

Inhibition of the *in vitro* Growth of *Salmonella enteritidis* D by Goat and Cow Milk Fermented with Probiotic Bacteria *Bifidobacterium longum* Bb-46

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Abstract

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This study was carried out to determine the influence of goat and cow milk fermented by *Bifidobacterium longum* Bb-46 on the pathogenic *Salmonella enteritidis* D strain. The basic hypothesis of this study was that fermented goat milk could possibly have a stronger inhibitory effect on the growth of *Salmonella enteritidis* D than fermented cow milk. The correlation between the inhibitory effect and some fermentation parameters (number of viable cells of *Bifidobacterium longum* Bb-46 and pH of fermented milk) was also analysed. *S. enteritidis* D strains were isolated directly from the faeces of an infant with diagnosed salmonellosis. The inhibitory effects of goat and cow milk fermented with *Bifidobacterium longum* Bb-46 were determined on Salmonella-Shigella agar after 0, 5, 10, 15, 20, and 25 h from the start of fermentation. *Bifidobacterium longum* Bb-46 count and pH values were also measured in samples of goat and cow milk during fermentation. The results obtained have shown a considerably higher inhibitory effect of fermented goat milk on the growth of *Salmonella enteritidis* D as compared to that of fermented cow milk. At the same time, higher acidity and CFU of *Bifidobacterium longum* Bb-46 were noted in fermented goat milk in all the phases of the fermentation process. The inhibitory effects of the fermented goat and cow milk on *Salmonella enteritidis* D growth increased rapidly with the fermentation time. The results indicated high sensitivity of *Salmonella enteritidis* D to acidity of both fermented milks. Consequently, a significant correlation between the inhibition degree and pH values of fermented goat and cow milk was noted.

Keywords: *Bifidobacterium longum* Bb-46; fermented goat milk; fermented cow milk; inhibitory effect; lactic acid fermentation; *Salmonella enteritidis* D

To have an impact on the colonic flora, it is important for probiotic strains to exhibit antagonism against pathogenic bacteria via antimicrobial substance production or competitive exclusion (SAARELA *et al.* 2000). Many authors suggested that low molecular weight metabolites and secondary metabolites may be more important than

bacteriocins, since they show a wide inhibitory spectrum against many harmful organisms (NIKUPAAVOLA *et al.* 1999; SAARELA *et al.* 2000). The role of bacteriocins in the pathogen inhibition is limited, since bacteriocins have inhibitory effects only against closely related species (HOLZAPFEL *et al.* 1998).

Among the bacteria, *Escherichia* and *Salmonella* are the most common causative agents responsible for diarrhea of infectious origin (GISMONDO *et al.* 1999; DE BUCK *et al.* 2004). The results of many *in vitro* studies indicated antagonistic effects of some probiotic lactobacilli and bifidobacteria against salmonellae (TUOMOLA *et al.* 1999; CAPLAN & JILLING 2000; PAVLOVIĆ *et al.* 2006). The antibacterial activity of *Lactobacillus acidophilus* LB SCS (COCONNIER *et al.* 1997) and *Lactobacillus acidophilus* (*johnsonii*) LA1 (BERNET-CAMARD *et al.* 1997) towards *Salmonella typhimurium* was also maintained *in vivo* in the infected mouse model.

In all above-mentioned studies, probiotics grew on different carbohydrate media, often with prebiotic addition. There is a poor scientific evidence for the antagonistic activity of milk fermented by probiotics against *Salmonella*. The main question is whether the concentrations of antibacterial substances and the number of viable cells of probiotic in fermented milk are sufficient for antagonistic activity.

Goat milk has many unique characteristics, which supports the contention of high qualities of dairy products from goat milk for human nutrition (HAENLEIN 2004). The physiological and biochemical facts of the unique qualities of goat milk are just barely known and little exploited (PARK 1994a). Unique characteristics of goat milk in comparison to cow milk include: better digestibility (JUAREZ & RAMOS 1986), higher buffering capacity (PARK 1991), smaller diameter of fat globules and better distribution in milk emulsion (MEHAIA 1995), higher content of short – chain fatty acids in the milk fat (BICKERSTAFFE *et al.* 1972), higher contents of zinc, iron and magnesium (PARK 1994b), a stronger lactoperoxidase (antimicrobial) system (ZAPICO *et al.* 1991) as well as better immunological and antibacterial characteristics (PARK 1994a). Higher amounts of the above mentioned minerals in goat milk may influence the growth of lactic acid bacteria, since they are part of some enzymatic complexes of lactose fermentation. A higher protein content could also be significant because *L. acidophilus* and bifidobacteria grow better in the presence of higher levels of some amino acids (valine, glycine, histidine) (MISRA & KULIA 1994; TAMIME *et al.* 1995).

