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Quality attributes of fermented whey-based beverage enriched with milk and probiotic strain

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Abstract

Beverages produced by fermentation of whey have significantly lower viscosity, milder flavour and less viability of probiotic microorganisms compared to those obtained by fermentation of milk. Therefore, it is necessary to choose an adequate combination of cultures and supplements that can enhance these characteristics of the final product. The main objectives of the paper were to study the influence of milk and additional probiotic strain *Lactobacillus rhamnosus* ATCC 7469 on quality attributes of fermented whey-based beverage containing commercial ABY-6 starter culture. To formulate beverage, that meet required criteria for probiotics, supplementation of whey with 30% milk and its fermentation by ABY-6 co-cultured with *L. rhamnosus* is advisable. The obtained whey-based beverage has desirable texture and sensory quality attributes similar to traditional products and meets consumers' demands. The beverage contains 7.49 log (CFU mL^{-1}) probiotic bacteria, expresses antioxidant activity of 45.1%, satisfactory sensory characteristics and has a shelf life of at least 20 days.

Key words: *Whey; ABY-6; Lactobacillus rhamnosus; viscosity; syneresis; antioxidants*

1. Introduction

Compared to various types of yoghurt and other fermented dairy products, whey is one of the least frequently consumed dairy products around the world. Its poor sensory attributes have a large negative effect on consumer acceptability. Consequently, whey is commonly used as a supplement, in the form of whey powder or whey protein concentrate.[1] Expensive processing procedures lead to the fact that Serbia exploits only 12% of whey, in contrast to the developed countries that exploit 95% of this by-product.[2] The fermentation of whey by commercial starter cultures, designed for yoghurt production, could be an alternative to increase sensory quality of whey. On the other hand, well known health benefits[3] of raw whey can be significantly improved by its fermentation. There is large number of scientific reports that confirms evidence of health benefits of microorganisms including its production of antioxidants.[4] Thus, application of starters that produce exopolysaccharides, antioxidants or posses probiotic properties, can significantly improves whey quality due to their positive influence on immune system as well as on gastrointestinal health. These benefits could be the key point for increase whey exploitation by its integration in human nutrition.

Due to the low level of total solid content (approximately 6%, by weight), relatively high lactose-glucose ratio and high acidity, consumers perceive whey-based beverages as watery, sweet-sour liquid with the poor mouthfeel.[5] Likewise, beverages produced by fermentation of whey have significantly lower viscosity, milder flavour and less viability of probiotic microorganisms compared to those obtained by fermentation of milk. Therefore, it is necessary to choose an adequate culture or supplements that can enhance these characteristics of the final product. One of the possible ways could be the use of

dairy starters in combination with high exopolysaccharide-producing strains or use of hydrocolloids.[5] On the other hand, in order to avoid hydrocolloids and preserve the completely natural composition of beverage, milk addition could be a good alternative. The aggregation of casein and whey proteins, during fermentation, leads to the formation of the gel that constructs the beverage structure, protects probiotic strains and improves overall quality of product.[6]

Cultures that are most frequently used as dairy starters are AB (*L. acidophilus* and *Bifidobacterium* spp.), ABC (*L. acidophilus*, *Bifidobacterium* spp. and *L. casei*), ABT (*L. acidophilus*, *Bifidobacterium* spp. and *S. thermophilus*) and ABY (*L. acidophilus*, *Bifidobacterium* spp., *S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus*).[7-10] Combination of these commercial cultures with strains marked as a good exopolysaccharide producers could improve quality of beverages in several ways. The presence of exopolysaccharides leads to the improvement of textural attributes (such as firmness and mouthfeel) of many food products. Many of them can form gels that will constitute food structure and enhance viscosity of solutions owing to their high molecular weight.[11] Exopolysaccharides may act as prebiotics, selectively metabolised by beneficial bacteria, enhancing their growth, activity and viability in food products as well as in the gastrointestinal tract.[12-15] In the addition to the improvement in probiotic character, the exopolysaccharides also improves aroma of the final product, by stimulating the growth of microorganisms that produces aromatic compounds. This is very important since many studies have shown that the flavour is the first elimination parameter in the selection of food, followed by consideration of the health aspects.[16,17] Probiotic beverages with

disagreeable sensory characteristics are not attractive to costumers even if its consuming has multiple benefits to their health.

There are a small number of literature reports about the use of commercial ABY cultures for fermented whey beverage formulation. There is practically no data on the characterisation of beverage obtained by fermentation of whey using commercial ABY-6 culture. In addition, there is no data concerning the possibility that additional *L. rhamnosus* ATCC 7469 strain can improve general quality of produced whey beverage. Therefore, the aim of our study was to evaluate the influence of milk and additional probiotic strain *L. rhamnosus* ATCC 7469 on the quality of whey-based beverage that contains commercial ABY-6 starter culture.

