4.50%. *Lactobacillus rhamnosus* GG (ATCC53103), *Enterococcus faecalis* ATCC29212, *Escherichia coli* ATCC25922, *Pseudomonas aeruginosa* ATCC9027, *Salmonella enterica* subsp. *enterica* serovar Typhimurium ATCC14028, *Staphylococcus aureus* ATCC 43300 (MRSA – Methicillin-resistant *Staphylococcus aureus*) were purchased from ATCC (Manassas, Virginia, USA). Buffered peptone water, MRS agar and broth, XLD agar, Slanetz and Bartley agar, McConkey agar, Pseudomonas agar base with CN supplement, Mannitol Salt agar were obtained from Merck (Darmstadt, Germany). Phenolphthalein, 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), Folin-Ciocalteu's reagent, sodium carbonate, gallic acid, sodium nitrite, aluminium chloride, quercetin, and potassium persulphate were purchased from Sigma Aldrich (Darmstadt, Germany). Sodium chloride, sodium peroxide, ethanol, methanol, glucose, and fructose were supplied from Chempur (Piekary Śląskie, Poland). All reagents were of analytical grade.

Preparation and fermentation of flaxseed oil cake milk (FOCM)

The preparation of FOCM was carried out as described elsewhere (Łopusiewicz et al., 2020a). Initially, the FOC was mixed with distilled water in a ratio 1:10 (*w*/*w*). Subsequently, the mixture was heated at 90°C for 1 h with constant stirring (250 rpm), afterwards cooled down to 20°C. The extract was centrifuged (4000 rpm) for 30 min at 20°C (MPW-352R, MED Instruments, Warsaw, Poland), then the supernatant was filtered (Whatman No 1 filter paper, GE Healthcare, Buckinghamshire, UK) under vacuum to obtain a clear, milky fluid (FOCM – Flaxseed Oil Cake Milk). FOCM was homogenized for 5 min with homogenizer (SilentCrusherM, Heidolph, Germany) at 12000 rpm. After preparation, FOCM was dispensed into 4 containers (1000 mL volume, each containing 700 mL of FOCM), pasteurized by heating for 30 min at 60°C, then cooled down in refrigerator (6°C) one day before fermentation. *L. rhamnosus* GG was grown overnight in MRS broth at 37°C for 24 h in anaerobic conditions, obtained by using BD GasPak™ EZ sachets (Becton, Dickinson and Company, Sparks, Maryland, USA) in Parafilm (Bemis, Neenah, Wisconsin, USA) sealed glass jars. After incubation, fermentation broth was divided into three batches in sterile Falcon tubes (50 mL). Cells were harvested by centrifugation (2000 rpm for 5 min), washed with sterile physiological saline (0.9% NaCl) and centrifuged again. Afterwards, the bacterial biomass was resuspended in 10 mL of sterile physiological saline in each particular tube, to obtain approximately 2.0×10^6 CFU/mL (served as an inoculum). Three variants of fermented FOCM were prepared, and labelled as: FOCM-LGG (FOCM with addition of LGG); FOCM-LGG-1%G (FOCM + LGG + 1% of glucose) and FOCM-LGG-1%F (FOCM + LGG + 1% of fructose). After inoculation all variants were incubated in anaerobic conditions at 42°C for 24 h. The non-fermented reference sample FOCM-C (control FOCM devoid of any additive) was treated the same way.

Artificial contamination of FOCM samples after fermentation

After fermentation, the samples were artificially contaminated with pathogenic bacteria. Particular cultures from overnight grown solid plates (aerobic conditions, 37°C) were diluted in sterile physiological saline to obtain bacterial cells concentrations of 4 on the McFarland scale. Then, 1 mL of particular pathogen suspensions were aseptically added to fermented samples, vigorously shaken and incubated at 6°C for 48 h. Non-fermented samples were also inoculated with particular pathogenic bacteria for comparison of their survivability.

Microbial analyses

To enumerate the bacterial counts, the samples (1 mL) were collected and diluted with 9 mL of sterile buffered peptone water. Subsequently, ten-fold serial dilutions were prepared (Łopusiewicz et al., 2019). LGG counts were determined on MRS agar after incubation at 37°C under

anaerobic conditions for 72 h (collected after fermentation, 5 h, 24 h and 48 h of cold storage). Pathogenic bacteria counts were determined after 5 h, 24 h and 48 h of cold storage (incubation at 37°C under aerobic conditions for 24 h): *E. coli* on McConkey agar, *E. faecalis* on Slanetz and Bartley agar, *P. aeruginosa* on Pseudomonas agar base agar with CN supplement, *S. enterica* on XLD agar and *S. aureus* on Mannitol Salt agar. The enumeration of microorganisms was performed in triplicate and the viable cell counts were expressed as CFU/mL of the samples.

