# Lactic acid bacteria in silage – growth, antibacterial activity and antibiotic resistance

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# Lactic acid bacteria in silage – growth, antibacterial activity and antibiotic resistance

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**ABSTRACT**

Ensiling is a method that has been known for hundreds of years and been used in Sweden since 18th century. Ensiling is a better way to preserve forage than hay making because the method is not as rain sensitive. Lactic Acid Bacteria (LAB) are the organisms responsible for the preservation; they ferment Water Soluble Carbohydrates (WSC) under anaerobic conditions and produce lactic acid, which lowers the pH. These conditions inhibit growth of other microorganisms. To control the ensiling process, improve quality and to inhibit non-wanted microorganisms, LAB can be used as additives in silage making. The aim of this study was to follow two strains of LAB, *Lactobacillus plantarum* M14 and *L. coryniformis* Si3, in silage during 90 days to see how they grow, and also, after 90 days see if they have improved the quality of the silage. Both strains were labelled with selectable antibiotic resistance markers, and used to inoculate grass-dominated crop in mini silos. The growth of the labelled strains was followed on agar plates containing antibiotics and with PCR. After 60 days, a contamination of both labelled strains was discovered in the control silo. Further investigation showed that the contamination had been present since the packing of the silos, which calls for a revision of the methods used to inoculation and packing. Following the growth of the labelled strains showed differences between them but both strains were able to outgrow the epiphytic flora and probably dominate the fermentation.

When using LAB as feed additives or starter culture in silage, antibacterial properties in addition to antifungal properties are desirable. Four strains of LAB were tested against one strain of *Clostridium butyricum* and three strains of *C. turybutyricum* with the agar well method. All of the four strains tested were able to inhibit two or more clostridial strains.

Antibiotic resistance can be transferred from non-pathogenic bacteria like LAB to pathogenic bacteria and thereby cause problems in the human and veterinary medicine. It is important that LAB strains that are going to be used as feed additives do not carry resistance genes that can be transferred. The occurrence of antibiotic resistance among LAB was determined with three different methods and culture media in this study. The epiphytic flora of grass was screened against six antibiotics. LAB from the culture collection of Department of Microbiology, SLU were screened against antibiotics in different concentrations and finally four strains of LAB were tested with VetMIC™ plates to determine their MIC value of 16 antibiotics. There are differences between the methods used but it can be seen that almost all strains tested seems to be resistant to or have high MIC values for tetracycline often exceeding the breakpoint. Similarly, the MIC values for chloramphenicol were close to the breakpoint. Tetracycline and streptomycin seems to be the two antibiotics most affected by method and media. It is very important to find a method and media suitable for testing antibiotic resistance among LAB in a way that can be repeated and also reliable in laboratories all over the world.

*Keywords:* Silage, ensiling, lactic acid bacteria, *Lactobacillus,* antibacterial activity, *Clostridium*, antibiotic resistance, MIC values

# CONTENTS

**INTRODUCTION** 7
Lactic acid bacteria 7
Silage 7
Anti microbial properties of lactic acid bacteria 10
Antibiotic resistance 10

**MATERIALS AND METHODS** 12
Bacteria and growth conditions 12
Ensiling study 13
*PCR analysis* 14
Determination of antibacterial activity 14
*Agar well method* 14
*Overlay method* 15
Antibiotic resistance in the epiphytic flora of the grass 15
Determination of MIC-values with agar dilution method 15
Determination of MIC-values with broth dilution method 16

**RESULTS AND DISCUSSION** 16
Ensiling study 16
Antibacterial activity 19
Antibiotic resistance 20
*Antibiotic resistance in the epiphytic flora* 20
*Antibiotic resistance among LAB with known antifungal activity* 20
*Influences of method and growth media* 22

**CONCLUSIONS** 23

**REFERENCES** 24

**ACKNOWLEDGEMENTS** 26

**APPENDIX 1** 28
**APPENDIX 2** 29
**APPENDIX 3** 30
**APPENDIX 4** 31
**APPENDIX 5** 32
**APPENDIX 6** 33

# INTRODUCTION

Food and feed have been preserved by fermentation for a very long time; at first humans had no idea that microorganisms were responsible for what happened. Today we know that Lactic Acid Bacteria (LAB) and yeasts are part of the fermentation of many different foods and feed products (Adams & Moss, 2000). LAB are the microorganisms responsible for fermentation of Sauer kraut, sour dough, olives, salami and silage. LAB also cause the sour taste of yoghurt (Stiles, 1996). LAB is used in fermentation processes because of its ability to inhibit other microorganisms and because the lactic acid and other metabolites give the products a pleasant flavour and aroma (Adams & Moss, 2000).

## Lactic acid bacteria

LAB are a genetically distinct group of bacteria that share the same properties: Gram positive, non-sporulating rods or cocci, catalase negative, acid tolerant, fermentative (lactic acid is the main metabolite) and prefer growing under anaerobic conditions but are aero tolerant (Wessels *et al.*, 2004). *Lactococcus, Leuconostoc, Pediococcus, Lactobacillus* and *Streptococcus* are example of bacterial genera that are members of the LAB group (Adams & Moss, 2000). LAB can be found in different nutrient rich habitats like on mucosal membranes of humans and animals, on plants and in many food and feed systems (Holzapfel *et al.*, 2001).

The fermentation pattern of LAB can be described as homo- or heterofermentative depending on how they ferment carbohydrates under non-limited conditions. Homofermentative LAB use the glycolysis pathway with lactic acid as the main product. Heterofermentative LAB use the 6P-gluconate pathway or phosphoketolase pathway and the main end products are equal amount of lactic acid, carbon dioxide and ethanol (Adams & Moss, 2000).

## Silage

Forage is the most important source of nutrient for grazing animals and it is important that the animals have access to good quality feed. During the winter in Sweden animals are fed preserved forage and high quality is essential to maintain animal health and to obtain good quality meat and milk. The most common way to preserve forage used to be haymaking, which is a method dependent on good weather. Rain makes the grass wet, nutrients are drawn out and the risk of rotting and mould growth increases.

Ensiling is not as sensitive to bad weather as haymaking and has been known since ancient times and is today the most common way to preserve forage (McDonald *et al.* 1991). Ensiling has been used in Sweden since the eighteenth century but it is during the last 50 years it has been fully developed (McDonald *et al.*, 1991). The Swedish Board of Agriculture estimate that 90% of the forage crop is preserved as silage and only 10% as hay (Wikberger, 2005).

Ensiling is a preservation method where LAB ferment Water-Soluble Carbohydrates (WSC) to organic acids, mainly lactic acid, under anaerobic conditions. The production

of organic acids leads to decrease in pH and the grass is preserved (Weinberg & Muck, 1996). The low pH in combination with anaerobic condition and undissociated acids prevents growth of unwanted bacteria, moulds and yeasts (Scudamore & Livesey, 1998). LAB exist naturally on grass (epiphytic flora) and ensiling starts when air is excluded, for example when the grass is filled in silos or packed in plastic film (McDonald *et al*., 2002).

The silage process has four phases (Driehuis & Oude Elfink, 2000; Weinberg & Muck, 1996):

- Aerobic phase - oxygen is still present in the plant material but it is consumed by respiration of the plants and by aerobic microorganisms. pH is 6.0-6.5.

- Fermentation - oxygen is consumed and anaerobic microorganisms like lactic acid bacteria, but also clostridia that not are wanted in the silage, start to grow. pH decreases to 3.8-5.0.