2. Materials and methods

2.1. Culture and media

Commercial lyophilized dairy starter culture that is known as 'Lactoferm ABY 6' used in this study was supplied by Biochem s.r.l. (Monterotondo, Roma, Italy). Starter culture is mixture of *Streptococcus salivarius* ssp. *thermophilus (*80%), *Lactobacillus acidophilus (*13%), *Bifidobacterium bifidum* (6%), *Lactobacillus delbrueckii* ssp. *bulgaricus* (1%). The culture that consists of 10 g lyophilised starter powder is the one currently used in dairy industry. The culture was maintained according to the manufacturer's instructions at -18 ^{o}C until use (no longer than 20 mounts). For each experiment, 1% (w/*v*) of starter culture was gently dissolved in sterilised skim milk (0.5% fat) and activated 30 min at 42 ^{o}C. Concentration of viable probiotic cells (*L. acidophilus and B. bifidum*) in activated culture was 5.58 ± 0.06 log (CFU mL^{-1}).

The strain *Lactobacillus rhamnosus* ATCC 7469, used in this study, was supplied by American Type Culture Collection (ATCC, Rockville, USA). Stock culture was stored at -18 ^{o}C in 3 mL vials containing De Man Rogosa Sharpe (MRS) broth (Fluka, USA) and 50% (*v/v*) glycerol as a cryoprotective agent. To prepare the laboratory culture, a drop of stock culture was transferred to 3 mL of MRS broth and incubated for 18 h under anaerobic conditions at optimal growth temperature (37 ºC). The working culture was pre-cultured twice in MRS broth prior to experimental use. Concentration of viable cells in activated culture was 7.78 ± 0.165 log (CFU mL^{-1}).

After the activation, desired inoculum level of each culture was added into the fermentation medium, in accordance with the requirements of the experimental procedure (see section 2.2).

Whey remained after cheese production and sterile skim milk with 0.5% fat were obtained from domestic dairy plant Imlek a.d. (Belgrade, Serbia). After collection, the whey was stored at -18 ^{o}C until use (no longer than one week). The chemical composition of whey was: total solids 9.8 ± 0.03 % (w/v); protein 2.6 ± 0.012 % (w/v); fat 1.05 ± 0.08 % (w/v) and lactose 5.6 ± 0.114 % (w/v).

2.2. Experimental procedure

Based on preliminary experiments (data not shown) 30% milk was used for beverage formulation as concentration that appropriate for appreciably sensory quality improvement. Whey (0% milk, *v/v*) and whey-milk mixture (30% milk, *v/v*) were pasteurized at 60 ^{o}C for 60 min, cooled at fermentation temperature (42 ^{o}C) and inoculated with following level of activated cultures. Four different beverages were formulated: AW (0% milk, ABY-6 6%, *v/v*), AM (30% milk, ABY-6 6%, *v/v*), RW (0%

milk, ABY-6 4%, *L. rhamnosus* 2%, *v/v*), RM (30% milk, ABY-6 4%, *L. rhamnosus* 2%, *v/v*).

The flasks containing 300 mL of formulated beverage were prepared for each point of analyses. Prepared samples were incubated at 42 °C in a water bath. During the incubation, samples (2 ml) were taken every 1h for determination of pH value. The fermentations were carried out for 4h until pH = 4.6 ± 0.2 was attained. After 4h fermentations were stopped by quick cooling. The fermented beverages were stored at 4 °C for 28 days. Analysis of the titratable acidity (TA, ˚SH), pH value, viable cell count (log (CFU mL^{-1})), syneresis (%), viscosity (cP), antioxidant activity (%) and overall acceptability was carried out during fermentation and 28 days of storage.

2.3. Chemical analysis

The titratable acidity was determined by the Soxhlet-Henkel method,[18] and the pH value was measured using a pH meter (Inolab, WTW 82362, Wellheim, Germany).

2.4. Microbiological analysis

One milliliter of fermented sample was diluted with 9 mL of sodium chloride (0.85%, w/v), and mixed uniformly. Subsequent serial dilutions were prepared and viable cell count was determined using pour plate technique. MRS-maltose (MRSM) agar and anaerobic incubation at 37 ˚C for 48 h were used for the enumeration of viable cell count of probiotic bacteria (*L. acidophilus* and *B. bifidum* in AW and AM beverages; *Lb. acidophilus*, *B. bifidum* and *L. rhamnosus* in RW and RM beverages).[19]

2.5. Texture analysis

2.5.1. Viscosity

160 The apparent viscosities were determined at 8˚C according to modified method.[20] A
161 Brookfield DV II+ Pro viscometer (Brookfield Engineering Lab Inc, Stoughton, MA)
162 was used. A spindle N˚61 was set to 10 rpm. The viscosity measurements were
163 continuous over 30 s required to collect 70 data points. Data points were averaged per
164 sample per replication. The apparent viscosity was determined on three cups of sample
165 per replication. Three replications were conducted and values are expressed in cP.