Determination of total solids content (TSC), pH and titratable acidity (TA)

Total solids content (TSC) was analysed using the drying method (105°C for 24 h) (Horwitz, 2000). The pH of non-fermented and fermented samples was measured directly at 25°C using a pH-meter (CP-411, Elmetron, Zabrze, Poland). The TA determination in samples after fermentation and during cold storage (expressed as g of lactic acid per 100 mL of the sample), consisted of mixing 10 mL of sample with 10 mL of distilled water and titration with 0.01 M NaOH solution, using phenolphthalein (0.1%, *w/v* in 95% ethanol) as an indicator. All tests were carried out in triplicate.

Color and viscosity analysis

Color of the samples was measured with Konica Minolta CR - 5 colorimeter (Konica Minolta, Osaka, Japan). The values measured were L* (white 100 and black 0), a* values (redness positive and greenness negative), and b* values (yellowness positive and blueness negative). The viscosity measurements were performed using a rheometer (AR G2, TA Instruments Ltd., New Castle, DE, USA). The samples were analysed at 20°C using a stainless steel cone plate having a diameter of 62 mm. Steady-state flow measurements were carried out at a shear rate 50 s^{-1} and the viscosity values were obtained from the TA Rheology Advantage Data Analysis equipment software V 5.4.7. Analyses were carried out at three independent times and presented as average values with ± standard deviation.

Preparation of FOCM supernatants

The samples were transferred into 1.5-mL Eppendorf tubes and centrifuged at 14,000 rpm/min for 10 min at 20°C (Centrifuge 5418 Eppendorf, Warsaw, Poland) to obtain clear fluids for analyses. The supernatants of the particular type of sample were mixed and filtered through 0.22-μm nylon membrane filters (Merck, Darmstadt, Germany). The obtained clear fluids were used for further analyses.

Analysis of total polyphenolics content (TPC)

The total polyphenolics content was determined by Folin-Ciocalteu method as described elsewhere with a slight modification (Tong et al., 2019). The supernatants (100 μL) were mixed with 6 mL of distilled water and 0.5 mL of Folin-Ciocalteu's reagent. After 3 min, 1.5 mL of saturated Na_2CO_3 solution was added and the mixture was incubated for 30 min in darkness at 40°C. The absorbance was measured at 765 nm (UV-Vis Thermo Scientific Evolution 220 spectrophotometer). The concentration of TPC was calculated as mg of gallic acid equivalents (GAE) per mL of sample (mg GAE/mL). All tests were carried out in triplicate.

Determination of total flavonoids content (TFC)

The total flavonoids content (TFC) of each sample was estimated as described by Tong et al. (2019) with own modification. 250 μL of supernatant was mixed with 1 mL of distilled water and 75 μL of 5% $NaNO_2$ solution. After 5 min, 75 μL of 10% $AlCl_3$ solution was added, and the mixture was allowed to stand for 6 min before the addition of 250 μL of 1 M NaOH. The total volume mixture was made up to 3 mL with distilled water, and then the absorbance was measured at 510 nm. Quercetin was used for a calibration curve, and the results were expressed as mg of quercetin equivalents (QE) per mL of the sample (mg QE/mL). All tests were carried out in triplicate.

Analysis of DPPH/ABTS radicals scavenging activity

DPPH, $ABTS^{+\cdot}$ radicals scavenging activities were determined according to the procedures as described elsewhere (Łopusiewicz et al., 2019). In brief, the DPPH radical scavenging activity was determined by mixing 1 mL of the supernatants with 1 mL of 0.01 mM DPPH methanolic solution. The absorbance was measured at 517 nm. Three mL of $ABTS^{\cdot+}$ solution were mixed with 50 μL of the supernatants and the absorbance was measured at 734 nm.

Statistical analysis

All data were expressed as mean ± standard deviation (SD). Statistical significance was determined using an analysis of variance (one-way ANOVA) followed by Fisher's LSD post-hoc testing with a significance threshold of $p < 0.05$. All analyses were performed with Statistica version 10 (StatSoft Polska, Kraków, Poland).

RESULTS AND DISCUSSION

The changes of total solids content, pH and titratable acidity as a result of FOCM fermentation with LGG

Average values of total solids content (TSC), pH, Titratable Acidity (TA) and Viscosity are summarized in Table 1. It was noticed that the physicochemical properties of FOCM were modified by the fermentation process. After fermentation, the highest TSC was observed in

Table 1: Total solids content (TSC), viscosity, pH and titratable acidity (TA) of fermented beverages and unfermented (control) sample

Sample	TSC (%)	Viscosity (MPa·s)	pH	TA (g lactic acid/100 mL)
FOCM	2.91 ± 0.46^{c}	27.20 ± 0.28^{c}	5.97 ± 0.00^{d}	0.17 ± 0.00^{a}
FOCM-LGG	2.08 ± 0.50^{a}	17.40 ± 0.14^{b}	4.36 ± 0.01^{c}	0.46 ± 0.00^{b}
FOCM-LGG-1%G	3.01 ± 0.85^{c}	13.05 ± 0.92^{a}	3.53 ± 0.01^{a}	1.47 ± 0.01^{d}
FOCM-LGG-1%F	2.71 ± 0.37^{b}	13.15 ± 0.78^{a}	3.55 ± 0.00^{b}	1.35 ± 0.01^{c}

