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(54) Title: METHODS AND COMPOSITIONS FOR IMPROVING AEROBIC STABILITY OF SILAGE

(57) Abstract

A method for treating silage to enhance aerobic stability by inhibiting growth of microorganisms selected from yeasts, molds and spore-forming bacteria is disclosed. The method comprises treating silage or feed with a composition comprising *Lactobacillus buchneri*, LN 3957, or the antimicrobial components produced thereby. According to the invention, a strain of *Lactobacillus* has been purified and isolated which is nontoxic, safe and which improves aerobic stability of silage.

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TITLE: METHODS AND COMPOSITIONS FOR IMPROVING AEROBIC STABILITY OF SILAGE

FIELD OF THE INVENTION

This invention relates generally to the silage process and to microorganisms and use of the same in treating animal feed and silage to enhance aerobic stability of the same.

BACKGROUND OF THE INVENTION

The ensiling process is a method of moist forage preservation and is used all over the world. Silage accounts for more than 200 million tons of dry matter stored annually in Western Europe and the United States alone. The concept involves natural fermentation, where lactic acid bacteria ferment water soluble carbohydrates to form organic acids under anaerobic conditions. This causes a decrease in pH which then inhibits detrimental microbes so that the moist forage is preserved. The process can be characterized by four different phases.

Upon sealing in the storage unit, the first phase is aerobic, when oxygen is still present between plant particles and the pH is 6.0 to 6.5. These conditions allow for continued plant respiration, protease activity and activity of aerobic and facultative aerobic microorganisms.

The second phase is fermentation which lasts several days to several weeks after the silage becomes anaerobic. Lactic acid bacteria develop and become the primary microbial population thereby producing lactic and other organic acids, decreasing the pH to 3.8 to 5.0.

The third phase is stable with few changes occurring in the characteristics of the forage so long as air is prevented from entering the storage unit.

The final phase is feedout when the silage is ultimately unloaded and exposed to air. This results in reactivation of aerobic microorganisms,

primarily yeast, molds, bacilli and acetic acid bacteria which can cause spoilage.

Aerobic instability is the primary problem in silage production. Even before storage units are open for feedout, silage can be exposed to oxygen because of management problems (i.e., poor packing or sealing). Under these types of aerobic conditions, rapid growth of yeast and mold cause silage to heat and spoil, decreasing its nutritional value.

Aerobic instability can be a problem even in inoculated silage that has undergone what would traditionally be considered a "good" fermentation phase, namely a rapid pH drop, and a low terminal pH. The yeast which contribute to instability in these conditions may be those which are tolerant of acid conditions and can metabolize the lactic acid produced by lactic acid bacteria during fermentation.

Management techniques that can be used to help prevent this condition involve using care to pack the silage well during the ensiling process and, also, using care in removing silage for feeding to minimize the aeration of the remaining silage.

The susceptibility of silage to aerobic deterioration is determined by physical, chemical, and microbiological factors. Management (compaction, unloading rates) largely effects the movement of oxygen into silage. During feedout, air can penetrate 1 to 2 m behind the silage face so that exposure to oxygen is prolonged. Fermentation acids and pH inhibit the rate of microbial growth but spoilage rates are affected also by microbial numbers and the rate of aerobic microbial growth on available substrates.

It is possible to use both chemical and biological additives in making silage to promote adequate fermentation patterns especially under sub-optimal conditions. Biological additives comprise bacterial inoculants and enzymes. Bacterial inoculants have advantages over chemical additives because they are safe, easy to use, non-corrosive to farm machinery, they do not pollute the environment and are regarded as natural products. Silage inoculants containing principally homofermentative lactic acid bacteria have become the

dominant additives in many parts of the world. Their function is to promote rapid and efficient utilization of a crop's water soluble carbohydrates resulting in intensive production of lactic acid and a rapid decrease in pH. Inoculants also reduce aerobic spoilage and improve animal performance.

Several problems, however, with lactic acid bacteria inoculants have been encountered. These primarily include failure to dominate fermentation and failure to inhibit adverse microbial activity. Lactic acid bacteria inoculants have been plagued by such things as inoculants being infected by phage, inoculant strains not growing well on certain crops, technical problems associated with the lactic acid bacteria not being viable at the time of application, and of course the epiphytic lactic acid bacteria population. Because these types of homofermentative lactic acid bacteria inoculants do not always prevent or reduce undesirable microbial activity, several new approaches have been tried.

