INFLUENCE OF RAW MILK QUALITY ON *LACTOBACILLUS ACIDOPHILUS* MULTIPLICATION AND PROBIOTIC YOGHURT PRODUCTION

Eva Csutak

University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca, Romania
e-mail: szkkeva@yahoo.com

Abstract

Considering that the quality of raw milk is a prerequisite condition to obtain a good quality probiotic yoghurt, our studies aimed the measurement of milk factors which can affect the multiplication of probiotic lactic acid bacteria (LABs) Lactobacillus acidophilus (LA-5. The probiotic strains Bifidobacterium BB-12 and Lactobacillus acidophilus LA-5-we used for trials- are tested probiotics by Christian Hansen company. We studied comparatively raw and pasteurized milk, their chemical composition and the correlations between the spontaneous microbial flora (NTG) found in milk samples and the impact of this flora on the multiplication of LABs. We investigated as well the effect milk proteins, added prebiotics(lactose, molasses) on pH and LAB development, the influence of NTG (number of total germs), NCS (somatic cells number) in raw milk before and after pasteurization, on lactic fermentations and LA-5(Lactobacillus acidophilus) Generally multiplication of LA-5 strains was reversely correlated with NTG values. There is a direct correlation between presence of prebiotics and probiotic bacteria activity.

Key words: yoghurt, probiotics, lactic acid, NTG, pasteurization

INTRODUCTION

Yoghurt is a long time known and appreciated dairy product, obtained traditionally by the spontaneous or induced lactic fermentation of milk. The microbiology of lactic-producing bacteria and the fermentation biochemistry and technology of yoghurt is well documented [1], [2], [3], [6], [13].

The term "probiotic" is known since 1903 when the benefic actions of *Lactobacillus acidophilus* strains were observed in human intestine, and the term of "prebiotic" is known since 1961, and define the substances, generally natural ingredients or microorganisms which improve the intestinal equilibrium and defense against pathological bacteria [4], [7], [11], [15].

Yoghurt, by its high content in lactic acid bacteria (LABs) possesses antimicrobial activity *in vitro* against a wide variety of Gram-positive and Gram-negative bacteria, as well as some fungi. The exact cause of inhibition is not known, but may be due to the antagonist action of LAB species which prevent the adherence, establishment, replication, and/or pathogenic action of certain enteropathogenes. To improve continuously the quality of yoghurts, preservation of probiotic characteristics and the shelf-life of live LABs, with improved capacity of fermentation, are needed [9], [10], [12], [17], [18].

Among many strains, *Lactobacillus acidophilus* is best candidates to be used, alone or in combinations as lactic fermenting microorganisms with high probiotic activity (Kailaspathy, 1997). An important factor which influence the development and survival rate of probiotic LAB is the milk quality and its bacterial flora. It is known that the quality of raw milk in Romania is still an unsolved problem, since the number of total germs and of somatic cells found in milk is higher than the permitted level in European Union (NTG <100000/ml, NCS<400000/ml) [2], [3].

Considering that the quality of raw milk is a prerequisite condition for obtaining a good quality probiotic yoghurt, our studies aimed the measurement of main milk factors which can affect the multiplication of probiotic-forming bacteria *Lactobacillus acidophilus*

(LA-5) We studied comparatively the raw and pasteurized milk, the correlations between the spontaneous microbial flora found in milk samples and the impact of this flora on the multiplication of probiotic bacteria. Added prebiotics are influencing the multiplication of LA-5, and in this way may be decreased raw milk qualities effects.

MATERIALS AND METHODS

The samples of cow milk originated from the region of Harghita county and the tests were made at NIZO, Netherlands and at S.C. Gordon Prod company's authorized lab. For experiments we used *Lactobacillus acidophilus (LA-5)* strain provided by Christian Hansen company. The media used for the storage and determinations of bacterial multiplication were MRS agar for LA-5 the nutritive, sorbitol agar (Sanimed) used for the determination of NTG .