Values are means ± standard deviation of triplicate determinations. Means with different lowercase in the same column are significantly different at $p < 0.05$.

sample FOCM-LGG-1%G (3.01 ± 0.85%), whereas the lowest value was noted for sample FOCM-LGG (2.08 ± 0.50%). Those values are lower than reported for FOC-kefir beverage (Łopusiewicz et al., 2019), and lower than TSC content in semi-fat cow milk (Atalar, 2019). During fermentation, an increase of FOCM acidity, as a result of metabolic activity of LGG was observed. The TA values of all samples significantly increased after fermentation ($p < 0.05$), whereas pH values significantly decreased ($p < 0.05$). The fermentation significantly reduced pH to the range of 3.53 ± 0.01 (FOCM-LGG-1%G) – 4.36 ± 0.01 (FOCM-LGG) in comparison to non-fermented sample ($p < 0.05$). Final pH values caused by metabolic activity of LGG reported by Valík et al., were 4.00 and 6.50, in MRS broth and animal (cow) milk, respectively (Valík et al., 2008). The highest TA (1.47 ± 0.01 g of lactic acid per 100 mL of product), and the lowest pH (3.53 ± 0.01) was observed for sample FOCM-LGG-1%G, and is comparable to values reported for FOC-kefir beverage (Łopusiewicz et al., 2019), but lower than reported for yogurt-like FOCM (Łopusiewicz et al., 2020a). In contrast, Alegre et al. (2011) observed that TA was not influenced by LGG in fresh-cut apples. Glucose and fructose affected the acidification of FOCM positively, which was expected since they are simple, easily fermented basic nutrients for LGG (Hedberg et al., 2008; Bernat et al., 2014). However, a significant reduction of pH and increase of TA was also observed in sample FOCM-LGG in comparison to the unfermented sample ($p < 0.05$). Less effective fermentation efficiency may be linked with lower nutrition content (Kocková et al., 2013). Other authors also repeatedly reported significant pH reduction of LGG-fermented products (including plant-based milks) (Bernat et al., 2014; Williams and Hekmat, 2017; Kehinde et al., 2020).

The changes of viscosity and color as a result of FOCM fermentation with LGG

It was found, that fermentation significantly reduced viscosity of the samples ($p < 0.05$), as presented in Table 1. Similar findings were observed for yogurt-like fermented FOCM (Łopusiewicz et al., 2020a), by Jia et al. (2016) who fermented goat milk with LGG, and Lorusso et al. (2018) who fermented quinoa flour with *L. rhamnosus* SP1. The lowest viscosity was observed for sample FOCM-LGG-1%G (13.05 ± 0.92 MPa·s). The highest viscosity of the unfermented sample may be explained by the content of flaxseed mucilage which was probably partially utilized by LGG (Łopusiewicz et al., 2019). Also, according to Jia et al. (2016) protein gel in food matrix may be dehydrated with reduced pH, thus it lowered the water-retaining capability of FOCM and decreased the viscosity. In previous study FOC was evaluated as a potential substrate for the production of a novel kefir-like fermented beverage. However, in this work FOC was used "as a whole" to obtain variants of beverages containing, 5%, 10% and 15% of FOC (*w*/*w*), which had rather semi-solid consistency (similar to jelly), also increased viscosity was observed in FOC-kefir beverages, which was linked with production of viscous polysaccharide kefiran (Łopusiewicz et al., 2019). On the other hand, Bernat et al. (2014) reported that fermentation by LGG did not affect the viscosity of hazelnut milk. Table 2 summarizes the color parameters of both non-fermented and fermented samples. It was noticed, that fermentation significantly increased lightness (L*) and redness (a*) values, and decreased b* (yellowness) value of FOCM-LGG sample, whereas values of samples FOCM-LGG-1%G and FOCM-LGG-1%F increased ($p < 0.05$). Those changes can be attributed to pH variations, and oxidation of some pigments presented in the raw material (Łopusiewicz et al., 2019). Those results are partially in agreement with previous results, as FOC fermentation with kefir cultures as well as FOCM fermentation with yogurt culture increased L* values. Dos Santos et al. (2019) also observed significantly increased lightness of fermented soymilk beverages. On the other hand, a decrease of a* values and increase/decrease of b* (depending on FOC concentration) was reported (Łopusiewicz et al., 2019, 2020a).