- Storage phase - few changes occur in the silage if no oxygen enters the silo

- Feeding out phase - the silage is exposed to oxygen when animals are fed. When oxygen enters, aerobic microorganisms start to grow again and the silage will be destroyed due to an increase in pH and growth of yeasts and moulds.

Bad silage can be dangerous for animals; it may contain yeasts, moulds, mycotoxins, pathogenic bacteria like *Listeria* and spore forming bacteria like *Clostridium botulinum, C. butyricum* and *C. tyrubutyricum* (Wilkinson, 1999). There is also a risk of finding spores from *Bacillus* and growth of enterobacteria might occur.

*C. botulinum* produces a toxin; botulinum, that is the most powerful neurotoxin found in nature. Botulinum causes botulism, fatal muscle paralysis (Adams & Moss, 2000). *C. tyrubutyricum* and *C. butyricum* in silage might cause problems in milk products, for example late blowing in hard cheeses. Late blowing is a phenomenon caused by butyric acid fermentation of the bacteria*;* cheeses swell and explode due to gas formation (Klijn, 1995). Spores from *Bacillus* spp. can be found in silage and they are able to pass through the gastrointestinal tract of the animals unaffected and end up in faeces. The spores might then be transferred to the milk via faecal contamination of the udder and they also survive the processing of the milk and cause spoilage and food borne illness (Te Giffel *et al*., 2002). Almost all moulds and yeasts are strict aerobes and these are not a problem if oxygen is excluded. The problem with growth of moulds and yeasts occur when oxygen enter the silage due to leakage in the plastic film or outtake from the silo when feeding the animals (McDonald *et al*., 1991). Enterobacteria are facultative anaerobes, which means that they can grow both in presence and absence of oxygen, they ferment sugars and the end product is acetic acid. They are also able to degrade amino acids (McDonald *et al.*, 1991)

Badly preserved silage is silage were clostridia, enterobacteria or both have dominated the fermentation. The amount of LAB, lactic acid and WSC content are low while acetic acid and butyric acid levels are high. This kind of silage is preserved with too low dry matter or too low levels of WSC (McDonald *et al*., 2002). A badly preserved silage usually have pH-values between 5.0 and 7.0, which is due to formation of acetic acid

and butyric acid, acids that are weaker than lactic acid and not able to lower the pH to the same extent. In this kind of silage the levels of ammonia-N are high, the unwanted bacteria degrades amino acids and releases ammonia-N, amines, keto acids and fatty acids and the nutritional value is decreased (McDonald *et al*., 1991). High pH is not always equal to bad silage; it depends on the dry matter content of the grass. Silage with pH 4.9 can be considered good if the dry matter is over 45% but bad if the dry matter is 30% (Fig. 1).

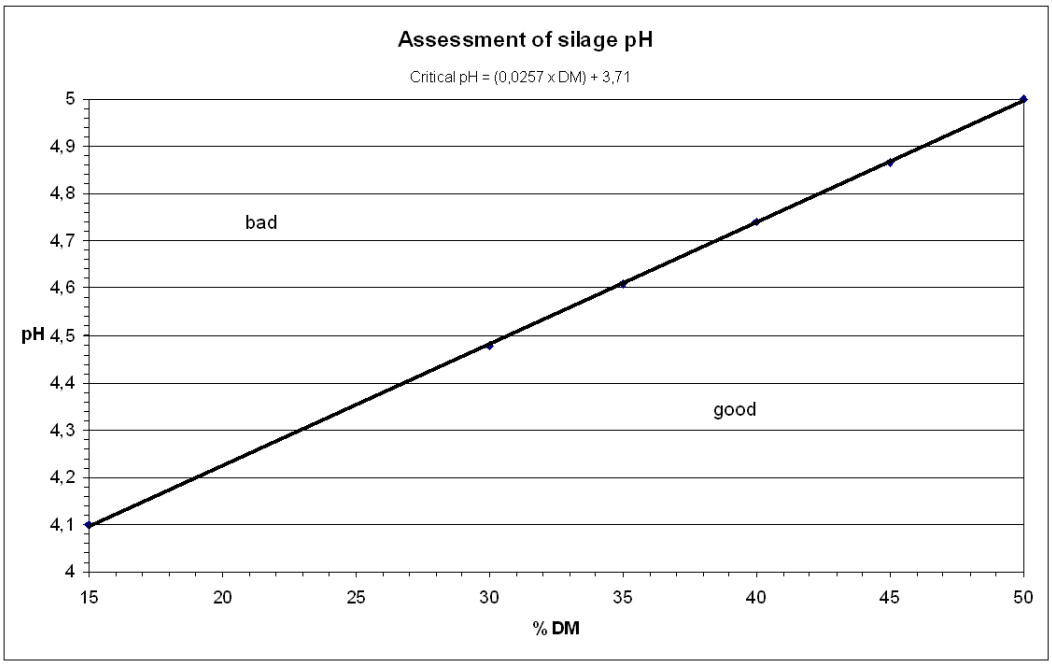


*Figure 1*: The influence of pH and dry matter on the quality of silage (modified from Weiβbach, 1996).

Silage can also be destroyed due to leakage of oxygen into the silo. Silages of this category should always be considered toxic because of growth of moulds, yeasts and aerobic bacteria and should not be fed to animals (McDonald *et al*., 2002).

To improve the silage and the efficiency of the preservation and in turn animal performance, LAB can be used as additives or starter cultures in the silage making process. The most common LAB used as starter cultures are *Enterococcus faecium, Lactobacillus plantarum, L. acidophilus, Pediococcus acidilactici* and *P. pentosaceus* (Weinberg and Muck, 1996). The inoculant should have several properties to be a suitable part of a starter culture (Weinberg & Muck, 1996):

- Homofermentative lactic acid bacterium
- Competitive to the epiphytic flora and fast growing
- Produce large amounts of lactic acid in short time
- Acid tolerant
- Ability to grow at temperatures up to 50°C and in low water activity.

**Anti microbial properties of lactic acid bacteria**

Chemicals have been used for a long time to preserve food. Today consumers are more concerned of how chemicals affect the body and the environment and they want food that is minimally processed with as little added chemicals as possible (Schnürer & Magnusson, 2005). At the same time the shelf life shall be the same as with chemical preservatives, as shall the taste and look. LAB can be used as bio preservatives because they may have both antifungal and antibacterial properties (Schnürer & Magnusson, 2005). When LAB ferments food or feed they produce lactic acid that is the main substance that inhibit microbiological growth due to the lowering of pH (Adams & Moss, 2000). LAB produce a lot of other substances for example several bacteriocins, acetic acid, propionic acid and phenyl lactic acid (Stiles, 1996; Magnusson & Schnürer, 2005; Valerio *et al* 2004).

Bacteriocins are peptides or proteins that are bactericidal and they are often active only against bacteria closely related to the bacterium that produced them. Nisin is the only bacteriocin that is approved of as a food and feed additive but other bacteriocins produced by lactic acid bacteria has the potential to be used as food and feed preservatives (Stiles, 1996). Acetic and propionic acid produced by heterofermentative LAB reduces growth of fungi and bacteria in combination with lactic acid by acidifying the cytoplasm of the microorganism (Schnürer & Magnusson, 2005). Phenyl lactic acid was discovered as one of the main factors for pro-longed shelf life in bread and also for anti fungal effects. It is also one of the metabolites forming cheese flavour (Valerio *et al*., 2004). Ström *et al* (2002) isolated a strain of *L. plantarum* that produced antifungal cyclic dipeptides; furthermore they have described the production of an antifungal acid in a *L. plantarum* strain.