The concept of heterofermentative lactic acid bacteria in an inoculant has gained recent favor. The idea is that increased levels of undissociated volatile fatty acids, such as acetate, may inhibit other microbes that initiate aerobic deterioration. Heterofermenters have the ability to convert lactic acid to acetic acid in the presence of oxygen, and the acetate produced may inhibit other deleterious organisms. With such a mechanism, one-third of the lactic acid dry matter consumed will be lost as carbon dioxide. However a small loss of 1% or perhaps up to 2% dry matter may easily offset much larger losses by aerobic microorganisms. Concerns with heterofermentative lactic acid bacteria include effects on animal performance as well as the identification of appropriate strains useful for the procedure. Different strains of even the same species do not have identical properties and vary in their fermentation characteristics.

A review of the silage process and the use of inoculants can be found in FMS Microbiology Rev. 19 (1996) 53-68, Weinberg, ZNG., and Muck, RE, "New trends and opportunities in the development and use of inoculants for silage", the disclosure of which is incorporated herein by reference.

PCT publication WO 97/29644 discloses a single strain of *Lactobacillus buchneri* (NCIMB 40788) which was found to inhibit the growth of spoilage organisms in the storage of silage. Other attempts to identify heterofermentative organisms for silage inoculants have included (Wyss et al., 1991, "Einfluss von Luftstress und die Wirkung von spezifishen Zusatzen anf die arobe Stabilitat von Grasswelksilagen", Wirschaftseigene Futter, 37: 129-141), which used an inoculant comprising lactate and propionate producing organisms in wilted grass silage. Weinberg et al. (1995), "The effect of a propionic acid bacterial inoculant applied at ensiling, with or without lactic acid bacteria on the aerobic stability of Pearl-Millet and maize silages", J. Appl. Bacteriol., 78:430-436 disclosed the use of *Propionibacterium shermanii* in millet, corn, sorghum, and wheat silages. Propionic acid was produced only in a wheat silage in which the pH decline was delayed and thus aerobic stability was improved. In all other silages the pH decline was rapid and the propionic acid bacteria could not proliferate.

Another attempt included select strains of *Serratia rubidaea* and *Bacillus subtilis* along with *L. plantarum*. When used in bale grass silages the number of molds decreased significantly. Some improvement was also observed in high moisture ear corn. (Moran et al., (1993), "The development of a novel bacterial inoculant to reduce mold spoilage and improve the silage fermentation in big bale silage. In: Silage Research 1993, Proceedings of the Tenth International Conference on Silage Research (O'Kiely, P., O'Connell, M. and Murphy, J., Eds.) pp. 85-86, Dublin City University, Ireland). A similar composition to that for bale grass silage was developed for wheat silage which added *Pediococcus* strains to the composition. *Pediococcus* is capable of fermenting pentose sugars which result from hemicellulose hydrolysis in wheat silages. In a single trial with wheat silage, no improvement in the aerobic stability was observed.

The ensiling process is a complex one and involves interactions of numerous different chemical and microbiological processes. Further, different silages and different methods of ensiling present a variety of different needs.

As can be seen a need exists in the art for further improvement in compositions and methods to improve the aerobic stability of silage.

It is an object of the present invention to provide a method and composition which can be used as an inoculant to improve aerobic stability of silage.

It is yet another object of the invention to provide a microorganism which can be used as a heterofermentative inoculant to decrease lactic acid content and increase acetic acid content of silage.

It is a further object of the present invention to increase dry matter recovery of silage by reducing aerobic spoilage.

It is yet another object of the invention to provide an inoculant which is safe and nonhazardous for an additive to silage.

It is a further object of the invention to provide a natural additive composition for silage.

It is yet another object of the invention to provide quality silage material as determined by temperature, pH, dry matter recovery, nitrogen profile, color and microorganism count.

Other objects of the invention will become apparent from the description of the invention which follows.

SUMMARY OF THE INVENTION

According to this invention, forage materials which are to be stored are treated with a particular strain of *Lactobacillus buchneri* to preserve forage quality and to inhibit or retard aerobic deterioration.

Thus the invention provides a method of treating forage materials to enhance their preservation which comprises administering to the forage materials an effective amount of *Lactobacillus buchneri* strain LN3957, its functional equivalents, or the forage preserving compositions produced thereby.

As explained in more detail below, the microorganism of the invention has a unique effect, different from and/or extending beyond its ability to

produce volatile fatty acids, such as acetic, propionic and lactic acids, that are normally produced in fermentation. The organism produces antimicrobial factors or effects such as hydrogen peroxide which are characterized by their ability to inhibit the growth of a variety of spoilage organisms.

These substances may be isolated and purified by methods known to those of ordinary skill in the art. As such, it may be used directly to treat animal feed or silage. In other words, it may not be necessary to use a microorganism as such in the compositions and methods of this invention.