The aim of this study was to determine the antagonistic effects of goat and cow milk fermented with *Bifidobacterium longum* Bb-46 against enteropathogenic *Salmonella enteritidis* D strains. The basic hypothesis of this study was that fermented

goat milk could possibly have a stronger inhibitory effect on the growth of *Salmonella enteritidis* D than fermented cow milk. Consequently, one of the primary objectives of this research was to compare the inhibition degree of fermented cow milk on the growth of *S. enteritidis* D with that of fermented goat milk. There is no clear scientific evidence of the antagonistic effect of goat milk fermented with the use of bifidobacteria against pathogenic bacteria, especially against *Salmonella* species. In order to establish the possible differences, *in vitro* microbiological experiments were conducted. Furthermore, the correlation between the inhibition degree and some fermentation parameters was determined in this study. Following up some earlier studies (BERNET-CAMARD *et al.* 1997; HOLZAPFEL *et al.* 1998; SLAČANAC *et al.* 2005), the inhibition degree was connected to CFU of *Bifidobacterium longum* Bb-46 and pH value of fermented milk. Correlations were determined during the whole fermentation process. The results entirely confirm the hypothesis that goat milk fermented using *Bifidobacterium longum* Bb-46 has a higher inhibitory potential than is that of cow milk. Furthermore, high correlation between the inhibition degree and the measured fermentation parameters of cow and goat milk (CFU of *Bifidobacterium longum* Bb-46 and pH value of fermented milk) was also determined.

MATERIAL AND METHODS

Isolation of *Salmonella enteritidis* D. The strain *Salmonella enteritidis* D was isolated from a patient affected by salmonellosis. The CFU of *S. enteritidis* D from faeces were determined and enumerated on chromogenic URI SELECT-4 agar, as well as on *Salmonella* selective Salmonella-Shigella agar (BIOLIFE, Italy). *S. enteritidis* D was cultured on Salmonella-Shigella Agar at 37°C for 48 hours. The standard microbiological methods for the determination and enumeration were used (PRESCOTT *et al.* 1996).

Fermentation of goat and cow milk. UHT commercial goat and cow milk with 3.2% fat content were used to prepare the fermented goat and cow milk. Before inoculation, milk was treated by UHT (140°C/2–3 s). The average chemical composition of UHT goat and cow milk was determined using FT 120 MILKOSCAN (FOSS Electric, Denmark). Ten samples of both types of milk were analysed. The monoculture *Bifidobacterium longum* Bb-46

(Chr. Hansen, Denmark) was used to inoculate the goat and cow milk at 37°C for 25 h (TAMIME & MARSHALL 1997).

Analyses during fermentation. The pH values during fermentation were measured using an MA 235 pH/Ion Analyser (METTLER TOLEDO).

The viable count of *Bifidobacterium longum* Bb-46 was determined on modified *Bifidobacterium medium* (according to Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany) in anaerobic jars at 37°C for 48 hours. The modification was performed by adding 13.5 g/100 ml bacteriological agar (Agar Bios Special LL, Biolife, Italy) and 3 g/100 ml LiCl. The viable count of *Bifidobacterium longum* Bb-46 and pH values were determined every five hours during fermentation. All measurements were performed in 5 replicates.

Determination of the degree of inhibition. Two original *in vitro* methods were used to determine the inhibition of *S. enteritidis* D in samples of fermented milk, described in SLAČANAC *et al.* (2004). The Streak-Plate technique was used to prepare pure cultures of *S. enteritidis* from a mixed population on URI SELECT-4.

In the first method, a known number of *S. enteritidis* D cells (24-h-old culture on nutrient agar) was prepared. From a 10^{-6} dilution, 0.1 ml of inoculum was spread on the surface of Salmonella-Shigella agar using a glass spreader. 0.1 ml of fermented milk was then spread evenly using a glass spreader. The agar plates were then incubated at 37°C for 24 h and the number of *S. enteritidis* D (CFU/ml) was calculated.

