Volume 118, Nos. 1–3, July–September 2004, ISSN: 0273–2289

Applied Biochemistry and Biotechnology

Editor-In-Chief: **Ashok Mulchandani**

Emerging Frontiers at the Interface of Chemistry and Biology

Proceedings From the International Conference on the Interface of Chemistry and Biology April 28–30, 2003, Trivandrum, India

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0273-2289/04/118/0001/$25.00

Lactic Acid Bacteria Used in Inoculants for Silage as Probiotics for Ruminants

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Received March 27, 2003; Revised September 1, 2003; Accepted September 2, 2003

Abstract

Many studies have shown the beneficial effects on ruminant performance of feeding them with silages inoculated with lactic acid bacteria (LAB). These benefits might derive from probiotic effects. The purpose of the current study was to determine whether LAB included in inoculants for silage can survive in rumen fluid (RF), as the first step in studying their probiotic effects. Experiments were conducted in the United States and Israel with clarified (CRF) and strained RF (SRF) that were inoculated at 10^6–10^8 microorganisms/mL with and without glucose at 5 g/L. RF with no inoculants served as control. Ten commercial inoculants were used. The RF was incubated at 39°C and sampled in duplicates at 6, 12, 24, 48, 72, and 96 h for pH and LAB counts. The results indicate that with glucose the pH of the RF decreased during the incubation period. In the SRF, the pH of the inoculated samples was higher than that of the controls in most cases. This might be a clue to the mechanism by which LAB elicit the enhancement in animal performance. LAB counts revealed that the inoculants survived in the RF during the incubation period. The addition of glucose resulted in higher LAB counts.

Index Entries: Lactic acid bacteria; silage inoculants; rumen fluid; ruminant performance; probiotics.

Introduction

Ensiling is a method of preserving moist forage that is widely used in North America, Europe, Israel, and elsewhere. It is based on natural fermentation whereby lactic acid bacteria (LAB) ferment water-soluble

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carbohydrates into organic acids, mainly lactic, under anaerobic conditions. As a result, the pH decreases, inhibiting detrimental anaerobes and preserving the nutritional value and palatability of the moist forage.

Inoculants that include LAB are often used as silage additives to enhance lactic acid fermentation and, hence, to better preserve the ensiled crops. Most commercially available inoculants contain homofermentative LABs, which are fast and efficient producers of lactic acid and thus improve the silage fermentation. Among the homofermentative LAB most frequently used are *Lactobacillus plantarum*, *Enterococcus faecium*, *Pediococcus acidilactici*, *Pediococcus pentosaceus*, and *Lactobacillus acidophilus*. Heterofermentative LAB are also sometimes included in inoculants for silage, because they produce volatile fatty acids (VFA), which inhibit the yeasts and molds that are activated on aerobic exposure of the silage. The recommended application rates of these products are usually 10^5–10^6 viable cells/g, which are often sufficient to enable the inoculant LAB population to overtake those of the epiphytic LAB and become the predominant population in the silage. The results of many studies involving silage inoculants are summarized by McDonald et al. *(1)* and in various reviews (*see* ref. *2*).

The results of various studies indicated that feeding cattle with silages that had been treated with certain LAB also improved ruminant performance. In that context, it should be mentioned that certain LAB are also believed to induce probiotic effects in humans (e.g., *see* refs. *3* and *4*). Improvements in animal performance are in many cases the principal economic justification for inoculant use. In 25–40% of the reported trials, inoculants exhibited substantial effects on performance. Average increases in intake, live-weight gain, milk production, and feed efficiency were 4–11, 7–11, 3–5, and 9%, respectively *(5,6)*.

A considerable number of animal experiments with low-dry-matter (DM) grass silage inoculated with *L. plantarum* MTD1 were performed in Northern Ireland (e.g., *see* refs. *7–9*). Many studies on MTD1 found improvements in both silage fermentation and animal performance. However, in some studies there was an improvement in animal performance although there was no apparent effect on fermentation (in some cases, both the control and treated silages were of good quality; in others, clostridial fermentation occurred in both). In some studies there was an improvement in fermentation but no apparent effect on animal performance. Rooke and Kafilzadeh *(10)* compared the effects of three LAB strains on silage quality and animal performance. All three strains improved fermentation similarly, but only MTD1 resulted in a significant increase in DM intake in wethers, which indicates that this phenomenon might be strain specific.