To make measurements, the raw milk, after cooling, was inoculated with both bacterial strains at three dilutions (10^{-1}, 10^{-2} and 10^{-3}) and incubated for 72 hrs. The counting of bacteria was made after 48 and 72 hrs of incubation. For LA-5 the incubation was made at 43°C. All samples were done in duplicate. For preparing of probiotic yogurt pasteurized milk on 95°C was cooled to 43°C, and inoculated with LA-5 probiotic strain. After 3 hours of incubation were measured the parameters presented in results.

To determine the NTG we used the bacterial counter IBCM Bactocount and to count the NCS, we used the Somatos tester NCS. Somatos tester used for NCS determination is an equipment, based on difference of viscosity of milk with different number of somatic cells. The equipment was bought from Viola company, from Bucharest. The pH was determined using the lab pH meter WTW315i. PH meter WTW 315i is imported by Viola company, produced by WTW company. By photometry we determined the lactic acid and lactates from our samples (yoghurt, yoghurt with molasses).

With added phosphoric acid the lactates are transformed to lactic acid.

Form the prepaired samples we obtain a dilution 10-2, and from this diluated sample with a pipette we measure 50 µl to a test-tube with bung. To the diluated yoghurt sample we add 4 ml 3:1 H2SO4: H2O, and 100µl hydro-kynon solution with etanol 12.5%.The test-tube must be agitated (shaked), and boiled in a water-bath about 20 minutes (together with an etalon and a standard sample), until the color of sample will become yellow.

After cooling the content of test-tube, by photometry-on 405 nm- we determine the lactic acid content.

Etalon sample is prepaired from 50µl of distillated water, and the standard solution from 50-50 micro-liter of standard.The lactic acid content of product was tested a University of Kaposvar, and at Gordon Prod's lab.

Bactocount IBCm is an automated instrument using flow cytometry (FCM) for the rapid enumeration of individual bacteria in raw milk. The milk is sampled and dispensed into individual vials manually. An incubation reagent made up with a clarification buffer, a proteolytic enzyme, and a fluorescent marker are then added in manually in order to lyse the somatic cells, solubilize the fat globules and proteins, permeabilize the bacteria and stain their DNA. The vials are then placed on an incubator at 50 °C. The fluorescence marker intercalates rapidly and selectively into all the bacteria double-stranded nucleic acid. The mixture is then sonicated manually during the incubation on two different occasions. The sonication process is used to help the chemical breakdown of the interfering particles, disrupt die remaining bacteria colonies to improve the detection of individual bacteria and reduce the background fluorescence. The cell debris, devoid of nucleic acid, are excluded from the analysis.

After the incubation period the mixture is then transferred manually from the incubation plate to the sample intake pipette. The mixture is then transferred to the flow cytometer where the bacteria are aligned and exposed to an intense laser beam and fluoresce. The fluorescence signal is collected by the optics, filtered, and detected with a photo multiplier. The fluorescence pulses intensity and height are recorded and used as gating parameters. The sorted pulses

are dien translated into individual bacteria count after instrument calibration.

RESULTS AND DISCUSSION

The composition of milk samples (raw and pasteurized) before to be inoculated with probiotic strains:

The main characteristics of raw milk comparing with the pasteurized milk used in experiments are presented in Table 1.and the composition of native proteins found in whey originating from raw and pasteurized milk are presented in Table 2. The fat, protein, lactose content of milk are measured in percent, the microorganisms number in CFU/ml(colony formating unit).

Table 1.
Main chemical parameters which characterize the milk samples (raw and pasteurized)

Milk samples	Total protein %	Lactose %	Fat %	Density Kg/l	Aerobic psyhotrophic microorganisms (CFU/ml)	TSR9 (CFU/ml)	Phosphatase activity
Raw milk	3.28	4.41	3.80	1.029	$1.4*10^7$	$1.7*10^4$	positive
Pasteurized milk	3.27	4.53	3.77	1.029	$9.6*10^4$	$1.3*10^4$	negative

No significant differences between density, proteins, lactose and fat contents were observed for raw vs pasteurized milk, but a significant decrease of aerobic psyhotropic microorganims, TSR9 density.

Table 2.
Analysis of native protein fractions found in native whey originating from raw and pasteurized milk.

