Examining the possibilities for application of pea milk in obtaining fermented probiotic foods

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Examining the possibilities for application of pea milk in obtaining fermented probiotic foods: *Lactic Acid Bacteria are able to coagulate animal milks. Fermentation with only vegetable milk does not lead to the production of tasty and quality dairy products but mixes of animal and vegetable milks seem to be preferable for obtaining fermented product of better quality. The Lactobacillus strains Lactobacillus acidophilus Ar, Lactobacillus acidophilus 1A29$_3$, Lactobacillus plantarum 226-15, Lactobacillus casei ssp. casei Shirota D, Lactobacillus plantarum BB22; the starter MZ$_2$ and the yeast strain Saccharomyces cerevisiae ssp. diastaticus 25-G have been selected because of their ability to grow in media containing skimmed cow's milk and pea milk (from Pisum sativum), mixed in different ratios. This leads to obtaining quality nontraditional fermented products – beverages and yoghurt. The obtained products have high concentrations of viable cells and moderate titratable acidity and they can be regarded as functional foods.*

Key words: *Lactobacillus, Saccharomyces, diet foods and beverages, pea milk*

INTRODUCTION

Food fermentation is a process in which raw materials such as meat, vegetables or milk are converted into fermented foods thanks to the metabolic activities of selected microorganisms. Some components from the medium (raw material) are used by these microorganisms to synthesize desirable by-products such as aromatic components [5] and to increase the organoleptic quality of a product. Selected strains of microorganisms with desirable properties are employed for obtaining each type of food products. This is followed by selection of symbiotic combinations between the selected microorganisms and their usage as starters for the inoculation of the raw materials. The best fermentation conditions are then applied to optimize and control the growth of the microorganisms. This type of fermentation is called "controlled fermentation". The control of the process is necessary for all materials but it is especially important for raw materials with high risk of contamination such as milk.

The family of Lactic Acid Bacteria (LAB) that includes the genera *Streptococcus*, *Lactobacillus, Leuconostoc, Pediococcus* and *Lactococcus* is the main microbial family used in this kind of fermentation because of their probiotic properties [8] and their diversity [2, 4], which is especially characteristic for the genus *Lactobacillus* [6]. LAB can be isolated from variable sources such as non-food sources, naturally fermented yoghurts or faecal mass. LAB when used in controlled fermentation, are also referred to as starter cultures. Their ability to convert carbohydrates (most of the lactose in the raw milk) into lactic acid and other by-products is essential for the acidification of the medium and for the coagulation of the milk resulting in yoghurts with desirable rheological properties.

In the majority of fermented products, animal milks constitute the only raw material, so the proteins and carbohydrates in the medium are exclusively of animal origin. The formation of physical and chemical bonds between proteins and carbohydrates is necessary for the production of a good dairy product [9]. With the increasing cost of animal breeding, proteins from vegetables seem to become a good alternative to this problem. The first vegetable tested as an alternative protein source was soybean *Glycine max* and it was a success because of its special properties, for example its good capacity to bind calcium [3]. Some soybean products are already commercialized. Inspired by this success, researchers continued the studies with vegetable proteins and now a new vegetable draws the attention of food industry scientists - pea *Pisum sativum* [1]. In order to extract the gliadins and glutenins (essential proteins in pea) from the whole pea seed, several processes can be used. The most commonly performed method is pea crushing and protein concentration by drying to obtain an isolate which will be then re-suspended to

obtain “pea milk”. Pea milk is mixed with skimmed cow’s milk in different ratios and utilized by the microorganisms as a “new raw material”.

The aim of the present work is to investigate the potential application of pea milk to obtain fermented probiotic foods which includes two steps: 1) the selection of pure lactobacilli and yeast cultures and yoghurt starters that can develop in a medium containing pea milk; and 2) finding the most appropriate ratio between skimmed cow’s milk or 0.5% NaCl solution and pea milk for the production of quality food products using the selected probiotic starters and strains.

MATERIALS AND METHODS

1. Microorganisms. In the present work the following strains of lactic acid bacteria and yeasts are used: genus *Lactobacillus*: *Lactobacillus acidophilus* Ar, *Lactobacillus acidophilus* $1A29_3$, *Lactobacillus casei* ssp. *casei* Shirota D, *Lactobacillus plantarum* 226-15, *Lactobacillus plantarum* BB22, Starter MZ_2, Starter MSH; yeasts: *Saccharomyces cerevisiae* var. *diastaticus* 25 - G.