166 **2.5.2. Syneresis**

167 Syneresis of fermented samples was determined according to the method.[21] The
168 fermented samples (20.0 mL) were centrifuged at 1000 rpm for 10 min at 4 ± 1 °C.
169 Collected supernatant was drained, weighed and the following equation was used for
170 syneresis calculation:

171 $$\text{Syneresis}\,(\%) = \frac{\text{Weight of supernatant (g)}}{\text{Weight of fermented sample (g)}} \times 100\% \quad (1)$$

172 **2.6. Sensory analysis**

173 Sensory analysis of fermented beverage samples was conducted after 1, 7, 14, 21 and 28
174 days of storage according to the modified method.[22] Fifty-five untrained panellists (35
175 being women and 20 men, age between 25 and 55) from the faculty, including teachers,
176 students and staff were randomly selected and invited to participate in the sensory
177 evaluation of fermented whey-based beverages on the basis overall acceptability. The
178 participants were asked to assess the overall acceptability of the four different fermented
179 beverages: AW, AM, RW and RM. Each questionnaire consists of four questions: name,
180 age, sex and overall acceptability for four consumed products.
181 The samples were presented monadically at 4 ± 1 ˚C, in individual plastic cups coded
182 with 3-digit numbers, serving 20 mL samples to each panellist. The participants were

183 given four samples at a time at storage temperature (4 ± 1 °C), a pencil, a questionnaire
184 and a glass of cold water to rinse their mouths between samples. They have been asked to
185 mark an value on the questionnaire scale which best represents how much they liked or
186 disliked each of four samples with respect to overall acceptance, using a 9-point hybrid
187 hedonic scale where 1 = disliked extremely; 5 = neither liked nor disliked and 9 = liked
188 extremely. The sensory analysis was consisted of 275 questionnaires distributed into 5
189 sessions (5 times of storage). Prior to serving all samples were subjected to counts of
190 yeasts, molds and coliforms to evaluate the hygienic and sanitary conditions of the
191 products.

192 **2.7. Antioxidant activity**

193 Antioxidant activity of fermented whey-based beverages was determined by its ability to
194 scavenge DPPH (1,1-diphenyl-2-picrylhydrazyl) radical, which was measured according
195 to the modified method.[23] A stock solution of 0.1 mM DPPH (Sigma-Aldrich, Australia)
196 was prepared by dissolving in methanol. After 4h fermentation samples were macerated
197 with methanol and centrifuged at 8000 rpm for 20 min at 4 ˚C. Methanol (1.5 mL) and
198 DPPH (1.0 mL) were added to the supernatant (0.5 mL). Control sample was prepared by
199 mixing methanol (1.5 mL) and DPPH (1.5 mL), while methanol was used as blank
200 sample. Mixtures were allowed to stand 30 min in dark, at room temperature. The
201 antioxidant activity was analyzed by reading the absorbance at 517 nm. Scavenging
202 activity was calculated using the following equation:

$$\text{DPPH scavenging activity}\,(\%) = \left[(\text{Ac} - \text{Aa})/\text{Ac}\right] \times 100 \quad (2)$$

203

204 Where Aa and Ac represent absorbance of sample and control, respectively.

205 **2.8. Statistical analysis**

The experiments were performed in triplicate. All values are expressed as mean ± standard deviation. Mean values were analysed using two-way ANOVA. The Tukey post hoc test was performed for means comparison (Origin Pro 8 (1991-2007), Origin Lab Co., Northampton, USA). Differences were considered significant at $P < 0.05$.

3. Results and discussion

3.1. Chemical analysis

Fermentation of whey by commercial cultures designed for yoghurt production could be an interesting way of including whey in human consumption. Changes in pH and titratable acidity (˚SH) during fermentation and storage period are specific for every product and depend on the microorganisms used for formulation as well as of the substrate composition.

A gradual decrease of pH was observed in all samples during 4h fermentation as well as during 28 days of storage (Fig. 1). Values of pH were ranged from 4.34 to 4.51 in all samples after fermentation. Statistically significant difference ($P < 0.05$) in pH was recorded in samples AW (4.51) and RW (4.37). Observed difference, means that *L. rhamnosus* leads to significant drop of pH in sample formulated without milk. In samples supplemented with 30% milk (AM and RM) applied culture does not have statistically significant ($P > 0.05$) influence on pH. Comparing samples fermented by ABY-6 (AW and AM) and samples fermented by ABY-6 co-cultured with *L. rhamnosus* (RW and RM) regarding the milk content, it was observed that milk supplementation does not significantly ($P > 0.05$) affects pH value after fermentation.

Compared to the pH values obtained after fermentation, pH values at the end of storage period were considerably lower, ranged from 3.82 to 3.89 in all samples (Fig.1). A

possible explanation of this behaviour could be that the used strains are capable to save their productivity during storage. It can be also assumed that buffering capacity of milk[24] is enough to suppress significant pH decreasing after fermentation but not after 28 days of storage. *L. rhamnosus* has statistically significant effect ($P < 0.05$) on pH in sample RW, as well as in sample RM.

On the other hand, milk supplementation does not significantly affect the pH after 28 days of storage. The obtained results are different to those reported by others[25,26] who reported pH of about 4.20 after 35 days of storage, when the initial pH value was about 4.60. Therefore, we could say that *L. rhamnosus*, as a strain with high lactic acid productivity,[27] significantly affects pH of whey-based beverage during 28 days of storage regardless of milk addition.

Based on the results, pH decreases faster in samples with *L. rhamnosus* during the fermentation as well as during storage period. As reported in the literature,[28] this strain is characterised by excellent proteolytic activity with high amount of free amino acids (FAA) produced during process of cheese production. Due to this specific ability, it provides amino acids to the strains present in ABY-6 culture and probably increases their metabolic activity. In addition, the increased strains activity leads to the production the higher amount of lactic acid and faster decrease of pH in these samples during fermentation as well as during storage period.