DETAILED DESCRIPTION OF THE FIGURES

Figure 1 is a graph depicting silage characteristics of forage treated with different treatments including the *L. buchneri* strain of the invention. (LN3957 - the strain of the invention; LU1184+1188 - 50:50 mixture of LU1884 AND 1188; UL1001+1188 - 50:50 mixture of UL1001 and 1188; LU0211+1188 - 50:50 mixture of LU0211 and 1188; LU1884 - the experimental strain of *Lb. curvatus*; UL1001 - the experimental strain of *Lb, sake*; LU0211 - the experimental strain of *Lb, curvatus*; 1188 - the commercial product containing Lactobacillus plantarum LP286, LP287, LP318, LP319, LP346, and Enterococcus faecium SF202 and SF301; and control - uninoculated.)

DETAILED DESCRIPTION OF THE INVENTION

According to the invention a microorganism has been isolated and purified which improves the aerobic stability of ensiled forage. A specific strain of the species *Lactobacillus buchneri* has been shown to enhance aerobic stability of silage by not only metabolizing lactic acid but also by producing a substance which is toxic to microorganisms that contribute to causing aerobic instability in silage.

In the present invention, the inhibition of organisms responsible for spoilage is accomplished by treating the silage with organisms of the species *Lactobacillus buchneri*, especially the strain *Lactobacillus buchneri* LN3957 or

with compositions containing *Lactobacillus buchneri* strain LN3957 or closely related organisms, and as well by treatment with effective mutants or equivalents of *Lactobacillus buchneri* strain LN3957 and compositions containing same.

The compositions which are used in the method of the invention may be in either liquid or dry form and may contain additional bacterial strains. In solid treatment forms, the composition may comprise the *Lactobacillus buchneri* together with a carrier. The carrier may be in the nature of an aqueous or nonaqueous liquid or a solid. In solid forms, the composition may contain solid carriers or physical extenders. Examples of such solid carriers, solid diluents or physical extenders include malto-dextrin, starches, calcium carbonate, cellulose, whey, ground corn cobs, and silicone dioxide. In short, the carrier may be organic or an inorganic physical extender. The solid composition can be applied directly to the forage in the form of a light powder dusting, or if it is disbursed in a liquid carrier it can successfully be sprayed on the forage.

Typical compositions useful for treating silage according to this invention contain 10^2-10^{12} viable organisms/gm, preferably 10^7-10^{10} viable organisms/gm, and more preferably 10^9-10^{10} viable organisms/gm in soluble formulations. For granular formulations preferred is 10^4-10^{10} and most preferred is 10^7-10^8.

The treatment range for silage is typically 10^7-10^{17} viable organisms/ton, preferably 10^9-10^{15} viable organisms/ton, and more preferably 10^{10}-10^{12} viable organisms/ton.

Those of ordinary skill in the art will know of other suitable carriers and dosage forms, or will be able to ascertain such, using routine experimentation. Further, the administration of the various compositions can be carried out using standard techniques common to those of ordinary skill in the art.

As used herein the term "strain" shall be interpreted to include any mutant or derivative of strain LN3957 deposited with the ATCC as accession number 202118, which retains the functional activity of improving aerobic

stability of forage as described and defined by the methods and examples disclosed herein.

The microorganism of the invention was purified and isolated from grass silage. After much experimentation it was discovered from testing about 4000 isolates.

After purification and isolation of the specific strain, taxonomic studies were done to identify the strain. It was identified as *Lactobacillus buchneri* and given the prototype number LN3957. According to the invention, this strain, compositions comprising this strain, or the factors produced by this strain, are used to treat forage materials.

Materials that are suitable for ensiling or storage, according to the methods of the invention, are any which are susceptible to aerobic spoilage. The material will usually contain at least 25% by weight dry matter. Such materials include rye or traditional grass, maize, including high moisture corn, whole plant corn, Lucerne, wheat, legumes, sorghum, sunflower, barley or other whole crop cereals. The silage may be in bales (a form particularly susceptible to aerobic spoilage), oxygen limiting bags, bunkers, upright stave silos, oxygen limiting silos, bags, piles or any other form of storage which may be susceptible to aerobic spoilage. Alternatively, the invention may be used with any susceptible animal feed, whether solid or liquid, e.g. for pigs, poultry or ruminants.

The activity associated with this invention may be found in other strains of *L. buchneri*, in other species of *Lactobacillus*, e.g. *L. kefir, L. parakefir* and *L. parabuchneri, L. brevis, L. sake, L. curvatus* and possibly also in other genera. This can be established by routine experimentation, on the basis of the information herein.

