

Inhibitory Effect of Goat and Cow Milk Fermented by *Bifidobacterium longum* on *Serratia marcescens* and *Campylobacter jejuni*

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Abstract

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This study was performed to determine the influence of fermented goat and cow milk produced by the use of *Bifidobacterium longum* Bb-46 on pathogenic *Serratia marcescens* and *Campylobacter jejuni* strains. The correlation between the inhibitory effect and some fermentation parameters (the number of viable probiotic cells and pH of fermented milk) was also determined. *Bifidobacterium longum* counts and pH values were also measured in milk samples during fermentation. The results showed that the inhibitory effect of *Bifidobacterium longum* Bb-46 fermented goat milk on *Serratia marcescens* increased with the fermentation time. The highest inhibitory effect of fermented cow milk occurred in the middle course of fermentation. Statistically significant correlation between the inhibition degree of *Serratia marcescens* growth and pH values of fermented goat milk was noted as opposed to the correlation between the inhibition degree of *Serratia marcescens* growth and pH values of fermented cow milk which was not statistically significant. All samples of goat and cow fermented milk exhibited inhibitory effects on the growth of *Campylobacter jejuni*.

Keywords: *Bifidobacterium longum*; *Campylobacter jejuni*; fermented goat and cow milk; inhibitory effect; *Serratia marcescens*

Until the beginning of the 20th century, *Serratia marcescens* was considered to be a harmless saprophyte. It is known now that *S. marcescens* causes 1.4% nosocomial septic infections out of which 26% are lethal, 2% infections of lower respiratory tract, urinary tract, and surgical wounds. In addition, *S. marcescens* causes meningitis, endocarditis with high lethality, and endophthalmitis (MARINELLA & WARWAK 1998). Considerable therapeutic problems are caused, by unpredictable antimicrobial

susceptibility of *S. marcescens*. Multiple resistant strains of *S. marcescens* are often found in hospital environments (MANFREDI *et al.* 2000).

Infections caused by the pathogenic *Campylobacter jejuni* are among the most significant causes of acute bacterial gastroenteritis. In developing countries, species of *Campylobacter* genus cause diarrhea and even children deaths (ALLOS 2001). Furthermore, in developing countries, the incidence of *Campylobacter*-caused diarrhea is

2–7 times higher as compared to the “traditional” diarrheal infections caused by *Salmonella*, *Shigella* or *E. coli* O157:H (BLASER *et al.* 1983; SLUTSKER *et al.* 1997).

Probiotics are “living microbial food/feed supplements which beneficially affect host human/animal by improving its intestinal microbial balance” (FULLER 1989); over the period of the last two decades, probiotic bacteria have been used in the fermented milk production due to their favourable impact on human health. Healthy bowel microflora maintenance can protect the body from GI disorders and bowel inflammation (MITSUOKA 1982; HAENEL & BENDIG 1995; SALMINEN *et al.* 1998). Probiotic microorganisms interact with pathogenic bacteria and bowel microflora by the production of antimicrobial substances and competitive inhibition (SAARELA *et al.* 2000). Low molecular mass metabolites such as hydrogen peroxide, lactic acid, acetic acid, and flavour compounds produced by probiotic bacteria inhibit the growth of strains of different *Clostridium*, *Escherichia* and *Helicobacter* species (SKYTTÄ *et al.* 1993; HELANDER *et al.* 1997; NIKU-PAAVOLA *et al.* 1999).

Although no significant difference exists in the energetic values between goat and cow milk, goat milk is nutritionally more valuable (HAENLEIN 2004). Higher amounts of short and medium chain fatty acids and the smaller diameter of fat globules increase the digestion of goat milk (MEHAIA 1995). Antibacterial and immunological properties of goat milk are distinctively better than those of cow milk and increase its therapeutic value (PARK 1994; HAENLEIN 2004). Additionally, some authors reported that goat milk has a stronger antimicrobial lactoperoxidase system than cow milk (ZAPICO *et al.* 1991).

In the production of fermented milk, goat milk is rarely used although in the fermented form it has a higher nutritional value and digestibility (LOEWENSTEIN *et al.* 1980; MARTÍN-HERNÁNDEZ *et al.* 1992; BOŽANIĆ *et al.* 1998).

The primary goal of this work was to determine the antagonistic effects of goat and cow milk fermented by the probiotic strain *Bifidobacterium longum* Bb-46 on the selected pathogenic strains of *Serratia marcescens* and *Campylobacter jejuni*. Secondly, the aim was also to ascertain whether pH value and *Bifidobacterium longum* Bb-46 cell concentration affect the inhibition degree of the selected pathogenic strains. The main hypothesis was that goat milk fermented by the probiotic strain

has a distinctively different effect on pathogenic bacteria in comparison with cow milk. For this purpose, *in vitro* microbiological experiment was performed.

