



# Antagonism of lactic acid bacteria towards Staphylococcus aureus and Escherichia coli on agar plates and in milk

W Fang, M Shi, L Huang, J Chen, Y Wang

## ► To cite this version:

W Fang, M Shi, L Huang, J Chen, Y Wang. Antagonism of lactic acid bacteria towards Staphylococcus aureus and Escherichia coli on agar plates and in milk. Veterinary Research, 1996, 27 (1), pp.3-12.  
hal-00902393

HAL Id: hal-00902393

<https://hal.science/hal-00902393>

Submitted on 11 May 2020

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Original article

## Antagonism of lactic acid bacteria towards *Staphylococcus aureus* and *Escherichia coli* on agar plates and in milk

W Fang\*, M Shi, L Huang, J Chen, Y Wang

Department of Veterinary Medicine, College of Animal Sciences,  
Zhejiang Agricultural University, Hangzhou, Zhejiang 310029, China

(Received 10 October 1994; accepted 22 May 1995)

**Summary** — The antagonistic effect of lactic acid bacteria (LAB, including *Lactobacillus acidophilus*, *L bulgaricus*, *L casei* and *Streptococcus thermophilus*) on *Staphylococcus aureus* and *Escherichia coli* was evaluated on MRS agar with the deferred and cross-streaking techniques, and in milk with the plate counting method. All LAB were repressive to *S aureus* and *E coli* on the agar medium. However, their suppressive activity was significantly reduced when the agar medium was buffered to pH 7.2. In normal milk, *L acidophilus* strains A and B, *S thermophilus* and its combinations with *L acidophilus* A and *L bulgaricus* 6032 were inhibitory to *S aureus*, while in mastitic milk, only *S thermophilus* and its combinations showed inhibition. *L acidophilus* A and *L bulgaricus* 34104 were repressive to *E coli* growth in normal milk. *S thermophilus* and its combinations were inhibitory to *E coli* in both the normal and mastitic milk samples. These results indicate that the antagonistic activity of LAB on pathogenic bacteria varied with the type of media in which the tests were done, and that testing of in vitro antagonism in milk would be more informative than that in artificial media for in vivo tests concerning the possible roles of competitive microbiological ecology in mastitis control.

bacterial antagonism / lactic acid bacteria / *Staphylococcus aureus* / *Escherichia coli*

**Résumé** — Antagonisme de bactéries lactiques contre *Staphylococcus aureus* et *Escherichia coli* sur gélose et dans le lait. L'effet antagoniste de bactéries lactiques (BL, y compris *Lactobacillus acidophilus*, *L bulgaricus*, *L casei* et *Streptococcus thermophilus*) sur *Staphylococcus aureus* et *Escherichia coli* a été évalué sur une gélose MRS grâce aux techniques d'inoculation différenciée et d'inoculation croisée, et dans du lait par la méthode de comptage sur gélose. Toutes les BL étaient répres-

\* Current address: Department of Pharmacology and Toxicology, Faculty of Veterinary Medicine, University of Helsinki, Hämeentie 57, Fin-00014, Helsinki, Finland

sives vis-à-vis de *S aureus* et d'*E coli* sur gélose. Cependant, leur effet suppresseur était considérablement réduit lorsque le milieu gélosé était tamponné à pH 7,2. Dans du lait normal, les souches A et B de *L acidophilus*, *S thermophilus* et ses combinaisons avec *L acidophilus* A et *L bulgaricus* 6032 inhibaient *S aureus*, alors que, dans du lait de mammite, seul *S thermophilus* et ses combinaisons provoquaient une inhibition. *L acidophilus* A et *L bulgaricus* 34104 inhibaient la croissance d'*E coli* dans du lait normal. *S thermophilus* et ses combinaisons inhibaient *E coli* à la fois dans les échantillons de lait normal et de lait de mammite. Ces résultats indiquent que l'activité antagoniste de BL sur des bactéries pathogènes varie avec les types de milieux dans lesquels les tests sont effectués : les tests d'antagonisme *in vitro* seraient plus informatifs s'ils étaient effectués dans le lait plutôt que dans un milieu artificiel, en vue d'étudier *in vivo* les rôles possibles de l'écologie microbiologique compétitive dans le contrôle des mammites.