DEPOSITS

A deposit of the microorganism *Lactobacillus buchneri* LN3957 is and has been maintained by Pioneer Hi-Bred International, Inc., 7100 NW 62nd Avenue, Johnston, Iowa 50131-1000, since prior to the filing date of this

application. Access to this deposit will be available during the pendency of the application to the Commissioner of Patents and Trademarks and persons determined by the Commissioner to be entitled thereto upon request. Upon allowance of any claims in the application, the Applicant(s) will make available to the public without restriction a deposit of the same with the American Type Culture Collection (ATCC), Rockville, Maryland, 20852. The microorganism deposited with the ATCC will be taken from the same deposit maintained at Pioneer Hi-Bred and described above. Additionally, Applicant(s) will meet all the requirements of 37 C.F.R. § 1.801 - 1.809, including providing an indication of the viability of the sample when the deposit is made. This deposit of *Lactobacillus buchneri* LN3957 will be maintained without restriction in the ATCC Depository, which is a public depository, for a period of 30 years, or 5 years after the most recent request, or for the enforceable life of the patent, whichever is longer, and will be replaced if it ever becomes nonviable during that period.

The following examples serve to further describe and define the invention, and are not intended to limit the invention in any way.

EXAMPLES

Example 1

Buxtehude Whole Plant Corn Silage Trials

An experiment was conducted to inoculate whole plant corn forage with the heterofermentative strain LN3957 as well as bacteria that exhibit anti-yeast activity in bench-top assays, to determine whether aerobic stability of the silage is improved.

Methods

Trials were carried out by the standard methods used in European silage research studies (see Example 3). Whole plant corn (hybrid 'Noveta')

was chopped and ensiled at 28.3 to 39.5% dry matter. PVC silos 4" x 14" were used with 50% compaction rate (approx. 100kg dry matter/m3) and two 48 hr periods of air infusion at 4 and 6 weeks post-ensiling. There were four locations and two replicate silos per treatment per opening day. Table 1 summarizes the treatments used.

Table 1. Treatments

Treatments:	Description:
Control	Uninoculated
1188	Commercial Product containing *Lactobacillus plantarum* LP286, LP287, LP318, LP319, LP346, and *Enterococcus faecium* SF202 and SF301
LU0211	Experimental strain of *Lb. curvatus*
UL1001	Experimental strain of *Lb. sake*
LU1884	Experimental strain of *Lb. curvatus*
LU0211+1188	Mixture of LU0211 and 1188 (50:50)
UL1001+1188	Mixture of UL1001 and 1188 (50:50)
LU1884+1188	Mixture of LU1884 and 1188 (50:50)
LN3957	The strain of the invention

Results

Table 2 shows the pooled result of four trials and Figure 1 is a graph depicting the results. Aerobic stability of the 'anti yeast bacteria' treated silages was similar to the untreated control silage. Silage treated with the commercial product 1188 was less stable than control silage in this study. Whole plant corn silage treated with LN3957 had 30 to 40 h higher aerobic stability than 1188 or control treatments.

Table 2. Treatment LSMeans – Day 65

TRT	pH	Stability (h)	Aerobic Loss (%)
1188	4.00	11.13	12.85
CONTROL	4.02	24.13	13.62
LN3957	3.90	63.25	8.06
LU0211	3.98	21.13	11.92
LU0211+1188	3.97	18.75	12.12
LU1884	3.92	25.88	11.60
LU1884+1188	3.94	39.38	10.96
UL1001	3.93	31.38	9.40
UL1001+1188	3.94	17.63	13.19

Example 2

U.S. Whole Plant Corn Silage Trials

The purpose of this trial was to look at different conditions and treatments that might affect aerobic stability of whole plant corn silage. Two different packing densities were tested along with bacterial inoculants including LN3957 and UL1001, an isolate of Lactobacillus sake which had some antiyeast activity in culture.

Methods

Trials were carried out at the Pioneer Livestock Nutrition Center (Polk City, Iowa) using Pioneer Brand Hybrid 3394. Whole plant corn was harvested and chopped at 31.9% dry matter.

Inoculation

1132 and 1174 were commercial products in soluble form. UL1001 was prepared by our contract manufacturer. LN3957 was grown and freeze-dried by Pioneer's Fermentation Lab. The treatments were solubilized and were applied on the forage with a 30cc syringe fitted with a 16 gauge needle. All treatments except propionic acid were applied at a rate of 1ml/lb of forage for a final inoculum level of 1.0 e5 CFU/g. Propionic acid was applied at a rate of 5ml/2.2lb of forage. UL1001 and 1132 were combined at a 50:50 ratio.

Packing

For each treatment, 4"x14" standard PVC experimental silos were packed at our Normal (100%) packing Density[4.6 lb. (231 kg DM/m3)] or at 70% of our Normal (70%) packing Density [3.2 lb.(160 kg DM/m3].