The changes of total phenolic content, total flavonoid content and DPPH/ABTS radicals scavenging activity As a result of FOCM fermentation with LGG

The total phenolic content (TPC), total flavonoid content (TFC) and DPPH/ABTS radicals scavenging activities are listed in Table 3. In general, TPC and TFC increased after fermentation in comparison to non-fermented sample ($p < 0.05$). After fermentation, the highest TPC and TFC was detected in sample FOCM-LGG-1%F (16.91 ± 0.02 mg GAE/mL and 2.18 ± 0.01 mg QE/mL, respectively). The observed TPC values were higher than reported for quinoa flour fermented with *L. plantarum* (Lorusso et al., 2018), and for yogurt-like FOCM beverage (Łopusiewicz

et al., 2020a), but lower than in kefir-like beverage produced from FOC (Łopusiewicz et al., 2019). Also, the TFC content was lower than reported for FOC-kefir (Łopusiewicz et al., 2019), but higher than reported for yogurt-like FOCM (Łopusiewicz et al., 2020a). However, the increase of TPC and TFC as a result of plant-based beverages fermentation have been previously reported (Lorusso et al., 2018; Łopusiewicz et al., 2019, 2020a). In fact, it was reported that due to enzymatic activity of some LAB strains, polyphenolic compounds (that were bounded to proteins and cell wall carbohydrates of plant material can be delinked), which results in their improved bioavailability and bioactivity (Valero-Cases et al., 2020). The effect of fermentation on the TPC, TFC, and antioxidant activity of plant matrices has been reported (Lorusso et al., 2018; Łopusiewicz et al., 2019). Some phenolic compounds, showing antimicrobial activity can be also increased or produced *ex novo* by LAB. Some studies reported the production of phenyllactic acids by LAB, and the antimicrobial activity of these compounds has been widely documented on pathogenic microorganisms (Ricci et al., 2019). Antioxidant activity has been reported to be concomitant with production of polyphenolics and flavonoids (Łopusiewicz et al., 2019, 2020a). In fact, as can be seen in Table 3 the DPPH, ABTS scavenging activities of raw samples significantly increased as a result of fermentation ($p < 0.05$). The highest DPPH radical inhibition was noticed from sample FOCM-LGG (94.50 ± 0.86%), whereas the ABTS radical inhibition was found to be the highest in sample FOCM-LGG-1%F (99.31 ± 0.01%). Those results are comparable with the results of other authors who reported high antioxidant activity of fermented plant beverages using various *Lactobacillus* strains (Mauro et al., 2016; Lorusso et al., 2018; Minervini et al., 2020). Previously, it was suggested that the increasing antioxidant activity during fermentation using LAB cultures may be due to the mobilization and production of individual phenolic compounds (Valero-Cases et al., 2020). On the other hand, fermentation enhances the total antioxidant capacity, also because of the proteolytic activity of the microflora in the specified protein fractions (Łopusiewicz et al., 2020b). Thus, it is reasonably to suggest, that the antioxidant activity of developed beverages may be attributable, in part with production/mobilization of phenolic compounds as well as formation of bioactive peptides and amino acids with antioxidant capacity (Łopusiewicz et al., 2019; Łopusiewicz et al., 2020b).

Table 2: Color values of fermented beverages and unfermented (control) sample

Sample	L*	a*	b*
FOCM	56.76 ± 0.07[a]	-1.40 ± 0.03[d]	19.26 ± 0.13[b]
FOCM-LGG	64.84 ± 0.14[b]	-1.12 ± 0.09[a]	18.34 ± 0.03[a]
FOCM-LGG-1%G	66.30 ± 0.29[c]	-1.13 ± 0.20[b]	21.62 ± 0.26[d]
FOCM-LGG-1%F	66.63 ± 0.13[c]	-1.23 ± 0.07[c]	21.00 ± 0.08[c]

Values are means ± standard deviation of triplicate determinations. Means with different lowercase in the same column are significantly different at $p < 0.05$.

The LGG viability during cold storage in conventional and artificially contaminated samples

As regards the probiotic survivability, food matrix is considered as one of the major factors regulating colonization (Bernat et al., 2014; Łopusiewicz et al., 2019). It can play an important role in protecting microorganisms from the stomach environment. Food matrix might increase bacteria survival, primarily by functional ingredients, such as prebiotics, that could interact with them (Moreira et al., 2017; Łopusiewicz et al., 2019; Sireswar et al., 2019). Generally, plant-based products (including agricultural residues) are reported to be good matrices for developing non-dairy fermented foods (Dueñas and García-Estévez, 2020; Valero-Cases et al., 2020; Tsafrakidou et al., 2020). Our study confirmed that FOCM formulations are an appropriate matrix to develop functional non-dairy products - LGG survivability was meaningfully stable over storage period both in conventional (Table 4) as well as artificially contaminated samples (Table 5). As a result of fermentation LGG concentrations 7.34 ± 0.03 log CFU/mL (FOCM-LGG), 7.16 ± 0.00 log CFU/mL (FOCM-LGG-1%G) and 7.00 ± 0.03 log CFU/mL (FOCM-LGG-1%F) were noticed. It was also observed that during storage time, the number of LGG in almost all samples increased ($p < 0.05$) (Table 4 and Table 5). It should be emphasized that LGG counts were maintained in the product at the recommended probiotic level $>10^6$ CFU/mL, and are comparable with the results of other authors using plant products to produce fermented beverages (Mpofu et al., 2016; Szparaga et al., 2019). High survivability of LGG in acid conditions was expected due