MATERIAL AND METHODS

Pathogenic bacteria. *Serratia marcescens* was isolated from the urethra of a patient with urinary tract infection. The samples of urethra swabs were inoculated on Blood agar base with horse blood (Merck, Germany), and *Serratia marcescens* was determined after the incubation under aerobic conditions at 37°C for 48 hours.

C. jejuni was isolated from a patient with campylobacteriosis on Campylobacter Blood Free Medium CCDA Bolton (Biolife, Italy). The plates were incubated for 48 h at 37°C under microaerophilic conditions (Anaerobic jar with Anaerocult C; Merck, Germany). The standard microbiological methods were used (PRESCOTT 1999).

Both pathogens examined were determined by the API system (BioMérieux, Marcy l’Etoile, France).

Fermentation of goat and cow milk. For the fermentation of goat and cow milk, the commercial available UHT cow (with 3.2% of milk fat) and goat (with 3.2% milk fat) milks were used. The chemical composition of goat and cow milks was determined by MILCOSCAN FT 120 (Foss Electric, Denmark). 30 samples of both types of milk were analysed. The average chemical composition is presented in Table 1.

The DVS culture of *Bifidobacterium longum* Bb-46 (Chr. Hansen, Denmark) was used to inoculate the goat and cow milk at 37°C for 25 hours.

Analysis during fermentation. pH value and electrochemical potential of H⁺ ions during fermentation were measured on MA 233 pH/Ion Analyzer (Mettler Toledo). The number of viable cells of *B. longum* Bb-46 (CFU) in fermented milk was determined after incubation (3 days at 37°C) on MRS agar in Anaerobic jar with Anaerocult A (Merck, Germany). The viable count of *Bifidobacterium longum* Bb-46 and pH values were determined after every 5 hours of fermentation. All measurements were carried out 5 times.

Degree of inhibition. *In vitro* method was used in order to determine the degree of inhibition of *S. marcescens* and *C. jejuni* in the samples of fermented milk, such as described by SLAČANAC *et al.* (2004). Briefly, a known number of test cells

Table 1. Chemical composition and acidity of goat and cow milk used for the production of fermented goat and cow milk by the use of *Bifidobacterium longum* Bb-46 culture

Composition and acidity (g/100 g)	Goat milk		Cow milk	
	\bar{x}	SD	\bar{x}	SD
Total solids	11.45	0.128	11.69	0.031
Ash	0.84	0.040	0.69	0.014
Fat	3.20	–	3.20	–
Lactose	4.24	0.037	4.75	0.029
Proteins	3.17	0.085	3.05	0.036
Acidity	194	0.088	pH = 6.64	0.061
	pH = 6.55 8.05 °SH	0.131	7.23 °SH	0.047

SD – standard deviation; \bar{x} – mean value of 30 determinations

(24 h old culture on nutrient agar) was prepared. From 10^{-6} dilution, 0.1 ml of the inoculum was spread on the surface of agar plates (blood agar with horse blood for *S. marcescens* and *Campylobacter jejuni* on Campylobacter Blood Free Medium CCDA Bolton) with a glass spreader. Then, 0.1 ml of the fermented milk was spread evenly with a glass spreader. The blood agar plates were then incubated at 37°C for 24 h and the number of *S. marcescens* (CFU/ml) was calculated. *Campylobacter jejuni* was incubated at 37°C for 48 h under microaerophilic conditions (Anaerobic jar with Anaerocult C; Merck, Germany) and the influence of fermented goat and cow milk was observed.

Campylobacter jejuni grows on agar plates in low and spreading colonies that are uncountable. Therefore, a qualitative method was performed for determining the inhibition using the comparison of control growth and the growth after spreading the fermented milk.

Inhibition of pathogens by supernatant of fermented goat and cow milk (Antibiotic sensitivity test). The Antibiotic sensitivity test was conducted according to the Kirby-Bauer method on Mueller-Hinton agar (Merck, Germany) (PRESCOTT 1999). The samples of fermented milk were centrifuged at $2222 \times g$ (4436 rpm) for 10 min at 4°C before the antibiotic assay. The clear supernatant was applied in drops (40 μ l) on the antibiogram susceptibility disc (diameter 12.7 mm; Schleicher & Schuell, Germany) and put on Mueller-Hinton plates inoculated with *S. marcescens* and *C. jejuni* (PRESCOTT 1999) which were subsequently incubated at 37°C for 24 h (48 h for incubation

of *C. jejuni* in microaerophilic conditions). The diameters of the inhibition zones around the discs were measured.

