Romanian Biotechnological Letters *Vol. 15, No. 6, 2010*

ORIGINAL PAPER

A new functional dairy product containing an optimized mixture of *Lactococcus* bacteria, kefir and brewer's yeasts

Received for publication, May 4, 2010
Accepted, December 15, 2010

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Abstract.

Functional dairy products (FDP), especially those with probiotic properties, became very popular the last decade, due to both their health-promoting properties. Our studies aimed the elaboration of a new prototype of a FDP, based on a complex mixture of microorganisms with synergistic action. Meanwhile, we aimed to valorise the bioactive potential of brewer's yeast, as a nutritional complement beside the lactobacilli (Lactococcus) and kefir yeast in the FDP. By two steps experiments we elaborated different functional dairy product (FDP) variants based on mixtures of Lactococcus bacteria, kefir and brewer yeasts, in different ratios in order to obtain an optimized composition. The physical-chemical parameters to qualify these products were pH, total acidity and syneresis factor, their modifications during incubation and shelf life did not vary significantly, but the presence of the brewer yeast brought added-value to the final product and determined the less acidic pHs than for typical yogurt.

Keywords: functional dairy product, kefir, brewery' yeast, *Lactococcus lactis,* optimisation

Introduction

Functional foods, beside their rich nutrient composition, contribute to the human health by their biologically active compounds, especially nowadays, becoming attractive and essential against pathological risks resulting from the multitude of aggressive factors which disturb the physiological balance systems [1]. The bioactive components of functional foods derived from dairy products (e.g. probiotics and prebiotics) are yet known to improve the metabolic turnover balance of the human metabolism, well documented by many reports which demonstrate the beneficial effects on gut and immune system [2-6], the consumers being increasingly interested to use these products [7].

The brewer yeast has been included in functional foods or food supplements since 80's, due to its natural balance between proteins, vitamins B, essential minerals and a antioxidant biomolecules which act as redox modulators [8,9], therefore assuring not only an energetic and nutritional value, but also a therapeutic value [10, 11]. Brewer's yeast obtained as a by-product of beer fermentation, proved to be the best functional yeast which is grown on high-value malt worth and synthesizes greater quantities of vitamins and biologically active substances, more than in other substrates [12]. Modern diets including yeast products have shown important improvements of the health of persons suffering of digestive, hepatic, inflammatory as well on nervous system disturbances [13]. The fermentation technology and specificity of microorganisms used for inoculation are key-elements which affect the quality of a final product (flavor, viscosity, microbial viability and chemical features) [14-16]. Lactic bacteria multiply and produce lactic acid at lower rate when they are co-cultivated with yeasts [17, 18] and their acidity increases during lactic acid biosynthesis [19].

The purpose of our studies was the elaboration of a new functional dairy product (FDP), based on a complex mixture of microorganisms with synergistic action, such as lactobacilli, kefir yeast and brewer yeast. We optimized the manufacturing process of the FDP by pilot and laboratory experimental studies. Firstly, we used different concentrations of starter cultures inoculated in milk, co-cultivated with different proportions of yeasts, until a final prototype product was obtained, of high quality and good flavor. In two-step experiments we optimized the ratios between the three types of microorganisms in order to obtain a balanced, stable composition with good flavouring and functional properties. We followed the general quality parameters of the experimental compositions, such as pH, acidity and syneresis, as well their changes during shelf-life (storage at 4º C, from 1 to 21 days). We reported that the presence of *Saccharomyces* yeast in the product does not negatively affect its physical and chemical qualities. Based on the national and international standards and regulations governing the field, we decided to classify this dairy product prototype as functional dairy product (FDP).