Table 3: Total Polyphenolics (TPC), Total Flavonoids (TFC) contents and DPPH/ABTS radicals scavenging activity of fermented beverages and unfermented (control) sample

Sample	TPC (mg GAE/mL)	TFC (mg QE/mL)	DPPH (%)	ABTS (%)
FOCM	10.58 ± 0.11[a]	1.67 ± 0.02[a]	74.92 ± 0.49[a]	79.60 ± 0.11[a]
FOCM-LGG	12.20 ± 0.01[b]	1.92 ± 0.04[b]	94.50 ± 0.86[c]	88.28 ± 0.23[b]
FOCM-LGG-1%G	13.83 ± 0.27[c]	2.07 ± 0.01[c]	84.11 ± 0. 74[b]	99.28 ± 0.05[c]
FOCM-LGG-1%F	16.91 ± 0.02[d]	2.18 ± 0.01[d]	93.95 ± 0.86[c]	99.31 ± 0.01[c]

Values are means ± standard deviation of triplicate determinations. Means with different lowercase in the same column are significantly different at $p < 0.05$.

Table 4: LGG counts after fermentation and during storage time in conventional (non-contaminated) samples. *0 - time after fermentation; 5, 24, 48 – time during cold storage

	Time (hours)*			
	0*	5	24	48
Sample	LGG counts (log CFU/mL)			
FOCM-LGG	7.34 ± 0.03^{Ac}	7.63 ± 0.29^{Bb}	8.30 ± 0.02^{Cb}	8.69 ± 0.01^{Db}
FOCM-LGG-1%G	7.16 ± 0.00^{Ab}	7.55 ± 0.01^{Bab}	8.45 ± 0.04^{Ca}	8.71 ± 0.01^{Db}
FOCM-LGG-1%F	7.00 ± 0.03^{Aa}	7.43 ± 0.10^{Ba}	8.63 ± 0.01^{Cc}	8.08 ± 0.08^{Da}

Values are means ± standard deviation of triplicate determinations. Means with different lowercase in the same column are significantly different at $p < 0.05$.

Table 5: LGG counts during storage time in artificially contaminated samples. *EF – LGG co-incubated with *E. faecalis*; EC – LGG co-incubated with *E. coli*, PA – LGG co-incubated with *P. aeruginosa,* SE – LGG co-incubated with *S. enterica*, SA – LGG co-incubated with *S. aureus*

	Time (hours)		
	5h	24h	48h
Sample	LGG (+EF)* (log CFU/mL)		
FOCM-LGG	6.65 ± 0.08^{Aa}	7.93 ± 0.03^{Ba}	7.86 ± 0.02^{Ba}
FOCM-LGG-1%G	7.65 ± 0.01^{Ab}	8.47 ± 0.01^{Bb}	8.37 ± 0.02^{Bb}
FOCM-LGG-1%F	7.21 ± 0.03^{Ab}	8.43 ± 0.01^{Bb}	8.19 ± 0.01^{Cb}
	5h	24h	48h
Sample	LGG (+EC)* (log CFU/mL)		
FOCM-LGG	7.87 ± 0.31^{Ab}	7.97 ± 0.03^{Ba}	8.03 ± 0.05^{Ca}
FOCM-LGG-1%G	6.43 ± 0.06^{Aa}	8.34 ± 0.03^{Bb}	8.69 ± 0.05^{Cb}
FOCM-LGG-1%F	7.07 ± 0.16^{Aa}	8.42 ± 0.01^{Bb}	8.90 ± 0.02^{Cc}
	5h	24h	48h
Sample	LGG (+PA)* (log CFU/mL)		
FOCM-LGG	6.59 ± 0.02^{Aa}	7.91 ± 0.02^{Ba}	7.98 ± 0.03^{Ba}
FOCM-LGG-1%G	7.65 ± 0.12^{Ab}	8.42 ± 0.02^{Bc}	8.63 ± 0.01^{Cb}
FOCM-LGG-1%F	7.60 ± 0.06^{Ab}	8.25 ± 0.05^{Bb}	8.71 ± 0.01^{Cb}
	5h	24h	48h
Sample	LGG (+SE)* (log CFU/mL)		
FOCM-LGG	6.51 ± 0.04^{Aa}	6.96 ± 0.02^{Bc}	8.18 ± 0.05^{Ca}
FOCM-LGG-1%G	7.34 ± 0.12^{Ab}	8.44 ± 0.03^{Ba}	8.50 ± 0.01^{Cb}
FOCM-LGG-1%F	7.71 ± 0.23^{Ab}	7.99 ± 0.04^{Bb}	8.68 ± 0.05^{Cc}
	5h	24h	48h
Sample	LGG (+SA)* (log CFU/mL)		
FOCM-LGG	7.55 ± 0.05^{Aa}	7.92 ± 0.07^{Ba}	8.07 ± 0.01^{Ca}
FOCM-LGG-1%G	7.73 ± 0.08^{Ab}	8.55 ± 0.03^{Bb}	8.54 ± 0.04^{Bb}
FOCM-LGG-1%F	8.11 ± 0.01^{Ac}	8.61 ± 0.01^{Bb}	8.63 ± 0.06^{Bb}