**antagonisme bactérien / bactérie lactique / *Staphylococcus aureus* / *Escherichia coli***

## INTRODUCTION

Lactic acid bacteria (LAB), which are the normal flora of digestive and urogenital tracts (Watkins and Kratzer, 1983; Watkins and Miller, 1983; Tannock, 1984; Conway et al, 1987; Bruce and Reid, 1988; Fielding, 1989), have been reported to be antibacterial *in vitro* towards a variety of pathogenic bacteria such as *S aureus*, *S uberis* and coliforms (Dahiya and Speck, 1968; Collins and Aramaki, 1980; Gilliland and Ewell, 1983; McGroarty et al, 1988; Hechard et al, 1990; Rammelsberg and Radler, 1990; Reinheimer et al, 1990; Fang et al, 1993). Such bacterial antagonism could arise from the combined effects of several mechanisms during their growth in the media. These may include: i) lactic acid production leading to a decrease in pH and hydrogen peroxide ( $H_2O_2$ ) formation, both of which are known to be inhibitory against Gram-positive and Gram-negative bacteria; ii) competition for the available nutrients; and iii) the production of specific proteins called bacteriocins with a narrow inhibitory spectrum against some Gram-positive bacteria (Muriana and Klaenhammer, 1987; McGroarty et al, 1988; Hechard et al, 1990; Rammelsberg and Radler, 1990; Reinheimer et al, 1990).

In view of these properties, LAB have been used as probiotics or introduced into some dairy products as sources of beneficial

microorganisms to compete with the enteropathogens for control of diarrhea in human and animals (Hammes and Tichaczek, 1994). Previous studies showed that colonization of mammary glands by minor pathogens (eg, *S epidermidis* or *Corynebacterium bovis*) could confer protection against infections by major pathogens such as *S aureus* and streptococci (Rainard and Poutrel, 1988; Matthews et al, 1991). Similarly, it appears that LAB might play a part in mastitis control.

However, there have been contradictory reports concerning their efficacy. Fielding (1989) claimed that a probiotic product was effective on clinical mastitis by reducing pH of the milk and by competing for nutrients. On the contrary, Greene et al (1991a, b) reported that intramammary infusion of a probiotic product (lactobacillal species) was not effective in the treatment of clinical and subclinical mastitis. To be a possible candidate for mastitis control, LAB should, we believe, meet several criteria. It should be antagonistic against pathogenic bacteria in milk, resistant to the indigenous antibacterial factors of milk, and capable of surviving within the mammary gland. However, there seems to be a paucity of information regarding these basic aspects. The present study was carried out to investigate the *in vitro* inhibitory activity of several LAB strains against *S aureus* and *E coli* on MRS agar and in milk.

## MATERIALS AND METHODS

### Bacteria and their subculture

*E. coli* 83903 (serum resistant strain) was purchased from the China National Institute for Control of Veterinary Drugs and Bioproducts. *S. aureus* 1.89 was from the Institute of Microbiology, Chinese Academy of Sciences. Both reference pathogens were not of mastitis origin but grew well in normal and mastitic milk (Fang et al., 1993) and could induce mild clinical mastitis in our experimental mastitis models. *L. acidophilus* A was kindly provided by the China North-eastern College of Agricultural Sciences, Shenyang, China. *L. acidophilus* B was isolated from 'Lactophilus' 477224, product of Suomessa Finnpharma OY Organon AB, Helsinki, Finland, and was a gift from Prof M Sandholm. *L. bulgaricus* 6032 and *L. casei* 6028 were purchased from the Institute of Food Science, Chinese Ministry of Light Industry. The China National Institute for Control of Veterinary Drugs and Bioproducts also provided *L. bulgaricus* 34104 and *L. casei* 34103. *S. thermophilus* was purchased from the Wuxi College of Light Industrial Sciences, Jiangsu, China.

The above bacterial strains were subcultured three times in brain-heart broth (*E. coli* and *S. aureus*) and MRS (de Man, Ragosa, Sharpe) broth (LAB) at 37 °C for 18–24 h, and stored as stock cultures (with 10% glycerol) at –20 °C. The stock cultures were thawed before use and passed twice in the appropriate broth media at 37 °C for 16–18 h. Filter-sterilized whey (from mastitic milk centrifuged at 45 000 g, 60 min, 4 °C, Ultracentrifuge Model L8-55M, Beckman Co, USA) was added to the broth media (50% by volume) for adaptation in the second passage of the bacteria used for antagonism tests in mastitic milk.

### Normal and mastitic milk

Normal milk samples with somatic cell counts (SCC) less than  $2 \times 10^5$ /mL (Coulter Counter Model ZM, Coulter Electronics Ltd, UK) were collected aseptically into Erlenmeyer flasks from the quarter of a cow that was free from bacterial infections. Mastitic milk samples were aseptically taken from the mastitic quarter that was infected with

*S. dysgalactiae* (subclinical with SCC between 4–5  $\times 10^6$ /mL during the period of sampling). Both the normal and mastitic milk samples were collected during the morning milking on the day of each experiment.