2. Media:

2.1. Sterile skimmed milk with titratable acidity 16-18ºT

2.2. MRS-broth

2.3. MRS-agar

2.4. Malt-agar

2.5. Medium for the determination of molds and yeasts in milk and milk products at 25°C according to ISO 6611/2004

2.6. Medium for the determination of staphylococci (*Staphylococcus aureus*) - Baird Parker agar base (BS EN ISO 6888-1:2005 + A1:2005)

2.7. Medium for the determination of *Salmonella* sp. by horizontal fixing method for the isolation of *Salmonella* ssp. according to BS EN ISO 6579/2003

2.8. Medium for the determination of coliforms (*E. coli*) - Chromocult TBX agar (ISO 16649-2:2001)

2.9. Medium for the determination of the total aerobic and facultative anaerobic microorganisms - PCA-agar - according to BS ISO 6610:2002

2.10. Pea milk. Smooth pea seeds imported from China are used to obtain pea milk. The dried peas are soaked in water for 24 hours. The peas are mixed with water and the mixture is processed with direct steam from the steam generator in the Russian apparatus “Soevaya korova”. Grinding of the seeds and water vapor extraction at 110°C for 1 hour is achieved using the steam supply. The resulting extract is filtered and the filtrate is used as pea milk.

3. *Determination of the titratable acidity.* The Thorner method is used for the determination of the acid formation ability [7] of the lactic acid bacteria. One °T is equal to 1 cm^3 0.1 N NaOH, needed for the neutralization of an equivalent amount of organic acid contained in 100 cm^3 of culture medium. To 10 cm^3 of each sample (bacteria developed in skimmed milk or liquid medium) are added 20 cm^3 of distilled water. The mixture is titrated with 0.1 N NaOH using phenolphthalein as an indicator until the appearance of a pale pink color that persists for one minute.

4. *Determination of the concentration of viable cells of lactic acid bacteria and yeasts.* The strains of lactic acid bacteria and yeasts are cultured separately for a certain period of time at a certain temperature. Appropriate serial dilutions in saline solution of the cultural suspensions are prepared and the spread plate method is applied. 0.1 cm^3 of the last three dilutions is used to inoculate LAPTg10-agar (for the enumeration of lactic acid bacteria), malt extract-agar (for the enumeration of yeasts) or the respective elective solid medium (for the enumeration of the specific microorganisms). The inoculated Petri dishes are incubated for 3 days at optimum temperature for the growth of the respective microorganisms until the appearance of countable single colonies. The count of the colonies is then used to estimate the number of bacteria in the original sample.

5. The organoleptic evaluation is performed according to BS 15612-83.

RESULTS AND DISCUSSION

The ability of probiotic lactobacilli strains, starters for yogurt production with the probiotic strain *Lactobacillus delbrueckii* ssp. *bulgaricus* MZ_2 and the yeast strain *Saccharomyces cerevisiae* ssp. *diastaticus* 25-G to grow in pea milk is investigated in a series of experiments. It has been found that the use of pea milk alone for the preparation of food products is not possible due to the high content of starch in it. Therefore, as a medium for the development of strains of lactobacilli, starters for yoghurt and yeasts different dilutions of the pea milk in other culture media (10% of skimmed cow milk or 0,5% NaCl solution) are prepared, in order to determine the desired composition of the culture medium for the preparation of nontraditional dairy products.

For the initial screening of the optimal composition of the peamilk-containing culture medium pea milk, prepared from peas in an apparatus via steam extraction, and skimmed cow's milk or 0.5 % solution of NaCl are used.

Table 1. Characteristics of the nontraditional yoghurts and beverages produced with pea milk. TA – titratable acidity