Values are means ± standard deviation of triplicate determinations. Means with different lowercase in the same column are significantly different at $p < 0.05$. Means with different uppercase in the same raw are significantly different at $p < 0.05$.

to its relatively high resistance to stomach environment as well as low pH products (Hedberg et al., 2008; Tayo and Akpeji, 2016; Moreira et al., 2017; Westerik et al., 2018; Sireswar et al., 2019). Moreover, it is noteworthy that LGG was able to utilize FOCM in cell synthesis and acid production without external nutrients supplementation in variant FOCM-LGG. The fact that LGG in the fermented FOCM remained highly concentrated might be due to the prebiotic effect of flaxseed fiber (HadiNezhad et al., 2013; Łopusiewicz et al., 2019). HadiNezhad et al. (2013) reported that flaxseed soluble dietary fiber acts as a good prebiotic, enhancing LAB growth in kefir model. Another important factor determining microorganisms viability and their metabolic activity was the product storage temperature (6°C). It is widely known that refrigerated conditions are one of the pivotal points maintaining LAB viability in fermented beverages, increasing their shelf-life (Łopusiewicz et al., 2019; Sireswar et al., 2019).

Evaluation of LGG as a protective culture

The survivability of pathogenic bacteria in fermented/non-fermented FOCM samples are presented in Table 6, whereas changes of TA of the samples during refrigerated storage are listed in Table 7. In general, the viability of bacteria in non-fermented FOCM was stable, except *P. aeruginosa* which increased after 24 h and 48 h, and *S. aureus*, which counts decrease was noticed ($p < 0.05$). It was found that co-incubation of LGG in FOCM samples resulted in significant reduction of all pathogenic bacteria counts ($p < 0.05$). This was probably due to lactic acid and other organic acids produced by LGG during the fermentation process. This observation is in line with results of Mpofu et al. (2016), who used LGG as protective culture in traditional African fermented beverage. LGG was reported to be an excellent starter for fermented and non-fermented dairy products manufacturing, which have many probiotic functions for adults and children (Kocková et al., 2013; Jia et al., 2016; Sun et al., 2019). It was also used not only as probiotic but also as protective culture in fermented and non-fermented, non-dairy products including ready-to-eat products such as plant milks (Bernat et al., 2014), meat products (Erkkilä et al., 2000), cereals, pseudocereals and legumes (Kocková et al., 2013; Williams and Hekmat, 2017), fresh-cut fruits, fruit juices and beverages (Alegre et al., 2011; Tayo and Akpeji, 2016; Moreira et al., 2017; Sireswar et al., 2017, 2019; Iglesias et al., 2018). The most efficient reduction of pathogenic counts was observed in samples supplemented with glucose and fructose. Complete reduction of *S. enterica* was observed after 5 h, *E. coli* after 24 h and *E. faecalis* after 48 h of co-incubation with LGG in samples FOCM-LGG-1%G and FOCM-LGG-1%F. Complete reduction of *P. aeruginosa* was noticed after 24 h of co-incubation with LGG in sample FOCM-LGG-1%F, whereas this effect was observed in sample FOCM-LGG-1%G after 48 h. Only in the case of *S. aureus* no complete reduction over storage time was noticed. Significant decrease of all pathogenic counts was also observed in samples FOCM-LGG, but no complete reduction was observed ($p < 0.05$).

Table 6: Pathogenic bacteria counts during storage time in artificially contaminated samples

