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SURVIVING OF COMMERCIAL PROBIOTIC STRAIN *Lactobacillus rhamnosus* GG IN SLOVAK COW LUMP CHEESE EXPERIMENTALLY INOCULATED WITH *Listeria innocua*

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ABSTRACT

Cow lump cheese represents a traditional Slovak cheese. It belongs to fresh types of cheeses. The aim of this study was to test surviving of commercial probiotic strain *Lactobacillus rhamnosus* GG in cow lump cheese experimentally infected with *L. innocua*; (listeriae are contaminants) and to check the suitability of GG strain as additive for this product. The counts of GG strain in cow lump cheeses were well balanced during whole experiment. It was found in the counts from 5.48 ± 0.15 to 7.77 ±1.50 log10 cfu/g. Its maximum in cheese was 7.77 ± 1.30 log10 cfu/g on day 7 with stability up to day 14. The identity of GG strain isolated from cheeses was confirmed by PCR. The counts of other lactic acid bacteria were also well balanced during the whole experiment in the experimental cheeses with stability up to day 14. Only in E1/GG cheese, the highest number of LAB was detected (10.60 ±1.26 log10 cfu/g). The count of *L. innocua* LMG 13568 was not influenced. The pH and lactic acid values were not negatively influenced. Visually, the GG cheese provided a good structure (consistency).It can be disputed that shelf-life of the product could be maintained by this way and/or the product itself with GG strain can be consumed as afunctional food or to serve as a probiotic strain carrier.

Keywords: Cheese, probiotic, *Lactobacillus rhamnosus*, *Listeria innocua*

INTRODUCTION

Milk consumption among human population belongs every time on top place in dairy industry. This is in close relation with consumption of cheeses. Cheese is milk product containing milk protein-casein, milk fat and the other substances such as lactose, minerals, etc. Cow lump cheese represents a traditional Slovak cheese which is allotted to fresh types of cheeses. It is semi-soft, full-fat cheese **(Grieger, Burdová, 1987**). This cheese is very popular among consumers. At recent time, functional foods are very popular and requested by consumers on the market. These foods can be enriched with different types of additives which have to improve the quality, protection or popularity of the product itself and also its suitability for market and consumers **(Sokol *et al.*, 2004).** Among additives, probiotic bacteria are the most often incorporated **(Ouwehand *et al.*, 2004).** *Lactobacillus rhamnosus* GG (ATCC53103) is commercially known probiotic strain legislatively used in many dairy products **(Tynkkynen *et al.*, 1998).** Microbiological safety of the products belongs to the most important criteria to assess the food quality **(Sokol *et al.*, 2004).** Therefore, the aim of this study was to test surviving of *Lactobacillus rhamnosus* GG strain in cow lump cheese which was experimentally infected with *L. innocua*; listeriae are frequent causative agents of milk contamination via e.g. cow mastitis (**Bourry *et al.*, 1995).**

MATERIAL AND METHODS

Bacterial strains, media and growth conditions

Lactobacillus rhamnosus GG (ATCC 53103, 10^9 cfu/g kindly provided by Dr. Ouwehand, Danisco, Finland) was marked by rifampicin (**Strompfová *et al.*, 2003**) to differ it from the other lactic acid bacteria. The strain (0.1%) inoculum was cultivated in MRS broth (Merck, Germany) overnight at 37º C. The colonies count was performed by the standard microbiological dilution method according to ISO, plating on MRS agar and cultivated as mentioned formerly. *Listeria innocua* LMG13568 (kindly provided by prof. Luc De Vuyst, University of Brussel, Belgium), 0.1 % inoculum (10^6 cfu/mL) was cultivated in Trypticase soy broth (Becton and Dickinson, Cockeysville, USA) at 32º C for 48 h and enumerated on Oxford agar (Becton and Dickinson).

