IRSTI 34.27.19 https://doi.org/10.26577/ijbch.2022.v15.i2.07

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(Received 12 April 2022; received in revised form 24 June 2022; accepted 29 June 2022)

Study of the antioxidant activity of plant extracts and their effect on the growth of lactic acid bacteria isolated from kumis

Abstract. This article describes the effect of plant extracts on the growth and reproduction of lactic acid bacteria (LAB) *Lactobacillus acidophilus* strain 0015k-1 and *Lactobacillus delbrueckii subsp. bulgaricus* strain 018k-3, isolated from the traditional fermented milk drink – kumis. In this study, analyses were carried out on plant extracts obtained from *Rubus caesius* L. (gray blackberry), *Rubus idaeus* L. (common raspberry) and *Punica granatum* L. (common pomegranate) with water as principal extragent. The focus was on the antioxidant activity and the total content of polyphenols. Antioxidant activity was measured using amperometry with quercetin taken as the calibration standard. It was determined that the extract from *Rubus caesius* L. had the highest content of antioxidants (3.30 mg/dm^3) followed by pomegranates (2.40 mg/dm^3) and then raspberries (2.37 mg/dm^3). The total polyphenol content was highest in gray blackberries (8.20%) followed by raspberries (2.99%) and pomegranates (2.78%). To test the prebiotic activity of aqueous plant extracts, LAB were cultured in milk with and without plant components (10%, 15% and control samples) until a clot was formed. The colony-forming unit (CFU) titer was determined for each sample. The results of the *in vitro* test showed a significant growth of bacterial colonies in samples containing plant extracts (10%) compared to control samples. However, a slight inhibition of LAB growth was observed when the content of plant extracts was increased from 10% to 15%.

Keywords: Blackberry, raspberry, pomegranate, antioxidant activity, polyphenols, prebiotics, lactic acid bacteria.

Introduction

Current trends in the promotion of nutrition supplements for improved and healthier bodily functions has seen a rise in the advertising and use of functional ingredients, such as prebiotics (indigestible fibre that can be broken down by gut microbiome) and probiotics (live microorganisms cultured as a supplement). Evolving trends and scientific research promotes the use of prebiotics to feed probiotics or "synbiotics", which have a mutually reinforcing effect on the body's metabolic process [1;2].

The concept of synbiotics is very tempting; however, their mutually reinforcing effect must be proven. When creating functional products with the addition of prebiotics, it is important to determine which prebiotic is most appropriate for the particular product being developed. Consideration should be given to the specific characteristics of prebiotics, including their nutritional and technological properties, such as resistance to processing conditions, minimum and maximum concentrations yielding the desired effects and potential side effects [3].

A prebiotic is "a selectively fermented ingredient that allows certain changes, both in the composition and/or activity of the microbiome of the GIT, beneficial to the host". Traditionally, prebiotics have been characterized as polysaccharides with a chain of indigestible carbohydrate fragments with unique β- and terminal α-bonds, which are present in food items in the form of plant cell wall polysaccharides and resistant starches. These fibers pass through the upper GIT intact as there are no enzymes that initiate the hydrolysis and depolymerization of complex carbohydrates. They also reach the lower colon intact where they stimulate the growth of beneficial bacteria and promote metabolism [4].

The prebiotics present in synbiotic mixtures should fulfill at least one of their two possible functions. Firstly, they should act as prebiotics, that is,

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stimulate the number and activity of endogenous beneficial microbes in the gut microbiome. Secondly, to serve as a "lunch box" for probiotics hence they should maintain the viability of beneficial microbes (probiotics) as they travel through the hostile environment of the gastrointestinal tract (GIT), in other words a synergistic approach [5]. However, the function of prebiotics as a lunch box suggests that probiotics can use them as a substrate, and this is not always true. Some products contain lactobacillus probiotics with a bifidogenic prebiotic; in this case, the probiotic is not able to benefit from the use of the prebiotic as a growth substrate. Currently, not all commercially available probiotics are capable of fermenting fructooligosaccharides (FOS) or galactooligosaccharides (GOS) [4, p. 5].