The effects on animal performance of LAB inoculants in silages other than grass are varied. Kung et al. *(11)*, who used two different inoculants in corn silage, observed tendencies to higher fat-corrected milk yields and higher DM intake only with MTD1, which agrees with the results obtained in low-DM grass silage. Sanderson *(12)* did not obtain any enhancement of fiber degradability when he used corn silage inoculated with *L. plantarum*

and *E. faecium*. Recently, Salawu et al. *(13)* observed increased rates of *in situ* degradation of nitrogen and fiber in bicrop pea/wheat silage inoculated with *L. plantarum* and *Lactobacillus buchneri*.

The evidence cited indicates that inoculants sometimes have a positive probiotic effect on ruminant performance, the mechanism of which is as yet unclear. It could be that LAB interact with rumen microorganisms in such a way that their activity is enhanced and fiber degradability is improved. Another possibility is that LAB produce bacteriocins in the silage, and that these might inhibit detrimental microorganisms, both in the silage and in the rumen.

The first step in studying the probiotic mechanism of LAB in ruminants is to determine whether they survive in rumen fluid (RF). The purpose of the present study was to determine whether LAB used in inoculants for silage can survive in RF. We summarize the findings of experiments performed in both the United States and Israel.

Materials and Methods

RF was collected for each experiment from two fistulated Holstein cows in each location. In the United States, the cows were fed on a total mixed ration containing 30% DM alfalfa silage, 30% corn silage, 10% soluble soybean meal, 30% ground shell corn, and supplemental vitamins and minerals. In Israel, the dry cows were fed on 6 kg of wheat hay and 4 kg of DM total mixed ration containing 30% concentrated grains, 35% wheat and corn silage, 15% soybean and sunflower meal, 20% byproducts (cotton seed, wheat bran, and gluten feed), and supplemental vitamins and minerals. The RF was strained (SRF) through cheesecloth or clarified (CRF) by setting for 1 h in Imhoff cones, centrifuging at 26,000*g* for 1 h, and passing through a hollow-fiber dialysis cartridge. CRF was used only in the United States.

The RF (SRF or CRF) was subdivided into sterile Erlenmeyer flasks, each of which was inoculated with a commercial LAB silage inoculant (*see* next section). The CRF was inoculated at 10^6 colony-forming units (CFU)/mL, and the SRF at 10^7 and 10^8 CFU/mL. RF with no LAB inoculant served as a control. The inoculated RF was further subdivided and to one half was added sterile 50% (w/v) glucose solution to a final concentration of 5 g/L. The various treatments were added (7–9 mL) to sterile vials that were flushed with CO_2 before sealing. The tubes were incubated at 39°C. At 6, 12, 24, 48, 72, and 96 h after inoculation, two vials from each treatment were sampled for analysis.

Inoculants

The following commercial inoculants for silage were used:

1. *L. plantarum* MTD1 (Ecosyl, Yorkshire, UK).
2. *P. pentosaceus* (Ecosyl).
3. *L. plantarum* (Agri-King, Fulton, IL).

4. *L. pentosus* (Agri-King).
5. *P. pentosaceus* (Agri-King).
6. *E. faecium* (C) (Agri-King).
7. *E. faecium* (Q) (Agri-King).
8. *L. buchneri* (Biotal Canada Limited, Calgary, AB, Canada).
9. 11A44 Pioneer™ containing *L. buchneri* (Pioneer Hi-Bred, Johnston, IA).
10. 1188 Pioneer™ containing *L. plantarum* and *E. faecium* (Pioneer Hi-Bred).

The number of LAB cells in the dry products was determined before the experiments by suspending the inoculants in deionized water and pour plating serial dilutions into Rogosa SL agar or MRS agar (Difco Becton Dickinson, Sparks, MD). MRS agar was used for all products containing *E. faecium*. The inoculants were applied by suspending an adequate weight (according to the LAB number in the product) in 100 mL of tap water and using 1 mL of the suspension to treat 200 mL of RF, or by adding the adequate weight directly to 200 mL of RF.

The enumeration of LAB was done with pour plates. MRS agar was used for the *Enterococcus* spp.; all other inoculated treatments were enumerated on Rogosa SL agar. Plates were incubated at 30°C for 3 d. Statistical analysis of data from a given time point was performed with the GLM procedure of SAS (SAS Institute, Cary, NC), and where factors were significant, differences between treatments were determined by the least significant difference method ($p < 0.05$).

