Applied and Environmental Microbiology, Mar. 2009, p. 1790–1792 Vol. 75, No. 6
0099-2240/09/$08.00+0 doi:10.1128/AEM.01943-08

Interaction between Lactic Acid Bacteria and *Mycobacterium bovis* in Ethiopian Fermented Milk: Insight into the Fate of *M. bovis*▽

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Received 20 August 2008/Accepted 12 January 2009

***Mycobacterium bovis* causes tuberculosis in animals and humans. Infected cows can transmit the bacillus to humans via milk. Milk also contains lactic acid bacteria (LAB). LAB isolated from milk were put in milk cultures together with spiked *M. bovis*. Different LAB had different abilities to reduce *M. bovis* counts, as *M. bovis* was undetectable in some while it persisted in two of the cultures.**

Mycobacterium bovis causes tuberculosis (TB) at both pulmonary and extrapulmonary sites in animals and humans (3, 4, 12). One way that transmission of *M. bovis* from animals to humans occurs is via consumption of contaminated milk. In Ethiopia, >80% of the population lives in the countryside, farming and rearing cattle. Traditionally, milk is fermented at household temperatures, which can range from 15 to 24°C depending on the locality, in clay pots that are first cleaned and smoked to add flavor and aroma to the milk. Smoking slows the souring process, as do low temperatures (5). Fermented milk can be stored at household temperatures and consumed for up to 20 days (5).

Information on the prevalence of *M. bovis* infection with animal origin in humans in Ethiopia is scarce. One study (G. Ameni, P. Bonnet, and M. Tibbo, unpublished report) found a high infection rate in cattle from 12 dairy farms. In Ethiopia and elsewhere in Africa, *M. bovis* has been isolated from raw milk (3). In TB-afflicted children in Ethiopia, the proportion of extrapulmonary TB was found to be high in those who frequently drink raw milk (6), and reports from the WHO also showed that, overall, the notification rate of extrapulmonary TB was high (35% of new cases) (13).

Lactic acid bacteria (LAB) are found in various habitats, including milk (10, 11). Several reports on the enumeration and identification of LAB from different African fermented milk samples showed that mean LAB counts range between 10^7 to 10^9 CFU/ml of fermented milk (1, 5, 7, 9). LAB may inhibit pathogens in foods by producing antimicrobial peptides and/or acid, in addition to the competitive exclusion of pathogens (2, 8). This study made use of milk cultures with or without LAB, into which *M. bovis* was spiked, and was intended to give insight about the fate of *M. bovis* in milk during the natural fermentation process, as determined by plating.

Isolation of LAB, collection of milk, and growth of *M. bovis*. LAB were isolated earlier from fermented milk on LAB media (MRS agar, plate count agar [PCA], and M17 agar; Oxoid Ltd., Basingstoke, England), as summarized in Table 1, and were stored in a freezer for future use in milk cultures. Milk to be used for cultures was collected hygienically from a skin test-negative cow and placed into sterile containers. To produce bacterial suspension for spiking, *M. bovis* ATCC 19210 was grown in Middlebrook 7H9 broth containing 0.05% Tween 80, 10% oleic acid-albumin-dextrose-catalase, and 0.4% pyruvate. This laboratory-grown strain may not behave exactly like *M. bovis* from the milk of infected cattle, but this experiment serves as a starting point to assess the in vivo situation.

Milk culture setup. Preliminary experiments showed that heating milk at 80°C for 10 min kills all resident microflora. A summary of the milk culture setup, including whether heat treatment was used, LAB was added, and *M. bovis* was spiked, is given in Table 2. Milk was divided into 1-liter portions each in 2-liter presterilized dark glass, screw-cap bottles, heat treated, and cooled. One milliliter of *M. bovis* (optical density at 580 nm, 0.700) culture was spiked into milk and gently shaken. Then, 100 μl of individual (milk cultures 1 to 5) or mixed (milk culture 6) LAB (optical density at 600 nm, 0.600) was added. The bottles, along with the controls (milk cultures 7 and 8), were kept at room temperature (20 to 22°C) in a cardboard box within the P3 laboratory.