The forage was pressed to the selected density with a hydraulic press. The silos were fitted with Fernco quick caps at each end; the top one having a Bunsen valve to allow for gas escape. After filling the silos were kept in an environmentally controlled room (approximately 72°F) until opening.

Analyses

One pre-ensiled uninoculated forage sample and one inoculated forage sample from each treatment were taken for dry matter (DM), pH and microbial analysis. DM was determined by drying approximately 150 g of forage for 72 hours in a 55°C oven. pH was determined after macerating 11g of forage with 99 ml of sterile deionized water in a stomacher. Lactic acid bacteria were enumerated on modified MRS agar. Yeast and molds were enumerated on Sabouraud Dextrose (SAB) agar.

On Day 11 two standard PVC silos (100% and 70% Packing Density) from each treatment were opened. Samples were taken for pH and microbial analysis.

On Day 95 three standard PVC silos (100% and 70% Packing Density) from each treatment were opened. Samples were taken for pH and lactic and

acetic acid analysis. Aerobic stability was determined by placing 2.5 lb. of silage into a plastic-lined polystyrene cooler and placing a temperature probe in the center of the silage mass. The coolers were kept in a temperature controlled room. Ambient temperature and silage temperature were measured every three hours for one week and recorded by a datalogger. ROT for the silage was defined as the time in hours it took for the silage temperature to rise 1.7°C above ambient. Average ambient temperature for this trial was 29.7°C. Cumm_DD is the integration of the area between the actual temperature curve and a line drawn at the ambient temperature. Max Temp is the maximum temperature recorded.

Treatments

Treatments:	Description:
Control	Uninoculated
1132	Commercial Product containing *Lb. plantarum LP286*, LP287, LP329, LP346, LP347, and E. faecium SF202 and SF301
1174	Commercial Product containing *Lb. plantarum* LP286, LP287, LP346, LP347, and *E. faecium* SF202 and SF301
UL1001	Experimental strain of *Lb. sake*
UL1001+1132	Mixture of UL1001 and 1132 (50:50)
LN3957	Strain of the invention
Positive Control	Propionic Acid

Results and Discussion

pH

Mean pH values are shown in Table 1. Day 11 values are the average of duplicate samples. Day 95 values are the average of triplicate samples. The initial pH of the forage was 5.97.

Table 1. Mean pH Values for Day 11 and Day 95 Silage from Two Packing Densities (PD)

	Day 11		Day 95	
	100% PD	70% PD	100% PD	70% PD
Control	3.76	3.73	3.76	3.77
1132	3.66	3.66	3.64	3.67
1174	3.68	3.66	3.68	3.68
UL1001	3.73	3.72	3.74	3.74
1001+1132	3.67	3.66	3.65	3.67
LN3957	3.75	3.76	3.90	3.95
Propionic Acid	3.68	3.66	3.70	3.71

This data shows that all the treatments, including the heterofermenter LN3957, gave a good fermentation (pH< 4.0). There were differences in the pH values between treatments with all packing methods. 1132 usually had the lowest pH and LN3957 had the highest pH. Packing density had no effect on pH.

Lactic and Acetic Acid

Table 2 shows the average amount of lactic and acetic acid found in the silage extracts on Day 95. The calculated lactic/acetic ratios are given in Table 3.

Table 2. Average Amounts of Lactic Acid and Acetic Acid Detected in Day 95 Silage (Packing density = PD)

	Lactic Acid (ug/ml)		Acetic Acid (ug/ml)	
	100% PD	70% PD	100% PD	70% PD
Control	1223.8	1124.9	353.8	400.8
1132	1200.4	1268.9	235.6	302.6
1174	1243.0	1274.9	282.1	325.7
UL1001	1206.9	1220.6	357.0	444.8
1001+1132	1253.2	1439.4	267.3	362.6
LN3957	739.0	749.2	900.1	991.5
Propionic Acid	1093.9	957.1	321.6	272.8

Table 3. Ratio of Lactic Acid to Acetic Acid in Day 95 silage

	Lactic/Acetic Ratio	
	100% PD	70% PD
Control	3.5	2.8
1132	5.1	4.2
1174	4.4	3.9
UL1001	3.4	2.7
1001+1132	4.7	4.0
LN3957	0.8	0.8
Propionic Acid	3.4	3.5

Silage treated with LN3957 contained less lactic acid and more acetic acid, which resulted in lower lactic/acetic acid ratios than the other treatments. 1132 had the highest lactic/acetic ratio and the lowest pH.