Statistical analysis. All the experimental results were statistically analysed at 95% confidence level for means using the descriptive statistics in Excel 2000. The comparison of pH values and *Bifidobacterium longum* Bb-46 counts during fermentation of goat and cow milk was made by ANOVA (two factors without replication) in Excel 2000. The points in Figures 1–2 were represented as the mean values \pm SD (Statistica 7.0).

The comparison between the results of inhibition of *Serratia marcescens* by the *Bifidobacterium longum* Bb-46 fermented goat and cow milk with the changes in pH and CFU was made by Basic Statistic/Tables, Correlation matrices model in Statistica 7.0. The coefficient of variation (CV) was used to analyse the microbiological results. The coefficient of variation values were calculated according to the equation (SHELLEY *et al.* 1987): $CV(\%) = (SD/\bar{x}) 100$.

RESULTS AND DISCUSSION

Scientific reports on bifidobacterial growth in goat milk are rare (BOŽANIĆ *et al.* 1998; SLAČANAC *et al.* 2004). The results obtained in this work suggest that *Bifidobacterium longum* Bb-46 grows better in goat milk than in cow milk (Figure 2). The pH values of goat milk decreased more rapidly (Figure 1) and a higher number of viable cells *Bifidobacterium longum* Bb-12 (Figure 2) was found during the fermentation of goat milk. The

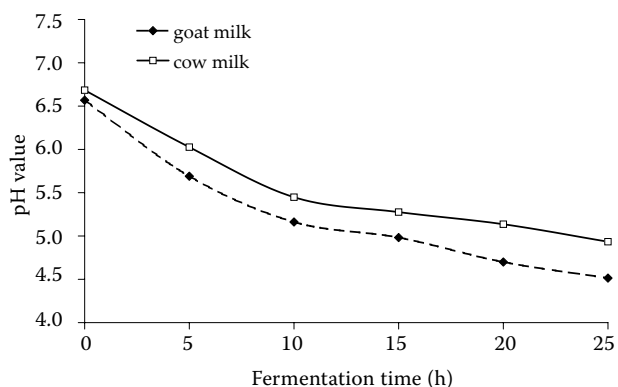


Figure 1. Changes of pH values during the fermentation of goat and cow milk by *Bifidobacterium longum* Bb-46

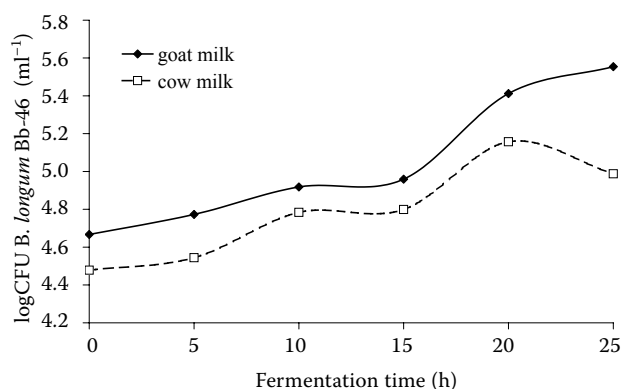


Figure 2. Changes of CFU of *Bifidobacterium longum* Bb-46 during the fermentation of goat and cow milk

results of ANOVA show statistically significant differences between the goat and cow milk in pH values and CFU of *Bifidobacterium longum* Bb-46 during fermentation ($P < 0.05$; Table 2). Some authors indicated that the higher fermentation activity of lactic acid bacteria in goat milk is due to its specific composition and structure (LOEWENSTEIN *et al.* 1980; BYLUND 1995; ANTUNAC *et al.* 2000). However, it was not a foregone conclusion on the basis of the overall composition of goat and cow milk (Table 1). A higher content of whey proteins (Table 1) could be significant because bifidobacteria are growing better in the presence of higher levels of some amino acids presents in lactoglobulins and lactoalbumins (ARUNACHALAM 1999). Furthermore, the possible reasons for the higher growth rate of *Bifidobacterium longum* Bb-46 in goat milk could be a higher amount of some minerals and short chain fatty acids, as well as the easier protein digestibility (ALICHANDIS & POLYCHRONIADOU 1997).