Titratable acidity of samples ranged from 16.1 to 24.2 ˚SH after fermentation, and from 23.2 to 35.4 ˚SH after 28 days of storage. As shown in Fig 2, the highest titratable acidities of 24.2 ˚SH and 35.4 ˚SH were observed in sample RM after fermentation and after 28 days of storage, respectively. Based on the observed results we could observe

that the presence of milk and *L. rhamnosus* increases titratable acidity of whey-based beverages. It is interesting to note that milk significantly ($P < 0.05$) affects titratable acidity of samples AM and RM, in contrast to the non-significant ($P > 0.05$) effect of milk on pH of above samples. The possible explanation could be the fact that the productivity of both cultures in the presence of milk proteins was enhanced, but produced lactic acid cannot be recorded by measuring pH.

Lactic acid has significant impact on the flavour of fermented milk products. A beverage is considered to have a good quality if it has a titratable acidity of approximately 44 ˚SH. In our study, due to very short fermentation time (4h) and whey as poor substrate, strains present in ABY-6 culture are not able to produce satisfactory amount of lactic acid. The addition of highly productive strain and milk enhances amount of lactic acid present in produced beverage (Fig. 2). However, titratable acidities of the fermented whey-based beverages in this study were below value 53.0 ˚SH at which unpleasant acid taste could be detected.[29,30]

3.2. Microbiological analysis

The preferred option for whey fermentation is the use of culture containing probiotic strains. Probiotics in form of fermented dairy products are metabolically active products, which pass through some modifications during their shelf life, such as loss of culture viability and overall sensory quality. Whey does not contain an abundance of nutrients, but its enrichment can create the conditions present in the gastrointestinal tract, which is the natural habitat of probiotic bacteria and thus lead to improvements of their growth and viability. The changes in viable cell count of probiotic bacteria in beverages

formulated with whey and whey-milk mixture, fermented by ABY-6 and ABY-6 co-cultured with *L. rhamnosus* for 4h and stored for 28 days are shown in Fig. 3.

As indicated in Fig. 3 viable cell count of probiotic bacteria (*L. acidophilus* and *B. bifidum*) ranged from 4.88 to 5.19 log (CFU mL^{-1}) in samples AW and AM, respectively, after 4 h of fermentation. It suggests that milk have significant ($P < 0.05$) influence on growth of ABY-6 culture. Regardless of the positive effect of milk, both samples fermented by ABY-6 starter culture (AW and AM) did not meet the requirement (>6.0 log (CFU mL^{-1}) to be considered as probiotics.[31] Same statistically significant ($P < 0.05$) positive influence of milk observed in samples fermented by ABY-6 co-cultured with *L. rhamnosus* where the viable cell count of probiotic bacteria was ranged from 6.69 log (CFU mL^{-1}) in sample RW to 7.51 log (CFU mL^{-1}) in sample RM (Fig. 3). That confirms that milk has significant influence on the growth of these probiotic strains. According to earlier reports,[32] the remarkable effect of milk on the growth of microorganisms was recorded and it is caused mainly by presence of milk protein during the fermentation of whey-milk base. We could say that milk proteins protect probiotic strains, enhance their growth and probably viability.

Co-culturing of ABY-6 with probiotic strain *L. rhamnosus* significantly ($P < 0.05$) increases viable cell count of probiotic bacteria regardless of the presence of milk. The reached count of viable probiotic bacteria was for about 1.5-2.3 log units higher in samples RW and RM than in samples AW and AM that contained only *L. acidophilus* and *B. bifidum* as probiotics (Fig. 3). Based on these results, addition of milk and highly productive probiotic strain *L. rhamnosus*, with excellent growth capability, improves the probiotic character of produced whey-based beverage. Maximal viable cell count of

297 probiotic bacteria (7.51 log (CFU mL^{-1})) was reached in sample RM (30% milk, ABY-6
298 4%, *L. rhamnosus* 2%, *v/v*) after 4 h of fermentation.
299 The rate of population reduction was significantly ($P < 0.05$) slower in samples
300 supplemented with milk during 28 days of storage, regardless of culture. The observed
301 results suggest that milk slows the probiotic viable cell count reduction.
302 As shown in Fig. 3, samples RW and RM have significantly ($P < 0.05$) higher probiotic
303 viable cell count than samples fermented AW and AM during the whole storage period.
304 Sample RM had significantly ($P < 0.05$) higher probiotic viable cell count (6.30 log (CFU
305 mL^{-1})) than sample RW (6.10 log (CFU mL^{-1})) at the end of storage period. Both samples
306 RW and RM meet the requirement (> 6.0 log (CFU mL^{-1}) to be considered as probiotics.
307 The obtained results are consistent to those reported in our previous research,[33] which
308 suggests that synergistic effect of proteins and polysaccharides can positively affect
309 growth and viability of probiotic bacteria. Sample RM supplemented with 30% milk and
310 fermented by ABY-6 co-cultured with *L. rhamnosus*, achieved the maximal probiotic cell
311 count of 7.51 log (CFU mL^{-1}) after 4 h fermentation and held that count of viable
312 probiotic bacteria during 28 days of storage.

