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The effect of *Lactobacillus buchneri* and *L. plantarum*, applied at ensiling, on the ensiling fermentation and aerobic stability of wheat and sorghum silages

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The effect of applying *Lactobacillus buchneri* (LB), alone or in combinations with *L. plantarum* (LP) and yeasts at ensiling, on the ensiling fermentation and aerobic stability of wheat and sorghum silages was studied under laboratory conditions. Treatments comprised LB, LP, yeasts, LB + yeasts, LP + yeasts, LB + LP and B-589 (a lactic acid bacterial strain isolated from wheat silage in Israel) alone. The treatments were also applied to sterilized aqueous extracts of wheat which were incubated at 30°C for 10 days. The pH of all treatments was below 4.0 already on day 4 of the experiment. Silages treated with LB had higher acetic acid concentrations than those treated with LP: 32–34 *vs* 16–18, and 28–34 *vs* 4–7 g kg^{-1} in the experiments with wheat and sorghum, respectively. Similar results were obtained in wheat extracts. In the aqueous phase, marked differences in pH decrease were noticed among the treatments: 4.4 in LB, 6.0 in the yeast, and 3.7 in LP and B-589 (from day 3 and onwards). In both crops LB resulted in aerobically stable silages when applied alone or with LP and yeasts, whereas LP resulted in unstable silages upon aerobic exposure; the stability of the LB-treated silages is attributed to the higher acetic acid concentrations. The isolated strain (B-589) did not exhibit any advantage with regard to aerobic stability.

Keywords: *Lactobacillus buchneri*; *Lactobacillus plantarum*; silage; aerobic stability

Introduction

Ensiling is a conservation method for moist crops. It is based on natural fermentation under anaerobic conditions, whereby epiphytic lactic acid bacteria (LAB) convert water-soluble carbohydrates (WSC) into organic acids, mainly lactic acid (LA). As a result, the pH decreases and the forage is preserved.

It is possible to apply bacterial inoculants at ensiling in order to promote adequate fermentation patterns. In many parts of the world commercial silage inoculants are available; they usually comprise strains of homofermentative LAB such as *Lactobacillus plantarum* (LP), *Enterococcus faecium*, and *Pediococcus* species. When used, such inoculants often result in a faster decrease in pH, lower final pH values, higher lactate:acetate ratios (obtained by increasing lactic and decreasing acetic acid production), lower ammonia nitrogen (mainly because of reduced protein breakdown as a result of a faster decrease in pH) and a 1–2% improvement of dry matter (DM) recovery [10].

However, homofermentative LAB inoculants sometimes impair the aerobic stability of silages by enhancing the aerobic deterioration of mature cereal silages by increasing numbers of yeast and fungi [5,6,11]. Such inoculants enhanced the aerobic deterioration of mature cereal silages by increasing numbers of yeasts and fungi [11]. Aerobic deterioration of silage is not only associated with high DM losses, but also with a risk of mycotoxin production in the feed by aerobic fungi; such mycotoxins are detrimental to animal health. Weinberg *et al* [11] hypothesized that 'high levels of residual WSC, combined with high LA concentrations and lack of sufficient concentrations of protective volatile fatty acids (VFA, such as acetic, propionic and butyric) in the silages inoculated with homofermentative LAB were associated with aerobic spoilage. This is because both WSC and LA are substrates for fungi and yeasts and VFA often inhibit these organisms'. Legume silages are probably stabilized by indigenous factors which are inhibitory to fungi (eg, saponins).

These findings stimulated the search for bacterial strains which might be suitable as silage inoculants and might also protect the silage upon aerobic exposure. Aerobic stability is important because silage is exposed to air during storage and feedout. One option is to add a heterofermentative LAB that would produce VFA in the silage and so stabilize it during aerobic exposure. In this process some DM might be lost as CO_2; however, such losses may be smaller than the losses caused by aerobic microorganisms during exposure to air. *L. buchneri* (LB) is one of the heterolactic microorganisms which might be used in silage in order to improve its aerobic stability.

Moran [9] compared the effects of LB and LP in low-DM grass silage. LB resulted in unsatisfactory fermentation, with high DM losses; LP did not have a marked effect compared with the control, and no mention was made of the effect of LB on aerobic stability.