The second method was conducted under different microbiological conditions. After the inoculation of milk with *B. longum* Bb-46, 10 ml was put in a sterile tube which was then inoculated with 0.1 ml of *S. enteritidis* D culture (24-h-old culture on nutrient agar) and incubated at 37°C for 24 hours. Subsamples were taken every five hours during fermentation. The number of *S. enteritidis* cells in 1 ml of fermented milk (CFU/ml) was determined by placing 0.1 ml of inoculum from a 10^{-6} dilution on the surface of Salmonella-Shigella agar. The inoculum was spread and the plates were incubated at 37°C for 24 h, and CFU/ml was then calculated.

Antibiotic sensitivity test. The antibiotic sensitivity test was conducted according to the Kirby-Bauer method on Mueller-Hinton agar (DURAKOVIĆ 1996). Three antibiotics were tested in the control series:

norfloxacin, tetracyclin, and kinolon (Biolife, Italy). Samples of fermented milk were centrifuged at $222 \times g$ for 10 min at 4°C before the antibiotic assay. The clear supernatant was dropped on antibiogram susceptibility discs (diameter 12.7 mm; producer Schleicher & Schuell, Germany) which were put on Mueller-Hinton plates inoculated with *S. enteritidis*. The plates were incubated at 37°C for 24 h and the diameters of the inhibition zones around the discs were measured (SLAČANAC *et al.* 2004).

Statistical analysis. All the results were statistically analysed using the Descriptive statistics in Excel 6.0, at the 95% confidence level for the means. The comparison of pH values and *Bifidobacterium longum* Bb-46 counts during fermentation between goat and cow milk was made by ANOVA (two factors without replication) in Excel 6.0. The comparison between the results of inhibition of *S. enteritidis* D by the fermented goat and cow milk with the changes in pH and CFU was made using a linear correlation matrices method in Statistica 6.0. The coefficient of variation (CV) was used to analyse the microbiological results (SHELLEY *et al.* 1987).

RESULTS AND DISCUSSION

The average chemical compositions of goat and cow milk are reported in Table 1. Very small differences in the overall composition between UHT goat and cow milk were noted. Goat milk had inconsiderably lower average content of lactose, but a higher level of whey proteins in comparison to cow milk. Furthermore, SD values in Table 1 suggest very low variations in the composition of 20 samples of goat and cow milk.

Few reports exist on bifidobacterial growth in goat milk. Some authors have indicated that goat milk is a better substrate for lactobacilli growth than cow milk (LOEWENSTEIN *et al.* 1980; ABRAHAMSEN & RYSSTAD 1991; ALICHANDIS & POLYCHRONIADOU 1997; ANTUNAC *et al.* 2000; SLAČANAC *et al.* 2004, 2005). The results presented in Figures 1 and 2 show that *Bifidobacterium longum* Bb-46 grows better in goat milk than in cow milk. The pH values of goat milk decreased more rapidly (Figure 1) and higher numbers of viable cells *Bifidobacterium longum* Bb-46 (Figure 2) were found during the fermentation of goat milk. The results of ANOVA show statistically significant differences between the goat and cow milk in pH values ($F = 148.10$) and CFU of *Bifidobacterium longum* Bb-46

Table 1. Chemical composition (g/100 g) and acidity of the cow and goat milk used to produce fermented goat and cow milk using *B. longum* Bb-46

	Goat milk			Cow milk		
	\bar{x}	range	SD	\bar{x}	range	SD
Total solids	11.41	11.22–11.90	0.240	11.36	11.27–11.42	0.083
Ash	0.79	0.76–0.89	0.033	0.72	0.69–0.73	0.014
Fat	3.20	3.20	–	3.20	3.20	–
Lactose	4.25	4.20–4.35	0.036	4.90	4.87–4.96	0.053
Proteins	3.35	3.16–3.47	0.214	3.10	3.03–3.19	0.045
Whey proteins	0.69	0.61–0.74	0.043	0.58	0.52–0.63	0.031
Acidity	pH = 6.53	pH = 6.46–6.63	0.065	pH = 6.66	pH = 6.60–6.69	0.057
	8.10°SH	7.91–8.23 °SH	0.160	7.18°SH	7.12–7.38°SH	0.130

SD – standard deviation; \bar{x} – mean value of 20 determinations

($F = 13.91$) during the fermentation (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 (ANTUNAC *et al.* 2000). However, it was not a foregone conclusion on the basis of comparison of the overall compositions of goat and cow milk (Table 1). A higher content of whey proteins in goat milk (Table 1) could be significant because bifidobacteria grow better in the presence of higher levels of some amino acids present in lactalbumins and lactoglobulins (TAMIME *et al.* 1995).