Yeast and Mold Counts

Tables 4 and 5 show Day 11 yeast and mold counts. On Day 11 LN3957 and Propionic Acid had lower yeast and mold counts than the other treatments.

Table 4: Yeast CFU/g (wet matter basis) Packing Density = PD

	Day 0	Day 11	
	100% PD	100% PD	70% PD
Control	1.85E+06	2.20E+05	3.20E+05
1132	3.40E+06	4.68E+05	2.04E+06
1174	4.40E+06	3.13E+05	5.15E+05
UL1001	4.45E+06	5.30E+05	3.72E+05
1001+1132	4.65E+06	2.78E+05	1.68E+05
LN3957	7.95E+06	1.96E+05	5.89E+04
Propionic Acid	2.15E+06	1.48E+05	6.71E+04

Table 5: Mold CFU/g (wet matter basis) Packing Density = PD

	Day 0	Day 11	
	100% PD	100% PD	70% PD
Control	1.65E+06	1.25E+03	1.04E+03
1132	1.10E+06	9.33E+03	3.46E+03
1174	6.00E+05	2.64E+03	6.50E+02
UL1001	5.50E+05	2.75E+03	3.32E+03
1001+1132	5.50E+05	2.37E+03	3.05E+03
LN3957	3.00E+05	3.00E+01	<E+02
Propionic Acid	2.00E+05	<E+02	<E+02

Aerobic Stability

Average values for the 3 measures of aerobic stability - ROT, Cumm_DD and Max Temp - are given in Table 6.

Table 6: Average ROT, Cumm_DD and Max Temp Values

	ROT		Cumm_DD		Max Temp	
	100%	70%	100%	70%	100%	70%
	Packing Density	Packing Density	Packing Density	Packing Density	Packing Density	Packing Density
Control	32	28	138.8	86.4	37.6	34.5
1132	26	34	117.6	80.2	36.7	34.6
1174	28	28	139.3	130.5	38.6	39.3
UL1001	32	30	89.9	54.0	34.9	33.2
1001+1132	30	28	107.0	55.4	36.2	33.7
LN3957	56	90	57.6	59.9	33.9	35.9
Propionic Acid	42	28	102.2	76.7	35.9	35.9

At both packing densities, LN3957 inoculated silages had the best (highest) ROT values; except for LN3957 (at 70% packing density) all ROT values were less than 75 hours.

LN3957 treated silage had the lowest CUMDD and Max Temp values at 100% packing density, and lower CUMMDD than control, 1174 and 1132 treated silage at 70% density.

Table 7: Yeast CFU/g (wet matter basis)in Silage After Exposure to Air

	Time After Exposure to Air			
	0 Hrs.	24 Hrs.	48 Hrs.	93 Hrs.
Control	5.36E+04	2.56E+07	5.70E+08	1.13E+09

1132	6.08E+04	8.85E+06	2.55E+07	7.86E+08
UL1001	4.73E+04	1.63E.07	1.35E+07	4.77E+08
LN3957	nd	5.00E.03	5.55E.06	6.14E+07
Propionic Acid	4.95E+02	7.56E+03	1.67E+06	8.54E+07

Table 7 shows that silage treated with LN3957 and propionic acid had lower yeast populations 24-93 hr after exposure to air than control silage or silage treated with 1132 or UL1001.

Example 3

Protocol for Determining Aerobic Stability

DETERMINATION OF AEROBIC DETERIORATION SYSTEM VÖLKENRODE

H. Honig

The system is based on the linear correlation existing between temperature rise and intensity of CO_2-production, which again can be transformed into DM-(Glucose-) losses via the respiration formula. The conversion factors given later relate to the test set-up used here. Higher insulation and larger forage amounts will give higher temperature rise at the same losses.

Containers:

1 l aluminum tins, 100 mm diameter, 150 mm high, covered with plastic sheet, 10 mm diameter hole in tin bottom and covering sheet, 60 mm styrofoam insulation to the sides, 30 mm to top and bottom.

Gas flow:

Gas flow is secured by the difference in specific weight of the CO_2, produced during the process, and the surrounding air. The hole diameter is sufficient for the necessary gas exchange as is shown by comparative measurements with the "Sapromat-system" (Compensating the O_2 deficit automatically at demand). Tin cover should be plastic to avoid excessive drying of the surface layers of material.

Temperature measurement:

Thermo couple connected to a 100 channel automatic printer. Measurements are taken at 6-hour intervals and averaged daily.

Basic temperature:

Containers are stored in a 20°C controlled temperature room. If material is colder at the beginning, it should be given time to adjust to 20°C before applying insulation.