Driehuis *et al* [2] reported that in maize silage, LB treatment resulted in conversion of lactic acid into other products, mainly acetic acid. The improved aerobic stability was attributed to the change in the VFA profile. Another

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study [3] indicated that LB degraded lactic acid to acetic acid and to 1,2-propanediol in maize silage, and suggested a metabolic pathway for this process; improved aerobic stability is attributed to both these products.

The purpose of the present work was to study the effects of the application of *L. buchneri* to wheat and sorghum silages, on the ensiling fermentation, yeast population and aerobic stability. This was done by application of the microorganisms both to laboratory silages and to sterilized wheat extracts.

Materials and methods

Ensiling wheat in glass jars

Wheat at the milk stage of maturity was chopped to 2 cm and ensiled in 1.5-L glass jars (Weck, Wher-Oftlingen, Germany) equipped with a lid that enables gas release only. Each jar was filled with about 800 g (wet weight) of chopped wheat, without a headspace, and stored at ambient temperature (25–27°C). Three jars per treatment were sampled on days 4, 12 and 65, for chemical and microbiological analyses. At the end of the experiment, the silages were subjected to an aerobic stability test lasting 5 days, in a system described in Ashbell *et al* [1]. In this system, CO_2 production, change in pH, number of yeasts and molds and visual appraisal serve as spoilage indicators.

Lactic acid bacteria and yeasts were used in the present experiments. Yeasts were used in order to challenge the aerobic stability of the silages: *Lactobacillus plantarum* (LP), ATCC 8014 (American Type Culture Collection, Manassas, VA); *Lactobacillus buchneri* (LB), NRRL B-1837 (USDA Northern Regional Research Center, Peoria, IL; donated by Dr LK Nakamura); *Lactobacillus* sp TUB B-589 (Technical University of Budapest, Hungary; isolated from wheat silage in Israel); *Hansenula subpelliculosa* OKI 595 (H) (National Institute of Public Health, Budapest, Hungary); TUB Y-56 (Technical University of Budapest, Hungary, isolated from air-exposed wheat silage in Israel). All microorganisms were stored as freeze-dried cultures. Before use, the lactobacilli were suspended in a sterilized broth the composition of which was (g L^{-1}): sucrose (BDH 102745C, BDH, Poole, UK), 20; corn steep liquor 50% solids (Sigma c-4648, Sigma, St Louis, MO, USA), 10; yeast extract (Difco, Detroit, MI, USA), 2; KH_2PO_4 l; $MgSO_4 \cdot 7H_2O$ and NaCl, 0.5 each; a solution of trace elements, containing manganous, zinc, cobalt and ferrous salts was also added. The pH of the broth was 5.5. The yeasts were suspended in potato dextrose broth (Difco). The suspensions were incubated at 30°C, and enumerated 3 days prior to use by plating serial dilutions of the lactobacilli on pour plate Rogosa agar (Oxoid CM627, Oxoid, Basingstoke, UK), and of the yeasts on spread plate malt extract agar (Difco) acidified with lactic acid to pH 4.0. The plates were incubated for 3 days at 30°C.

Treatments used: Control (no additives), LP, LB, Y-56, LP + Y-56, LB + Y-56 and B-589 alone. The number of lactobacilli in the suspensions was 1.5×10^9, and of the yeasts 10^7 ml^{-1}. The treatments were applied by spraying 50 ml of each suspension over 15 kg of the chopped wheat, spread over a 1×4-m area, and mixing thoroughly.

The lactobacilli were applied at 5×10^6 (which is similar to the recommended application rate of commercial LAB inoculants), and the yeasts at 3×10^4 CFU g^{-1}, respectively.

Experiments with aqueous wheat extract

Chopped wheat was extracted by mixing it with distilled water for 30 min in plastic bags at a ratio 1:2.5. The extract was filtered through gauze and distributed among tubes closed by rubber stoppers equipped with gas-release valves. The tubes, each containing 30 ml extract, were autoclaved at 121°C for 15 min. After cooling, 0.5 ml of the microorganism suspensions was added aseptically to each extract. Thus, the tubes contained *ca* 1.5×10^8 and 10^5 lactobacilli and yeasts, respectively. The treatments applied were the same as those in glass jars. The tubes were incubated at 30°C; there were six tubes per treatment, three of which were sampled after 3 days and the remaining three after 7 days. The latter tubes remained in the incubator for an additional 3 days with stoppers loosened, to simulate aerobic exposure.