**Antibiotic resistance**

Antibiotic resistance among bacteria is a growing problem and is considered to be a major public health issue. The reason for the increase in antibiotic resistance is the exaggerate use of antibiotics in human medicine as well as the use in animals for growth promotion (Mayrhofer *et al*., 2004). Like other bacteria LAB can be antibiotic resistant and this might be a problem. LAB themselves are not pathogenic but they can transfer antibiotic resistance genes to pathogenic bacteria that infect humans or animals (Herreros *et al*., 2005). Antibiotic resistant LAB might be transferred from animals to raw food products like meat and milk, and then end up in unpasteurized products, for example salami and cheese (Gevers *et al*., 2002; Herreros *et al*., 2005). LAB are often resistant to commonly used antibiotics like β-lactams, imidazole and aminoglycosides (Herreros *et al*., 2005). Before a strain of LAB can be used as a feed additive it must undergo antibiotic resistance screenings. (Question N° EFSA-Q-2004-079, 2005).

The Scientific Committee of Animal Nutrition (SCAN) has set up microbiological breakpoints for 13 antibiotics of veterinary and human clinical importance (Table 1). If a strain is to be used as a feed additive, it has to have a MIC value (Minimal Inhibitory Concentration) equal to or lower than the breakpoint. (Question N°EFSA-Q-2004-079, 2005). If the MIC value is higher than the breakpoint the strain can be approved if the resistance can be shown to be non transferable.

*Table 1:* Microbiological breakpoints for *Lactobacillus* spp. and *Lactobacillus plantarum* for the 13 antibiotics that SCAN consider as important (Question N° EFSA-Q-2004-079, 2005)

| **Antibiotic** | **Breakpoint (µg/ml)** *Lactobacillus* spp. | **Breakpoint (µg/ml)** *Lactobacillus plantarum* |
|---|---|---|
| **Ampicillin** | 4 | 4 |
| **Chloramphenicol** | 4 | 8 |
| **Clindamycin** | 4 | 4 |
| **Erythromycin** | 4 | 4 |
| **Gentamycin** | 8 | 64 |
| **Kanamycin** | 16 | 64 |
| **Linezolid** | 4 | 4 |
| **Neomycin** | 16 | 32 |
| **Streptomycin** | 16 | 64 |
| **Tetracycline** | 8 | 32 |
| **Trimethoprim** | 8 | 8 |
| **Vancomycin** | 4* | - |
| **Virginiamycin** | 4 | 4 |

* Investigation of vancomycin is not required for heterofermentative *Lactobacillus* species

Intrinsic resistance is also called natural resistance and it is inherent to a bacterial species or genus. The intrinsic resistance is typical for all strains of that species. This type of resistance is believed to have a minimal potential to be transferred to other bacterial species. If the antibiotic resistance gene has been obtained from another bacterium or if it is a mutation in indigenous genes, the resistance is acquired. This type of resistance is believed to have a high potential to be transferred to other bacteria (Question N° EFSA-Q-2004-079, 2005).

The resistance genes can be transferred between bacteria in three different ways, by conjugation, transformation or transduction. Conjugation is transfer of plasmids where cell-to-cell contact is required. A plasmid is a genetic element that replicates independently of the host chromosome and it normally carries non-essential genes but still is useful to the bacteria. When the plasmid also carries genes for its own transfer it can transfer a copy of itself from the host cell to a recipient cell, which in turn becomes a donor cell as it receives the plasmid. This type of plasmids can be spread very rapidly in bacterial populations and is responsible for a great deal of the spread of antibiotic resistance genes (Madigan *et al*., 2003).

Transformation is a form of transfer where a recipient cell takes up free DNA fragments from the surroundings (Maloy *et al*., 1994). Free DNA is released from dead bacterial cells. Transduction is the transfer of bacterial DNA carried from one bacterium to another by a phage particle (transducing particle). This phenomenon is widespread but not all phage are able to transduce and not all bacteria are transducible (Madigan *et al*., 2003; Maloy *et al*., 1994). In addition, phages are host specific and normally infect only closely related bacteria. As different from plasmids, linear DNA does not replicate in a bacterium and has to recombine into the chromosome

## MATERIALS AND METHODS

### Bacteria and growth conditions

Strains of LAB and Clostridia used in the experiments are listed in table 2. LAB were grown on MRS agar or broth (Oxoid Ltd., Basingstoke, England) under anaerobic conditions (GasPak System, BBL, Cockeysville, MD, USA) at 30˚C unless stated otherwise. Clostridia were grown on Reinforced Clostridial Agar, RCA (Oxoid) or in Reinforced Clostridial Medium, RCM (Merck, Darmstadt, Germany) under anaerobic conditions (GasPak System) at 30˚C. *Enterococcus faecalis* was grown on blood agar plates (Swedish Veterinary Institute) at 37˚C.

*Table 2:* Strains and species of bacteria used, the source of isolation and reference.

| Strain | Species | Source | Reference |
|---|---|---|---|
| **L4** | *Lactobacillus plantarum* | Unpasteurized cheese | Unpublished |
| **M6** | *Lactobacillus plantarum* | Lilac flowers | Magnusson *et al.* 2003 |
| **M14** | *Lactobacillus plantarum* | Lilac flowers | Magnusson *et al.* 2003 |
| **M14 (pLV100)** | *Lactobacillus plantarum* | Lilac flowers | This study |
| **M37** | *Lactobacillus plantarum* | Dandelion flower | Magnusson *et al.* 2003 |
| **M248** | *Lactobacillus plantarum* | Chestnut flower | Magnusson *et al.* 2003 |
| **M262** | *Lactobacillus plantarum* | Chestnut flower | Magnusson *et al.* 2003 |
| **M939** | *Lactobacillus plantarum* | Grass silage | Ström *et al* 2005 |
| **M123** | *Lactobacillus coryniformis* | Dandelion | Magnusson *et al.* 2003 |
| **M275** | *Lactobacillus coryniformis* | Coltsfoot flower | Magnusson *et al.* 2003 |
| **M282** | *Lactobacillus coryniformis* | Hepatica flower | Magnusson *et al.* 2003 |
| **M291** | *Lactobacillus coryniformis* | Rowan leaves | Magnusson *et al.* 2003 |
| **M303** | *Lactobacillus coryniformis* | Coltsfoot flower | Magnusson *et al.* 2003 |
| **M311** | *Lactobacillus coryniformis* | Dandelion flower | Magnusson *et al.* 2003 |
| **Si3** | *Lactobacillus coryniformis* | Grass | Magnusson *et al.* 2003 |
| **Si3Cb** | *Lactobacillus coryniformis* | Grass | This study |
| **M18** | *Pedicoccus pentosaceus* | Chestnut flower | Magnusson *et al.* 2003 |
| **M24** | *Pedicoccus pentosaceus* | Clover | Magnusson *et al.* 2003 |
| **M392** | *Pedicoccus pentosaceus* | Grass silage | Unpublished |
| **203** | *Clostridium butyricum* | Mejerierna, Lund | Kask 1996 |
| **208** | *Clostridium tyrubutyricum* | Mejerierna, Lund | Kask 1996 |
| **211** | *Clostridium tyrubutyricum* | Mejerierna, Lund | Kask 1996 |
| **213** | *Clostridium tyrubutyricum* | Mejerierna, Lund | Kask 1996 |
| **ATCC29212** | *Enterococcus faecalis* | Reference strain | CCUG* |