It was indicated that various fermented dairy products inhibit the growth of different strains of *Salmonella in vitro* (SAARELA *et al.* 2000). There was no previous scientific evidence for the inhibi-

tory effect of fermented goat milk on the growth of *Salmonella* strain.

The results obtained in this work exhibited a marked inhibitory effect of fermented goat milk on the growth of *Salmonella enteritidis* D colonies, rather than fermented cow milk. Tables 3 and 4 report the results on the degree of inhibition of *S. enteritidis* by the fermented goat and cow milk. All samples of goat milk fermented with *Bifidobacterium longum* Bb-46 inhibited the growth of *S. enteritidis* on Salmonella-Shigella agar (method 1). The degree of inhibition is most often linked to the number of lactic acid-producing bacteria, as well as reduction in the pH value during fermentation (OUWEHAND *et al.* 1999). The results shown in Table 3 and Figures 1 and 2 are in complete

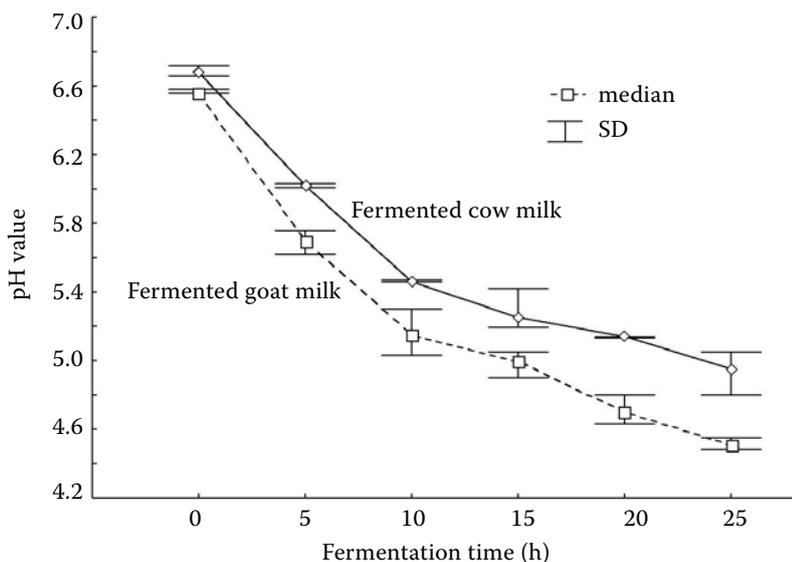


Figure 1. Changes of pH values during the fermentation of goat and cow milk with *Bifidobacterium longum* Bb-46

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

Source of variation	SS	Df	MS	$F_{\text{calculated}}$	P -value	F_{critical}
For Figure 1						
Rows*	4.96	5.00	0.99	148.10	2×10^{-5}	5.05
Columns**	0.30	1.00	0.30	44.34	1.2×10^{-3}	6.61
Error	0.03	5.00	0.01			
Total	5.29	11.00				
For Figure 2						
Rows*	0.89	5.00	0.18	13.91	0.01	5.05
Columns**	0.19	1.00	0.19	14.67	0.01	6.61
Error	0.06	5.00	0.01			
Total	1.14	11.00				

*single variations by the every hour of fermentation process; **differece in overall inhibition degree between fermented goat and cow milk

correlation with this theory. The highest degree of inhibition on the *in vitro* growth of *S. enteritidis* was shown by samples of goat milk fermented for 20 and 25 h (Table 3). These samples contained the highest numbers of viable *B. longum* Bb-46 bacteria and had the lowest pH value. The correlation between the degree of inhibition of *S. enteritidis* growth and pH value of fermented goat milk was statistically significant ($r = -0.77$). A high correlation between the degree of inhibition of *S. enteritidis* growth and the number of viable cells of *B. longum* Bb-46 in goat fermented milk was also noted ($r = 0.84$).