### Antagonism on the agar plates

The deferred method (Barefoot and Klaenhammer, 1983) was used on the MRS agar plates (1.5%) of three types, one buffered with PBS (pH 7.2), one treated with catalase (500 µg/mL), and one left untreated. Briefly, each LAB culture was streaked (0.5 cm in width) across the surface of two pre-poured MRS agar plates. The plates were incubated at 37 °C for 24–36 h with 5% CO<sub>2</sub>. The agar layer was inverted onto the cover of the Petri dishes and the uninoculated surfaces were seeded in duplicate with the overlay soft nutrient agar (0.75%) containing *S. aureus* or *E. coli*, which were adjusted to  $10^6$  cfu/mL. The inhibitory width was observed after re-incubation at 37 °C for 18 h.

The cross-streaking technique (Woodward et al., 1987) was followed with certain modifications. Each of the LAB cultures was streaked (0.5 cm in width) down the center of two MRS agar plates as described above. The plates were incubated at 37 °C for 24–36 h with 5% CO<sub>2</sub>. When growth of the colonies became confluent, the LAB streak was removed by scraping with sterile cotton swabs. The residual growth was eliminated by inverting the plate over a chloroform-saturated filter paper. After aeration for 1–2 h to let the remaining chloroform to evaporate, *S. aureus* or *E. coli* culture ( $10^6$  cfu/mL) was streaked in duplicate at right angles to the original LAB streak. The inhibition width was recorded after the plates had been re-incubated at 37 °C for 18 h.

### Antagonism in milk samples

For bacterial antagonism tests in milk, all the bacteria were harvested by centrifugation (800 g, 15 min) and were resuspended in sterile saline. The bacterial suspensions were adjusted by photometry to a turbidity (620 nm, 1 cm light path) of 0.4 for LAB (about  $1.5 \times 10^8$  cfu/mL), and 0.15 for *S. aureus* and *E. coli* (about  $1.0 \times 10^8$  cfu/mL).

Aliquots (50 mL) of the freshly collected normal and mastitic milk samples were transferred into

sterilized Erlenmeyer flasks. The milk samples were inoculated in duplicate with 1.5 mL of each of LAB and 200 µL of *S aureus* or *E coli*. The control milk samples were inoculated with the pathogenic bacteria only. One millilitre from each flask was taken immediately after shaking for enumeration of *S aureus* or *E coli* as the zero hour counts. Both the inoculated and control milk samples were then incubated at 37 °C for 12 h, after which 1 mL was transferred from each flask for bacterial enumeration. The standard plate counting method was followed (Messer et al, 1985), using Chapman Stone Medium for *S aureus* and MacConkey Agar Medium for *E coli* (Fang et al, 1993). The pH values of the milk samples before inoculation and after 12 h incubation was also determined (pH I-70, Beckman Co, USA).

### Statistical analysis

The inhibition width of duplicate streaks or seeded layers of pathogens on two parallel agar plates was averaged. Duncan's multiple range test was used to compare the difference of inhibition among three different types of agar media (Pharm/PCS, version 4.0, MicroComputer Specialists, NY, USA). The bacterial counts of duplicate plates, at appropriate dilutions, of two parallel milk samples at hour zero were averaged and adjusted relative to the counts of the control milk, and their counts at hour 12 calibrated corresponding to this adjustment coefficient. The inhibition index (II) was expressed as the division of the counts ( $\log_{10}$  cfu/mL) of the control samples at hour 12 by those of the test samples. Thus, an index larger than 1.0 implies inhibition. Analysis of variance was used to evaluate the significance of inhibition (Excel 4.0, Microsoft Corporation, Redmont, WA, USA).

## RESULTS

All LAB strains repressed the growth of *S aureus* and *E coli* on the agar plates as determined with both the deferred method and the cross-streaking technique (table I). The inhibition seemed to be dependent on the condition of their growth on the agar. Those with affluent growth exhibited better

inhibition, as in the case of *L casei* strains 6028 and 34103, and *L acidophilus* A. There was less inhibition for all LAB in PBS-buffered MRS agar plates as compared with the untreated controls ( $P < 0.05$ ). Supplementation of catalase in the agar medium reduced the inhibition of *S aureus* of varying extent by LAB, with only two LAB strains being significantly affected ( $P < 0.05$ , table I).

*L acidophilus* strains A and B, *S thermophilus* and its combinations with *L acidophilus* A and *L bulgaricus* 6032 were inhibitory to the growth of *S aureus* in normal milk. In mastitic milk, only *S thermophilus* and its combinations exhibited inhibition (table II). Table III shows that *S thermophilus* and its combinations were also repressive to *E coli* in mastitic milk, and repressive to this pathogen in normal milk were *L acidophilus* A, *L bulgaricus* 34104, *S thermophilus* and its combinations. Instead of showing inhibition, as on the agar, the two *L casei* strains promoted the growth of both pathogens in either normal or mastitic milk. There appeared to be no synergic inhibition with the combinations of *S thermophilus* and lactobacilli.