* Culture Collection University of Gothenburg

For the ensiling study *Lactobacillus plantarum* M14 and *Lactobacillus coryniformis* Si3 were labelled with antibiotic resistance to make it possible to follow their growth in the silage. *L. plantarum* M14 was transformed with the plasmid pLV100 (Sorvig *et al.*, 2005) giving the transformant M14(pLV100). The plasmid contains a gene coding for resistance against chloramphenicol. The plasmid also contains genes coding for two plasmid maintenance proteins *orf* 2 (PemI like protein) and *orf* 3 (PemK like protein) (Sorvig *et al.*, 2005; Zhang *et al.*, 2004). To obtain the mutant *L. coryniformis* Si3Cb selection on antibiotic containing plates was used. The mutant is spontaneously resistant against rifampicin and streptomycin. It has earlier been shown that both labelled strains are stable for at least 50 subsequent generations, i.e. that the plasmid or resistance genes

are not lost during growth in the absence of selection. It has also shown that the labelled strains have the same properties as the wild types concerning growth, carbohydrate fermentation as tested using API 50 CH (BioMerieux, France) and antifungal effect.

**Ensiling study**

Grass dominated crop from Kungsängen Research Centre, Uppsala, was harvested and pre dried to a DM of 43.9%. The grass was placed on plastic sheets and inoculated with bacterial suspensions of either *L. coryniformis* Si3Cb or *L. plantarum* M14(pLV100). The bacteria were suspended in peptone water (Oxoid, 0.2% w/v) to a concentration of 1.25 x $10^9$ cells/ml to give a final concentration of 5 x $10^6$ cells/g grass. The control was sprayed with sterile peptone water. Five hundred and eighty g +/- 10 g of the material was packed in glass jars (1.7 l), the jars were sealed with a lid and water lock (Fig. 2). From all three treatments, three PVC silos were filled with 1500 g material and sealed with a lid and water lock (Fig. 2). As controls the same crop was inoculated with a suspension of *L. coryniformis* Si3 or *L. plantarum* M14 at a concentration of 5 x $10^6$ cells/ g grass and mixed. One thousand five hundred g of the material was packed in PVC silos and sealed with lid and water lock. All silos were incubated at 20°C.

*Figure 2:* Glass silos (left) and PVC silos (right) used in the ensiling study

Fresh grass was analysed for the amount of epiphytic LAB and occurrence of moulds and yeasts. The grass was also screened for epiphytic LAB with resistance to chloramphenicol and rifampcin/streptomycin that would hamper identification of the labelled bacteria.

Glass silos were opened at day 1, 2, 3, 5, 10, 20, 30 and 60 to follow the growth of LAB in the silage. At each day, three silos from each treatment (control, Si3Cb and M14 (pLV100)) were opened. The top 5 cm of the grass was removed and 20 g of material was mixed with 180 g sterile peptone water in the Stomacher at normal speed for 120 s. The fluid was serially diluted in peptone water. To follow the growth of the labelled strains, the diluted material was spread on MRS-plates with the relevant antibiotic (Table 3). The total number of LAB in the silage was determined on MRS-

plates. The purpose of using plates with antibiotics was to measure the amount of the added bacterial strain and also see that the added bacteria were alive. By combining that with plain MRS the total amount of LAB in the silage were detected.

*Table 3:* Treatment of grass and which plates were used for detection of different LAB. Cl = chloramphenicol, Rif = rifampicin and step = streptomycin

| | **M14(pLV100)** | **Si3Cb** | **Peptone water** |
|---|---|---|---|
| **MRS + Delvocid** | X | X | X |
| **MRS +Cl (15µg/ml)** | X | | X |
| **MRS + Rif (40µg/ml) / Strep(400µg/ml)** | | X | X |

After 90 days, the PVC silos were opened, the amount of LAB was analysed with the same method as day 1-60. The aerobic stability was determined by filling 400 g silage in sterile PVC tubes with ends sealed with a fibre cloth. The tubes were placed in a block and a thermometer was placed in each tube to register the temperature every second hour for 7 days. A rise in temperature indicates growth of aerobic organisms.

### *PCR analysis*

To further confirm that the bacteria growing in presence of respective antibiotics were the labelled strains, colonies were subjected to PCR-analysis.

Primers pLV100:1 (5'-ggt aag cca agg tga tat att tta tg-3') and pLV100:2 (5'-aag agg gat aaa ttt cat atc ctt tc-3') were used to identify *L. plantarum* M14(pLV100) and primers Lc:1 (5'-cgt aat tag gcg ttt aaa agg gc-3') and Lc:2 (5'-atc aac ttt gat gtt acg gac gg-3') were used to identify *L . coryniformis* Si3Cb.

ReadyToGo PCR beads were used according to the manufacturers instruction. Primers 1 and 2 were added to a final concentration of 2µM. Ten µl of the mix was added to a PCR tube and a small amount of colony material was added. For detection of *L. plantarum* M14(pLV100) a colony of *L. plantarum* M14 was used as a negative control and a colony of *L. plantarum* M14(pLV100) was used as positive control. For detection of *L. coryniformis* Si3Cb, a *L. plantarum* M14 colony was used as negative control and a *L. coryniformis* Si3Cb colony as positive control. The PCR was done under following conditions: 95 °C, 20 s; 47 °C, 20 s; 72 °C, 1 min for 30 cycles. Gel electrophoresis was performed in 1% agarose gel for *L. plantarum* M14(pLV100) and 2% for *L. coryniformis* Si3Cb in TBE buffer, stained with ethidium bromide and photographed in UV light.

## Determination of antibacterial activity

### *Agar well method*

In this study four LAB strains; *L. plantarum* M14, *L. coryniformis* M123, *L. plantarum* M939 and *L. coryniformis* Si3 were screened against four strains of *Clostridium* (see table 2). Clostridia were grown over night at 30°C in RCM. LAB were grown over night at 30°C in MRS broth. One hundred ml RCA was inoculated with suspension of clostridia to a concentration of $10^5$-$10^6$ cells/ml and poured into three Petri dishes. After 30-60 minutes four wells about 6 mm in diameter were cut out in the agar in each dish

and a small amount of soft agar was pipetted into the wells. Fifty µl of an over night culture of LAB in MRS was pipetted into the wells. The plates were incubated under anaerobic conditions at 30°C for 48 h after which the inhibition zone (clear zone around the wells) was measured.

***Overlay method***

The same clostridia and LAB as in the agar well method were used. LAB was streaked in two 2 cm long lines on MRS plates and incubated under anaerobic conditions at 30°C for 48 h. Soft agar containing 0.05% malt extract was inoculated with clostridial cells to an amount of $10^5$ cells/ml, 10 ml was poured over the plates with LAB and incubated under anaerobic conditions at 30°C for 48 h. After incubation the plates were examined for clear zones around the bacteria and given scores according to table 4.

*Table 4:* The scores used for evaluation inhibition with the overlay method (Magnusson & Schnürer, 2001)

| **Inhibition area (%)** | **Score** |
|---|---|
| **No inhibition** | - |
| **Weak inhibition above the LAB** | (+) |
| **0,1-3** | + |
| **3-8** | ++ |
| **>8** | +++ |

**Antibiotic resistance in the epiphytic flora of grass**

One hundred ninety-two colonies isolated from the epiphytic flora in the silage experiment were grown in 96 micro wells plates in MRS. Bacteria in groups of 48 were replica plated onto 6 MRS agar plates containing antibiotics at concentrations given in table 5 and incubated at 30°C for 48 hours.