Strain	Medium	Microscopic pattern	Characteristics of the yoghurt/ beverage	TA, °T
Lactobacillus acidophilus Ar	10% skimmed cow milk : pea milk = 1: 4 +1% glucose	Long rods	Insipid, with no good taste	12
Lactobacillus acidophilus Ar	10% skimmed cow milk : pea milk = 1: 4	Greater in number, but shorter rods	Insipid, with a hint of pea	11
Lactobacillus acidophilus Ar	10% skimmed cow milk : pea milk = 1: 1 +1% glucose	Short rods	A beverage with nice taste and no hints	26
Lactobacillus acidophilus $1A29_3$	10% skimmed cow milk : pea milk = 1: 1 +1% glucose	Long, big, thick rods	A beverage with pleasant taste	41
Lactobacillus plantarum 226-15	10% skimmed cow milk : pea milk = 1: 4 +1% glucose	More cells, short rods	No hints	76
Lactobacillus plantarum 226-15	10% skimmed cow milk : pea milk = 1: 4	More short rods	Has a hint of pea	61
Lactobacillus casei ssp. *casei* Shirota D	10% skimmed cow milk : pea milk = 1: 4	Rich microscopic pattern. A lot of rods	Very sour	68
Lactobacillus plantarum 226-15	0,5% NaCl : pea milk = 1 : 4 + 1% glucose	A lot of short rods	The two concentrates are mixed in a ratio of 1: 2 (lactobacilli : yeasts) and a very nice tasting boza is obtained. It is better to have more yeast.	13
Saccharomyces cerevisiae ssp. diastaticus 25 – G	0,5% NaCl : pea milk = 1 : 4 + 1% glucose	Two strains of yeasts, budding		
Lactobacillus plantarum BB 22	10% skimmed cow milk : pea milk = 1 : 1 +1% glucose	A lot of short rods	The two concentrates are mixed in a ratio of 1: 2 (lactobacilli : yeasts) and a better tasting boza than the previous one is obtained. It is better to have more yeasts.	29
Saccharomyces cerevisiae ssp. *diastaticus* 25 – G	10% skimmed cow milk : pea milk = 1 : 1 +1% glucose	Two strains of yeasts, budding		
Starter MZ_2	10% skimmed cow milk : pea milk = 1 : 4	Rods prevail	Insipid yoghurt	36.27
Starter MZ_2	10% skimmed cow milk : pea milk = 1: 4 +1% glucose	Cocci and rods, but rods prevail	Good yoghurt	28.5
Starter MZ_2	10% skimmed cow milk : pea milk = 1 : 2 + 1% glucose	Great picture	Good yoghurt	55
Starter MSH	10% skimmed cow milk : pea milk = 1 : 4	More cocci, short rods	More sour yoghurt	26
Starter MSH	10% skimmed cow milk : pea milk = 1 : 4 + 1% glucose	Less cocci, more rods	Better yoghurt	41
Starter MSH	10% skimmed cow milk : pea milk = 1 : 2 + 1% glucose		Nice beverage with no side hint	42

The various strains are grown in optimal for each of the strains nutrient medium (for the representatives of the genus *Lactobacillus* - MRS broth and for the yeast strain – malt extract-agar) for 17 hours at the optimum temperature for each of the used strains of lactobacilli or yeasts (*Lactobacillus acidophilus* strains, *Lactobacillus casei* ssp. *casei* Shirota D - 37°C; *Lactobacillus plantarum* strains, *Saccharomyces cerevisiae* ssp. *diastaticus* 25-G - 30°C). The yoghurts with the starters are incubated at 41±1°C for 4-5 hours until the coagulation of the milk. The results of the experimental studies are shown in Table 1.

Two medium controls are prepared: Control 1 (Composition - 0,5% NaCl solution : pea milk = 1 : 1) and Control 2 (Composition - (skimmed cow milk : pea milk = 1 : 4) + 1% glucose). Both controls have titratable acidity 5°T. The number of insemination microorganisms in both the two controls (C1 and C2) is determined (Table 2).

Table 2. Microflora of the control samples

Indicator	TBA, cfu/cm^3	Specific microorganisms, cfu/cm^3			Molds and yeasts, cfu/cm^3
		E.coli (TBX-agar)	*St.aureus*	*Salmonella* sp.	
C1	<1	<10	Not found	Not found	<10
C2	<1	<10	Not found	Not found	<10

Experimental evidence suggests that the best yoghurt and beverages are prepared using the medium containing skimmed cow's milk and pea milk in a ratio of 1 : 1 and the medium containing skimmed cow's milk and pea milk in a ratio of 1 : 4. The best boza is prepared using medium consisting of skimmed cow's milk and pea milk in a ratio of 1 : 1 and medium consisting of pea milk and 0.5 % NaCl solution in a ratio of 1 : 1.

A second series of yoghurts and beverages are prepared using the selected nutrient media. The results of these experiments are shown in Table 3.