Currently, there is a narrow range of confirmed prebiotic substances, among which galactans and fructans, for example inulin, predominate. The desire to stimulate a wider group of commensal organisms has led to the development of new candidate prebiotic compounds (Figure 1).

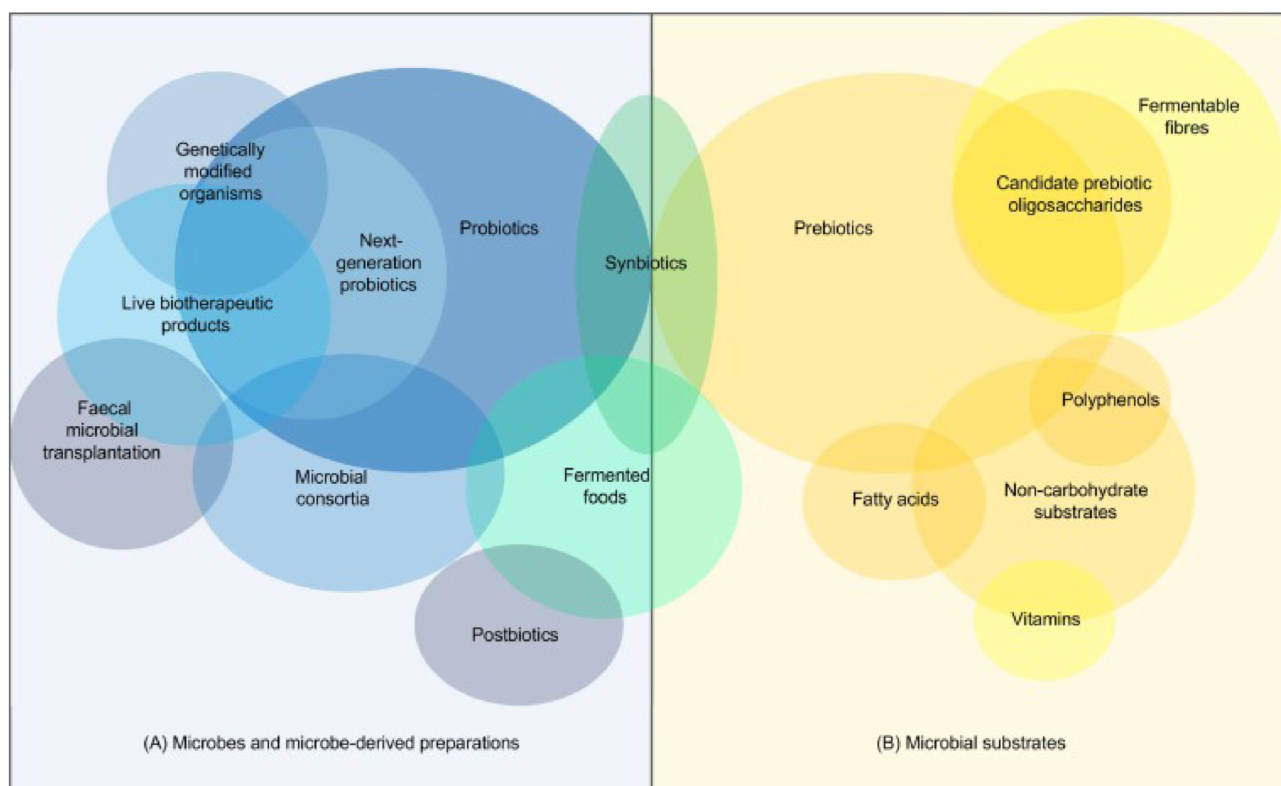

Figure 1 – Probiotics, prebiotics, and adjacent fields

These include carbohydrate-based substances derived from plants – source of traditional prebiotics such as inulin but may also include substances that mimic animal-derived substrates (e.g., milk oligosaccharides; O-linked glycans found in mucins), yeast substances and many non-carbohydrate substances including polyphenols, fatty acids, herbs, and other micronutrients [6].