Materials and Methods

1. Microorganisms

The microorganisms used to form the inoculum were represented by a bacterial starter culture and two yeast cultures. We used a mesophilic bacterial culture FD-DVS CHN-22 (provided by Chr. Hansen) **(C)** of *Lactococcus lactis (ssp.cremoris, ssp. lactis and ssp lactis biovar diacetylactis)*, *Leuconostoc mesenteroides subsp. cremoris,* containing 10^{10} colony forming units per mL (cfu/mL). A first yeast culture consisted on 10^{10} cfu/ml *Debaryomyces hansenii* kefir yeasts LAF 3 **(K)**, provided by Chr. Hansen. Both of these starter cultures were freeze-dried powders and Direct Vat Set (DVS). The brewer's yeast (*Saccharomyces cerevisiae)* **(B)** represented the second yeast culture (10^{10}cfu/mL), separated from the secondary fermentation of beer, provided by a local brewer, having a cellular viability of 96%.

2. First step experiment

In the pilot station of the University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca, Romania) aliquots of 2 L fat 1.8% (w/w) skimmed, pasteurized milk, cooled at 30°C, was inoculated with starter cultures **C** and **K** and brewer's yeast **B**, and incubated at 29-30°C for 12 hr, pre-cooled at 18-20°C for 1hr, cooled again at 4-6°C for 10 hr and stored up to 21 days at 0-4°C. Two replications of all batches and samples were performed. The milk sample was inoculated with 1 mL reactivated mesophilic culture (C) and 2 mL *Saccharomyces* yeast (B) **(trial C+B)** or with 1 mL (C) and 2 mL kefir yeast (K) **(trial C+K)**, or with 1 mL (C) and 1 mL (K) and 1 mL (B) **(trial C+K+B).** The volumetric ratios (expressed in mL) between milk and microorganisms (C, K, and B) were 2000:1:0:2 (trial C+B), 2000:1:2:0 (trial C+K) and 2000:1:1:1 (trial C+K+B). All samples were made in triplicate.

3. Second step experiment

Based on the first step experiment and its results, we tried to optimize the fermentation of C±K±B mixtures in aerobic and anaerobic conditions, using specific ratios between the microorganism cultures, as it is shown in Table 1. In this case, we used 0.5 L of skimmed milk, 1.8% (w/w) fat, which was inoculated and fermented in aerobic conditions at 29-30°C for 12hr (S_1-S_4) and the same milk quantity inoculated and fermented in anaerobic conditions (S5-S8), 20% (v/v) CO_2 at 29-30°C for 12 hr. The volumetric ratios (expressed in mL) between milk and microorganisms (C,K,B) can be seen in Table 1. They used increasing proportions of K yeast (S2-S3-S4, S6-S7-S8) and respectively high B (S1 and S5), in order to correlate the quality of the product with its sensorial properties. All samples were made in triplicate.

4. Physical-chemical and microbiological analysis

The physical and chemical parameters of the FDP (obtained in both experiments) were

monitored during shelf-life (1-21 days), stored at 0-4°C. The pH was measured using an electronic pH-meter (Hanna Instruments Inc.). The total acidity was determined by samples titration with N/10 NaOH solution and was expressed in Thőrner degrees, (1°T = mL of NaOH 0.1 N used to neutralize 100 mL sample). The degree of syneresis was expressed as % (w/w) of free whey and was measured from the FDP weight before (M_1) and after (M_2) the whey release through filtration under vacuum for 10 min [20], using the formula:

$$\% \text{ free whey} = [(M_1-M_2)/M_1]*100$$

In order to monitor the density and viability of the lactic bacteria and yeasts, a tryptone-water (Difco) mixture (1 g/L) was used to prepare the dilutions for the microbiological analysis. Lactic acid bacteria were counted in M17 medium (Difco), a selective medium for lactococci (Terzaghi & Sandine, 1975), at pH 7.2 ± 0.2, after incubation under anaerobic conditions, 5% (v/v) CO_2, at 30 °C for 18–24 hr. Yeasts were enumerated in WLN (Wallerstein Laboratoires Nutrient medium) at pH 5.5 ±0.3 in a selective medium for *brewer yeast,* after incubation at 25°C for 48hr. The density of microorganisms was determined by direct counting in the Thoma Chamber, while the viability of the brewer's yeast was determined by differential coloration**.** Three replications of all measurements were carried out for each sample.

Table 1: Different compositions used by inoculation with *Mesophilic culture (C), Kefir yeast (K) and/or brewer yeast (B)* and fermented in aerobic (S_1-S_4) or anaerobic conditions (S_5-S_8).