Native whey Proteins	α-lactalbumin (g/l)	ß-lactoglobulin (g/l)	BSA (g/l)	Immuno globulin (g/l)	Total whey protein (g/l)	De-natured whey-protein %
Raw milk	0.97	3.19	0.29	0.50	4.96	0
Pasteurized milk	0.95	2.96	0.18	0.27	4.35	12.2

Regarding the native whey proteins, significant decreases in BSA (bovine serum albumin), immunoglobulin and total protein were observed for pasteurized milk, as well the increase of denatured proteins (up to 12.2%) (Table 2).

Relations between the NTG found in raw and pasteurized milk and their effects on the multiplication probiotic strain of LA-5:

We observed that the initial milk NTG, and as well NCS influenced significantly the probiotic bacteria evolution (Table 3). We found out that pasteurization at 95°C was not enough efficient and even after 95°C pasteurization, the presence of microorganisms can affect the probiotic development.

Samples of raw milk we used for trials are presented in table 3.

The influence of the NTG found in raw and pasteurized milk and their effects on the multiplication (expressed in %) of LA-5 at concentrations of 0.025g/l is presented on fig. 1. and table 4. Incubation temperature of milk: 43ºC.

Table 3.
Determination of raw milk parameters used for evaluation of NTG influence on probiotic bacteria multiplication

Nr. of sample	Name of collector or collecting center	NCS, CFU/ml	Protein content, %	Fat content, %	Lactose content, %
1	Torok Antal	76300	3.42	4.14	4.69
2	Turdeni	103000	3.46	4.08	4.71
3	Gordon's Farm	92000	3.38	4.08	4.67
4	Hadnagy Antal	116000	3.44	4.09	4.69
5	Dobeni	96000	3.45	4.11	4.71
6	Farm 3	13200	3.42	4.16	4.72
7	Cobatesti	84000	3.41	4.08	4.68
8	Kiss Antal	123000	3.39	4.09	4.71

Table 4.
Influence of raw milk NTG on LA-5 bacteria multiplication

Nr. of sample	NTG of milk saple by IBCm BactoCount, CFU/ml	NTG of pasteurized milk, CFU/ml	Titrable acidity, Th°	Lactic acid content, %	Number of LA-5, by inoculation on MRS agar	pH,
1	10621099	10200	71	0.69	2360000	4.79
2	1589781	8100	78	0.72	2640000	4.68
3	357856	6400	84	0.78	2730000	4.56
4	1702388	7600	81	0.73	2620000	4.65
5	989569	6350	82	0.76	2700000	4.59
6	91392	4850	88	0.88	2830000	4.55
7	272785	6100	76	0.72	2690000	4.67
8	114321	5150	84	0.87	2838000	4.61