## DISCUSSION

All LAB exhibited inhibition of varying degrees to *S aureus* and *E coli* on the agar plates (table I). Their inhibitory effect markedly decreased on the PBS-buffered agar as compared with the untreated agar ( $P < 0.05$  in all cases), suggesting that the lactic acid produced during their growth was responsible, to a considerable extent, for their antagonistic activity. The decreased inhibition of *L acidophilus* B and *L bulgaricus* 34104 against *S aureus* on the catalase-supplemented MRS agar (table I,  $P < 0.05$ ) might indicate that they were H<sub>2</sub>O<sub>2</sub> producers. There seemed to be no difference in inhibition between the conventional MRS agar and the MRS agar supplemented with

**Table I.** Inhibition of *S. aureus* and *E. coli* by LAB strains on the agar media.

		Inhibition width (mean $\pm$ SD, cm)*					
		Staphylococcus aureus			Escherichia coli		
		Agar 1	Agar 2	Agar 3	Agar 1	Agar 2	Agar 3
<i>L. acidophilus</i> A	DM	3.35 $\pm$ 0.26 <sup>a</sup>	2.55 $\pm$ 0.26 <sup>b</sup>	3.1 $\pm$ 0.24 <sup>a</sup>	3.8 $\pm$ 0.38 <sup>a</sup>	2.0 $\pm$ 0.26 <sup>b</sup>	3.65 $\pm$ 0.21 <sup>a</sup>
	CS	2.65 $\pm$ 0.26 <sup>a</sup>	1.9 $\pm$ 0.17 <sup>b</sup>	2.35 $\pm$ 0.24 <sup>a</sup>	3.25 $\pm$ 0.21 <sup>a</sup>	1.85 $\pm$ 0.26 <sup>b</sup>	3.15 $\pm$ 0.24 <sup>a</sup>
<i>L. acidophilus</i> B	DM	2.55 $\pm$ 0.21 <sup>a</sup>	1.65 $\pm$ 0.24 <sup>b</sup>	1.95 $\pm$ 0.26 <sup>b</sup>	3.15 $\pm$ 0.17 <sup>a</sup>	2.15 $\pm$ 0.21 <sup>b</sup>	3.2 $\pm$ 0.26 <sup>a</sup>
	CS	2.2 $\pm$ 0.18 <sup>a</sup>	1.4 $\pm$ 0.16 <sup>b</sup>	1.7 $\pm$ 0.22 <sup>b</sup>	2.75 $\pm$ 0.24 <sup>a</sup>	1.65 $\pm$ 0.21 <sup>b</sup>	2.65 $\pm$ 0.19 <sup>a</sup>
<i>L. bulgaricus</i> 6032	DM	1.95 $\pm$ 0.24 <sup>a</sup>	1.2 $\pm$ 0.14 <sup>b</sup>	1.65 $\pm$ 0.19 <sup>a</sup>	2.4 $\pm$ 0.18 <sup>a</sup>	2.0 $\pm$ 0.22 <sup>b</sup>	2.3 $\pm$ 0.25 <sup>a</sup>
	CS	1.5 $\pm$ 0.26	1.35 $\pm$ 0.13	1.3 $\pm$ 0.19	2.35 $\pm$ 0.26 <sup>a</sup>	1.6 $\pm$ 0.22 <sup>b</sup>	2.45 $\pm$ 0.29 <sup>a</sup>
<i>L. bulgaricus</i> 34104	DM	2.0 $\pm$ 0.18 <sup>a</sup>	1.55 $\pm$ 0.19 <sup>b</sup>	1.6 $\pm$ 0.22 <sup>b</sup>	2.65 $\pm$ 0.24 <sup>a</sup>	2.2 $\pm$ 0.23 <sup>b</sup>	2.7 $\pm$ 0.29 <sup>a</sup>
	CS	1.65 $\pm$ 0.24 <sup>a</sup>	1.2 $\pm$ 0.22 <sup>b</sup>	1.35 $\pm$ 0.25 <sup>ab</sup>	2.45 $\pm$ 0.34	2.1 $\pm$ 0.24	2.4 $\pm$ 0.22
<i>L. casei</i> 6028	DM	3.3 $\pm$ 0.26 <sup>a</sup>	2.5 $\pm$ 0.28 <sup>b</sup>	3.25 $\pm$ 0.25 <sup>a</sup>	3.85 $\pm$ 0.29 <sup>a</sup>	3.15 $\pm$ 0.21 <sup>b</sup>	3.95 $\pm$ 0.26 <sup>a</sup>
	CS	3.0 $\pm$ 0.3 <sup>a</sup>	2.1 $\pm$ 0.15 <sup>b</sup>	2.75 $\pm$ 0.2 <sup>a</sup>	3.65 $\pm$ 0.26	3.3 $\pm$ 0.32	3.75 $\pm$ 0.29
<i>L. casei</i> 34103	DM	3.1 $\pm$ 0.22 <sup>a</sup>	2.25 $\pm$ 0.21 <sup>b</sup>	2.9 $\pm$ 0.24 <sup>a</sup>	3.9 $\pm$ 0.18 <sup>a</sup>	3.25 $\pm$ 0.31 <sup>b</sup>	4.0 $\pm$ 0.22 <sup>a</sup>
	CS	2.5 $\pm$ 0.22 <sup>a</sup>	2.0 $\pm$ 0.21 <sup>b</sup>	2.4 $\pm$ 0.18 <sup>a</sup>	3.6 $\pm$ 0.25 <sup>a</sup>	2.1 $\pm$ 0.29 <sup>b</sup>	3.45 $\pm$ 0.31 <sup>a</sup>