Cow lump cheese manufacturing process

Cow lump cheese was manufactured in 20-liter vats from milk using the standard technology for this type of cheese. Pre-heated milk vats were divided into E1 inoculated with 0.1% inoculum of *L. rhamnosus* GG strain (10^9cfu/mL), E2-*L. rhamnosus* GG experimentally contaminated with 0.1 % inoculum of *Listeria innocua* LMG13568 (10^6 cfu/mL), E3-*L. innocua* LMG13568) and the reference cheese vat (without strains). Appropriate amount of cream culture, rennet as well and 40% $CaCl_2$ was added calculated for 20 L. Curds were cut (15 min), scalded, pressed, cheese lumps were formed, they were put to drop off (18-20ºC) and cheeses were stored in cold room for 14 days. Sampling (10g) for microbiological analysis was provided on day 0-1 (start of the experiment), on days 2, 7 and 14. Cheese homogenates in 90 ml of sterile Ringer solution (treated in Stomacher-Masticator PK 400, IUL, Spain) were prepared. To check lactic acid and pH values, sampling was provided on day 0-1, 2,3,6,7 and 8 (cheeses manufacturing).

Microbial analysis

To check the counts of *Listeriae*, decimal dilutions were transferred to a tube with Fraser broth (Becton and Dickinson, Cockeysville, USA); each dilution in 3 tubes and cultivated at 32º C for 48 h. Then 100 µL of broth was checked by plating on Oxford agar (Becton and Dickinson), cultivated at 32ºC for 48 and estimated by the most probable number technique (MPN). To check the counts of *Lactobacillus rhamnosus* GG strain, MRS agar (Becton and Dickinson) enriched with rifampicin (100 µg) was used. Other lactic acid bacteria were checked on MRS agar. Reference vat was checked to be contaminant bacteria absent (by plate count agar and blood agar, Becton and Dickinson). The bacterial counts were expressed in colony forming units per gram (cfu/g) ± SD.

PCR confirmation of *L. rhamnosus* GG strain

The grown colonies of GG strain were confirmed by PCR method (**Brandt and Alatossava, 2003).** The primers used were F-CAATCTGAATGAACAGTTGTC, R-TATCTTGACCAAACTTGACG. The amplification protocol was as followed:initial denaturation at 94º C for 2min, 35 cycles of 94º C for 30s, 55º C for 30s, 72º C for 30s, 72º C for 10 min. Techgene KRD thermocycler (Techne, the United Kingdom) was used. The PCR products (10 µL) were separated by electrophoresis in 0.8 % agarose gels (Sigma, Germany) buffered with 1xTAE (Merck, Germany) with 1 µg/mL ethidium bromide (Sigma). The molecular mass standard (Promega, USA) was used according to the manufacturers instructions. DNA from cells was prepared according to **Baelae *et al.* (2000).**

Lactic acid and pH measurement

Lactic acid (LA) analysis was performed by the isotachophoric method (ZKI-001) with detector and leading and termining electrolytes. As leading electrolyte 10^{-2} M HIS, 10^{-2} M HIS Cl, 0.1% MHEC 10 mL, pH6.0 were used and as termining electrolyte 5 x10^{-3} glutaric acid and 5 x10^{-3} TRIS, pH 7-9 were used. The standard was lactate calcium. The pH values were measured by the use of pH meter Jenway 3310 (England) with electrodes. These measurements were done on day 0-1, 2, 3, 6, 7 and 8.