	Time (hours)		
	5h	24h	48h
Sample	***E. faecalis* (log CFU/mL)**		
FOCM	7.65 ± 0.03^{Aa}	7.64 ± 0.03^{Ac}	7.74 ± 0.02^{Ac}
FOCM-LGG	6.53 ± 0.06^{Ab}	4.54 ± 0.09^{Bb}	3.23 ± 0.64^{Cb}
FOCM-LGG-1%G	5.09 ± 0.13^{Ac}	2.77 ± 0.06^{Ba}	0.00 ± 0.00^{Ca}
FOCM-LGG-1%F	5.48 ± 0.03^{Ac}	2.96 ± 0.05^{Ba}	0.00 ± 0.00^{Ca}
Sample	***E.coli* (log CFU/mL)**		
FOCM	7.15 ± 0.07^{Aa}	7.41 ± 0.01^{Bc}	7.56 ± 0.00^{Cc}
FOCM-LGG	5.50 ± 0.09^{Ab}	4.78 ± 0.03^{Bb}	3.90 ± 0.07^{Cb}
FOCM-LGG-1%G	4.65 ± 0.12^{Ad}	0.00 ± 0.00^{Ba}	0.00 ± 0.00^{Ba}
FOCM-LGG-1%F	4.46 ± 0.03^{Ac}	0.00 ± 0.00^{Ba}	0.00 ± 0.00^{Ba}
Sample	***P. aeruginosa* (log CFU/mL)**		
FOCM	6.95 ± 0.57^{Ab}	6.79 ± 0.07^{Ad}	8.12 ± 0.01^{Cc}
FOCM-LGG	6.19 ± 0.03^{Ab}	4.79 ± 0.01^{Bc}	2.32 ± 0.07^{Cb}
FOCM-LGG-1%G	4.26 ± 0.00^{Aa}	3.89 ± 0.21^{Ab}	0.00 ± 0.00 ^{Ca}
FOCM-LGG-1%F	4.37 ± 0.15^{Aa}	0.00 ± 0.00^{Ba}	0.00 ± 0.00^{Ba}
Sample	***S. enterica* (log CFU/mL)**		
FOCM	7.67 ± 0.23^{Ac}	6.63 ± 0.21^{Bc}	7.07 ± 0.10^{Cc}
FOCM-LGG	7.05 ± 0.01^{Ab}	2.82 ± 0.01^{Bb}	2.22 ± 0.06^{Bb}
FOCM-LGG-1%G	0.00 ± 0.00^{Aa}	0.00 ± 0.00^{Aa}	0.00 ± 0.00 ^{Aa}
FOCM-LGG-1%F	0.00 ± 0.00^{Aa}	0.00 ± 0.00^{Aa}	0.00 ± 0.00^{Aa}
Sample	***S.aureus* (log CFU/mL)**		
FOCM	6.02 ± 0.03^{Ab}	5.90 ± 0.03^{Bc}	5.29 ± 0.03^{Ca}
FOCM-LGG	5.94 ± 0.10^{Ab}	5.60 ± 0.13^{Ab}	3.98 ± 0.08^{Bc}
FOCM-LGG-1%G	5.64 ± 0.15^{Aa}	4.57 ± 0.09^{Ba}	3.61 ± 0.12^{Cb}
FOCM-LGG-1%F	5.49 ± 0.04^{Aa}	4.51 ± 0.07^{Ba}	3.65 ± 0.17^{Cb}

Values are means ± standard deviation of triplicate determinations. Means with different lowercase in the same column are significantly different at $p < 0.05$. Means with different uppercase in the same raw are significantly different at $p < 0.05$.

Table 7: Titratable acidity (TA) during storage time in artificially contaminated samples

	Time (hours)		
	5h	24h	48h
Sample	**TA – *E. faecalis* (g lactic acid/100 mL)**		
FOCM	0.13 ± 0.00^{Aa}	0.16 ± 0.00^{Ba}	0.17 ± 0.00^{Ca}
FOCM-LGG	0.45 ± 0.00^{Ab}	0.48 ± 0.02^{Bb}	0.43 ± 0.00^{Cb}
FOCM-LGG-1%G	1.29 ± 0.01^{Ad}	1.13 ± 0.01^{Bc}	1.17 ± 0.01^{Cc}
FOCM-LGG-1%F	1.25 ± 0.01^{Ab}	1.22 ± 0.01^{Bd}	1.25 ± 0.01^{Ad}
Sample	**TA – *E. coli* (g lactic acid/100 mL)**		
FOCM	0.14 ± 0.01^{Aa}	0.15 ± 0.01^{Aa}	0.17 ± 0.01^{Ac}
FOCM-LGG	0.35 ± 0.00^{Ab}	0.43 ± 0.00^{Bb}	0.48 ± 0.02^{Cb}
FOCM-LGG-1%G	1.26 ± 0.01^{Ac}	1.19 ± 0.01^{Bc}	1.28 ± 0.01^{Aa}
FOCM-LGG-1%F	1.35 ± 0.01^{Ad}	1.23 ± 0.01^{Bd}	1.33 ± 0.01^{Ca}
Sample	**TA – *P. aeruginosa* (g lactic acid/100 mL)**		
FOCM	0.13 ± 0.00^{Aa}	0.13 ± 0.00^{Aa}	0.17 ± 0.00^{Ba}
FOCM-LGG	0.34 ± 0.00^{Ab}	0.43 ± 0.00^{Bb}	0.45 ± 0.05^{Cb}
FOCM-LGG-1%G	1.15 ± 0.01^{Ad}	1.19 ± 0.01^{Bc}	1.29 ± 0.01^{Cc}
FOCM-LGG-1%F	1.19 ± 0.01^{Ac}	1.26 ± 0.01^{Bd}	1.29 ± 0.01^{Cc}
Sample	**TA – *S. enteridis* (g lactic acid/100 mL)**		
FOCM	0.13 ± 0.01^{Aa}	0.12 ± 0.00^{Aa}	0.18 ± 0.03^{Ba}
FOCM-LGG	0.39 ± 0.01^{Ab}	0.43 ± 0.00^{Bb}	0.46 ± 0.00^{Cb}
FOCM-LGG-1%G	1.15 ± 0.01^{Ac}	1.13 ± 0.01^{Ac}	1.24 ± 0.01^{Bc}
FOCM-LGG-1%F	1.23 ± 0.01^{Ad}	1.23 ± 0.01^{Ad}	1.28 ± 0.01^{Bd}
Sample	**TA – *S. aureus* (g lactic acid/100 mL)**		
FOCM	0.12 ± 0.00^{Aa}	0.14 ± 0.00^{Ba}	0.17 ± 0.00^{Ca}
FOCM-LGG	0.47 ± 0.00^{Ab}	0.43 ± 0.00^{Bb}	0.44 ± 0.01^{Bb}
FOCM-LGG-1%G	1.39 ± 0.01^{Ad}	1.18 ± 0.01^{Bc}	1.10 ± 0.01^{Cc}
FOCM-LGG-1%F	0.99 ± 0.01^{Ac}	1.25 ± 0.01^{Bd}	1.29 ± 0.01^{Cd}