Ensiling forage sorghum in glass jars

Forage sorghum FS5 (Dekalb, Plant Genetics, Lubbock, TX, USA) at the milk stage of maturity was chopped and ensiled in glass jars, as described above. Treatments included control, LP, LB, LP + LB, H, LP + H, LB + H, LP + LB + H. The treatments were applied by suspending 1 ml of microorganisms in 9 ml distilled water and spraying the suspension over 2.5 kg chopped forage. According to microbial counts, the lactobacilli and the yeasts were applied at 10^6 CFU g^{-1}. There were three jars per treatment. The jars were opened after 103 days of ensiling, and the silages were subjected to an aerobic stability test as described above.

Analytical procedures

Dry matter was determined by oven drying for 48 h at 60°C. Water-soluble carbohydrates were determined by the phenol sulphuric acid method [4]. Lactic acid, ethanol and volatile fatty acids were determined in aqueous extracts using a gas chromatograph with a semi-capillary FFAP column (Hewlett Packard, Waldbronn, Germany), over a temperature range of 45–230°C. Gas losses were calculated according to weight loss.

Microbiological evaluation included enumeration of lactobacilli and yeasts and molds, using the methods described above.

Statistical analysis included one-way analysis of variance and Duncan's multiple range test, which were applied to the silage results using the Statistical Analysis System (SAS, Cary, NC, USA).

Results

Fermentation profiles of the pure microorganisms indicated that the major fermentation product of LP and B-589 was lactic acid (2.5%), that of LB comprised both lactic and acetic acids (1.0 and 0.6%, respectively), and that of H and Y-56 was ethanol at concentrations of 0.5 and 0.07%, respectively.

Table 1 Chemical analyses of the final wheat silages (day 60)

Treatment	g kg^{-1} dry matter			
	WSC	Ethanol	Acetic acid	Lactic acid
C	39 ± 1^{a}	6 ± 1	25 ± 4^{c,d}	63 ± 5
LP	36 ± 7^{a,b}	7 ± 2	16 ± 1^{e}	61 ± 8
LB	9 ± 2^{e}	7 ± 3	32 ± 5^{a,b}	53 ± 6
Y-56	20 ± 6^{d}	6 ± 2	27 ± 3^{b,c}	52 ± 7
LP + Y-56	29 ± 2^{b,c}	6 ± 1	19 ± 2^{d,c}	50 ± 7
LB + Y-56	10 ± 2^{e}	6 ± 2	34 ± 2^{a}	55 ± 10
B-589	28 ± 3^{e}	5 ± 2	18 ± 3^{e}	53 ± 4

WSC, water-soluble carbohydrates.
Within a column, means followed by the same letter did not differ significantly ($P < 0.05$) in Duncan's multiple range test.

Ensiled wheat in glass jars

Fresh wheat contained 284 ± 3 and 60 ± 10 g kg^{-1} DM and WSC, respectively, 10^4 lactobacilli and <100 yeast CFU g^{-1}, and its pH was 6.4. After 4 days of ensiling the pHs of all silages were 3.9–4.1, regardless of treatment.

The DM content of the final wheat silages was around 275 g kg^{-1}, and the pH 3.8–3.9, the somewhat higher values being associated with the LB and LB + Y-56 treatments. Gas losses were around 0.5% for all treatments except for LB and LB + Y-56, where they were 0.8%. Table 1 summarizes the other results of chemical analyses. Residual WSC content was lowest and acetic acid content was highest in the LB and LB + Y-56 treatments. Differences in lactic acid content were not significant ($P < 0.05$).

Table 2 gives the microbiological data of the wheat silages during ensiling. In the early stages of fermentation (until day 12), the number of LAB increased to 10^8 CFU g^{-1}, and then decreased to 10^5 CFU g^{-1}, without marked differences among treatments. This is typical of silage fermentation. Yeast numbers were around 10^4 CFU g^{-1} in the early stages of ensiling; in the final samples (day 65), the Y-56 treatment had the largest yeast numbers; yeasts were not found in the C, LB, LB + Y-56 and B-589 treatments.