Sample code	***Aerobic incubation***				***Anaerobic incubation***			
	S 1	**S 2**	**S 3**	**S 4**	**S 5**	**S 6**	**S 7**	**S 8**
Mesophilic culture (C) mL	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Kefir yeast (K), mL	_	0.5	1	2	_	0.5	1	2
Brewer Yeast (B),mL	1	0.5	0.25	_	1	0.5	0.25	_
Ratios Milk:C:K:B	2000: 2:0:4	2000: 2:2:2	2000: 2:4:1	2000: 2:8:0	2000: 2:0:4	2000: 2:2:2	2000: 2:4:1	2000: 2:8:0

5. Statistical methods

All data were processed statistically, in order to identify the significant differences between samples and to find the optimised variants of fermentation protocols. The variance analysis (ANOVA, using the software Graph Pad Prism 5.00) was applied, considering a confidence interval of 95% ($p<0.05$) as threshold of significance.

Results and Discussion

1. First step experiment

Tables 2a, 2b and 2c include the mean values and standard deviations ($x \pm s$) obtained for pH, total acidity and % (w/w) of free whey in 1st step experiment conditions, when different functional dairy product prototypes were obtained (C+B, C+K or C+K+B) and stored 1,7, 14, and 21 days. Data represent the mean values from three independent experiments and their standard deviations**.** No significant differences were noticed between the different FDP prototypes. For all samples, the pH increased slightly during this period. Meanwhile, the total acidity decreased gradually during this period and the percentage of the whey increased,

demonstrating an increased syneresis (maturation of the coagulum). According to these parameters, non significant differences were noticed between C+B, C+K and C+B+K samples (p>0.05).

The microbiological analysis (Table 3a) of *Lactococcus* revealed that in experimental variants containing brewer yeast (C+B and C+K+B), its proliferation was inhibited, after the first 7 days of incubation, as we obtained during preliminary studies [21]. Therefore, *Lactococcus* count kept constant at a density of 10^3 cfu/mL for the rest of the shelf-life. In the control sample (C), the final density was higher than that of the experimental variants which contained brewer's yeast (10^4 *vs* 10^3 cfu/mL). By Bonferroni (two way ANOVA) test analysis we found a very significant decrease (**) p=0.0025 (p<0.01) between the density of *Lactococcus* between day 1, days 14, and 21 of shelf-life periods, respectively.

Table 2a. Mean values and standard deviations (x□ ± s) obtained for pH, in the 1st step experiment conditions, when different functional dairy product prototypes were obtained

Exp.1	pH				Significance p
	1 day	7days	14days	21days	
C+B	4.35±0.01	4.49± 0.01	4.61± 0.02	4.69±0.01	ns
C+K	4.33±0.01	4.45±0.02	4.66±0.02	4.71±0.015	ns
C+K+B	4.36±0.01	4.52±0.01	4.66±0.02	4.7±0.015	ns

Table 2b. Mean values and standard deviations (x□ ± s) obtained for total acidity, in the 1st step experiment conditions, when different functional dairy product prototypes were obtained

Exp.1	Total acidity, °T				Significance p
	1 day	7 days	14days	21days	
C+B	115±1.01	111± 1.2	105±1.3	103±1.52	ns
C+K	116±1.02	110±1.03	104±1.1	102±1.4	ns
C+K+B	115±1.1	109±1.05	104±1.2	102±1.02	ns

Table 2c. Mean values and standard deviations (x□ ± s) obtained for % free whey, in the 1st step experiment conditions, when different functional dairy product prototypes were obtained

Exp.1	Syneresis, %free whey				Significance p
	1 day	7 days	14days	21days	
C+B	0.5±0.15	1.2± 0.07	1.4± 0.05	1.8±0.15	ns
C+K	0.5±0.1	1.1±0.05	1.3±0.15	1.9±0.02	ns
C+K+B	0.5±0.07	1.1±0.1	1.4±0.15	1.9±0.04	ns

ns- non significant, p>0.05, Data represent the means ± SD of three independent experiments