Filling quantity:

100 g of DM is the basic filling quantity. As temperature rise shows a linear correlation to filling quantity in a range from 60 to 130 g DM, data can be corrected for small deviations. If material with high bulk density does not fill the 1 l volume, styrofoam disks with a center hole of 12 mm are put in as a substitute.

Loss calculation

DM content is the second factor besides filling quantity to be allowed for in the conversion from temperature rise to DM losses. The following table is based on extensive comparisons of temperature rise in the described set-up and simultaneous CO_2 determinations.

DM loss, % per day at 1°C temperature rise

a) Factor F_{DM100} (at 100 g DM filling weight)

% DM	0	1	2	3	4	5	6	7	8	9
10	0.450	0.438	0.425	0.413	0.400	0.388	0.375	0.363	0.350	0.338
20	0.325	0.315	0.306	0.279	0.287	0.278	0.268	0.259	0.249	0.240
30	0.230	0.226	0.222	0.218	0.214	0.210	0.206	0.202	0.198	0.194
40	0.190	0.186	0.181	0.177	0.172	0.168	0.163	0.159	0.154	0.150
50	0.145	0.140	0.136	0.131	0.127	0.123	0.118	0.114	0.109	0.105
60	0.100									

b) Factor F_{DM} (at variable filling weight (60...130 g))

$$F_{DM} = F_{DM100} \times \frac{100}{\text{DM filling weight (g)}}$$

Loss curve:

The daily determined losses are accumulated and plotted versus storage time. Normal storage time in Völkenrode is 9 days.

What is claimed is:

1. A composition for use as a silage inoculant comprising: a small but silage quality preserving amount of *Lactobacillus buchneri* LN3957 or effective mutants thereof, and carrier.

2. The composition of claim 1 wherein the composition contains from about 10^2 to about 10^{12} viable organisms per gram.

3. The composition of claim 1 wherein the composition contains from about 10^7 to about 10^{10} viable organisms per gram.

4. The composition of claim 1 wherein the composition contains from about 10^9 to about 10^{10} viable organisms per gram.

5. The composition of claim 1 wherein the suspension carrier is liquid.

6. The composition of claim 1 wherein the suspension carrier is solid.

7. The composition of claim 1 wherein said carrier is a solid carrier and is water soluble and selected from the group consisting of calcium carbonate, starch, and cellulose.

8. A method for treating animal feed or silage susceptible to the growth thereon of spoilage organisms selected from yeasts, molds and spore-forming bacteria, which comprises: adding to said feed an antimicrobial effect obtainable by fermentation of *Lactobacillus buchneri*, LN3957, said effect being obtained in addition to fatty acids normally produced in fermentation, and said effect being characterized by its ability to inhibit the growth of said spoilage organisms.

9. A method for treating silage, which comprises adding thereto an antimicrobial effect as defined in claim 8.

10. A method according to claim 8, wherein the silage is grass.

11. A method according to claim 8, wherein the silage is maize.

12. A method according to claim 8, wherein the silage is alfalfa.

13. A method according to claim 8, wherein the silage is wheat.

14. A method according to claim 8, wherein the silage is legumes.

15. A method according to claim 8, wherein the silage is sorghum.

16. A method according to claim 8, wherein the silage is sunflower.

17. A method according to claim 8, wherein the silage is barley.

18. A method according to claim 8, wherein said effect is added on ensiling.

19. A method according to claim 8, which comprises maintaining the silage for at least 30 days.

20. A method according to claim 2, wherein the silage is in a bale.

21. A method according to claim 2, wherein the silage is in an oxygen limiting bag.

22. A method according to claim 2, wherein the silage is in a bunker.

23. A method according to claim 2, wherein the silage is in a stave silo.

24. A method according to claim 2, wherein the silage is in oxygen limiting silos.

25. A method according to claim 2, wherein the silage is in bags.

26. A method according to claim 2, wherein the silage is in piles.

27. A method according to claim 2, wherein the silage is in any other form of storage which may be susceptible to aerobic spoilage.

28. A method according to claim 8, which comprises adding to the feed or silage a microorganism having the ability to produce said effect on fermentation.