Table 3 gives the results of the aerobic stability test. The LB and LB + Y-56 treatments resulted in the most stable silages as indicated by absence of pH change and of CO_2 production, and low yeast counts. The Y-56 treatment resulted in stable silages, whereas LP was the most unstable.

Table 2 Microbiological data of the wheat silages

Treatment	$\log_{10}$ CFU g^{-1} FM					
	LAB (days)			Yeast (days)		
	4	12	60	4	12	60
C	8.7	8.6	5.9	5.3	4.5	NF
LP	8.7	8.5	4.7	4.8	4.8	4.0
LB	8.5	8.3	5.8	4.6	3.3	NF
Y-56	8.7	8.7	8.7	4.3	3.2	8.6
LP + Y-56	8.5	8.5	4.0	4.9	4.6	3.0
LB + Y-56	8.7	8.4	5.5	4.9	3.0	NF
B-589	8.6	10.0	4.3	NF	3.9	NF

NF, not found.

Table 3 Results of the aerobic stability test of the wheat silages

Treatment	pH	CO_2 (g kg^{-1} DM)	Yeasts
C	4.0 ± 0.1^{b}	15 ± 11^{b}	8.0
LP	5.0 ± 1.0^{a}	38 ± 9^{a}	9.0
LB	3.8 ± 0.0^{b}	0^{c}	5.0
Y-56	3.9 ± 0.2^{b}	4 ± 4^{c,b}	8.7
LP + Y-56	4.0 ± 0.2^{b}	17 ± 9^{b}	9.9
LB + Y-56	4.1 ± 0.1^{b}	0^{c}	5.0
B-589	4.1 ± 0.3^{b}	17 ± 12^{b}	9.1

Yeast numbers are given as $\log_{10}$ CFU^{-1} FM.
Treatment C also had molds $\log_{10}$ CFU^{-1} FM = 5.0.
Within a column, means followed by the same letter did not differ significantly ($P < 0.05$) in Duncan's multiple range test.

Aqueous wheat extract

Table 4 gives the results of chemical analyses of the wheat extracts after 7 days of incubation. The values obtained after 7 days were not markedly different from those obtained after 3 and 10 days of incubation. The pH of the fresh extract was 6.6 and its WSC content 79 ± 9 g kg^{-1}. Ethanol levels were low in all samples and are not given. In the aqueous phase, pH differed among treatments: Y-56, LB and LB + Y-56 had significantly higher values than LP and B-589. Inoculation of yeasts together with the LP or LB treatment resulted in higher pH values than LAB alone. This is different from the fermentation in the jars, where epiphytic LAB had a marked effect. As expected, LP and B-589 resulted in the highest lactic acid and LB in the highest acetic acid production in the extracts.

Table 5 gives the microbiological data of the fermented wheat extracts. Yeasts were found only in those treatments inoculated with Y-56; LAB numbers on day 7 did not differ markedly among treatments (except for the uninoculated control), and on day 10, the LP and B-589 treatments contained the lowest LAB counts. We cannot explain the presence of LAB in the Y-56 treatment.

Ensiled sorghum in glass jars

The fresh sorghum contained 288 ± 0 and 149 ± 20 g kg^{-1} DM and WSC, respectively and its pH was 5.7.

The DM content of the final sorghum silages was around 290 g kg^{-1} in most treatments, and in LB and LB + H it was 270 g kg^{-1}. The final pH values were 3.7–3.8 in most treatments, and 3.9–4.0 in the control, and the LB and LB + H treatments. Gas losses were around 0.7–0.9% for all treatments, except for LB and LB + H where they were 1.6–1.7% ($P < 0.05$). Residual WSC content was lowest and acetic acid highest in the LB and LB + H treatments, similar to wheat (Table 6). The acetic acid concentration in silages treated with LP was lowest; treatments comprising both LP and LB resulted in lactic and acetic acid concentrations which were approximately average values of those obtained with the two types of inoculants separately.