When we counted the brewer yeast density during shelf-life (Table 3b), a significant increase (100 times) was noticed, reaching values of 10^6 cfu/ml after 21 shelf-life days, for both C+B and C+K+B samples. By Bonferroni (two way ANOVA) test analysis we found significant variations (p<0.01) for the density of brewer yeasts between day 1, days 7 and 14, followed by a stabilization until day 21. This experiment demonstrated that *Lactococcus* culture co-cultivated with brewer yeast, at ratios 1:2 (in C+B) and 1:1 (C+K+B) was inhibited by brewer yeast significantly, while kefir yeast had a moderate inhibitory effect. These results determined us to modify, in the 2nd experiment these ratios, in favor of *Lactococcus,* in order to avoid the too intense activity of brewer yeast against lactobacillus and kefir development.

Table 3a. Variation of *Lactococcus* bacteria density (cfu/mL) during shelf-life (1-21 days) in the conditions of the 1st step experiment.

Experiment 1	Period								
	1 day		7 days		14 days		21 days		
	x□	s	x□	s	x□	s	x□	s	p
C+B (ratio 1:2)	2×10^6	1.1	7×10^3	1.6	8×10^3	1.3	1.2×10^3	0.5	**
C+K (ratio 1:2)	3×10^6	1.2	5×10^5	1.2	2×10^5	0.7	8×10^4	0.9	**
C+K+B (ratio 1:1:1)	2.5×10^6	0.9	3.2×10^3	1.1	5.8×10^3	0.9	3.8×10^3	0.4	**

** *Very significant differences,* $p<0.01$.

Table 3b. Variation of brewer yeast's density (cfu/mL) during shelf-life (1-21 days) in the conditions of the 1st step experiment.

Experiment 1	Period								p
	1 day		7 days		14 days		21 days		
	x□	s	x□	s	x□	s	x□	s	
C+B (ratio 1:2)	2.5×10^4	0.8	1.5×10^5	0.5	2.8×10^6	0.5	3×10^6	1.1	**
C+K+B (ratio 1:1:1)	3×10^4	1.1	2×10^5	0.9	3×10^6	0.9	4×10^6	1.5	**

** *Very significant differences,* $p<0.01$

2. Second step experiment

Figures 1 represents the mean values and standard deviations obtained for pH, total acidity and % (w/w) free whey in the 2nd step experiment conditions, when different functional dairy product prototypes were obtained by aerobic (S1-S4) or anaerobic incubation (S5-S8) and stored 1, 7, 14, and 21 days. The volumetric ratios Milk: C: K: B were different, such as 2000:2:0:4 (S1 and S5), 2000:2:2:2 (S2 and S6), 2000:2:4:1 (S3 and S7), 2000:2:8:0 (S4 and S8). As can be seen, according to these ratios, the proportion of brewer yeast (B) decreased gradually, while the kefir yeast (K) increased. No significant differences were noticed between the pH, acidity and syneresis values, between the aerobic *vs.* anaerobic conditions. Data represent the means ± SD of three independent experiments.

Similarly, there were non significant differences (ns) between the values of all parameters for the four samples (S1-S4) which were incubated aerobically or anaerobically (S5-S8). The product can thus be processed by aerobic incubation, and the manufacturing process can be extended to the pilot scale, where incubation takes place in aerobic conditions. In the case of FDP, we speculate that the pH remains constant and the product fails to become more acidic due to the presence of yeasts. These pH values of FDP were higher than those reported by other authors [21] who reported the pH of kefir and *Lactococcus* bacteria, and can be explained by the impact of brewer yeast on buffering the culture media. The dynamic of *Lactococcus* bacteria proliferation (cfu/mL) was measured during shelf-life (1-21 days) of samples in aerobic (S1-S4) and anaerobic (S5-S8) conditions (Table 4a and 4b). We aimed to obtain an optimum ratio between the viable *Lactoccocus* and the viable *Saccharomyces cerevisiae* yeasts for the duration of the shelf-life by varying the amounts of microbial suspensions contained in the inoculated strain.