313 **3.3. Texture analysis**

314 The knowledge of rheological behaviour of whey-based beverages is a valuable tool in
315 design of processing technologies and predicting the product stability during storage. The
316 basic parameter, obtained during rheological study of liquid foods, is viscosity, used to
317 characterize the fluid texture.[34,35,36] The changes in syneresis and viscosity of the
318 beverages formulated with whey and whey-milk mixture, fermented by ABY-6 and

ABY-6 co-cultured with *L. rhamnosus* and stored for 28 days are shown in Fig. 4 and Table 1.

As indicated in Fig. 4, the viscosity of samples fermented by ABY-6 increases and reaches values 1.6662 cP (AW) and 2.8350 cP (AM) during the first two weeks of storage. After 14 days, the viscosity of sample AW starts to declines reaching the value 1.5518 cP after 28 days of storage. On the other hand, in the sample formulated with whey-milk mixture (AM) viscosity increases during whole storage period reaching the value of 2.9529 cP after 28 days of storage. We can observe, that the viscosity of fermented whey-based beverages is significantly ($P < 0.05$) related to the presence of milk in formulation. Strong influence of milk on texture of whey-based beverage is in accordance with the results reported by others[5] who found that casein content had high influence on the texture of fermented milk products. Produced lactic acid reduces the pH of milk to the isoelectric point (pH = 4.6) of casein and leads to the formation of protein gel. This observation is also supported by previous studies[37,38] that pointed out that an additional amount of milk can cause a stronger texture due to stronger network of protein gel.

In the samples fermented by ABY-6 co-cultured with *L. rhamnosus* viscosity increases in both samples (RW and RM) reaching values 1.6281 cP and 2.7732 cP, respectively, after two weeks of storage. In these samples viscosity values of 1.4852 cP for RW and 2.3755 cP for RM were observed at the end of storage period.

Different behaviour of samples fermented by ABY-6 and ABY-6 co-cultured with *L. rhamnosus* (AM and RM) could be explained by presence of highly productive strain *L. rhamnosus* both in lactic acid and exopolysaccharide as well. Lower pH values in sample,

342 that contains *L. rhamnosus,* contribute to lowering stability of protein gel formed during
343 fermentation. It is also interesting to note, that after 14 days of storage presence of *L.*
344 *rhamnosus* leads to a considerable increase in the content of lactic acid. Protein gels are
345 pH-sensitive and presence of lactic acid affects a polypeptide chain interaction, which
346 leads to the uptaking of water inside the gel. Uptaking of water inside the gel weakens its
347 structure and leads to the decrease in viscosity of these samples.[6]
348 Changes in syneresis during the storage were observed in all samples (Table 1). It
349 appeared that syneresis increases during the 14 days of storage for samples inoculated
350 with ABY-6 co-cultured with *L. rhamnosus* (RW and RM). After 14 days of storage
351 syneresis of RW and RM samples were 84.3% and 70.0%, respectively. Further,
352 syneresis starts to decline and values 80.2% for sample RW and 65.9% for sample RM
353 were reached after 28 days.
354 In the samples inoculated with ABY-6 increase in syneresis was observed during 14 days
355 of storage. After 14th day, syneresis in sample AW decreases, in contrast to the sample
356 AM where increase in syneresis was observed to the end of storage period. Syneresis of
357 AW and AM samples was 85.0% and 78.3%, respectively, after 28 days. During the
358 whole storage period, syneresis values of samples were significantly different ($P < 0.05$)
359 in favour of the sample supplemented with 30% milk. The observed results were
360 correlated with the above results obtained for viscosity. An increase in viscosity
361 correlates to the stronger protein gel that loses the ability to hold the whey. Whey drains
362 from the protein matrix and appears on the surface of fermented milk.[39]
363 Comparing beverages formulated with whey-milk mixture it was observed that sample
364 RM had significantly ($P < 0.05$) lower syneresis than sample AM. This result is in

accordance to those reported by others[40,41] who observed lower level of syneresis in yoghurt gels made by EPS producing starters compared to those made by EPS non-producing starters.

We could say, that addition of probiotic *L. rhamnosus* strain, beside the slight reduction of viscosity, leads to the decrease of syneresis as the first eliminating parameter for beverage selection by consumers. Exopolysaccharide produced by *L. rhamnosus*[42]can form weak polysaccharide-protein interactions instead of more stable protein-protein ones.[43,38] This contributes to the formation of weak gel structure[44] that easily hydrates and thus reduces the syneresis of these beverages compared to the beverages fermented by ABY-6.

3.4. Sensory analysis

From 55 randomly panellists taking part in the overall acceptability test, 36.3% were male and 63.6% were female. Approximately 67.5% were between 25-45 years old. The analysis of whey-based beverages was conducted after 1, 7, 14, 21 and 28 days of refrigerated storage at 4 ˚C. The changes in acceptability values of fermented whey-based beverages are presented in Table 2.

The results indicated that supplementation of whey by 30% milk significantly ($P < 0.05$) affects sensory acceptance of whey-based beverages (Table 2). Samples AM and RM showed high acceptability values during the storage period, with mean values between 7.80 and 8.38. These results suggest that milk addition helps to avoid the poor sensory characteristics perceptible to consumers. Nonetheless, the acceptability values were significantly ($P < 0.05$) higher for sample AM, compare to the sample RM. Co-culturing of ABY-6 with *L. rhamnosus* leads to the decreases of acceptability values of whey-

beverage during whole storage period. Based on our previous research[45] this problem can be solved by fortification of the whey-based beverage with various fruit bases that can enhance its sensory characteristics. Taking into consideration the fact that the count of viable probiotic bacteria is significantly higher in beverage that contain *L. rhamnosus*, we can observe, that the benefit of the strain addition is much greater than its relatively negative impact on the sensory profile of beverage. It is also necessary to emphasise the positive effect of *L. rhamnosus* on reduction of syneresis as a characteristic that largely determines the acceptability of the whey-based beverage by consumers.