Table 7 gives the microbiological data of the sorghum. The fresh crop contained about 10^7 CFU g^{-1} DM of lactobacilli, yeasts and molds; in the silages, the lowest numbers

Table 4 Chemical analyses of the wheat extracts after 7 days of incubation

Treatment	pH	g kg^{-1}		
		WSC	Acetic acid	Lactic acid
Fresh extracts	6.6 ± 0.0	79 ± 9		
C	6.6 ± 0.1^{a}	50 ± 11^{a}	0^{c}	0^{c}
LP	3.7 ± 0.0^{d}	32 ± 6^{b,c}	0.31 ± 0.06^{b}	2.88 ± 0.24^{a}
LB	4.4 ± 0.0^{c}	39 ± 4^{b}	0.52 ± 0.10^{a}	1.37 ± 0.23^{b}
Y-56	6.0 ± 0.2^{b}	35 ± 3^{b,c}	0.25 ± 0.01^{b,c}	0.95 ± 0.06^{b}
LP + Y-56	4.6 ± 0.3^{c}	35 ± 6^{b,c}	0.27 ± 0.04^{b,c}	1.82 ± 0.62^{b}
LB + Y-56	5.8 ± 0.2^{b}	37 ± 3^{b,c}	0.21 ± 0.02^{b,c}	0.98 ± 0.10^{b}
B-589	3.7 ± 0.0^{d}	28 ± 5^{c}	0.12 ± 0.11^{c}	3.73 ± 0.96^{a}

Within a column, means followed by the same letter did not differ significantly ($P < 0.05$) in Duncan's multiple range test.

Table 5 Microbiological data of the wheat extracts

Treatment	$\log_{10}$ CFU g^{-1} FM					
	LAB (days)			Yeast (days)		
	3	7	10	3	7	10
C	NF	NF	NF	NF	NF	NF
LP	7.3	7.5	6.3	NF	–	NF
LB	8.5	7.5	8.7	NF	NF	2.8
Y-56	5.0	8.0	7.7	5.8	5.9	4.2
LP + Y-56	6.7	7.9	8.4	6.3	6.1	5.7
LB + Y-56	8.1	7.1	8.2	5.9	6.4	7.0
B-589	6.7	7.3	6.9	NF	2.2	2.5

LAB, lactic acid bacteria; NF, not found; FM, fresh material.

of LAB were associated with treatments comprising combinations of LP and LB or LP, LB and H. With regard to yeast numbers in the silages, surprisingly, none was detected in treatments comprising H. Mold numbers were generally low.

Table 8 gives the results of the aerobic stability test of the sorghum silages. Similarly to the wheat silages, treatments associated with LP resulted in poor aerobic stability, whereas the control, LB and H treatments resulted in stable silages. In combinations of LP and LB, the latter inoculant was able to counteract the detrimental effect of LP on the aerobic stability.

Discussion

The experiments described were designed to test the efficacy of *Lactobacillus buchneri* as a silage inoculant intended to enhance aerobic stability of mature whole plant cereal silages. For that purpose, LB was added alone, or challenged in combinations with LP and yeasts. LP was chosen because it is usually contained in commercial silage inoculants and because it exerts a detrimental effect on the aerobic stability of such silages [11]. Yeasts were used to challenge aerobic stability of the silages because they are known to be involved in aerobic deterioration of silages [7,13]. The idea of using TUB B-589 as a silage inoculant in this study evolved from the belief that crop specificity of isolates might be important to the ensiling process [10]; however, it did not exhibit any advantage. The fact that yeasts were not found in the B-589 treatment (Table 2) did not imply that this strain inhibits yeasts or stabilizes the silage upon aerobic exposure (Table 3).

The results clearly indicate that the LB inoculant maintained the aerobic stability of both the wheat and the sorghum silages, and these findings are in agreement with those of Driehuis *et al* [2]. Furthermore, LB was able to protect the aerobic stability of the silages, even in the presence of LP and yeasts. This property can be explained by the higher acetic acid concentrations found in these treatments, which inhibit fungi [8] or with other metabolites produced by this microorganism [3]. In silages which are

Table 6 Chemical analysis of the final sorghum silages

Treatment	g kg^{-1} dry matter			
	WSC	Ethanol	Acetic acid	Lactic acid
C	24 ± 18^{a,b}	12 ± 1^{a}	16 ± 1^{c}	45 ± 12^{a,b,c}
LP	24 ± 12^{a,b}	18 ± 4^{a}	7 ± 1^{d,e}	54 ± 6^{a,b}
LB	0^{b}	13 ± 4^{a}	28 ± 5^{b}	37 ± 8^{c}
LP + LB	22 ± 10^{a,b}	6 ± 2^{b}	15 ± 7^{c}	51 ± 8^{a,b}
H	24 ± 6^{a,b}	5 ± 0^{b}	12 ± 2^{c,d}	57 ± 5^{a}
LP + H	33 ± 20^{a}	15 ± 6^{a}	4 ± 1^{c}	40 ± 3^{b,c}
LB + H	6 ± 11^{b}	13 ± 1^{a}	34 ± 3^{a}	35 ± 9^{c}
LP + LB + H	33 ± 12^{a}	5 ± 1^{b}	11 ± 2^{c,d}	43 ± 3^{b,c}