Results

The pH of the fresh RF was 5.6–5.8 in the experiments in the United States and 5.9–6.9 in Israel. This reflects differences between the two locations in the status of the cows and variations in their rations in both places. The number of LAB in the fresh SRF was about 10^6 CFU/mL in the United States and 10^4 to 10^5 CFU/mL in Israel. In the United States, without glucose addition, the pH decreases ranged from 0 to 0.1 U for CRF and from 0.1 to 0.3 U for SRF; with glucose addition they ranged from 0.03 to 0.4 U for CRF and from 0.3 to 0.5 U for SRF. In the experiments with SRF in Israel, without glucose the pH decreased by 0.1–0.4 U but then increased again; with glucose it decreased by 0.8 to 1.4 U. Final pH was affected significantly ($p < 0.05$) by glucose addition in all experiments and by inoculant in some experiments. In experiments with CRF, inoculant and glucose-inoculant interaction parameters were significant in most cases. In Israel (experiments with SRF), the minimal pH values obtained were affected mainly by the pH value of the fresh RF and by glucose addition. Increasing the inoculation rate from 10^7 to 10^8 CFU mL did not markedly affect the decrease in pH, but within an experiment it changed the order of the inoculants with respect to the lowest pH values obtained, especially with glucose addition. A surprising result was observed with SRF in the experiments in both Israel and the United States: in most cases the pH of the inoculated SRF was higher than the pH of the controls (Figs. 1 and 2).

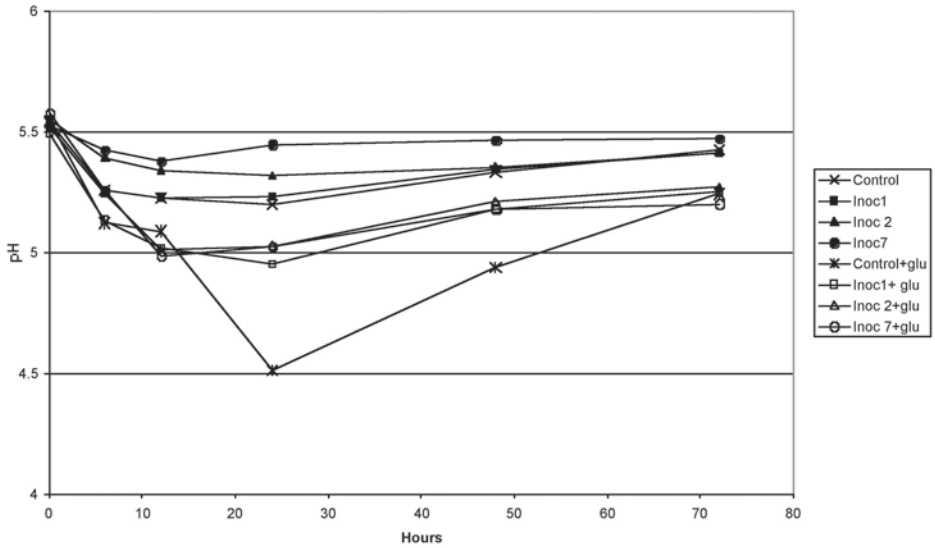

Fig. 1. Changes in pH in SRF during incubation with silage inoculants (from experiments in the United States).

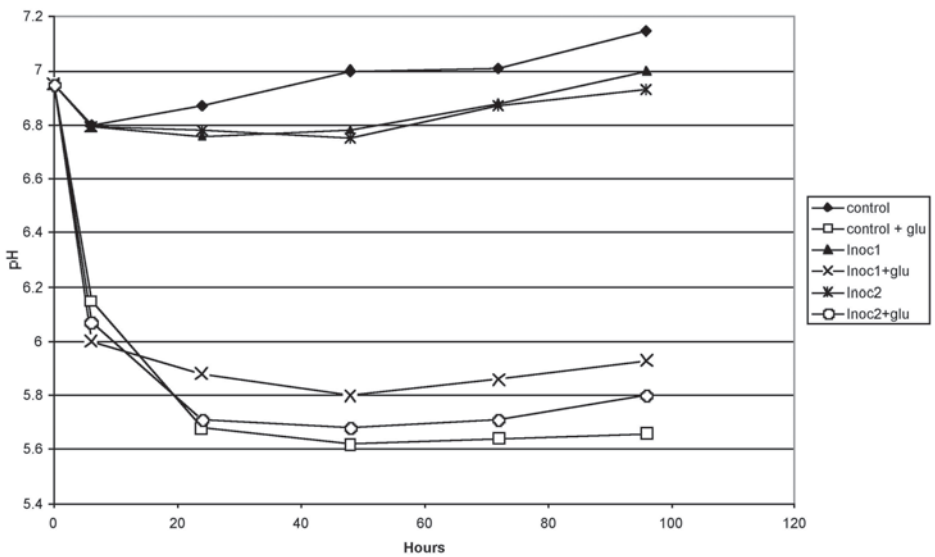

Fig. 2. Changes in pH in SRF during incubation with silage inoculants (from experiments in Israel).