Enumeration of *M. bovis*. On days 0, 3, 7, and 14, 10 ml from each milk culture was withdrawn after gentle agitation of the bottle, mixed with 5 ml phosphate-buffered saline (PBS) to aid in homogenization, and centrifuged at 1,000 × *g* for 90 min at 20°C using a Beckman GS-6 centrifuge and Beckman GH-3.8 rotor. The pellet and cream were resuspended in 2 ml PBS, 10-fold serial dilutions (1 ml into 9 ml PBS) were made, and 2 ml of the first and second dilutions treated by adding 7.2 μl of an antibiotic cocktail (consisting of chloramphenicol at 4 μg/ml, trimethoprim at 8 μg/ml, polymyxin B at 100 U/ml, ampicillin at 2 μg/ml, gentamicin at 0.5 μg/ml, and ketoconazole at 0.4 μg/ml [Sigma Chemical Co.]) and left standing for 1 h. Then, portions were spread onto Middlebrook 7H10 agar plates containing the antibiotic cocktail at the concentrations described above in triplicate. Plates were placed in a 37°C incubator. Counting of the colonies was done between the fourth and sixth weeks and was repeated up to six times. Plots of mean *M. bovis*

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▽ Published ahead of print on 23 January 2009.

TABLE 1. Media and conditions for LAB isolation from fermented milk and presumptive identification[a]

Isolate	Isolation condition			Method of presumptive identification								Presumptive identification
	M	T (°C)	A/An	CM	GS	C	NR	Growth in 5% NaCl	Growth at 45°C	Growth at 10°C	V	
LAB1	MRS	30	An	Coccobacilli	+	−	−	G	NG	G	S	Mesophilic LAB
LAB2	M17	30	A	Cocci	+	−	−	G	NG	G	S	Lactococci
LAB3	PCA	30	A	Coccobacilli	+	−	−	G	NG	NG	S	ND
LAB4	MRS	42	An	Rods	+	−	−	NG	G	G	S	*Lactobacillus* sp.
LAB5	MRS	42	An	Mostly cocci	+	−	−	NG	G	NG	S	*Streptococcus thermophilus*

[a] M, medium; T, temperature; A/An, aerobic/anaerobic; CM, colony morphology; GS, Gram stain; C, catalase; NR, nitrate reductase; +, positive; −, negative; V, vancomycin; G, growth; NG, no growth; S, sensitive; ND, not determined.

counts were generated using GraphPad Prism version 4.01 (GraphPad Software, Inc.).

In milk cultures 1, 2, and 3, *M. bovis* was undetectable by day 14, while it persisted in milk cultures 4 and 5 (Fig. 1A). In milk culture 6, which contained LAB mix, *M. bovis* was undetectable by day 7 (Fig. 1A). *M. bovis* continued to grow in milk culture 7, to which no LAB were added, whereas it was undetectable in milk culture 8, which contained indigenous LAB, by day 14 (Fig. 1B). All those plates without growth were inspected for a further 4 weeks (beyond 6 weeks). No growth was observed, indicating that LAB eliminated *M. bovis*. For these milk cultures, the lowest detectable limit of *M. bovis* was estimated to be 1 to 2 CFU/ml. At the concentrations of the antibiotic cocktail used, there was no significant difference in CFU between *M. bovis* plated with or without antibiotics ($P > 0.05$).

The *M. bovis* strain used for spiking the milk cultures was able to grow in milk in the absence of LAB, as evidenced by colony appearance in milk culture 7 and also as determined by plating 2 μl of the *M. bovis* suspension used for spiking onto Middlebrook 7H10 plates, which gave viable counts of 4.83×10^3 CFU/ml and 5.76×10^4 CFU/ml on days 3 and 7, respectively.