*Table 5:* The antibiotics and concentration used

| **Antibiotic** | **Manufacturer** | **Concentration in plates (µg/ml)** | **MIC values (µg/ml) according to SCAN*** |
|---|---|---|---|
| **Ampicillin** | Roche Diagnostics | 5 | 4 |
| **Chloramphenicol** | Sigma | 5 | 4 |
| **Erythromycin** | Sigma | 5 | 4 |
| **Kanamycin** | Sigma | 10 | 8 |
| **Streptomycin** | Merck | 10 | 8 |
| **Tetracycline** | Boehringer-Mannheim | 10 | 8 |

* The 2005 edition of the document was not available when this study was initiated therefore the values from the 2004 edition has been used.

**Determination of MIC-values with agar dilution method**

Seventeen strains from the LAB collection at the Department of Microbiology (Table 2) were used in this screening. Plates with Cation-reduced Mueller-Hinton agar (Becton, Dickinson & Co) with 5% horse serum and one of the six antibiotics (Table 5) at five

different concentrations (2, 4, 8, 16 an 32 μg/ml) were used. Overnight culture of the strains was replica plated onto the plates and incubated at 30°C for 48 h.

### Determination of MIC-values with broth dilution method

Four strains from the LAB collection at the Department of Microbiology were used in this screening (*L. plantarum* L4, *L. plantarum* M14, *L. plantarum* M939 and *L. coryniformis* Si3), here, also *E. faecalis* was used as a control. For this method VetMIC™ plates GP-mo-A and GP-mo-B (The Swedish National Veterinary Institute) were used according to the manufacturer's instruction. The plates were incubated at 37ºC for 48 h. When using these plates 16 antibiotics are tested; avilamycin (Av), cephalothin (Ct), chloramphenicol (Cm), clindamycin (Cl), enroflaxin (Ef), erythromycin (Em), fusidic acid (Fu), gentamicin (Gm), neomycin (Nm), oxacillin (Ox), oxytetracycline (Tc), penicillin (Pc), streptomycin (Sm), trimethoprim (Trim), vancomycin (Va) and virginiamycin (Vi).

Initially, CAMHB with 5% horse serum was used, later a novel medium called LAB Susceptibility test Medium (LSM) consisting of 90% Isosensitest broth and 10% MRS broth, adjusted to pH 6.7 were also tested in the VetMIC™ plates (Klare *et al.,* in press). The plates were incubated at 37ºC for 48 h.

## RESULTS AND DISCUSSION

### Ensiling study

LAB can be used as feed additives/starter cultures in silage production and the most important property is to compete with the epiphytic flora and dominate the fermentation process. In the ensiling study two strains of LAB that might be used as additives in the future were followed during the whole ensiling process to document their growth and ability to compete with the epiphytic flora. In the end, it is also important that the added bacteria are able to inhibit growth of spoilage organisms such as moulds, yeasts and undesired bacteria.

The result of the analysis of the grass showed that the epiphytic flora of LAB at day 0 were $1.3 \times 10^5$ CFU $g^{-1}$. Growth in the presence of antibiotics was identified as moulds and yeasts showing that there were no detectable chloramphenicol or rifampicin/streptomycin resistant LAB in the epiphytic flora. Moulds and yeasts were present in the amount of $3.95 \times 10^6$ CFU $g^{-1}$. The moulds and yeasts colonies were picked and streaked on MEA plates for identification. Four different moulds were found and identified using *Introduction to food- and airborne fungi* (Samson *et al*., 2002). One species belonged to the Zygomycete group and one was a *Cladosporium* species. Two *Penicillium* species, most likely *Penicillium commune* and *P. roqueforti* were present in the grass. Yeasts were identified using ID32 test (BioMerieux). Two different yeasts were found and one was identified as Candida species, most likely *Candida lambica* that is frequently found in silage. The second species could not be identified, but it was a yeast with extremely pink colour that grew best at low temperatures (+ 2ºC).

The PCR analysis was used to confirm that the labelled bacteria were growing on the plates with the respective antibiotics. pLV100 is a derivative of p256 containing the chloramphenicol resistance gene (*cat*) from pIP501 (Sorvig *et al*., 2005). The pLV100:1 and pLV100:2 primers are localised in the *cat*-gene and in a neighbouring p256-sequence respectively. Thus, a PCR-product is only obtained from strains harbouring the recombinant plasmid and neither strains containing p256 nor the *cat*-gene alone. Si3Cb primers are localised in a part of the reuterin operon that is unique for *L. coryniformis*-strains (unpublished). That is, we could only confirm that what was growing on the Rif/Str-plates were an *L. coryniformis* strain.

No colonies of LAB were found on the antibiotic plates from the control silo during the first 20 days at the dilutions used to determine the amount of the labelled strains. On day 30 and 60 lower dilutions were used and there were some growth on both types of antibiotics. PCR analysis of these colonies showed that some of them were the labelled bacteria, indicating contamination during packing the silos.

Grass from all three treatments from day 3, 5, 12, 20 and 30 had been stored in the freezer and could be used to determine the extent of the contamination. The same method as for the analysis on days 1-90 was used but all three kinds of agar plates were used for each treatment and less diluted material were spread on the plates. This showed that the silos with *L. plantarum* M14(pLV100) were not contaminated at all, the silos with *L. coryniformis* Si3Cb were contaminated with *L. plantarum* M14(pLV100) and the control silo was contaminated with both *L. plantarum* M14(pLV100) and *L. coryniformis* Si3Cb. In both cases the contamination was detectable from day three. This is consistent with the fact that the silos with *L. plantarum* M14(pLV100) were packed first followed by *L. coryniformis* Si3Cb and the control silo.

The control silos (peptone water, wt *L. coryniformis* Si3 and wt *L. plantarum* M14) are all contaminated with both *L. plantarum* M14(pLV100) and *L. coryniformis* Si3Cb and can not be used as reference material in the study. The contamination of *L. coryniformis* Si3Cb was 3% of the total amount LAB at day 30, at day 60 it was as high as 10% but at day 90 it was only 1‰ (see Appendix 1). The contamination of *L. plantarum* M14(pLV100) was 1% or less during the entire period. In the silos with *L. coryniformis* Si3Cb the contamination of *L. plantarum* M14(pLV100) was 1‰ or less and the contamination can be ignored because most likely it has not affected the silage and *L. coryniformis* Si3Cb has dominated the fermentation.

The method for the ensiling study obviously has problems. Despite the use of surface disinfectants to remove bacteria from the equipment used during packing and also covering the untreated crops with plastic, silos were contaminated. We believe that this was due to aerosol formation (small droplets of water carrying bacteria) during spraying and mixing despite the time that passed between the different treatments. When two or more bacterial strains are going to be used in the same study some changes have to be done to avoid contamination. Spraying is probably the only way to add the bacteria to the grass so the method has to be revised in other ways. If several stains are going to be used the simplest way is to start with the control and then separate the batches of grass and spray them with respective bacteria in separate places. Maybe this is not possible due to lack of space, then the easiest way is to do the packing on separate days (which on the other hand will make it impossible to use the same crop under the same

conditions) and clean the room in between. It can also be done outside at different locations.