\* Agar 1, normal MRS agar; agar 2, PBS-buffered MRS agar; and agar 3, catalase-supplemented agar. Means in the same row followed by different lower-case letters differ at  $P < 0.05$  level. DM: the deferred method. CS: the cross-streaking method.

**Table II.** Antagonism of LAB strains on *S aureus* in normal and mastitic milk.

	Staphylococcus aureus <sup>a</sup>					
	Normal milk			Mastitic milk		
	$\log_{10}$ cfu/mL	II <sup>b</sup>	pH <sup>c</sup>	$\log_{10}$ cfu/mL	II <sup>b</sup>	pH <sup>c</sup>
Control	7.45 ± 0.12		6.2	7.59 ± 0.12		6.2
<i>L acidophilus</i> A	7.23 ± 0.1	1.03*	3.7	7.62 ± 0.1	0.99	4.6
<i>L acidophilus</i> B	7.11 ± 0.09	1.05**	4.7	7.6 ± 0.09	0.99	4.3
<i>L bulgaricus</i> 6032	7.4 ± 0.11	1.01	5.5	7.36 ± 0.13	1.03	4.5
<i>L bulgaricus</i> 34104	7.41 ± 0.07	1.01	4.3	7.5 ± 0.12	1.01	5.1
<i>L casei</i> 6028	7.53 ± 0.09	0.99	6.1	8.09 ± 0.07	0.94**	5.9
<i>L casei</i> 34103	7.6 ± 0.12	0.98	6.0	7.86 ± 0.1	0.97*	5.7
<i>S thermophilus</i>	6.57 ± 0.13	1.13**	5.0	5.99 ± 0.12	1.27**	4.4
<i>S thermophilus</i> + <i>L acidophilus</i> A <sup>d</sup>	6.98 ± 0.09	1.07**	5.0	6.29 ± 0.1	1.21**	5.2
<i>S thermophilus</i> + <i>L bulgaricus</i> 6032 <sup>d</sup>	7.02 ± 0.06	1.06**	5.0	6.19 ± 0.11	1.23**	4.3

<sup>a</sup> Staphylococcal counts at hour 12 (mean ± SD), the initial counts were between 10<sup>5</sup>–10<sup>6</sup>/mL ( $\log_{10}$  cfu/mL = 5–6). <sup>b</sup> II = Inhibition index, \*  $P < 0.05$ ; \*\*  $P < 0.01$ ; same for table III. <sup>c</sup> pH value at hour 12, the initial pH = 6.42 in normal milk and 6.49 in mastitic milk, same for table III. <sup>d</sup> LAB combinations: 0.75 mL each of the bacteria for inoculation, same for table III.

catalase in the case of catalase-producing *E coli*. These findings were generally in agreement with the earlier reports that the lactobacillial antagonism might be ascribed in part to their lactic acid and H<sub>2</sub>O<sub>2</sub> production (Dahiya and Speck, 1968; Juffs and Babel, 1975; Collins and Aramaki, 1980; Fontaine and Taylor-Robinson, 1990; Hechard et al, 1990; Reinheimer et al, 1990).