3.5. Antioxidant activity analysis

Based on the aforementioned findings milk significantly affects the quality of the beverage. Thus, the beverage formulated with 30% milk was selected as acceptable. In addition, it was necessary to explore the effect of the EPS producing strain on antioxidant activity of fermented beverage formulated by 30% milk. The influence of *L. rhamnosus* on antioxidant activity of whey-based beverage formulated by 30% milk is shown in Figure 5.

The antioxidant activity was significantly higher ($P < 0.05$) in sample RM during fermentation as well as during the whole storage period. Additional exopolysaccharide produced by *L. rhamnosus*, probably stimulate ABY-6 strains to produce metabolic products such as bioactive peptides that contribute to the higher antioxidant activity of beverage. The obtained results are in accordance to the results reported by other researchers,[46] who found that the metabolic products of LAB obtained by utilisation of oligosaccharides contribute to the higher antioxidant activity of yogurt prepared by *S. thermophilus*, *L. delbrueckii* ssp. *bulgaricus* and *L. plantarum*. On the other hand, *L.*

411 *rhamnosus*, as a strain with high proteolytic activity[28] significantly contributes to the
412 production of antioxidant peptides.
413 In addition, it was found that in both samples (AM, RM) antioxidant activity decrease
414 from an initial value 46.0 and 51.2%, respectively, at the end of fermentation, to 38.1 and
415 44.1% by 14 days of storage (Figure 5). After two week of storage, antioxidant activity of
416 samples (AM, RM) starts to increases and reaches value 39.2 and 45.1%, respectively, at
417 21st day of storage. The obtained results are in agreement with earlier studies.[47] Increase
418 in antioxidant activity after 14th day of storage could be explained by release of
419 intracellular microbial enzymes by cell lysis that contribute to the antioxidant activity.
420 This observation is in accordance with literature reports[28] about increased peptidase
421 activities occurred during ripening of cheese produced by *L. rhamnosus* ATCC 7469.
422 Therefore, it could be assumed that proteolysis[48] and lactic acid production[49] as the
423 results of microbial activity during fermentation and refrigerated storage could be
424 additional sources of antioxidant activity.

425 **3.6. Conclusions**

426 The present study is the first report on use of commercial ABY-6 culture in whey
427 fermentation. Probiotic whey beverage was successfully formulated using milk and
428 commercial ABY-6 culture co-cultured with *L. rhamnosus*. Co-culturing of commercial
429 starter culture ABY-6 with probiotic *L. rhamnosus* strain increases viable cell count for
430 about 2.60 log units compared to the beverages obtained in fermentations performed by
431 ABY-6 culture. Milk helps to avoid the poor sensory characteristics perceptible to
432 consumers and in synergy with exopolysaccharides greatly improves the viscosity and
433 syneresis of beverage.

To formulate beverage that meets required criteria for probiotics (viable cell count > 10^6 CFU mL^{-1}) supplementation of whey with 30% milk as well as co-culturing of ABY-6 and *L. rhamnosus* is advisable. The obtained beverage contains 7.49 log (CFU mL^{-1}) probiotic bacteria, expresses antioxidant activity of 45.1%, satisfactory sensory characteristics, has a shelf life of at least 20 days, and it can be introduced in the market.

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463 **References**

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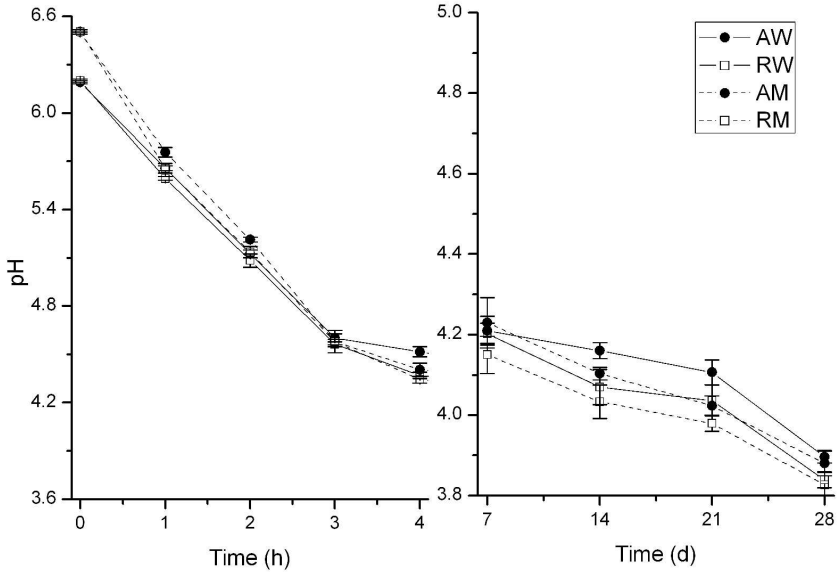
553 **Figures:**



554

555 **Fig. 1** Effect of milk and culture composition on pH value of whey-based beverages
556 during 4 h fermentation and 28 days of storage. AW (0% milk, ABY-6 6%, *v/v*), AM
557 (30% milk, ABY-6 6%, *v/v*), RW (0% milk, ABY-6 4%, *L. rhamnosus* 2%, *v/v*), RM
558 (30% milk, ABY-6 4%, *L. rhamnosus* 2%, *v/v*). Vertical bars represent the standard
559 deviation (n = 3) for each data point.