When analysing LAB on the MRS + delvocid plates from silos with *L. plantarum* M14(pLV100) and *L. coryniformis* Si3Cb almost all colonies picked were the labelled strains showing that both strains are able to dominate the fermentation in the silage.

The growth pattern differs between the both strains, *L. plantarum* M14(pLV100) grew fast in the beginning and between day 20 and 60 the number of living bacteria declined rapidly. *L. coryniformis* Si3Cb grew a bit slower at first but instead they did not die as quickly as *L. plantarum* M14(pLV100), see Appendices 2 and 3. The numbers of *L. plantarum* M14(pLV100) seems to increase from day 60 to 90. However, the values from the three silos at day 90 differed quite a lot ($4.1 \times 10^4$, $2.4 \times 10^5$ and $1.1 \times 10^6$) why this figure is uncertain. The fact that there are different types of silos used, for day 60 glass silos with smaller amount grass were used and for day 90 larger PVC silos were used affected the results. For example, the insulation properties may differ. In the future, both types of silos should be used for the last day investigated.

When analysing the silages after 90 days the amount of moulds and yeasts were determined (Fig. 3). On the plates two different types of yeasts were found but no moulds. In all silos the amount of fungi are lower than on the grass at day 0. The tested strains have known antifungal properties but they are both better of inhibiting growth of moulds than of yeasts. The differences between the treatments are small and there are also small differences between the labelled strains and the wild types. The amount of moulds and yeast in the silos with Si3Cb varied a lot, from $6 \times 10^5$ to $7 \times 10^6$, that is why the standard deviation is so large.

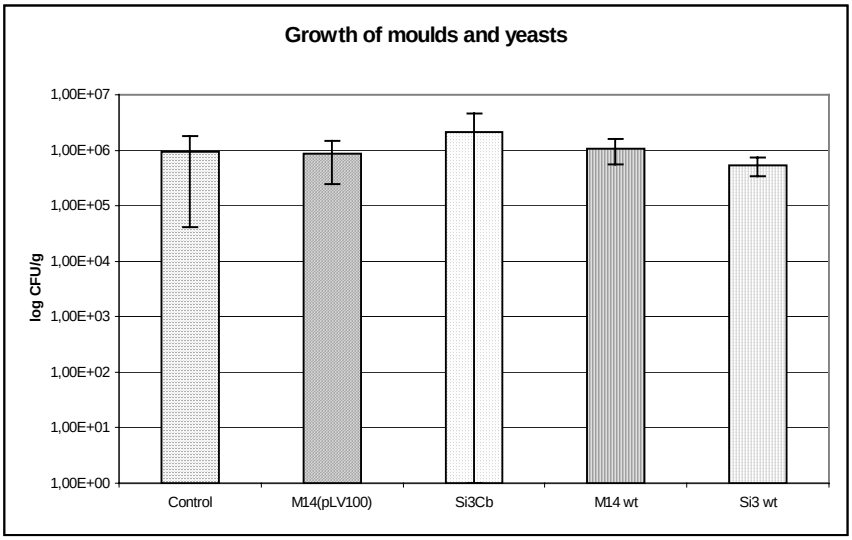


*Figure 3:* The amount of moulds and yeasts in the silages after 90 days.

The aerobe stability of the silages was determined by measuring the temperature in the silage every second hour during 7 days. The control silage reaches an increase of 5°C above room temperature after 0.75 day and the inoculated silages after 1.3-1.8 days. Desirable is that no increase in temperature takes place because it indicates growth of aerobe organisms in the silage. The temperature curve for *L. plantarum* M14 wt and

*L. plantarum* M14(pLV100) are almost identical, this shows that it is not a benefit or disadvantage to carry the plasmid. Between *L. coryniformis* Si3 wt and Si3Cb the differences are small, Si3Cb is a spontaneous double resistant mutant and the genetic background to the resistance is unknown. It cannot be excluded that other properties also are affected. The low aerobic stability of all silages suggests that the quality is low. However, no definite conclusions can be drawn until the chemical analyses of the respective silages are finished.

**Antibacterial activity**

Bacteria like clostridia are not wanted in the silage because they are able to cause food borne illness, including very dangerous conditions like botulism and problems in the production of food. Several strains of LAB have antibacterial properties and when using LAB as feed additive in silage, it is an advantage if that specific strain also has the ability to inhibit unwanted bacteria. In this study, strains that might be used as feed additives were tested for their antibacterial capacity against clostridia.

The antibacterial activity against *C. butyricum* and *C. tyrubutyricum* was determined with two different methods, the agar well method and the overlay method. After incubation for 48 h a clear zone around some of the wells with LAB was visible (Fig. 4) and the diameter of the zone was measured. *L. coryniformis* M123 inhibited all four clostridial strains when the agar well method was used and *L. plantarum* M14 inhibited all except *C. tyrubutyricum* 208. *L. plantarum* M939 and *L. coryniformis* Si3 inhibited *C. butyricum* 203 and *C. tyrubutyricum* 213 but not *C. tyrubutyricum* 208 and *C. tyrubutyricum* 211. *C. tyrubutyricum* 208 seemed to be the hardest strain to inhibit (Table 6).

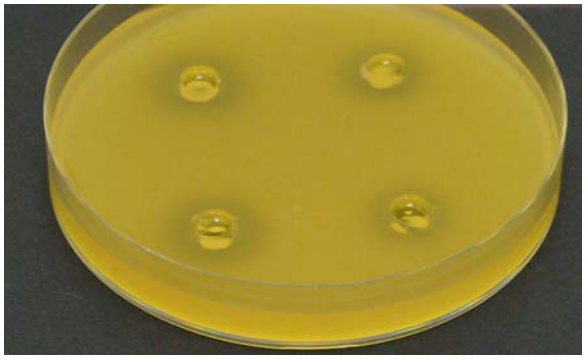

*Figure 4:* Inhibition of *Clostridium tyrubutyricum* 213 by LAB visible as a clear zone around the wells. Top from left M14 and M123, bottom from left M939 and Si3.

*Table 6:* Inhibition of growth of Clostridia by LAB in MRS broth expressed as clear zones around the wells (mm) including well (6 mm)

| | *C. butyricum* **203** | *C. tyrubutyricum* **208** | *C. tyrubutyricum* **211** | *C. tyrubutyricum* **213** |
|---|---|---|---|---|
| ***L. plantarum* M14** | 13 | No inhibition | 10 | 13 |
| ***L. coryniformis* M123** | 13,3 | 10,5 | 9 | 12 |
| ***L. plantarum* M939** | 16 | No inhibition | No inhibition | 13,3 |
| ***L. coryniformis* Si3** | 11,7 | No inhibition | No inhibition | 10,7 |

Unfortunately the overlay method did not work in the way it was used here. The clostridia did not grow at all on the plates with LAB or only very little in the edges of the agar plates and it was impossible to measure the inhibition. It was no problem for the clostridia to grow in the soft agar, on the control plate (without LAB) they covered the plate, and they also manage to grow on plain MRS. Clostridia are sensitive to low pH and when LAB are allowed to grow on the plates for 48 h before clostridia are added they will produce lactic acid which diffuse in the agar and lowers the pH. Clostridia are not able to grow under these conditions and the method has to be change. Probably addition of a suitable buffer to the MRS plates will help to maintain the pH and thereby it will be possible to see if LAB are able to inhibit clostridia with some compound other than lactic acid. There was no problem with the agar well method when the two bacteria started to grow at the same time.