However, their spent media (bacterial cell-free filtrates) showed no inhibition against *S aureus* and *E coli* even after the

plates filled in with the media were first stored at 4 °C for 10 h for improved diffusion (well-diffusion method, data not shown). One reason might be that the lactic acid present in the spent media was not enough to reduce pH of the agar through diffusion and thus to inhibit the bacterial growth. The LAB on the agar plates might have produced more lactic acid during their active growth phase, which was enough to reduce pH of the agar for the inhibitory effect. Another possibility would be that the antibacterial factors produced during their growth in the

**Table III.** Antagonism of LAB strains on *E coli* in normal and mastitic milk.

	Escherichia coli <sup>a</sup>					
	Normal milk			Mastitic milk		
	<i>Log</i> <sub>10</sub> cfu/mL	II <sup>b</sup>	pH <sup>c</sup>	<i>Log</i> <sub>10</sub> cfu/ml	II <sup>b</sup>	pH <sup>c</sup>
Control	8.12 ± 0.04		6.2	7.54 ± 0.1		6.0
<i>L acidophilus</i> A	7.86 ± 0.08	1.03**	3.7	7.65 ± 0.11	0.99	5.1
<i>L acidophilus</i> B	8.05 ± 0.08	1.01	4.6	7.76 ± 0.1	0.97*	5.0
<i>L bulgaricus</i> 6032	8.11 ± 0.09	1.0	4.9	7.69 ± 0.1	0.98	4.7
<i>L bulgaricus</i> 34104	7.81 ± 0.07	1.04**	4.7	7.53 ± 0.11	1.0	5.1
<i>L casei</i> 6028	8.24 ± 0.1	0.99	5.5	7.99 ± 0.11	0.94**	5.2
<i>L casei</i> 34103	8.23 ± 0.09	0.98	5.5	8.16 ± 0.1	0.92**	5.3
<i>S thermophilus</i>	7.69 ± 0.09	1.06**	5.0	6.82 ± 0.09	1.11**	4.3
<i>S thermophilus</i> + <i>L acidophilus</i> A	7.75 ± 0.05	1.05**	4.8	7.14 ± 0.11	1.06**	4.3
<i>S thermophilus</i> + <i>L bulgaricus</i> 6032	7.98 ± 0.07	1.02*	5.0	7.12 ± 0.09	1.06**	4.2

<sup>a</sup> *E coli* counts at hour 12 (mean ± SD), the initial counts were between 10<sup>5</sup>–10<sup>6</sup>/mL (*log*<sub>10</sub> cfu/mL = 5–6). <sup>b, c</sup> See table II.

broth medium were short-lived or removed by filter-sterilization.

In milk, only some LAB strains or their combinations (ie, *S thermophilus* + *L acidophilus* or + *L bulgaricus*) exhibited inhibition that had a statistical difference ( $P < 0.05$  or  $P < 0.01$ ) as compared with the control samples (tables II and III). Even with these effective strains, there was no satisfactory inhibition when the bacterial counts of *S aureus* and *E coli* in the associated bacterial cultures at hour 12 were compared with their initial counts (10<sup>5</sup>–10<sup>6</sup>/mL) at hour zero. These results are different from some previous reports on the inhibition of

pathogenic or psychrotrophic bacteria by LAB (Gilliland and Speck, 1972, 1974; Juffs and Babel, 1975; Collins and Aramaki, 1980; Gilliland and Ewell, 1983; Feresu and Nyati, 1990; Reinheimer et al, 1990). In these studies, however, the milk samples were either autoclaved before inoculation or the associated cultures in raw milk samples were maintained at low temperature (4 °C). These conditions were different from ours.

The discrepancy of results among various reports is, therefore, understandable. Firstly, heat treatment would destroy the indigenous antibacterial factors and affect the growth patterns of both the LAB and the

pathogenic bacteria. It is evident that the inhibitory effect would be dependent on the relative growing strength of the two different bacteria in the same milk environment. *S aureus* and *E coli* seemed to grow better than LAB in both the normal and mastitic milk samples, and mastitic milk appeared to inhibit the growth of LAB (Fang et al, 1993). This might explain why LAB were unable to exert satisfactory inhibition against the pathogenic bacteria (especially in mastitic milk) even though the size of their inocula was about seven-fold more than that of the latter (tables II and III). Secondly, there would be no bacterial growth when the milk samples are maintained at 4 °C. Yet, some LAB might produce H<sub>2</sub>O<sub>2</sub> during storage even at low temperatures to inhibit the growth of psychrotrophic bacteria (Dahiya and Speck, 1967; Juffs and Babel, 1975; Collins and Aramaki, 1980; Gilliland and Ewell, 1983). This means that the difference in incubation temperature of the associated cultures may account for different results among reports.

Tables II and III show that all the milk samples with associated bacterial cultures had lower pH than the controls. The reduced pH may be responsible in part for the inhibitory effect in milk as in the agar media (table I), but it does not mean that reduction of the milk pH would be certain to cause inhibition. Associated cultures with some of the LAB exhibited no inhibition at all in normal or mastitic milk irrespective of the reduced pH. This also implies that the antagonistic effect of some LAB in milk might result from the combined activity of factors such as competition for nutrients, H<sub>2</sub>O<sub>2</sub>, lactic acid and possibly bacteriocins.