Values are means ± standard deviation of triplicate determinations. Means with different lowercase in the same column are significantly different at $p < 0.05$. Means with different uppercase in the same raw are significantly different at $p < 0.05$.

This is probably linked with not sufficient acidity of samples without sugars supplementation. In fact, as listed in Table 7, TA of samples FOCM-LGG-1%G and FOCM-LGG-1%F was significantly higher than in samples FOCM-LGG over storage period ($p < 0.05$), which contributed most to the effectiveness of pathogen reduction. As listed in Table 7, the fluctuations of pH and TA values of all samples were observed over the storage period ($p < 0.05$). These changes were expected due to high viability of microorganisms over storage time. The acidity of fermented products is commonly maintained or increased during storage, a fact that is attributed to the persistent metabolic activity of LAB (Lorusso et al., 2018; Łopusiewicz et al., 2019, 2020a; Szparaga et al., 2019). The reduction of pH is attributed to production of organic acids, which during fermentation is of great importance due to inhibitory effects against spoilage and pathogenic microorganisms (De Keersmaecker et al., 2006; Yan and Polk, 2012; Abdel-Daim et al., 2013). The multifactorial antimicrobial activity of LGG was reported and is primarily linked with production of organic acids (mainly lactic acid) as well as other low molecular weight compounds such as peptides, nitric oxide, and hydrogen peroxide, supporting the activity of lactic acid (Meurman et al., 1995; Servin, 2004; Fayol-Messaoudi et al., 2005). The mechanism of antimicrobial activity of organic acids in their undissociated forms is based on entering the pathogenic bacterial cells and dissociate inside cytoplasm, eventually decreasing of intracellular pH and the accumulation of the ionized organic acids leading to pathogen death (Erkkilä et al., 2000; De Keersmaecker et al., 2006; Zhang et al., 2011). This effect is observed mainly in Gram-negative pathogens (due to different cell wall composition) (Fayol-Messaoudi et al., 2005), which may explain less reduction efficiency of Gram-positive *E. faecalis* and *S. aureus*. However, Tayo and Akpeji (2016) observed no inhibitory activity against *S. aureus* in LGG-probioticated pineapple juice. Particularly, different levels of LGG antagonistic activity against *E. coli* (Servin, 2004; Mpofu et al., 2016), *Salmonella* spp. (De Keersmaecker et al., 2006; Makras et al., 2006; Iglesias et al., 2018), *Enterococcus* spp. (Sun et al., 2010), *Pseudomonas* spp. (Johnson-Henry et al., 2008) and other important pathogens (such as *Clostridium* spp., *Listeria monocytogenes*, *Campylobacter jejuni*,

Bacillus cereus, *Streptococcus pneumoniae* and *Haemophilus influenzae*) was reported (Balejko et al., 2009; Alegre et al., 2011; Mpofu et al., 2016; Iglesias et al., 2018).

CONCLUSIONS

Taking into account the increasing complexity of the needs of different typologies of consumers, including vegan/vegetarian and subjects with intolerance/allergy to dairy products, an approach in this work was applied to obtain probiotic drink from flaxseed oil cake milk, using *Lactobacillus rhamnosus* GG, acting as protective agent against pathogenic bacteria. As it was demonstrated, the flaxseed milk formulations are an appropriate matrix to develop functional non-dairy products with high content of bioactive compounds. We observed that LGG viability was relatively high during the storage period, which was able to effectively reduce pathogenic microflora (linked to the pH-lowering effect), add value to the beverages and they can be characterized as functional foods. There is a potential for these products to be used where non-dairy alternatives are desired. However, to corroborate the health benefits of fermented non-dairy drink consumption, further *in vivo* research, including human clinical studies addressing matrix combinations and doses in different populations, is needed. As it was demonstrated, valorization of underestimated food industry by-products represents an attractive path towards obtaining novel functional foods and achieving "zero waste" goals.

COMPLIANCE WITH ETHICAL STANDARDS

Conflict of interest

The authors declare that there are no conflicts of interest.

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AUTHORS' CONTRIBUTIONS

Łukasz Łopusiewicz: conceptualization, formal analysis, investigation, methodology, supervision, visualization, writing original draft
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