As opposed to fermented goat milk, fermented cow milk had a considerably lower inhibitory effect

on the growth of *S. enteritidis* on Salmonella selective agar (method 1, Table 3). The differences in the degree of inhibition between fermented goat milk and fermented cow milk were highly statistically significant (Table 4). Only the samples of cow milk fermented with *B. longum* Bb-46 for 20 h inhibited the growth of *S. enteritidis* in the range over 50% (Table 3). Furthermore, in fermented cow milk no correlation was found between pH values ($r = -0.49$) and CFU of *B. longum* Bb-46 ($r = 0.57$), and the degree of inhibition. These results could be in correlation with the results obtained in some previous works which indicated that some uropathogenic strains are not sensitive to the acidity of fermented cow milk (SLAČANAC *et al.* 2004). At the same

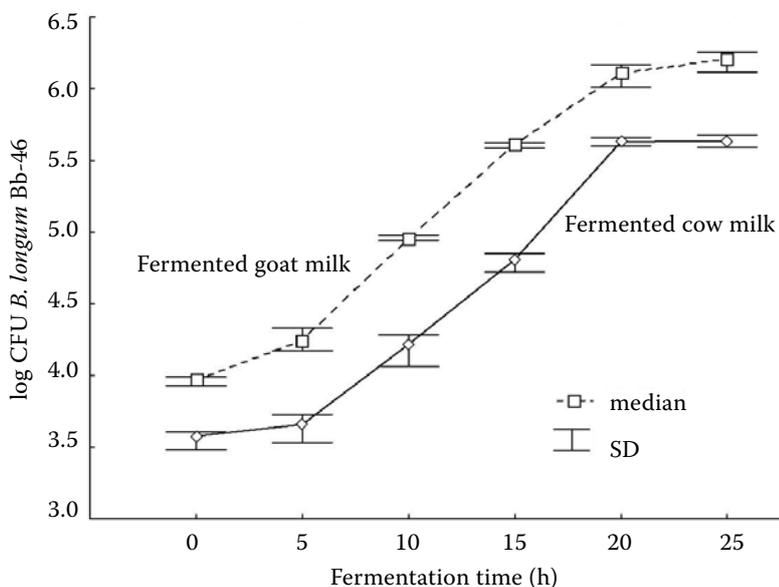
Figure 2. Changes of CFU of *Bifidobacterium longum* Bb-46 during the fermentation of goat and cow milk

Table 3. Inhibition of *Salmonella enteritidis* during fermentation of goat and cow milk with *Bifidobacterium longum* Bb-46 (number of samples = 6)

Fermentation time (h)	CFU of <i>S. enteritidis</i> (g ⁻¹) method 1	Inhibition (%)	CFU of <i>S. enteritidis</i> (g ⁻¹) method 2	CFU decrease during incubation – method 2 (%)	CV
Goat milk					
0	1.80 × 10 ⁸	12.20	2.91 × 10 ⁸	–	5.55
5	1.75 × 10 ⁸	14.63	1.08 × 10 ⁹	62.89	2.85
10	1.55 × 10 ⁸	24.39	1.61 × 10 ⁹	–	9.67
15	7.86 × 10 ⁸	25.14	1.49 × 10 ⁸	7.45	0.88
20	3.25 × 10 ⁸	68.77	3.96 × 10 ⁷	82.82	1.37
25	6.80 × 10 ⁸	35.23	3.48 × 10 ¹	99.99	7.83
Cow milk					
0	2.40 × 10 ⁸	0.00	5.14 × 10 ⁸	–	8.33
5	1.85 × 10 ⁸	9.76	1.31 × 10 ⁹	–	2.70
10	1.65 × 10 ⁸	19.51	2.34 × 10 ⁹	–	9.09
15	1.08 × 10 ⁹	0.00	7.79 × 10 ⁸	66.75	5.37
20	4.55 × 10 ⁸	56.67	2.35 × 10 ⁸	69.83	5.49
25	8.80 × 10 ⁸	16.19	3.82 × 10 ⁷	83.74	10.23

Control *S. enteritidis* count: 0–10 hours of fermentation = 2.05 × 10⁸ (CV = 2.44); 15–25 hours of fermentation = 1.05 × 10⁹ (CV = 2.39); – no inhibition

time, these results are in opposition to the defined characteristics of *Salmonella* (acidophobic).