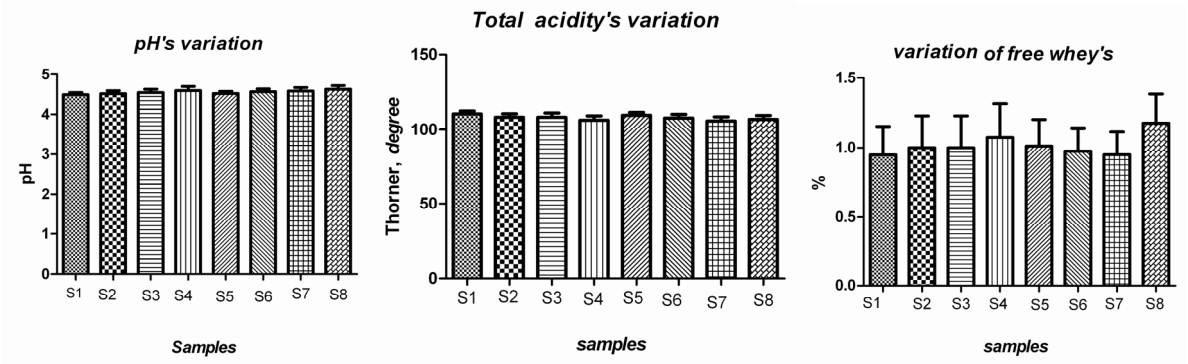

Figure 1. The variations of pH, total acidity and % (w/w) free whey in the 2nd step experiment conditions, where aerobic (S1-S4) and anaerobic (S5-S8) incubation conditions were considered.

Table 4a. Dynamics of *Lactococcus* bacteria proliferation (cfu/ml)during shelf-life (1-21 days) of samples. Aerobic (S1-S4) and anaerobic (S5-S8) conditions were considered (2nd step experiment).

2nd Experiment	Period							
	1 day		7 days		14 days		21 days	
	x□	s	x□	s	x□	s	x□	s
S 1 (C+B)	$3x10^5$	0.9	$1.2x10^5$	0.7	$8x10^4$	1.2	$4.5x10^4$	0.9
S 2 (C+K+B)	$3x10^6$	0.5	$2x10^5$	0.9	$2.8x10^4$	0.9	$8x10^4$	1.1
S 3 (C+K+B)	$1.8x10^6$	0.4	$3x10^5$	1.2	$5x10^5$	1.1	$2.3x10^5$	0.5
S 4 (C+K)	$2.5x10^6$	1.1	$5.5x10^5$	1.2	$3.2x10^5$	1.5	$1.1x10^5$	0.3
S 5 (C+B)	$6x10^5$	1.2	$1.6x10^5$	0.8	$8.4x10^4$	2.1	$3.2x10^4$	0.9
S 6 (C+K+B)	$3x10^6$	0.8	$3x10^5$	1.1	$1.9x10^5$	0.5	$1.4x10^5$	0.5
S 7 (C+K+B)	$4.3x10^6$	1.1	$2.8x10^5$	0.8	$2.1x10^5$	0.7	$1.5x10^5$	0.6
S 8 (C+K)	$2.8x10^6$	0.8	$1.6x10^5$	0.9	$1.8x10^5$	0.3	$2x10^5$	0.8

Data represent the means ± SD of three independent experiments

Table 4b. Dynamics of Brewer yeast proliferation (cfu/ml) during shelf-life (1-21 days) of B samples. Aerobic (S1-S4) and anaerobic (S5-S8) conditions were considered in the 2nd step experiment.