To assess if LAB were still present in the milk cultures, 100 μl from some of the milk cultures (2, 6, and 8) was plated onto MRS agar and PCA on day 21 of culture. Plates were incubated at 37°C for 48 h. Thus, milk cultures 2, 6, and 8 contained LAB at levels of 7.20×10^4, 4.80×10^4, and 1.60×10^4 CFU/ml, respectively. However, these counts were probably underestimates, since different LAB species have different growth requirements for temperature and aerobiosis or anaerobiosis. The elimination of *M. bovis* by LAB might be quickened if the diversity of LAB species increases.

It was beyond the scope of this work to determine the actual mechanisms of inhibition for *M. bovis* by LAB. However, the results of this experiment are consistent with previous experiments conducted in this laboratory with the same objective.

TABLE 2. Milk culture setup showing which was heat treated and to which LAB and/or *M. bovis* was added

Milk culture	Heat treatment	LAB added (isolate)	*M. bovis* spiked
1	Yes	Yes (LAB1)	Yes
2	Yes	Yes (LAB2)	Yes
3	Yes	Yes (LAB3)	Yes
4	Yes	Yes (LAB4)	Yes
5	Yes	Yes (LAB5)	Yes
6	Yes	Yes (LAB mix)	Yes
7	Yes	No	Yes
8	No	No	Yes

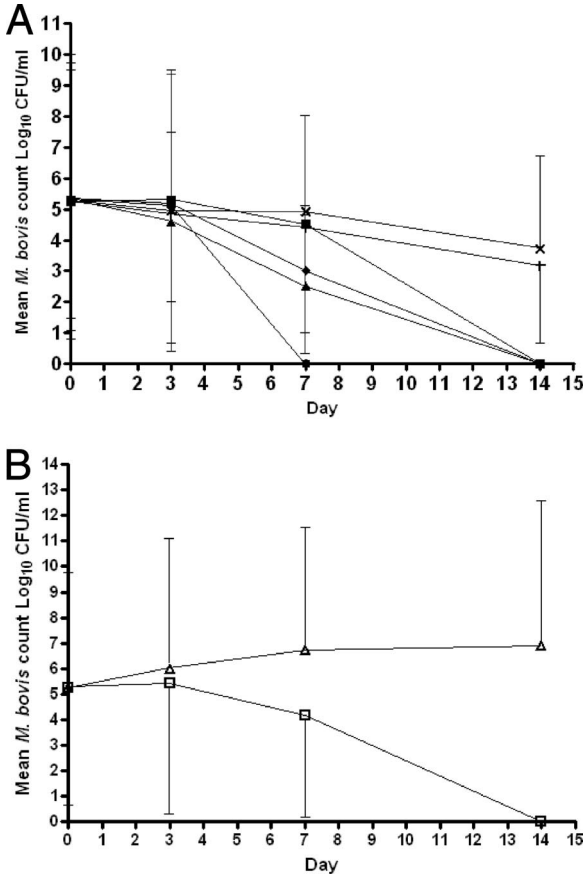

FIG. 1. *M. bovis* counts enumerated by plating dilutions of pellet and cream fractions of milk cultures from the LAB-*M. bovis* interaction study. Data presented at each time point are means ± standard deviations, where *n* is 3. (A) *M. bovis*-spiked cultures with LAB added. Symbols: ◆, LAB1 (milk culture 1); ■, LAB2 (milk culture 2); ▲, LAB3 (milk culture 3); ×, LAB4 (milk culture 4); +, LAB5 (milk culture 5); ●, LAB mix (milk culture 6). (B) *M. bovis*-spiked cultures without LAB added. Symbols: Δ, milk culture 7; □, milk culture 8 (contains indigenous LAB).

I thank Susanna Grundstrom, Morten Harboe, and Sven Hoffner for material assistance and Liya Wassie for technical assistance.

This work was supported by the AHRI core budget. AHRI is supported by NORAD and Sida.

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