The problem with traditional transport derivatives of prebiotics is that they also promote the growth of pathogenic bacteria [7] and have different bioavailability depending on the composition of the intestinal microbiota. Numerous studies have shown that pathogenic strains of *Escherichia coli* [8-12] and various pathogenic streptococci can use FOS as a substrate [13; 14]. In this respect, there is a need for alternative novel prebiotics with little or no side effects and that can either be incorporated into a probiotic food matrix or used alone to stimulate host intestinal LAB.

Current research is demonstrating a particular interest in polyphenolic compounds from various plant sources that have strong prebiotic activities, such as agave, blueberries, grapes, grape seeds, black currants, green tea, and many others [15-22]. There are also numerous studies that discuss the antimicrobial

properties of berry polyphenols and their inhibitory effect on the growth of certain pathogens [23; 24].

Polyphenols are a diverse group of secondary metabolites found in adequate amounts in a variety of plants [25]. They are called secondary metabolites because they do not participate in the growth, development, or reproduction of the plant. Their role is to protect against ultraviolet radiation (oxidative stress) and pathogens. They are also responsible for organoleptic properties such as color and taste, in particular the astringency of a particular plant product [26].

In the present study we determined the total content of antioxidants and polyphenols in plant extracts and their effect on the growth of LAB using *in vitro* method. Previously, similar work was done by Molan et al. in 2009 [27]. The authors cultivated LAB in De Man, Rogosa and Sharpe (MRS) medium supplemented with blueberry aqueous extracts. In 2017 P. Muniandy et al. [28] studied the effect of green, white and black tea on lactic acid, acid formation and the viability of *Streptococcus thermophilus* and *Lactobacillus spp*. in yogurt during refrigerated storage.

The distinguishing characteristic of this work is the use of a sterile milk as a potential food matrix for LAB to which different plant extracts of varying antioxidant capacity and polyphenol content are added. Furthermore, the LAB used in this study were isolated from the traditional fermented milk drink (kumis) and locally sourced plant materials.

Materials and methods

Objects of study. Rubus caesius L. (gray blackberry), *Rubus idaeus* L. (common raspberry) and *Punica granatum* L. (common pomegranate), *Lactobacillus acidophilus* strain 0015k-1, *Lactobacillus delbrueckii subsp. bulgaricus* strain 018k-3.

Antioxidant activity measurement. Measurement of antioxidant activity was performed on a flow-injection system with an amperometric detector «TsvetYauza-01-AA» (Khimavtomatika, Russia). The method measures the intensity of an electrical current that is produced during the oxidation of antioxidant molecules on the electrode surface at a certain potential, which, after amplification, is converted to a digital signal [29].

Sample preparation. The samples were prepared as follows: weighing, alcohol extraction with 70% ethanol agitated for 1 hour and finally solution filtration. The mass concentration of antioxidants was measured using a quercetin calibration curve. The curve was obtained using calibration standards of quercetin for which output signals were recorded.

Determination of polyphenol content. The content of polyphenols in water plant extracts was measured at a wavelength of 715 nm on a KFK-3-01 photoelectric photometer (OJSC "ZOMZ", Russia) [30].

Sample preparation. The samples were crushed, 50 mL of water was added and then the samples were boiled in a water bath for 2 hours. The mixture was cooled and filtered into a volumetric flask (100 mL), the volume was adjusted to the mark with purified water, and mixed. Then 5 mL of the resulting solution was placed in a flask (25 mL) and adjusted to the mark with purified water, mixed. After 5 mL of the resulting solution was placed in a flask with a capacity 50 mL, 1 mL of a solution of tungstate-phosphoric acid was added, the volume was adjusted to the mark with 15% sodium carbonate solution, mixed. The optical density of the resulting solution was measured 2-3 minutes after the addition of the last reagent at a wavelength of 715 nm in a cuvette with a wall thickness of 10 mm. Purified water was used as a reference solution.