The present study clearly shows that there was disagreement of the test results between the agar medium and milk. All LAB exhibited inhibition on the agar plates while only some of them were inhibitory in milk. Rather than showing strong inhibition as on the agar, two *L casei* strains promoted the growth of *S*

*aureus* and *E coli* in milk (tables II and III). According to our observations in this experiment and our previous results (Fang et al, 1993), the inhibitory effect may be related to the condition of LAB's growth in the media. *L casei* grew quite well on the agar media but poorly in milk compared with other LAB strains, while *L bulgaricus* did the reverse. This suggests that in vitro antagonistic testing in milk would be more informative than that in artificial media in directing in vivo tests on potential roles of competitive microbiological ecology in mastitis control.

However, there are several concerns about the 'introduction' of LAB into the mammary gland. One is the effect of low pH on udder health. Our later experiments showed that there were only two LAB strains that could be recovered in limited numbers (about 1–3 × 10<sup>3</sup> cfu/mL milk) for up to 5 d after intramammary infusion (1 × 10<sup>7</sup> cfu per quarter) (unpublished data). Instead of a decrease in pH as in milk in vitro (tables II and III), the pH of milk samples from infused quarters was slightly higher than that before infusion. This could be ascribed to suppression of LAB's growth within the gland due to constant influx of indigenous antibacterial factors, a condition different from that in vitro. Because of the constant host responses, it is actually less likely to have unlimited growth of any bacteria within the udder, creating a low pH in milk. For instance, there have been no reports of slurry milk (due to low pH) from quarters infected by *S agalactiae* having good lactose-fermenting ability. This may also apply to LAB. In this sense, selection of LAB should not be based on their acid-producing ability in vitro.

Another concern is the increase of SCC or total bacterial counts in milk following colonization of LAB within the udder during lactation. There would be paradoxical consequences of elevated SCC, reduced milk yield and milk hygiene, and possible therapeutic or preventive values. It is evident

that SCC would remain elevated along with the persistence of the infused LAB, as in the case of some minor pathogens such as *S epidermidis* or *C bovis* (Rainard and Poutrel, 1988; Matthews et al, 1991). However, none of the LAB strains in this study was unable to survive within the udder and SCC returned to the pre-infusion levels several days after disappearance of LAB from the milk samples (unpublished data). This might be one of the reasons for the failure of a probiotic product on mastitis although no information was given about the survival of LAB in milk from the infused quarters (Greene et al, 1991a, b).

Therefore, the hypothesis of bacterial competition for mastitis control could be justified on a certain LAB strain with antagonistic activity and resistibility (to the host response): i) if it is to be used to prevent dry cow mastitis by colonizing the selected LAB strain on the teats (Woodward et al, 1988); or ii) if there is no long-term persistence of the infused LAB strain when used to treat lactational mastitis. In the latter case, it seems less likely to find an LAB strain that could meet two incompatible requirements: survival and growth within the udder to compete with the pathogenic bacteria, and self-limiting withdrawal from the udder afterwards. Instead of using reference LAB strains or strains as feed additives (probiotic products), isolation of field LAB strains from the mouth of sucking calves or the teat surface of adult cows could be one possibility to find certain competent strains. It is recommended, then, that in vitro testing of their growth characteristics and antagonistic activity be done in milk and the pathogenic bacteria from mastitis cases be used for more informative results.

## ACKNOWLEDGMENTS

This work is part of the research supported by the International Foundation for Science, Stockholm, Sweden (Grant B/1724-1), and the Zhe-

jiang Provincial Natural Science Foundation (No 390049). Our thanks are due to M Sandholm (Finland) and A Hill (UK) for their advice.