After 72 h, LAB counts in CRF without glucose remained at 10^6 CFU/mL or decreased to 10^5 CFU/mL; with glucose their numbers increased to 10^7 to 5×10^7 CFU/mL. In SRF in the United States, LAB counts, including those in the controls, were within ± 1.5 log units of the initial values (Fig. 3). Glucose addition significantly raised LAB counts, but inoculant did not affect final LAB counts. In some cases, after 24 h LAB numbers decreased

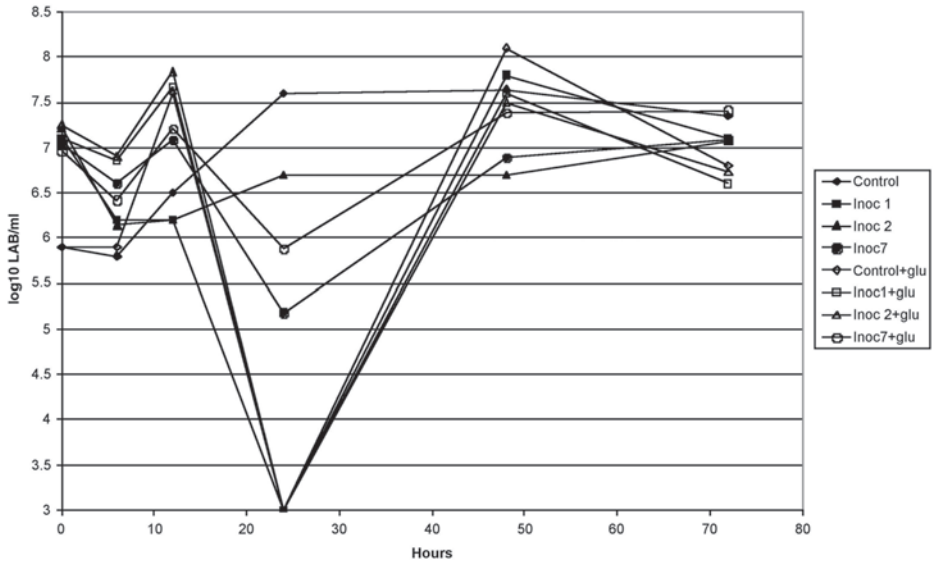

Fig. 3. LAB in SRF during incubation with silage inoculants (from experiments in the United States).

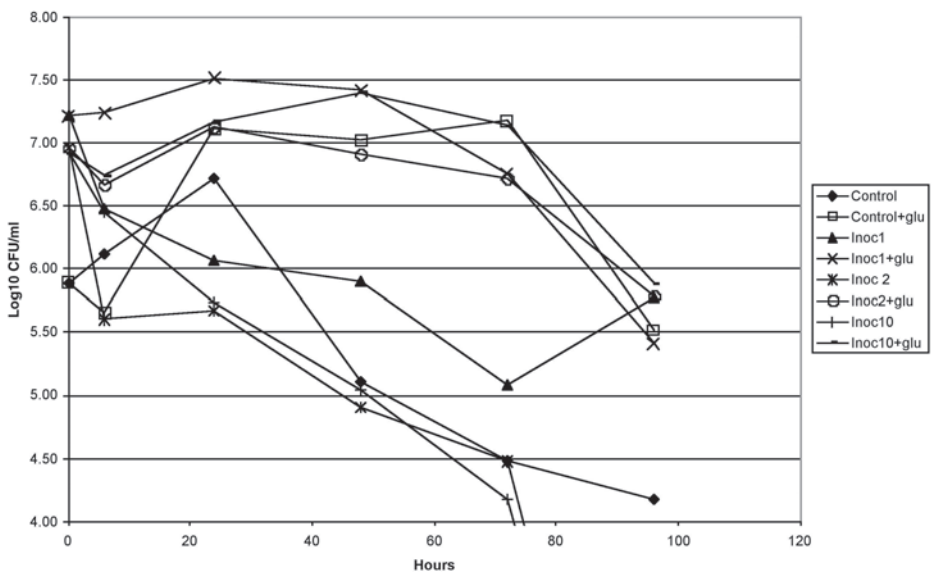

Fig. 4. LAB in SRF during incubation with silage inoculants (from experiments in Israel).