At the same time, the optical density of pyrogallol is measured 2-3 minutes after the addition of the last reagent and within 15 minutes after the dissolution of pyrogallol.

Preparation of crude aqueous plant extracts. The procedure for preparing plant extracts adopted and slightly modified from the work of Molan et al. [27, p. 1244]. Berries and pomegranate seeds were weighed (100 g), mixed with 100 mL of distilled water and grounded using a blender (Philips HR 16, China). The resulting solution was centrifuged (3000 rpm, 15 min) and the resulting plant liquid was filtered through an ash filter. To ensure the plant extracts were sterile, the liquid was passed through a membrane filter with a pore size of 0.22 μm. Aqueous plant extracts were stored at -20°C until use.

Explored bacterial strains. Lactobacillus acidophilus strain 0015k-1 and *Lactobacillus delbrueckii subsp. bulgaricus* strain 018k-3, collection numbers B-RKM 0511 and B-RKM-0509, respectively, were obtained from the culture collection of the Kazakh National Agrarian Research University (Almaty, Kazakhstan).

Lactobacillus acidophilus, strain 015k-1 was isolated from kumis (South Kazakhstan region, Turkestan, Kazakhstan, 2012). This bacterium is a Gram-positive rod with rounded ends, usually occurring as single cells, as well as in pairs or in short chains, 4.7-6.2μm x 0.6 μm in size. It is a catalase-negative, non-spore forming, immobile bacterium. It is a facultative anaerobe whose superficial colonies are curl-like, oily in consistency with a rough,

shiny, grayish-transparent surface. Deeper colonies take on the form of gray-white spiders. When grown on a liquid medium, turbidity with a precipitate is observed.

Lactobacillus delbrueckii subsp. bulgaricus strain 018k-3 was isolated from three-day-old kumis (Almaty region, Kegen district, Kazakhstan, 2012). This bacterium is a Gram-positive rod with rounded ends, usually occurring as single cells, but sometimes in short chains, 4-4.6µm x 0.9 µm in size. It is a catalase-negative, non-spore forming, immobile bacterium. It is a microaerophilic, facultative anaerobe that grows well on hydrolyzed milk, whey, MRS, wort media and their agar media.

Fermentation conditions. Several *in vitro* studies were conducted to evaluate the effect of aqueous crude plant extracts on the growth of these strains of LAB. The bacterial strains were grown at 37°C in milk hydrolysate (incubation period 24 hours or less). A sample of 0.5 mL of these cultures was added to 10 mL of skimmed milk containing 10% and 15% water-soluble plant extracts. The control sample was skimmed milk without plant extracts. The samples were incubated in a thermostat (JSC «Smolenskoye SKTB SPU» Russia) at 37°C until a clot was formed. The tubes were then cooled in a refrigerator. After cooling, CFU titers were determined. The control and the samples containing plant extracts were serially diluted 10 times in sterile distilled water. 1 mL dilution aliquots were inoculated onto the surface of agarized milk protein hydrolysate (AGM) plates. The incubation was at thermostat 37°C for 48 hours. The number of viable bacterial cells was then manually counted.

Results and discussion

Antioxidant activity. Quantitative determination of the antioxidant activity index was carried out by the method of absolute calibration according to the peak areas of standard antioxidant substances. The result is traditionally taken as the arithmetic mean of 5 measurements (relative standard deviation is not more than 5%). Based on the data obtained, a calibration graph was constructed (Figure 2), which is described by the equation Y=aX+b.

As demonstrated on Figure 2, *Rubus caesius* L. has the highest content of antioxidants (3.30 mg/dm^3), followed by *Punica granatum* L. (2.40 mg/dm^3) and *Rubus idaeus* L. (2.37 mg/dm^3).