## REFERENCES

- Barefoot SF, Klaenhammer TR (1983) Detection and activity of lactacin B, a bacteriocin produced by *Lactobacillus acidophilus*. *Appl Environ Microbiol* 45, 1808-1815
- Bruce AW, Reid G (1988) Intravaginal installation of lactobacilli for prevention of recurrent urinary tract infections. *Can J Microbiol* 34, 339-343
- Collins EB, Aramaki K (1980) Production of hydrogen peroxide by *Lactobacillus acidophilus*. *J Dairy Sci* 63, 353-357
- Conway PL, Gorbach SL, Goldin BR (1987) Survival of lactic acid bacteria in the human stomach and adhesion to intestinal cells. *J Dairy Sci* 70, 1-12
- Dahiya RS, Speck ML (1968) Hydrogen peroxide formation by lactobacilli and its effect on *Staphylococcus aureus*. *J Dairy Sci* 51, 1568-1572
- Fang WH, Shi MH, Huang LQ, Shao QJ, Chen J (1993) Growth of lactobacilli, *Staphylococcus aureus* and *Escherichia coli* in normal and mastitic milk and whey. *Vet Microbiol* 37, 115-125
- Feresu S, Nyati H (1990) Fate of pathogenic and non-pathogenic *Escherichia coli* strains in two fermented milk products. *J Appl Bacteriol* 69, 814-821
- Fielding J (1989) Probiotics and animal health. *Proc World Asso Vet Food Hygienists 10th Intl Sym, July 2-7, Stockholm, Sweden*, Relkam and Katalogtryck, Uppsala, 30
- Fontaine EA, Taylor-Robinson (1990) Comparison of quantitative and qualitative methods of detecting hydrogen peroxide produced by human vaginal strains of lactobacilli. *J Appl Bacteriol* 69, 326-331
- Gilliland SE, Speck ML (1972) Interactions of food starter cultures and food-borne pathogens: lactic streptococci versus staphylococci and salmonellae. *J Milk Food Technol* 35, 307-310
- Gilliland SE, Speck ML (1974) Antagonism of lactic streptococci toward *Staphylococcus aureus* in associative milk cultures. *Appl Microbiol* 28, 1090-1093
- Gilliland SE, Ewell HR (1983) Influence of combinations of *Lactobacillus lactis* and potassium sorbate on growth of psychrotrophs in raw milk. *J Dairy Sci* 66, 974-980
- Greene WA, Gano AM, Smith KL, Hogan JS, Todhunter DA (1991a) Comparison of probiotic and antibiotic intramammary therapy of cattle with clinical mastitis. *Bovine Practitioner* 26, 119-122
- Greene WA, Gano AM, Smith KL, Hogan JS, Todhunter DA (1991b) Comparison of probiotic and antibiotic

- intramammary therapy of cattle with elevated somatic cell counts. *J Dairy Sci* 74, 2976-2981
- Hammes WP, Tichaczek PS (1994) The potential of lactic acid bacteria for the production of safe and wholesome food. *Z Lebensm Unters Forsch* 198, 193-201
- Hechard Y, Dherbomez M, Cenatiempo Y, Letellier F (1990) Antagonism of lactic acid bacteria from goats' milk against pathogenic strains assessed by the 'sandwich method'. *Lett in Appl Microbiol* 11, 185-188
- Juffs HS, Babel FJ (1975) Inhibition of psychrotrophic bacteria by lactic cultures in milk stored at low temperature. *J Dairy Sci* 58, 1612-1619
- Matthews KR, Harmon RJ, Langlois, BE (1991) Effect of naturally occurring coagulase-negative staphylococci infections on new infections by mastitis pathogens in the bovine. *J Dairy Sci* 74, 1855-1859
- McGroarty JA, Angotti R, Cook RL (1988) *Lactobacillus* inhibitor production against *Escherichia coli* and coaggregation ability with uropathogens. *Can J Microbiol* 34, 344-351
- Messer JM, Behney HM, Leudecke LO (1985) Microbiological Count Method. In: *Standard Methods for the Examination of Dairy Products* (GH Richardson, ed), Port City Press Inc, 15th ed, Baltimore, MD, 133-146
- Muriana PM, Klaenhammer TR (1987) Conjugal transfer of plasmid-encoded determinants for bacteriocin production and immunity in *Lactobacillus acidophilus* 88. *Appl Environ Microbiol* 53, 553-560
- Rainard P, Poutrel B (1988) Effect of naturally occurring intramammary infections by minor pathogens on new infections by major pathogens in cattle. *Am J Vet Res* 49, 327-329
- Rammelsberg M, Radler F (1990) Antibacterial polypeptides of *Lactobacillus* species. *J Appl Bacteriol* 69, 177-184
- Reinheimer JA, Demkow MR, Candioti MC (1990) Inhibition of coliform bacteria by lactic cultures. *Aus J Dairy Technol* 45, 5-9
- Tannock DW (1984) Control of gastrointestinal pathogens by normal flora. In: *Current Perspectives in Microbial Ecology* (MJ Klug, CA Reddy, eds), Am Assoc Microbiol, Washington, DC, 374-382
- Watkins BA, Kratzer FH (1983) Effect of oral dosing of lactobacillus strains on gut colonization and liver biotin in broiler chicks. *Poultry Sci* 62, 2088-2094
- Watkins BA, Miller BF (1983) Competitive gut exclusion of avian pathogens by *Lactobacillus acidophilus* in gnotobiotic chicks. *Poultry Sci* 62, 1772-1779
- Woodward WD, Besser TE, Ward ACS, Corbeil LB (1987) In vitro growth inhibition of mastitis pathogens by bovine teat skin normal flora. *Can J Vet Res* 51, 27-31
- Woodward WD, Ward ACS, Fox LK, Corbeil LB (1988) Teat skin normal flora and colonization with mastitis pathogen inhibitors. *Vet Microbiol* 17, 357-365