In recent years, many authors pointed out that fermented milk with probiotics inhibits gram negative bacteria such as *Yersinia enterocolitica*, *Escherichia coli*, *Aeromonas hydrophila* and *Salmonella* spp. in *in vitro* experiments (HELANDER *et al.* 1997; SOOMRO *et al.* 2002). Although strong

antibacterial and immunological properties of goat milk have been indicated, little is known about the influence of fermented goat milk on pathogenic and potentially pathogenic microorganisms. The results presented in Tables 3 and 4 exhibited a higher inhibitory effect of fermented goat milk on the growth of *Serratia marcescens* colonies, rather than of fermented cow milk. All samples of fermented goat milk significantly more strongly inhibited the growth of *Serratia marcescens* on Blood agar than those of fermented cow milk (Tables 3 and 4). The samples of goat milk fermented for 15, 20, and 25 h inhibited the growth of *Serratia marcescens*. The highest antagonistic potential against *Serratia marcescens* was found in the samples of goat milk fermented for 20 hours (Table 3). Samples of fermented cow milk also inhibited the growth of *Serratia marcescens* but the inhibitory effect was less expressed in comparison to fermented goat milk. The results of ANOVA, presented in Table 5, show statistically significant differences in the degree of inhibition between fermented goat and cow milk during the fermentation process. Goat milk has a distinct antimicrobial impact and its specific composition may result in the increased antimicrobial compounds production (SEIFU *et al.* 2004; SLAČANAC *et al.* 2004). The results of

Table 2. Analysis of variance for the data given in Figures 1–2 (comparison between goat and cow milk; ANOVA, two factors without replication)

Source of variations	$F_{\text{calculated}}$	P -value	F_{critical}
Between pH values	40.820	0.001	6.608
Between CFU (<i>Bifidobacterium longum</i> Bb-46)	12.935	0.016	6.608

Table 3. Inhibition of *Serratia marcescens* by *Bifidobacterium longum* Bb-46 fermented goat milk in different fermentation phases. Number of samples (replicates) = 6

Fermentation time (h)	LogCFU <i>S. marcescens</i> (ml ⁻¹)	Inhibition (%)	CV(%)
0	8.04	1.9	7.9
5	7.90	2.8	8.3
10	7.93	3.2	7.14
15	7.44	7.3	8.33
20	7.41	13.88	5.88
25	7.98	9.2	6.67

CV(%) – coefficient of variation

LogControl *S. marcescens* (0–5 h of fermentation) = 8.1461 (CV = 6.47)

LogControl *S. marcescens* (10–15 h of fermentation) = 8.1761 (CV = 8.52)

LogControl *S. marcescens* (20–25 h of fermentation) = 8.1761 (CV = 3.83)

some authors have suggested that higher contents of short-chain fatty acids (SCFA) and medium-chained fatty acids (MCFA) are produced during fermentation of goat milk in comparison to cow milk (SLAČANAC *et al.* 2005). Higher contents of SCFA and MCFA, especially at lower pH values, could be the reason of the higher inhibitory effect of fermented goat milk.

Many studies indicated that the fermentation time, as well as the quantities of some metabolic products, have a great influence on the antagonistic activities of fermented milks. The work of SAARELA *et al.* (2000) supports this theory, however, some differences between fermented goat and cow milk were noted. The correlation between the degree of inhibition of *S. marcescens* and pH

values of fermented goat milk was higher than the correlation between the degree of inhibition of *S. marcescens* and pH values of fermented cow milk ($r = -0.87$ and -0.81 , respectively; $P < 0.05$). On the contrary, no statistically significant correlation was found between the degree of inhibition of *S. marcescens* and CFU of *B. longum* in goat milk ($r = 0.74$; $P < 0.05$). With fermented cow milk, the same correlation was considerably higher ($r = 0.94$; $P < 0.05$). As can be seen, the inhibition of *S. marcescens* growth was connected to pH values, apart from CFU of *B. longum*. Different tendencies were noted with fermented goat milk as compared to fermented cow milk. Accordingly, the possible reason could be the production of some antimicrobial compounds in goat and cow milk

Table 4. Inhibition of *Serratia marcescens* by *Bifidobacterium longum* Bb-46 fermented cow milk in different fermentation phases. Number of samples (replicates) = 6

Fermentation time (h)	LogCFU <i>S. marcescens</i> (ml ⁻¹)	Inhibition (%)	CV(%)
0	8.08	0.82	10.34
5	8.08	0.82	2.86
10	8.08	1.19	5.88
15	8.04	1.65	5.71
20	7.17	12.30	7.37
25	7.88	3.68	5.45

CV(%) – coefficient of variation

LogControl *S. marcescens* (0–5 h of fermentation) = 8.1461 (CV = 6.47)