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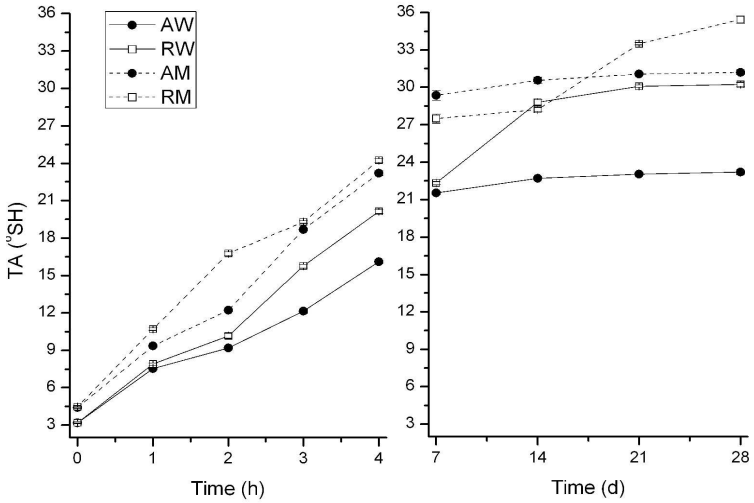

561

562 **Fig. 2** Effect of milk and culture composition on titratable acidity of whey-based
563 beverages during 4 h fermentation and 28 days of storage. AW (0% milk, ABY-6 6%,
564 *v/v*), AM (30% milk, ABY-6 6%, *v/v*), RW (0% milk, ABY-6 4%, *L. rhamnosus* 2%,
565 *v/v*), RM (30% milk, ABY-6 4%, *L. rhamnosus* 2%, *v/v*). Vertical bars represent the
566 standard deviation (n = 3) for each data point.

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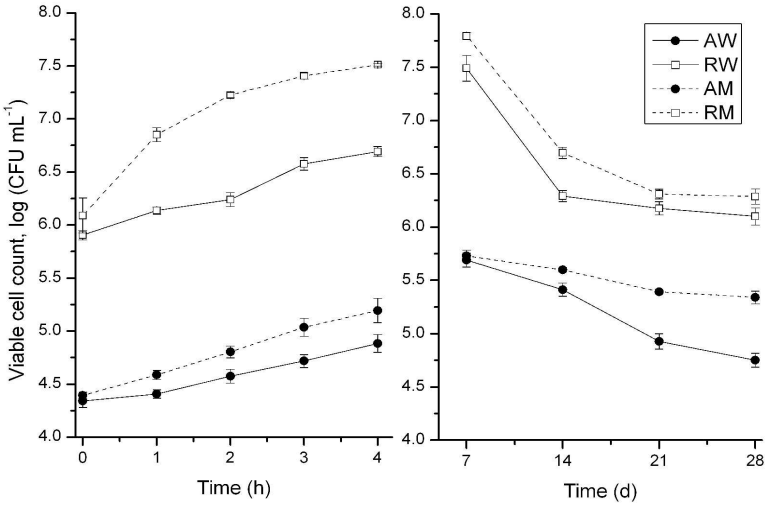

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570 **Fig. 3** Effect of milk and culture composition on viable cell count of probiotic bacteria in
571 whey-based beverages during 4 h fermentation and 28 days of storage. AW (0% milk,
572 ABY-6 6%, *v/v*), AM (30% milk, ABY-6 6%, *v/v*), RW (0% milk, ABY-6 4%, *L.*
573 *rhamnosus* 2%, *v/v*), RM (30% milk, ABY-6 4%, *L. rhamnosus* 2%, *v/v*). Vertical bars
574 represent the standard deviation (n = 3) for each data point.

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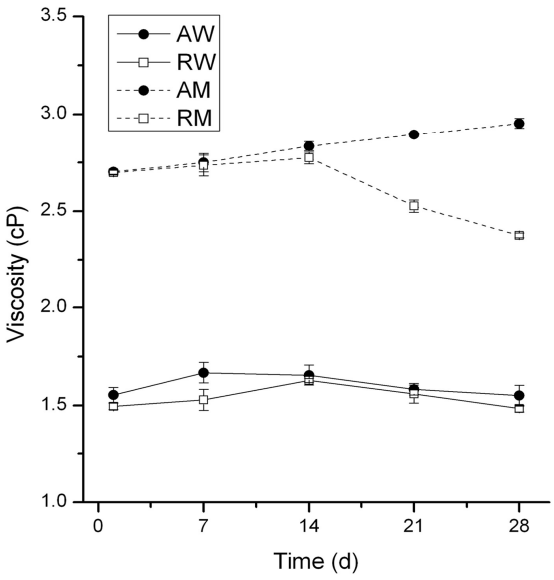

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578 **Fig. 4** Effect of milk and culture composition on viscosity of whey-based beverages
579 during 28 days of storage. AW (0% milk, ABY-6 6%, *v/v*), AM (30% milk, ABY-6 6%,
580 *v/v*), RW (0% milk, ABY-6 4%, *L. rhamnosus* 2%, *v/v*), RM (30% milk, ABY-6 4%, *L.*
581 *rhamnosus* 2%, *v/v*). Vertical bars represent the standard deviation (n = 3) for each data
582 point.