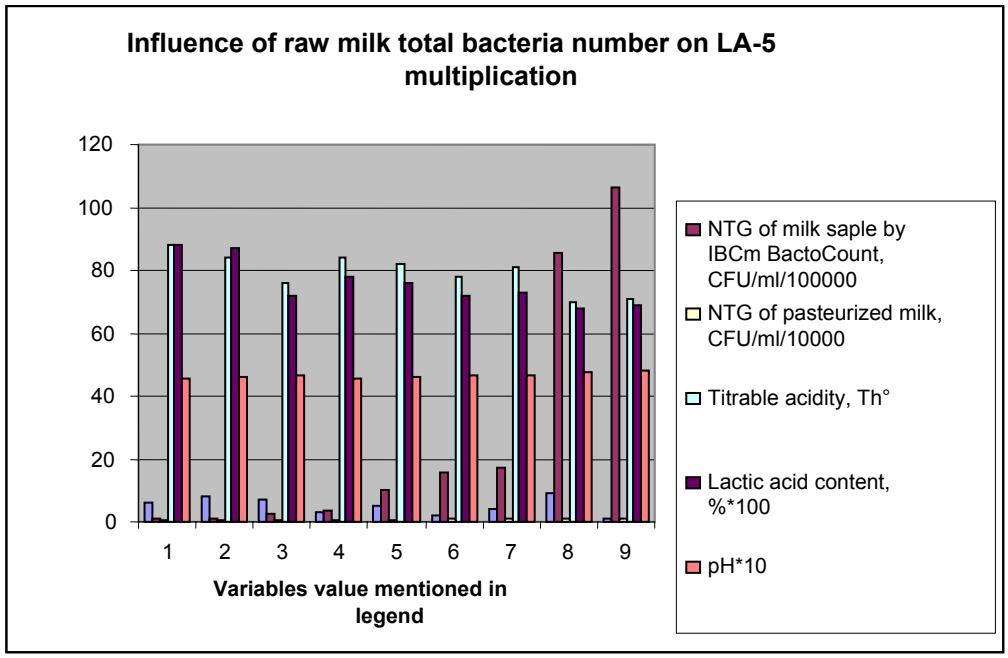

Fig.1. Influence of raw milk total bacteria number on LA-5 multiplication

Lactic acid content of probiotic yogurt with LA-5 is in direct relation with number of LA-5 bacteria, how it is presented on fig.2.

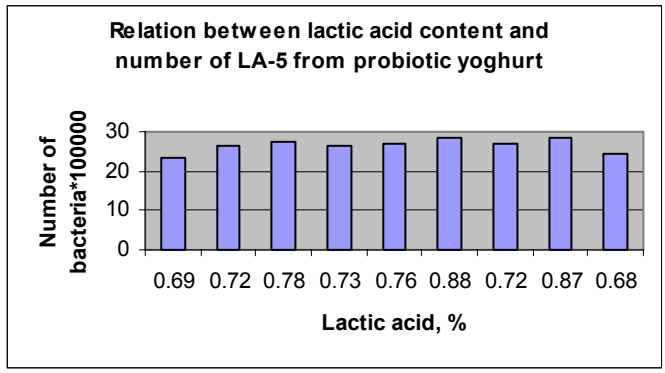

Fig.2. Relation between lactic acid content and number of LA-5 from probiotic yoghurt

For determining of total bacteria number bacteria number influence on LA-5 multiplication it was important selecting of raw milks with different total bacteria number. It was important that chemical properties of milk (fat content, protein content, lactose content) and its somatic cell number to be similar and low (lower than 200000 CFU/ml). For inoculation we used pure probiotic strains of LA-5. Influence of total bacteria number on LA-5 multiplication was measured off by determining of acidity, pH,NTG, lactic acid content of final product.

Raw milk's NTG influences directly total bacteria number of pasteurized milk. Acidity ,NTG, pH, lactic acid content of probiotic yoghurt obtained from raw milk with high NTG will be lower. Samples with NTG > 500000 CFU/ml after pasteurizing process will have a total bacteria number > 6000 CFU/ml, and this concurrent microflora will affect the multiplication of LA-5 probiotic strains.

Generally, the NTG in raw milk at the beginning of experiments were around 20 times higher than in pasteurized milk (where even after pasteurization at 95ºC, not all microorganisms were destroyed). In time, after inoculation with LA-5 the NTG decreased significantly, around 23 times (for raw milk) and 5 times (for pasteurized milk). It is obvious the competition between the initial contamination of milk and the effectiveness of LA multiplication.

CONCLUSION

The quality of raw milk in Romania needs to be improved, in order to obtain higher quality probiotic yoghurts. At his moment the number of total germs (NTG) is over the limits accepted by EU legislation, and it can induce technological problems during production.

In this context, the Gordon Prod company where our experiments were done, tried to investigate the impact of existing NTG and NCS in raw and pasteurized milk on LAB probiotics (*Lactobacillus acidophilus*). We found out that pasteurization at 95°C was not enough efficient and even after 98°C pasteurization, the presence of microorganisms can affect the probiotic development. Small increases of milk protein content seem not to influence the LABs development.

We investigated as well the effect milk proteins on pH and LAB development, the influence of NTG, NCS in raw milk before and after pasteurization, on lactic fermentations and LA activities.

Our further studies will be directed on the addition of milk powder (industrial), alone or in combination with other prebiotics such as inulins, or mono-and oligo-carbohydrates from molasses.

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