RESULTS AND DISCUSSION

***Lactobacillus rhamnosus* GG strain surviving in cheese**

L. rhamnosus GG was found in milk in sufficient amounts; in cow lump cheese as well (from 5.48 ± 0.15 to 7.77 ±1.30 log10 cfu/g, Table 1). Inoculating concentration of GG strain was 9.0 (log10) cfu/ml/g and on day 0-1 its count in E1 was 5.48 ± 0.15 cfu/g . This count was continually increased to reach maximum 7.77 ± 1.30 log10 cfu/g on day 7 with stability up to 14 days (Table 1). The counts of GG strain in cow lump cheeses were well balanced during whole experiment and even in the E2 cheese (GG strain with LMG strain) it was found in sufficient amount (from 4.69 ± 0.69 log10 cfu/g at the start of the experiment (0-1 day) with culmination on day 7 (6.55 ± 0.34 cfu/g); with stability up to the end of the experiment (days 14; 6.14±1.00 cfu/g, respectively, Table 1). In addition, the identity of *L. rhamnosus* GG - colonies isolated from cheeses was confirmed by PCR. The counts of other lactic acid bacteria (LAB) were also well balanced during the whole experiment in the experimental vats/cheeses, RCH including (Table 1) with stability up to day 14. Only in E1/GG cheese the highest number of LAB was detected (10.60±1.26 log10 cfu/g). It can be supposed that GG strain was the best established in that one E1 cheese; however, its establishment in E2 cheese was also sufficient. RCH was absent of spoilage (contaminant) bacteria.
The counts of *L. innocua* LMG 13568 were well balanced in E2 and E3 cheeses with culmination on day 2 (Table 1, 3.10 ±0.16 cfu/g, 3.34 ±0.82 cfu/g); however, the count of *L. innocua* LMG 13568 was not influenced by adding of the probiotic strain *L. rhamnosus* GG. Ability of GG strain to colonize dairy product was documented by us in our previous study, when a traditional Slovak product Bryndza cheese was inoculated with this probiotic strain. There the counts of GG strain reached 7.0 or 8.0 log 10 cfu/g on day 14 **(Lauková *et al.*, 2003).** The counts of other LAB counted there were similar to those presented by us in this experiment (9.0 respectively 10.0 log 10 cfu/g). Moreover, in Bryndza cheese coliforms and staphylococci were reduced with difference of 1 mathematical order. Protective effect of *L. rhamnosus* VT1 in milk was e.g. presented by **Koreňová *et al.*, (2006**). When *L. rhamnosus* GG was applied processing fermented dry sausage, it was found there in the count similar to ours (log10 7.0 cfu/g; **Erkkilä *et al.*, 2001**). The similar counts of probiotic strain as counted in our cow lump cheese were detected in Argentinian Fresco cheese inoculated with probiotic strain *L. acidophilus* **(Vinderola *et al.*, 2000). Oliveira *et al.* (2001)** tested the other *L. rhamnosus* strain LC 35 in relation with its milk supplementation and its effect on acidifying activity, textural properties and microbiological stability. The strain was tolerant and the product (yoghurt) possessed the good quality properties. *L. rhamnosus* GG was also experimentally tested in rabbits husbandry. There surprisingly similar counts were enumerated as in food; it again confirms its good adhesive ability (from 5.3 log 10 cfu/g to 6.1 cfu/g; **Simonová *et al.*, 2008**). Benefity of health after consumption of functional food (e.g. probiotic strain containing product) reported **Mikeš *et al.* (1995**). They referred reduction and optimization of cholesterol level in blood of volunteers consuming probiotic strain *E. faecium* M74. In this study, *L. innocua* LMG 13568 was not influenced; however, e.g. in tofu cheese inoculated with this strain and treated by enterocin A(P)-producing strain *E. faecium* EK13 (CCM7419) a reduction 1.17 log cycle was observed after 5 days of EK13 strain application (**Lauková *et al.*, 2002).** Two basic lines can be conducted by probiotic strain; to maintain shelf-life of the products and to improve consumers health by consuming the products containing those probiotic strains. Consumption of dairy products i.e. cheeses can control e.g. childrens health status. **Salminen *et al.* (1993)** reported that by rotavirus caused diarrhoea in children was weakened by *L. rhamnosus* GG treatment of children.

Table 1 The counts of *Lactobacillus rhamnosus* GG, *Listeria innocua* LMG 13568 and lactic acid bacteria (cfu/g ± SD).