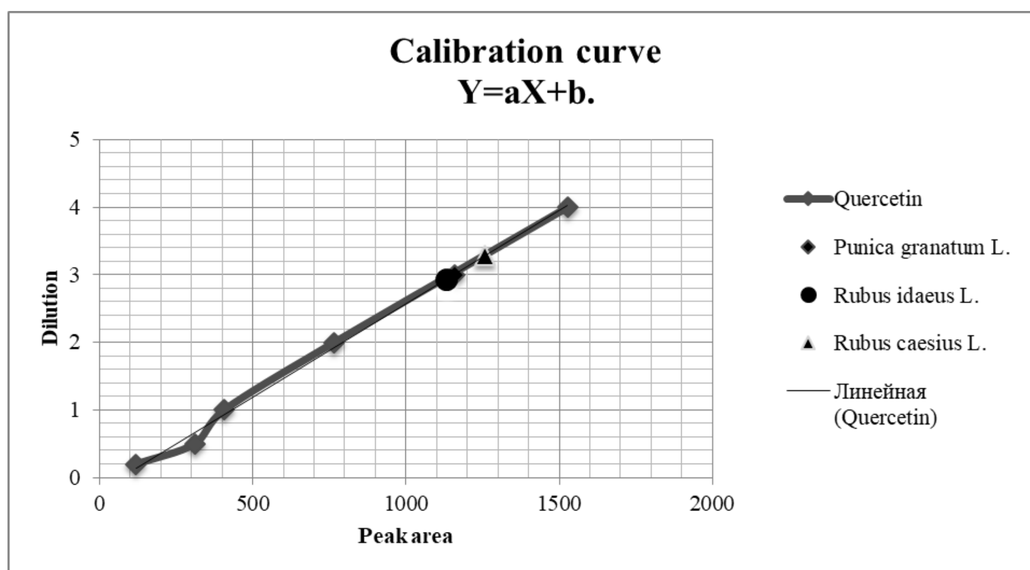

Figure 2 – Calibration curve for the measurement of antioxidant activity.
Note: X – mass concentration of quercetin, mg/dm³ and Y – quercetin signal (peak area)

Polyphenol content. Once the photometer has determined the optical density of the solutions, a calculation is necessary to quantify the total polyphenol content in terms of the percentage of pyrogallol (X). The following equation is used:

$$X\ (percentage\ of\ pyrogallol) = \frac{D1 \times mo \times 100 \times [25] \times 50 \times 5 \times 5 \times 100 \times 100}{Do \times m1 \times [5] \times 5 \times 100 \times 100 \times 50\ (100 - W)} = \frac{D1 \times mo \times 62.5 \times 100}{Do \times m1 \times (100 - W)}$$

where:

- D_1 is the optical density of the analyzed solution at a wave of 715 nm
- D_o is the optical density of the solution of pyrogallol at a wavelength of 715 nm
- m_1 is the mass of crushed raw materials in grams
- m_o is the mass of a sample of pyrogallol – 0.0500 g
- W is the moisture content of the raw material (%)

The total polyphenol content was highest in blackberries (8.20%) followed by raspberries (2.99%) and pomegranates (2.78%), Table 1. These results demonstrate that all plant samples have antioxidant properties as they contain a certain percentage of polyphenols.

Table 1 – Calculation of the total polyphenol content

Sample	Calculation
Punica granatum L.	$\frac{0.0246 \times 0.05 \times 62.5 \times 100}{0.07 \times 2.0813 \times 19} = 2.78\ \%$
Rubus idaeus L.	$\frac{0.0230 \times 0.05 \times 62.5 \times 100}{0.07 \times 2.2910 \times 15} = 2.99\ \%$
Rubus caesius L.	$\frac{0.0430 \times 0.05 \times 62.5 \times 100}{0.07 \times 1.9503 \times 12} = 8.20\ \%$

Fermentation time. The time taken for clot formation to occur was measured. The shortest time recorded for the fermentation of both strains inoculated with blackberry and raspberry extracts (10% and 15%) was 13 hours. The time taken for clot formation to occur for the control samples and those inoculated with pomegranate extracts was longer varying between 20-22 hours depending on the bacterial strain. The results are shown in Table 2.