2nd Experiment	Period							
	1 day		7 days		14 days		21 days	
	x□	s	x□	s	x□	s	x□	s
S 1 (C+B)	$4.5x10^4$	1.2	$1.2x10^5$	0.9	$8x10^5$	0.9	$2x10^6$	0.6
S 2 (C+K+B)	$5.8x10^4$	1.1	$1.2x10^5$	0.8	$7.8x10^5$	1.3	$1x10^6$	0.8
S 3 (C+K+B)	$5x10^4$	1.5	$8x10^4$	1.4	$4.5x10^5$	0.7	$9.5x10^5$	1.8
S 4 (C+K)	Absent							
S 5 (C+B)	$5.2x10^4$	0.9	$1.2x10^5$	0.5	$7.8x10^5$	1.5	$1.9x10^6$	1.9
S 6 (C+K+B)	$3.4x10^4$	0.9	$2.6x10^5$	1.1	$4.2x10^5$	0.8	$6x10^6$	0.7
S 7 (C+K+B)	$3.8x10^4$	1.1	$9.7x10^4$	1.9	$3x10^5$	0.5	$4.5x10^5$	0.9
S 8 (C+K)	Absent							

Data represent the means ± SD of three independent experiments

In the second experiment, we achieved a higher density of *Lactococcus* bacteria in

variants S3 and S4 ($2.3x10^5$ and $1.1x10^5$ cfu/mL, respectively) (Table 4a), while brewer yeast (Table 4b) had $9.5x10^5$ cfu/mL for S3 (aerobic conditions). In anaerobic conditions, the similar compositions (S7 and S8) had densities of 1.5×10^5 and 2×10^5 cfu/mL for *Lactococcus* bacteria, and 4.5×10^5 cfu/mL for brewer yeast (S7).

The analysis of the results for both groups of microorganisms showed that the best ratio between the number of viable *Lactococcus* and viable brewer's yeast was found in sample no. 3 (S3), in aerobic conditions. The data obtained for S3 was analyzed statistically by Bonferroni multiple comparison test, showing no significant differences between the density of *Lactococcus* ($p=0.9523$, $p>0.05$) and of yeasts ($p=0.8280$, $p>0.05$) during the shelf life. We therefore consider S3 to be a best combination of microorganisms (C+K+B) which contains concomitantly high densities of *Lactococcus* and brewer yeast.

In this sample S3, the density of *Lactococcus* bacteria fell during the first seven days of shelf life from a value of 10^6 cfu/mL to a value of 10^5 cfu/mL, and then remained constant at that level until the expiry date. The number of brewer yeasts remained 10^4 cfu/mL for the first seven days of shelf life and then jumped to 10^5 cfu/mL on day 14 and remained constant at that level until the expiry date.

In agreement with the findings reported by other researchers [19, 22] we reported a decrease of the *Lactococcus* density during the shelf-life. *Lactococcus* levels were lower than the levels found by other authors because of the presence of brewer yeasts in the inoculators. The yeasts density was similar to the level recorded by Irigoyen et al. [19], in spite of the fact that, in our study a selective medium wasn't used. We haven't found studies which referred exactly to the *Saccharomyces* yeast level in kefir products yet.

Conclusion

By two steps experiments we elaborated different functional dairy products (FDP) based on mixtures of *Lactococcus* bacteria, kefir and brewer yeasts, in different ratios.

In the first experiment, we demonstrated that *Lactococcus* culture co-cultivated with brewer yeast, at ratios 1:2 and 1:1 was inhibited significantly by brewer yeast, while kefir yeast had a moderate inhibitory effect on *Lactococcus*. In spite of the nutritional added-value brought by brewer yeast, the sensorial properties of these products were inferior (strong yeast flavor and bitter taste) [23].

According to this result, we modified the ratios between microorganisms and in the 2nd experiment we decreased gradually the proportion of brewer yeast, in favor of *Lactococcus.* We compared the density of *Lactococcus* and brewer yeast during the shelf life (up to 21 days) in anaerobic or aerobic conditions and found out the best FDP containing *Lactoccocus,* kefir yeast and brewer's yeast (variant S3), incubated especially in aerobic conditions. The physical-chemical parameters to qualify these products were pH, total acidity and syneresis factor, their modifications during incubation and shelf life did not vary significantly, but the presence of the brewer yeast brought added-value to the final product and determined the less acidic pHs than for typical *Lactococcus* yogurt.

The concomitant presence of all three microorganisms confer to this product best functional properties, expressed by stable quality parameters (pH, acidity, syneresis) during shelf- life, good and balanced density of each microorganism (10^5 cfu/mL).

Further measurements will focus on the investigation of probiotic characteristics showed by this new product.

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