WSC, water-soluble carbohydrates.
Within a column, means followed by the same letter did not differ significantly ($P < 0.05$) in Duncan's multiple range test.

Table 7 Microbiological data of the sorghum silages

Treatment	$\log_{10}$ CFU g^{-1} FM		
	LAB	Yeasts	Molds
Fresh sorghum	6.3	7.1	6.5
C	6.8	NF	2.0
LP	5.8	3.3	NF
LB	6.4	2.0	3.3
LP + LB	5.0	3.6	2.0
H	6.9	NF	NF
LP + H	4.6	3.2	NF
LB + H	5.4	NF	NF
LP + LB + H	5.5	NF	NF

LAB, lactic acid bacteria; NF, not found; FM, fresh material.

Table 8 Results of the aerobic stability test of the sorghum silages

Treatment	pH	CO_2 (g kg^{-1} DM)	Yeasts	Molds
C	3.9 ± 0.1^b	0.6 ± 1.1^c	6.6	NF
LP	4.9 ± 0.1^a	31.4 ± 6.8^a	9.9	NF
LB	3.9 ± 0.0^b	1.3 ± 1.2^c	5.6	NF
LP + LB	3.7 ± 0.0^b	1.6 ± 1.5^c	6.4	4.6
H	3.8 ± 0.0^b	1.3 ± 1.1^c	5.1	NF
LP + H	4.6 ± 0.6^a	23.0 ± 7.1^b	9.3	3.5
LB + H	3.9 ± 0.0^b	1.5 ± 1.3^c	5.4	NF
LP + LB + H	3.7 ± 0.0^b	0.5 ± 0.9^c	6.4	3.2

Yeast and mold numbers are given as $\log_{10}$ CFU^{-1} FM (fresh material). NF, not found.
Within a column, means followed by the same letter did not differ significantly ($P < 0.05$) in Duncan's multiple range test.

sensitive to aerobic exposure, such as mature whole plant cereal silages, the protection provided by LB during feedout could override its relatively large fermentation losses. If LB is combined with LP (or probably with any other homofermentative LAB), fermentation losses are minimal.

It was surprising that neither of the yeast treatments enhanced aerobic deterioration. In the first experiment with wheat, a yeast isolated from local wheat silage (Y-56) was used, in the hope it would induce aerobic deterioration. When this did not happen, *Hansenula subpelliculosa*, a silage spoiler [7] was used in the second experiment with sorghum; in both cases the yeasts resulted in appreciable concentrations of acetic acid which explains the aerobic stability obtained. Such a trend had already been noticed by Woolford [12] who noted that 'yeasts were able to produce both lactate and acetate from fermentable sugars and to compete successfully with LAB for substrate'.

Sterilized wheat extracts were used as a model system to study the potential of microorganisms as silage additives. The advantage of such extracts is that it is possible to control the microbial population at the start of the experiment. Indeed, in the aqueous phase it was possible to follow differences in fermentation patterns between treatments, during the initial phase of fermentation, whereas in the whole-plant silages, in which the epiphytic LAB had an effect, the pH had already decreased to around 4.0 after 4 days, regardless of the treatment. In the extracts, the yeast and LB treatments resulted in higher pH values than with the LP or TUB-589 treatments. The lactate:acetate ratio obtained in the fermented extracts was similar to that obtained in the silages, in the respective treatments (Tables 1 and 4). This model system was not effective for studying changes occurring under aerobic exposure.

In conclusion, the LB inoculant was very effective in protecting the wheat and sorghum whole-plant silages exposed to air under laboratory conditions; it could serve as a silage additive along with homofermentative LABs. Such a combination might cover all stages of silage making and use. It is suggested that LB be tested under farm conditions.

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