## Antibiotic resistance

### *Antibiotic resistance in the epiphytic flora*

The breakpoint values presented in documents by SCAN have varied since the first document in 2002 (Appendix 6). To investigate how the antibiotic resistance in naturally occurring LAB in grass related to these values the epiphytic flora in the material used for ensiling was studied. One hundred ninety two colonies were picked at random and screened without further identification or characterization. The concentrations of the six antibiotics used were 20% higher than the values set by SCAN (Question Nº EFSA-Q-2004-079, 2004). MRS plates were used for practical reasons to ensure that all the strains would grow, however this medium is not really suitable for determination of antibiotic resistance.

No strains grew on ampicillin, erythromycin and tetracycline while nearly all strains grew on kanamycin and streptomycin. About 15% of the strains grew on chloramphenicol (Table 7).

*Table 7:* Results from the determination of antibiotic resistance in the epiphytic flora of the grass

| Antibiotic | Concentration in plates (μg/ml) | Amount of growing strains (of 192) | Percent |
|---|---|---|---|
| **Ampicillin** | 5 | 0 | 0 |
| **Chloramphenicol** | 5 | 28 | 14,6 |
| **Erythromycin** | 5 | 0 | 0 |
| **Kanamycin** | 10 | 176 | 91,7 |
| **Streptomycin** | 10 | 191 | 99,5 |
| **Tetracycline** | 10 | 0 | 0 |

### *Antibiotic resistance among LAB with known antifungal activity*

The LAB collection at the Department of Microbiology contains several strains with a known antifungal effect and 17 of these were chosen for determination of MIC values with the agar dilution method. Strains with antifungal properties might be of interest for use as food or feed additives instead of chemical preservatives and then it is important

that they not carry antibiotic resistance genes that can be transferred to pathogenic bacteria. For this study Cation Adjusted Mueller Hinton agar plates containing 5% horse serum was used. As different from MRS this media is approved for antibiotic resistance studies since it is believed to have minimal influences on the antibiotics. All strains used were able to grow on this medium in the absence of antibiotics. The MIC value was read as the lowest concentration that completely inhibited growth. Erythromycin was the only antibiotic on which none of the strains tested were able to grow. For most of the strains the MIC values for ampicillin, chloramphenicol, kanamycin, streptomycin and tetracycline were close to or over the breakpoints set by SCAN (Table 8).

In the study of antibiotic resistance in the epiphytic flora almost all strains were able to grow on agar plates with kanamycin and in the determination of MIC values all tested strains had MIC values over 32 μg/ml for kanamycin. Unfortunately kanamycin is not one of the antibiotics tested in the VetMIC™ plates.

*Table 8:* Results from the determination of MIC values (μg/ml) with agar dilution method. Amp = ampicillin, Cl = chloramphenicol, Ery = erythromycin, Kana = kanamycin, Strep = streptomycin and Tet = tetracycline

| Strain | Amp | Cl | Ery | Kana | Strep | Tet |
|---|---|---|---|---|---|---|
| ***L. plantarum* L4** | 8 | 16 | <2 | >32 | >32 | >32 |
| ***L. plantarum* M6** | 4 | 32 | <2 | >32 | >32 | >32 |
| ***L.plantarum* M14** | 4 | 16 | <2 | >32 | >32 | 32 |
| ***P. pentosaceus* M18** | 4 | 32 | <2 | >32 | >32 | >32 |
| ***P. pentosaceus* M24** | 8 | 32 | <2 | >32 | >32 | >32 |
| ***L. plantarum* M37** | 8 | 32 | <2 | >32 | >32 | >32 |
| ***L. coryniformis* M123** | 4 | 32 | <2 | >32 | >32 | 8 |
| ***L. plantarum* M248** | 4 | 32 | <2 | >32 | >32 | >32 |
| ***L. plantarum* M262** | 4 | 32 | <2 | >32 | >32 | >32 |
| ***L. coryniformis* M275** | 4 | 32 | <2 | >32 | >32 | 32 |
| ***L. coryniformis* M282** | S | 16 | <2 | >32 | >32 | 32 |
| ***L. coryniformis* M291** | 4 | 16 | <2 | >32 | >32 | 16 |
| ***L. coryniformis* M303** | 4 | 32 | <2 | >32 | >32 | 32 |
| ***L. coryniformis* M311** | 4 | 16 | <2 | >32 | >32 | 32 |
| ***P. pentosaceus* M392** | 4 | 32 | <2 | >32 | >32 | >32 |
| ***L. plantarum* M939** | 4 | 32 | <2 | >32 | >32 | >32 |
| ***L. coryniformis* Si3** | 4 | 16 | <2 | >32 | >32 | >32 |

Four strains (*L. plantarum* L4, *L. plantarum* M14, *L. plantarum* M939 and *L. coryniformis* Si3) from the LAB collection were of extra interest since they have very good antifungal properties and have in earlier studies shown that they improve silage quality, were picked for further antibiotic resistance tests with VetMIC™ plates. When using these plates it is possible to test several antibiotics at the same time. The plates were used with two different types of broth, CAMHB with 5% horse serum and LSM broth. CAMHB is as mentioned earlier approved for antibiotic resistance studies but it is known that some strains of LAB not grow as well as desired in this broth, why the LSM broth was developed within the EU-financed project Assessment and Critical Evaluation of Antibiotic Resistance Transferability in the Food Chain (ACE-ART). The project ACE-ART is focused on breakpoints for antibiotics for non-pathogenic bacteria belonging to the group LAB because they are important in food and feed industry and

might carry antibiotic genes that can be transferred to other bacteria (www. aceart.net). When using both media in the same testing system, they can easily be compared. For most of the antibiotics tested, the values were the same regardless of media used (Appendix 5). For *L. plantarum* strains all strains had MIC values over the breakpoint set by SCAN for tetracycline, for chloramphenicol the MIC values were just at the breakpoint or slightly above for all strains. For *L. coryniformis* Si3 the MIC value for chloramphenicol were at the breakpoint or over for CAMHB with 5% horse serum and at the breakpoint or lower with LSM broth. For tetracycline *L. coryniformis* Si3 has breakpoints of 16-32 μg/ml and the breakpoint is at 8 μg/ml. The control strain *E. faecalis* ATCC29212 was only tested in CAMHB with 5% horse serum and the MIC values obtained never differed more than two fold from values found in EUCAST Discussion Document E. Dis 5.1 (2003) (data not shown).

The tetracycline resistance among these strains needs to be further investigated (if they have MIC values above the breakpoint); it has to be determined whether the resistance is intrinsic or acquired. If the resistance is intrinsic there is probably no risk of transfer to other bacteria and the strain can be used as food or feed additive. If the resistance is acquired, it has to be investigated further to determine whether it can be possible to use the strain in commercial products.

***Influences of method and growth media***

A study by Huys *et al* shows that the culture medium affects the antibiotic resistance pattern among LAB when using the disc diffusion method (Huys *et al.,* 2002). In the present study different methods and media are used and this phenomenon can also be seen here for some of the antibiotics used. Streptomycin and tetracycline seems to be the two antibiotics most sensitive to which media used in the determination. For example, when using MRS plates all strains tested are sensitive to tetracycline but when using CAMH agar or broth and LSM broth nearly all strains are resistant to tetracycline. The same thing can be seen with streptomycin, when using MRS plates or CAMH agar plates all strains have higher MIC values than with CAMH broth or LSM broth.