Table 3. Concentration of viable cells and titratable acidity of the second set of foodstuffs obtained using pea milk on their first day. TA – Titratable Acidity

Microorganisms	Media	N, cfu/cm^3	TA, °T	Lactic acid, %
Lactobacillus acidophilus A29$_3$	10% skimmed cow's milk : pea milk = 1 : 4	$5x10^9$	67.00	0.621
Lactobacillus plantarum 226-15	10% skimmed cow's milk : pea milk = 1 : 1	$1x10^{14}$	37.94	0.342
Starter MZ$_2$	10% skimmed cow's milk : pea milk = 1 : 4	$2x10^{10}$	76.26	0.686
Starter MZ$_2$	10% skimmed cow's milk : pea milk = 1 : 1	$1.3x10^{11}$	89.28	0.804
Lactobacillus plantarum 226-15	0,5% NaCl : pea milk = 1 : 4	$2x10^9$	47.80	0.430
Lactobacillus plantarum BB 22	0,5% NaCl : pea milk = 1 : 4	$1.76x10^{11}$	26.78	0.241
Lactobacillus acidophilus Ar	10% skimmed cow's milk : pea milk = 1 : 1	$1.6x10^{12}$	47.43	0.427
Lactobacillus casei ssp.*casei* Shirota D	10% skimmed cow's milk : pea milk = 1 : 1	$1x10^{11}$	29.02	0.261
Saccharomyces cerevisiae ssp. *diastaticus* 25-G	0,5%NaCl : pea milk = 1 : 4	$9.2x10^{11}$	8.18	0.074

In culturing the strains *Lactobacillus acidophilus* 1A29$_3$, *Lactobacillus plantarum* 226-15 and *Lactobacillus acidophilus* Ar in culture medium containing skimmed cow's milk and pea milk, pea beverages for immediate consumption are obtained. The culturing of the starter MZ$_2$ in a medium containing skimmed cow's milk and pea milk results in pea yoghurts.

Lactobacillus plantarum BB22 is isolated from fermented cereals. It is used for the preparation of boza obtained by cultivation of the *Lactobacillus* and the yeast strain separately, and then the single strain cultural suspensions are mixed in a ratio of 1 : 2 respectively. The resulting beverage is weakly acidic with no pea off-flavor.

Two boza options are obtained: Option 1 contains *Lactobacillus plantarum* 226-15 and *Saccharomyces cerevisiae* var. *diastaticus* 25-G in a ratio of 1 : 2; and Option 2 contains *Lactobacillus plantarum* BB 22 and *Saccharomyces cerevisiae* var. *diastaticus* 25-G in a ratio of 1 : 2.

In addition to the beneficial effects of the pea milk the beverages with the species *Lactobacillus acidophilus, Lactobacillus plantarum, Lactobacillus casei* ssp. *casei* Shirota D are also with high concentrations of viable cells of probiotic bacteria (5.10^9-$1,6.10^{12}$ cfu/cm^3) and moderate titratable acidity (29.02-69.00ºT), which increase their biological value. The obtained yogurts are dietary probiotic products.

The content of the active cells in the produced yoghurts exceeds 10^9cfu/cm^3. The titratable acidity ranges from 76.26 to 89.28ºT. At the same time the ratio of cocci to rods ranges from 1 : 2 (in medium containing skimmed cow's milk and pea milk in a ratio of 1 : 4) to 1 : 1 (in medium containing skimmed cow's milk and pea milk in a ratio of 1 : 1). The obtained yoghurts contain a considerable amount of the probiotic strain *Lactobacillus delbrueckii* ssp. *bulgaricus* MZ_2.

Both types of fermented foods, yogurts and fresh peamilk beverages, are new dietary probiotic foods. Along with the prebiotic ingredients of the pea grains in the human gastrointestinal tract would enter significant amounts of useful microorganisms required to restore and maintain the balance of the microflora in the entire digestive tract, which is particularly important for the health of the organism.

CONCLUSION

A starter, lactic acid bacteria and a yeast strain capable of growing in a medium containing pea milk are selected. 2. Pea yogurt and pea acidophilic beverages with a high concentration of viable cells (over 10^9cfu/cm^3) and moderate titratable acidity with no pea off-flavor are obtained. 3. This is the first time to use pea as a substrate in the production of boza.

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