Table 2 – Fermentation time

Time	*Lb. acidophilus* strain 015k-1	*Lb. bulgaricus* strain 018k-3
13 hours	Blackberry extracts Raspberry extracts	Blackberry extracts Raspberry extracts
20 hours	Pomegranate extract 15%	Control sample, Pomegranate extracts (10% and 15%)
22 hours	Control sample, Pomegranate extract (10%)	-

In vitro evaluation of prebiotic activity of aqueous plant extracts. During the experiment, the results presented on Figure 3 were obtained. The figure 3 shows that the addition of 10% blackberry promotes the growth of *Lb. acidophilus* strain 015k-1 (230 CFU), *Lb. bulgaricus* strain 018k-3 (150 CFU) approximately doubling the number of CFU compared to the control samples of these strains (120 CFU and 70 CFU, respectively). However, it appears that the addition of 15% blackberry extract at inhibited cell growth for both strains.

The addition of raspberry extract inhibits the growth of *Lb. acidophilus* strain 015k-1. However, 10% raspberry extract stimulates the growth of *Lb. bulgaricus* strain 018k-3 (approx. 2.5 times more cells) but again once the concentration of raspberry extract increases cell growth is inhibited.

The addition of 10% pomegranate extract to milk with *Lb. acidophilus* strain 015k-1 slightly increased cell growth whilst increasing the concentration to 15% showed no impact. Similarly for *Lb. bulgaricus* strain 018k-3, the addition of 10% pomegranate extracts stimulated cell growth but again once the concentration of pomegranate extract increases cell growth is inhibited.

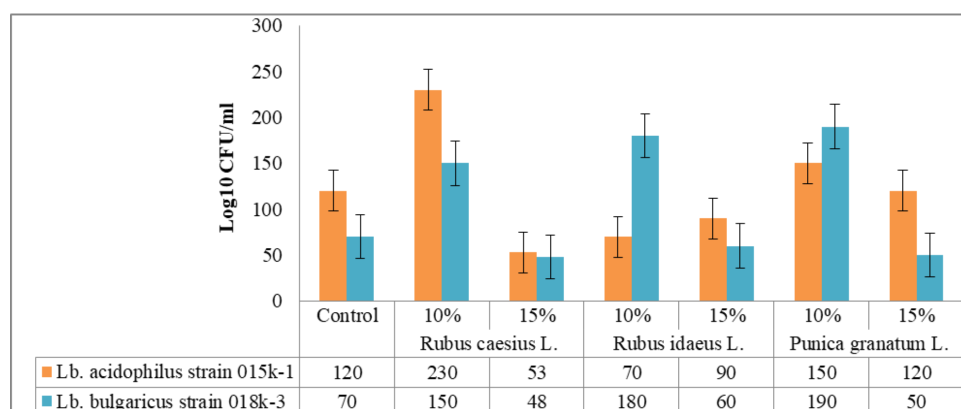

Figure 3 – Colony-forming units of the studied bacterial strains

Conclusion

The promotion of functional foods for gut health within the food industry is showing no signs of slowing down. However, given the limited research conducted to date and the lack of control of these so called "functional foods" it is highly probable that new synbiotic blends will not fulfil their functions. These synbiotic mixtures will possibly contain combinations of live and dead microorganisms combined with potential microbiome-modulating compounds such as fermentable substrates, vitamins and minerals, phytochemicals, and other plant-based material.

The results obtained in this study show for the first time that aqueous extracts of berries (blackberry and raspberry) and pomegranate are effective in stimulating the growth of LAB isolated from kumis under *in vitro* conditions. The use of plant extracts with a high polyphenol content and antioxidant activity will not only expand the range of synbiotic products, but it also has the potential to increase the number of viable beneficial bacterial cells in food products. However, further studies are necessary to monitor the changes in viable bacterial counts and the effect of pH and titratable acidity in the product during storage.

Acknowledgments

This work was supported by the grant of the program 267 "Increase availability of knowledge and scientific research" subprogram 101 "Program-targeted financing of scientific research and events" on the specifics 154 "Payment for research services" from the Ministry of Science and Higher Education, Republic of Kazakhstan.

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