29. Animal feed to which has been added an antimicrobial substance as defined in claim 8.

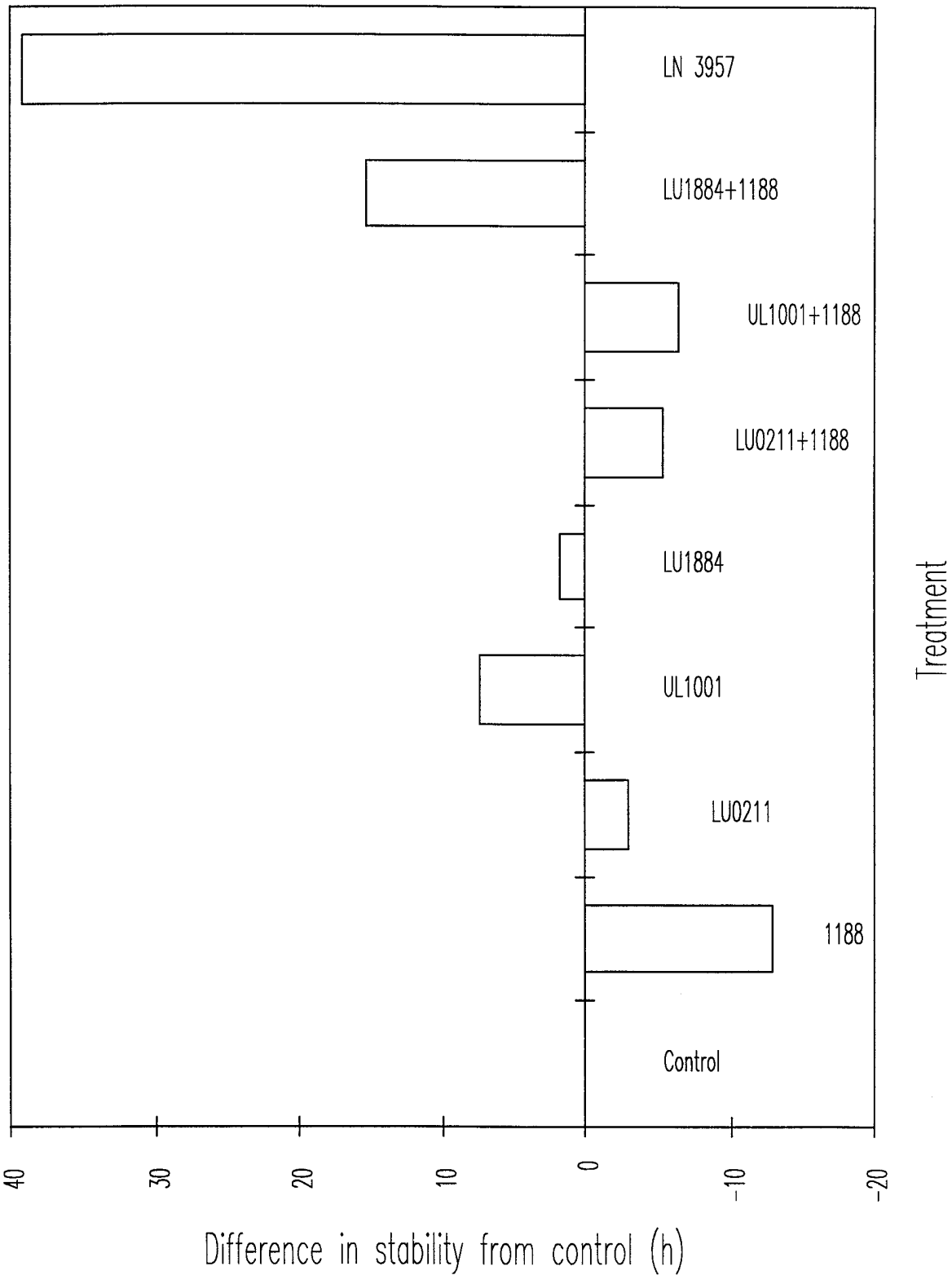
30. Animal feed to which has been added a microorganism as defined in claim 9.

31. Silage to which has been added an antimicrobial substance as defined in claim 8.

32. Silage to which has been added a microorganism as defined in any of claims 8.

33. A purified and isolated strain of *Lactobacillus buchneri*, strain LN3957, having ATCC accession number 202118.

Fig. 1


LN 3957
LU1884+1188
UL1001+1188
LU0211+1188
LU1884
UL1001
LU0211
1188
Control
Treatment
40
30
20
10
0
-10
-20
Difference in stability from control (h)

INTERNATIONAL SEARCH REPORT

Intern nal Application No
PCT/US 99/14451

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A23K1/00 A23K3/00 C12N1/20 //(C12N1/20,C12R1:225)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A23K C12N C12R

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 97 29644 A (BIOTAL LTD ;INSTITUUT VOOR DIERHOUDERIJ EN (NL); MANN STEPHEN PHIL) 21 August 1997 (1997-08-21) cited in the application the whole document --- -/--	1,8,9, 29-32

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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Date of the actual completion of the international search	Date of mailing of the international search report
15 October 1999	02/11/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	Dekeirel, M

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INTERNATIONAL SEARCH REPORT

Intern· ·nal Application No
PCT/US 99/14451

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
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