below detectable level (10^4 CFU/mL), and, instead, the agar was covered with many tiny colonies. Later in the incubation there was a recovery in LAB counts and the colonies appeared normal. The detectable level of 10^4 CFU/mL was determined arbitrarily and lower counts were considered as nonsurviving levels. We do not know as yet which organisms

formed the numerous tiny colonies, but they might have been LAB that did not develop into normal colonies. The interaction of glucose and inoculant was significant, and significant differences between the LAB counts from different inoculants with and without glucose were observed.

LAB counts in SRF in experiments in Israel decreased by 2 to 3 log units during incubation (Fig. 4). During most of the incubation period, LAB counts in the inoculated SRF plus glucose were higher than those in the controls, and higher inoculation rates tended to result in higher LAB counts. Without glucose, counts of some inoculants were already below the detectable level (log10 = 4) after 24 h, especially with the lower inoculation rates (inoculants 3–5).

Discussion

The present experiments were conducted within a broader investigation that aims to find out how LAB silage inoculants enhance ruminant performance. The first step is to determine whether such LAB can survive and grow under rumen-like conditions. The CRF was used first in order to determine whether the chemical composition of the RF is detrimental to the survival of LAB and to test the survival of the silage inoculants in RF without competition with microorganisms already present in the RF. However, the SRF provides a better simulation of the conditions prevailing in the rumen, because it allows competition between the inoculant LAB and the indigenous rumen microflora. The inoculation rates for each set of experiments (CRF or SRF) were chosen so that the LAB could grow or compete with the existing microorganisms. In addition, the inoculation rate for the SRF was comparable with the numbers of LAB ingested by cows that receive 45 kg of silage (wet weight) daily in their rations.

The results of our study indicate that the tested LAB were able to survive and, in many cases, to grow in both CRF and SRF. In some experiments with SRF, the numbers of LAB decreased during the incubation period. Sharp et al. *(14)* attributed loss of LAB in SRF to protozoa predation. As expected, glucose addition markedly enhanced the survival of the inoculant LAB in the RF, and their effect on the pH of the RF. Some strains grew better than others, but the differences were not consistent between the experiments that were conducted in the United States and in Israel, respectively.

The observation that the pH values of the LAB-inoculated samples of SRF were consistently higher than those of the respective controls was surprising, because in silage, LAB cause a rapid decrease in pH because of their production of organic acids, mainly lactic acid. The various strains also differed in their ability to buffer pH, both with and without supplemental glucose. This phenomenon suggests that the question of which rumen microorganisms predominate is more likely to be influenced by the mode of action of the inoculants in the RF than by direct fermentation of substrates by the LAB in the rumen. Certainly, higher rumen pH might

enhance the functionality of specific rumen microorganisms, especially in cases when the pH decreases following high-energy feeding *(15)*. In addition, improved digestibility of fiber in inoculated silage in cattle has been reported *(6)*, and this buffering effect may be a possible explanation because growth of ruminal fibrolytic bacteria is known to be inhibited at pH < 6.0 *(16)*. The study conducted in the United States indicated higher VFA concentrations in RF inoculated with *L. plantarum* MTD1, as compared with other inoculants *(17)*. How higher concentrations of VFA affect animal performance is not yet clear.

The pH values of the fresh RF differed among the various experiments performed in the United States and Israel, and these differences could have been caused by seasonal, or cow-to-cow variations, or differences in feeding. The question of how these variations might affect the LAB mode of action in the rumen is not yet clear and warrants more research. Our hypothesis is that lower pH values in the RF would favor LAB in their competition with rumen microorganisms.

To elucidate the mechanism by which LAB exert beneficial probiotic effects on ruminants, more research is needed to study their effect on fiber degradation and possible bacteriocin production. Preliminary tests have indicated that a few of the inoculants exhibited bacteriocin activity.

Conclusion

The results of our study indicate that silage inoculant LAB can survive in RF. LAB bring about some changes in the RF, e.g., in pH and VFA composition. How these changes affect animal performance is not yet clear and needs more research.

Acknowledgments

The Agricultural Research Organization, the Volcani Center, Bet Dagan, Israel, contributed (no. 401/03-E, 2003 series). This research was supported by research grant no. IS-3297-02 from BARD, The United States–Israel Binational Agricultural Research and Development Fund.

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