Samples	0-1	2	7	14
GG				
E1/GG	5.48 (0.15)	7.43 (1.56)	7.77 (1.30)	7.04 (1.50)
E2(GG+Li)	4.69 (0.69)	4.32 (1.50)	6.55 (0.34)	6.14 (1.00)
E3/Li	-	-	-	-
RCH	-	-	-	-
LAB				
E1/GG	6.55 (1.24)	8.23 (1.10)	9.07 (1.03)	10.60 (1.26)
E2(GG+Li)	6.66 (1.28)	8.27 (1.13)	9.04 (1.02)	9.71 (1.30)
E3/Li	6.73 (1.33)	8 .11 (1.05)	9.49 (1.22)	9.83 (1.68)
RCH	6.25 (1.12)	8.14 (1.07)	9.04 (1.02)	9.38 (1.17)
Li				
E1/GG	-	-	-	-
E2(GG+Li)	2.10 (0.00)	3.34 (0.82)	2.36 (0.53)	2.69 (0.64)
E3/Li	2.30 (0.14)	3 .10 (0.16)	2.43 (0.19)	2.30 (0.14)
RCH	-	-	-	-

Day-0-1, (at the start of the experiment), days -2, 7, 14; RCH-reference cheese; E1-*Lactobacillus rhamnosus* GG; E2-*L. rhamnosus* GG + *Listeria innocua* LMG13568; E3-*Listeria innocua* LMG13568GG; Microbiota are expressed in colony forming unit per gram (cfu/g ± SD).

Lactic acid values and pH

In GG cheese (E1) the values of lactic acid were almost the same measured in RCH; it means that addition of GG strain did not influence negatively LA values in cheese (Table 2); at the start of the experiment they were even slightly higher than in RCH. The „protective effect" of GG in E2 cheese (GG strain and LMG) could be supposed in relation with almost not changed LA values. On the other hand, in E3 cheese on day 3 very low value of LA was measured. It can be related with not autochnous strain as *L. innocua* is for cheese. The values of pH were not negatively influenced by additives; they were almost the same as in the reference cheese (Table 3). **Koréneková *et al.* (2007)** reported the higher amount of LA in milk inoculated with GG strain with maximum at 48h (on day 2) as was presented in this study. That is the most common that pH of the product is not negatively influenced by probiotic strains or their bacteriocins **(Lauková *et al.*, 2001).**Visually, the GG cheese provided a good structure (consistency).

Table 2 The values of lactic acid (g/l) ±SD

	0-1 day	2 d	3 d	6 d	7 d	8 d
RCH	3.80 (0.19)	4.10 (0.20)	2.50 (0.15)	1.00 (0.00)	0.70 (0.08)	0.55 (0.07)
E1	4.10 (0.20)	4.85 (0.22)	2.60 (0.16)	1.00 (0.10)	0.65 (0.08)	0.25 (0.05)
E2	3.95 (0.19)	4.90 (0.22)	3.70 (0.19)	0.65 (0.08)	0.75 (0.08)	0.55 (0.07)
E3	3.80 (0.19)	4.85 (0.22)	1.85 (0.03)	0.90 (0.09)	0.55 (0.07)	0.10 (0.01)

RCH-reference cheese; E1-*Lactobacillus rhamnosus* GG; E2-GG+LMG; E3-*Listeria innocua* LMG13568

Table 3 The pH values in cheeses

	0-1 d	2 d	3 d	6 d	7 d	8 d
RCH	5.21	4.96	4.90	4.88	4.86	4.84
E1	5.04	4.86	4.83	4.77	4.78	4.79
E2	5.06	4.94	4.88	4.95	4.98	4.99
E3	5.00	4.88	4.82	4.82	4.84	4.85

RCH-reference cheese; E1-*Lactobacillus rhamnosus* GG; E2-GG+LMG; E3-*Listeria innocua* LMG13568

CONCLUSION

Sufficient surviving and stability of *L. rhamnosus* GG strain in the traditional cow lump cheese experimentally infected with *L. innocua* LMG 13568 was shown in this study. The LA and pH values were not negatively influenced. It can be disputed that shelf-life of the product could be maintained by this way and/or the product itself with GG strain can be consumed as a functional food or to serve as a probiotic strain carrier.

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