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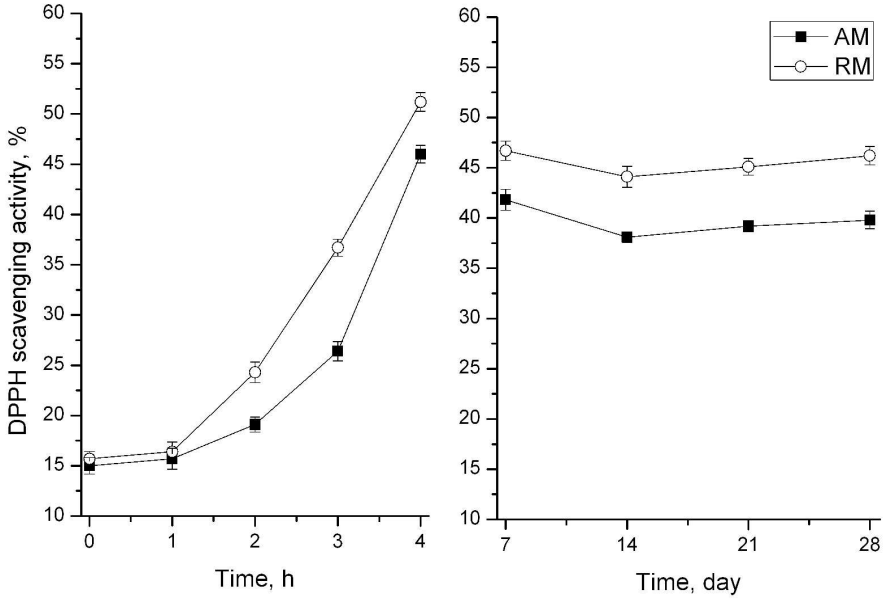

Fig. 5 Effect of culture composition on DPPH scavenging activity of whey-based beverages during 4 h fermentation and 28 days of storage. AM (30% milk, ABY-6 6%, *v/v*), RM (30% milk, ABY-6 4%, *L. rhamnosus* 2%, *v/v*). Vertical bars represent the standard deviation (n = 3) for each data point.

600 **Tables:**

601 **Table 1.** Effect of milk and culture composition on syneresis of whey-based beverages
602 during 28 days of storage. AW (0% milk, ABY-6 6%, *v/v*), AM (30% milk, ABY-6 6%,
603 *v/v*), RW (0% milk, ABY-6 4%, *L. rhamnosus* 2%, *v/v*), RM (30% milk, ABY-6 4%, *L.*
604 *rhamnosus* 2%, *v/v*).

Time (days)	Syneresis (%)[a]			
	Sample			
	AW	**RW**	**AM**	**RM**
1	88.3 ± 0.91	78.5 ± 1.05	67.5 ± 0.70	50.6 ± 0.60
7	90.1 ± 0.61	82.5 ± 1.05	72.3 ± 0.97	64.9 ± 0.85
14	91.2 ± 0.75	84.3 ± 1.15	75.1 ± 0.75	70.0 ± 0.68
21	87.9 ± 0.68	81.2 ± 0.95	76.1 ± 1.10	67.9 ± 0.80
28	85.0 ± 0.83	80.2 ± 0.58	78.3 ± 0.76	65.9 ± 0.86

605 [a] Data are the mean ± standard deviation calculated from three independent experiments (n=3).

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618 **Table 2.** Effect of milk and culture composition on acceptability values of whey-based
619 beverages during 28 days of storage. AW (0% milk, ABY-6 6%, *v/v*), AM (30% milk,
620 ABY-6 6%, *v/v*), RW (0% milk, ABY-6 4%, *L. rhamnosus* 2%, *v/v*), RM (30% milk,
621 ABY-6 4%, *L. rhamnosus* 2%, *v/v*).

Time (days)	Acceptability values [a]			
	Sample			
	AW	**RW**	**AM**	**RM**
1	6.20 ± 1.22	6.92 ± 1.11	8.52 ± 1.01	8.20 ± 0.76
7	6.08 ± 1.08	6.80 ± 1.19	8.52 ± 1.00	8.12 ± 1.01
14	5.96 ± 1.17	6.60 ± 1.15	8.40 ± 1.15	8.00 ± 1.04
21	5.80 ± 1.19	5.52 ± 1.29	8.32 ± 1.14	7.48 ±1.08
28	5.52 ± 1.16	5.16 ± 1.11	8.12 ± 1.13	7.20 ± 1.04
Mean	5.91 ± 0.26	6.20 ± 0.80	8.38 ± 0.17	7.80 ± 0.44

622 [a] Data are the mean ± standard deviation calculated from three independent experiments (n=3).