LogControl *S. marcescens* (10–15 h of fermentation) = 8.1761 (CV = 8.52)

LogControl *S. marcescens* (20–25 h of fermentation) = 8.1761 (CV = 3.83)

Table 5. Analysis of variance for the data given in Tables 3–4 (comparison of inhibitory effect between goat and cow milk; ANOVA, two factors without replication)

Source of variation	SS	<i>f</i>	MS	<i>F</i> _{calculated}	<i>F</i> _{critical}
Rows	28745.42	10	2874.54	117.48	2.97
Columns	1893.49	1	1893.49	77.39	4.96
Error	244.66	10	24.46		
Total	30883.58	21			

Rows = single variations by every 5 hours of fermentation process

Columns = difference in overall inhibition degree between fermented goat and cow milk

and an additional impact on pathogenic bacteria besides the influence of probiotic bacteria. Antimicrobial metabolites of probiotic bacteria and the drop of pH value, rather than cell count, are considered to be the main causes of the microbial inhibition (NIKU-PAAVOLA *et al.* 1999; SAARELA *et al.* 2000).

Campylobacter jejuni grows on agar plates in low and spreading colonies that are uncountable. Therefore, a qualitative method for determining the inhibition was performed using the comparison of the control growth and the growth after spreading fermented milk. To sum up, all samples of fermented goat and cow milk exhibited inhibitory effects (Table 6). No difference was observed between goat and cow milk. *Bifidobacterium longum* Bb-46 is a heterofermentative bacterium which produces acetic acid, ethanol, carbonile compounds and CO₂ with a possible antibacterial effect (TRATNIK 1998) as well as lactic acid. The possible cause of *Campylobacter jejuni* inhibition by *Bifidobacterium longum* Bb-46 (besides the pH drop) is H₂O₂ as

a product of lactose fermentation (TAMIME *et al.* 1995; TRATNIK 1998). *Campylobacter jejuni* shows extreme sensitivity to H₂O₂ (SMIBERT 1984).

The sensitivity of the selected pathogenic bacteria to tested antibiotics and fermented milk is reported in Table 7. A higher sensitivity of both bacteria was found in fermented goat milk as compared to fermented cow milk. Considerably larger inhibitory zones were measured for all the discs with the

Table 6. Inhibition of *Campylobacter jejuni* by *Bifidobacterium longum* Bb-46 in goat and cow milk in different phases of fermentation

Fermentation process	<i>Campylobacter jejuni</i>	
	goat milk	cow milk
Beginning	–	–
Middle	–	–
End	–	–

– less expressed growth in comparison to control

Table 7. Results of the tests of inhibition of *Serratia marcescens* and *Campylobacter jejuni* by supernatants of fermented goat and cow milk (Antibiotic sensitivity test)

Disc	Mean inhibition zone (mm)			
	<i>Serratia marcescens</i>		<i>Campylobacter jejuni</i>	
	goat milk	cow milk	goat milk	cow milk
Unfermented sample	+	+	+	+
Sample after 12.5 h fermentation	7.4 ± 0.19	3.1 ± 0.16	10.2 ± 0.15	6.3 ± 0.19
Sample after 25 h fermentation	5.3 ± 0.17	1.6 ± 0.10	8.3 ± 0.14	7.7 ± 0.15

Diameters in a control series of antibiotics (mm):

for *Serratia marcescens*: ceftibuten > 21 mm; ciprofloxacin > 21 mm; amoxicillin + clavulonic acid > 18 mm

for *Campylobacter jejuni*: ciprofloxacin > 21 mm; amoxicillin + clavulonic acid > 18 mm; azithromycin > 18 mm

+ inhibition zones not clearly expressed and difficult to measure

± represents SD of 5 replicates

samples from fermented goat milk. In the middle of the fermentation process, the zones around the discs were larger compared to those at the end of the fermentation process. The possible reasons are the metabolic activity of *Bifidobacterium longum* Bb-46 and its metabolites.

During fermentation, *B. longum* grew better in goat milk than in cow milk. The results obtained with *in vitro* microbiological method and the antibiotic sensitivity tests suggest a significantly higher antagonistic potential of fermented goat milk against *Serratia marcescens*. The degree of inhibition of *Serratia marcescens* revealed a high correlation with pH values of fermented goat milk, but no correlation with CFU of *B. longum* in fermented goat milk. In contrast to fermented goat milk, CFU of *B. longum* in fermented cow milk correlated with the degree of inhibition of *Serratia marcescens* growth. The results obtained showed a marked inhibitory effect of fermented goat and cow milk on the growth of *Campylobacter jejuni*.

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