The results obtained by the microbiological method 2 (Table 3) showed a marked inhibitory effect of both types of fermented milks on the growth of *S. enteritidis* colonies. In the fermented goat milk, *S. enteritidis* grew from the beginning of incubation to the 15th hour of fermentation. After 15 h of fermentation (pH = 4.6; CFU = 2.58 × 10⁵), the growth of *S. enteritidis* in goat milk completely stopped. The same applies to fermented cow milk. The growth of *S. enteritidis* in fermented cow milk was considerably inhibited after 15 h of

fermentation. In microbiological method 2, the differences in the degree of inhibition between fermented goat and cow milk were not statistically significant. However, the growth of *S. enteritidis* in fermented goat milk stopped completely after 20 h of fermentation (inhibition 100% after 25 h, 0 colonies of *S. enteritidis*). In fermented cow milk, the growth of *S. enteritidis* was strongly inhibited, but not completely stopped.

It is obvious that pH value and CFU of *B. longum* Bb-46 had an important role in the inhibition of *S. enteritidis* under microbiological conditions applied in method 2 (*S. enteritidis* inoculated

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

Source of variation	SS	Df	MS	$F_{\text{calculated}}$	P -value	F_{critical}
Rows*	4473.13	5.00	894.63	9.39	0.01	5.05
Columns**	826.02	1.00	826.02	8.67	0.03	6.61
Error	476.25	5.00	95.25			
Total	5775.40	11.00				

*single variations by each hour of the fermentation process; **difference in overall degree of inhibition between fermented goat and cow milk

Table 5. Results of antibiotic sensitivity tests

Disc	Inhibition zone (mm)	
	goat milk	cow milk
Unfermented sample	–	–
Sample after 12 h of fermentation	3.2	–
Sample after 24 h of fermentation	12.7	6.5

Diameters in a control series of antibiotics (mm): norfloxacin: > 25, tetracyclin: 20–25, kinolon: > 21; – no inhibition

in milk concurrently with probiotic *B. longum* Bb-46). There was a high correlation between the degree of inhibition and pH values, as well as CFU of *B. longum* Bb-46 in fermented goat and cow milk. The calculated correlation coefficients were as follows:

1. goat milk
 - inhibition degree with pH value ($r = -0.83$)
 - inhibition degree with CFU of *B. longum* Bb-46 ($r = 0.93$)
2. cow milk
 - inhibition degree with pH value ($r = -0.80$)
 - inhibition degree with CFU of *B. longum* Bb-46 ($r = 0.81$).

The antibiotic sensitivity tests are quite possibly the best way to express the *in vitro* antagonistic properties of some probiotics. The sensitivity of *S. enteritidis* to the antibiotics tested and fermented milk is reported in Table 5. *S. enteritidis* was found to be considerably more sensitive to fermented goat milk as compared to fermented cow milk. Similar results were previously reported in the study dealing with uropathogenic *E. coli* strain (SLAČANAC *et al.* 2004). Considerably larger inhibition zones were measured around all the discs with fermented goat milk samples. In the middle of the fermentation process, the zones around the discs with fermented goat milk were 3.2 mm; while around the discs with fermented cow milk no inhibition zones occurred. At the end of the fermentation process, the zones around the discs with fermented goat milk were about 95% larger than those around the discs with fermented cow milk.

CONCLUSION

Overall, the results have suggested a marked inhibitory effect of goat milk fermented with probiotic *Bifidobacterium longum* Bb-46 on the growth of *Salmonella enteritidis* D strain. The results obtained with microbiological methods 1 and 2 and

the antibiotic sensitivity tests showed a higher antagonistic potential of fermented goat milk in comparison to fermented cow milk. Additionally, the higher inhibitory potential of fermented goat milk was confirmed by antibiotic sensitivity test. The results of the analytical and microbiological analyses show that the pH values and the number of *Bifidobacterium longum* Bb-46 bacteria are in considerable correlation with the inhibition degree.

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