To sort this out we tested *L. plantarum* L4, *L. plantarum* M14, *L. plantarum* M939, *L. coryniformis* Si3 and one unidentified strain from the epiphytic flora against streptomycin and tetracycline in concentrations from 0-128 μg/ml in 96 micro wells plates in both MRS broth and LSM broth. All strains grew in presence of streptomycin at a concentration of 128μg/ml when using MRS broth, when using LSM media the strains were more sensitive and grew at concentrations of 32-64μg/ml. For tetracycline there was the same pattern, all strains grew in higher concentrations in MRS than in LSM. It can be established that in this study that all of the strains tested have MIC values lower than the breakpoints set by SCAN (except *L. coryniformis* Si3) and should therefore not be considered as resistant as they were in the VetMIC™ studies.

The occurrence of antibiotic resistance among LAB that are going to be used as food or feed additives has to be determined very accurately and it is important that the results are reliable and comparable from one study to another. Today there is no standardised media or methods available for LAB, which is needed to get accurate results comparable between different studies. Furthermore, the information on what methods should be used is very vague in the documents presented by SCAN. An agar or broth dilutions method should be used as opposed to disc diffusion methods and possible

media interference considered. Klare *et al.* (in press) has developed a standardised media, the Lactobacillus Susceptibility test Media used in broth dilutions tests in micro wells plates, that seems to be work well, is easy to prepare and hopefully this media and method can be the standardised method that is needed.

In Appendix 6, a comparison between three documents from SCAN is shown. From 2002 to 2005 the breakpoints for *Lactobacillus* spp. have change significantly. In the first document from 2002, are the same breakpoints for all lactobacilli. In 2004, the lactobacilli group has been separated into obligate homofermentative and heterofermentative and the breakpoints have been lowered for chloramphenicol, kanamycin, neomycin, streptomycin, tetracycline and trimethoprim. In 2005, *L. plantarum* has been given breakpoints and for kanamycin, neomycin and streptomycin the breakpoints have been increased again. The breakpoints for gentamicin are 8-fold higher, kanamycin and tetracycline is 4-fold higher and neomycin is 2-fold higher for *L. plantarum* than for other lactobacilli. No reasons are given for this. Neither is it mentioned on what basis the breakpoints are changed between the different documents. However, it is stated that the values will be reviewed on a regular basis and modified if necessary, if new data becomes available. This will have implications for the use of LAB as feed additives. For example, the MIC values presented in table 9 for *L. coryniformis* Si3 would be acceptable for an *L. plantarum* strain but not for a *L. coryniformis* strain without any obvious reason.

## CONCLUSIONS

The following conclusions can be drawn for this study:

The two labelled strains could be used to follow the growth of the inoculum in silage. Both strains competed well with the epiphytic flora and dominated from day one and throughout the study. However, the temperature in both silages increased indicating a low aerobic stability.

The agar well method should be used to determine antibacterial activity.

MRS medium should not be used for MIC determinations. Thus, the study of antibiotic resistance in the epiphytic flora should be repeated on another medium before any conclusions are drawn.

Methods based on the broth dilution seem to be preferable to agar dilutions methods when exact MIC values are determined, but replicating to agar plates will be useful when studying a large number of strains.

CAMHB with 5% horse serum and LSM broth seems to give comparable results, but LSM broth is easier to prepare.

The kanamycin resistance detected in the epiphytic flora and the antifungal strains from the culture collection has to be confirmed with broth dilution in CAMHB or LSM broth.

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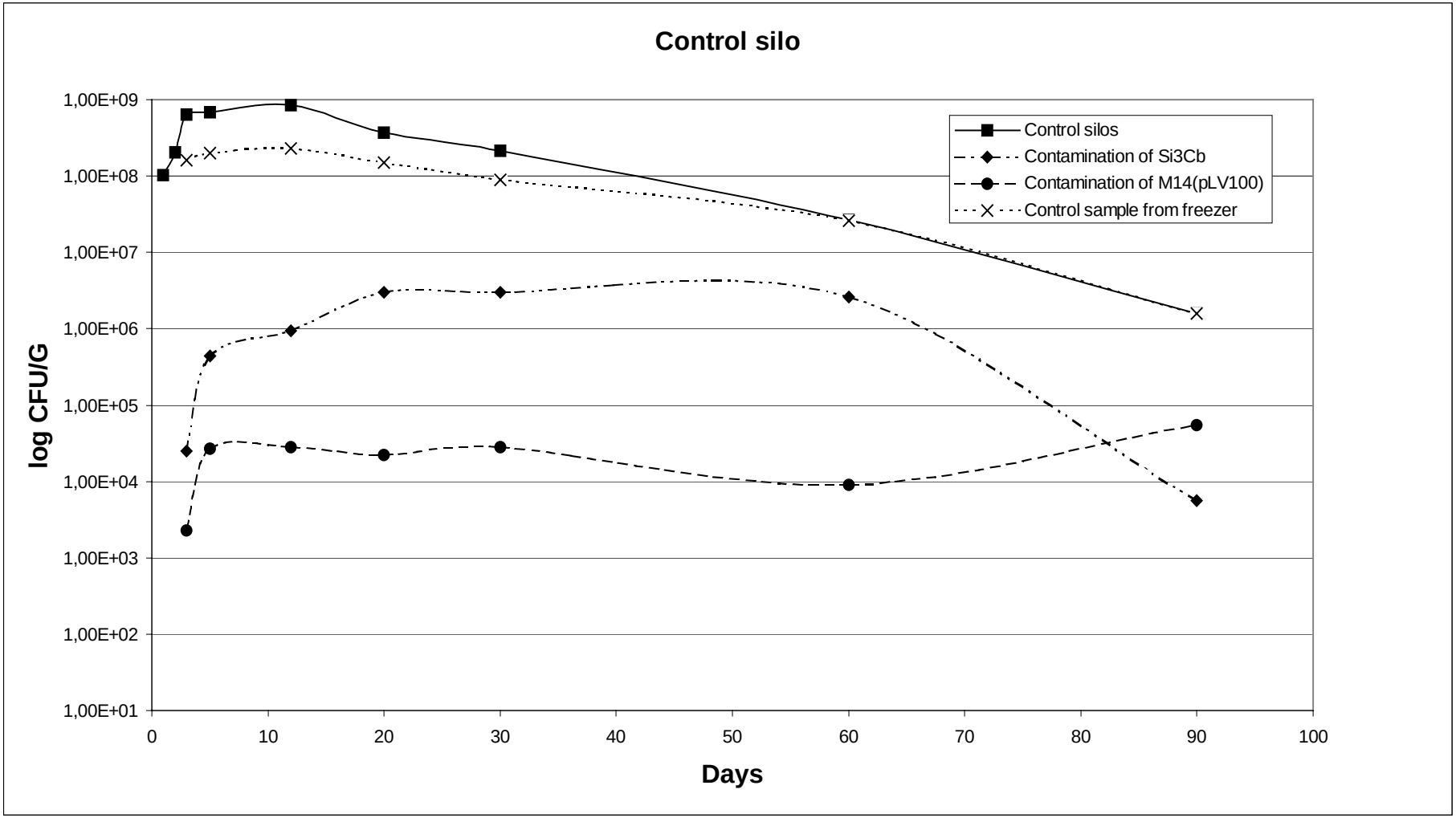
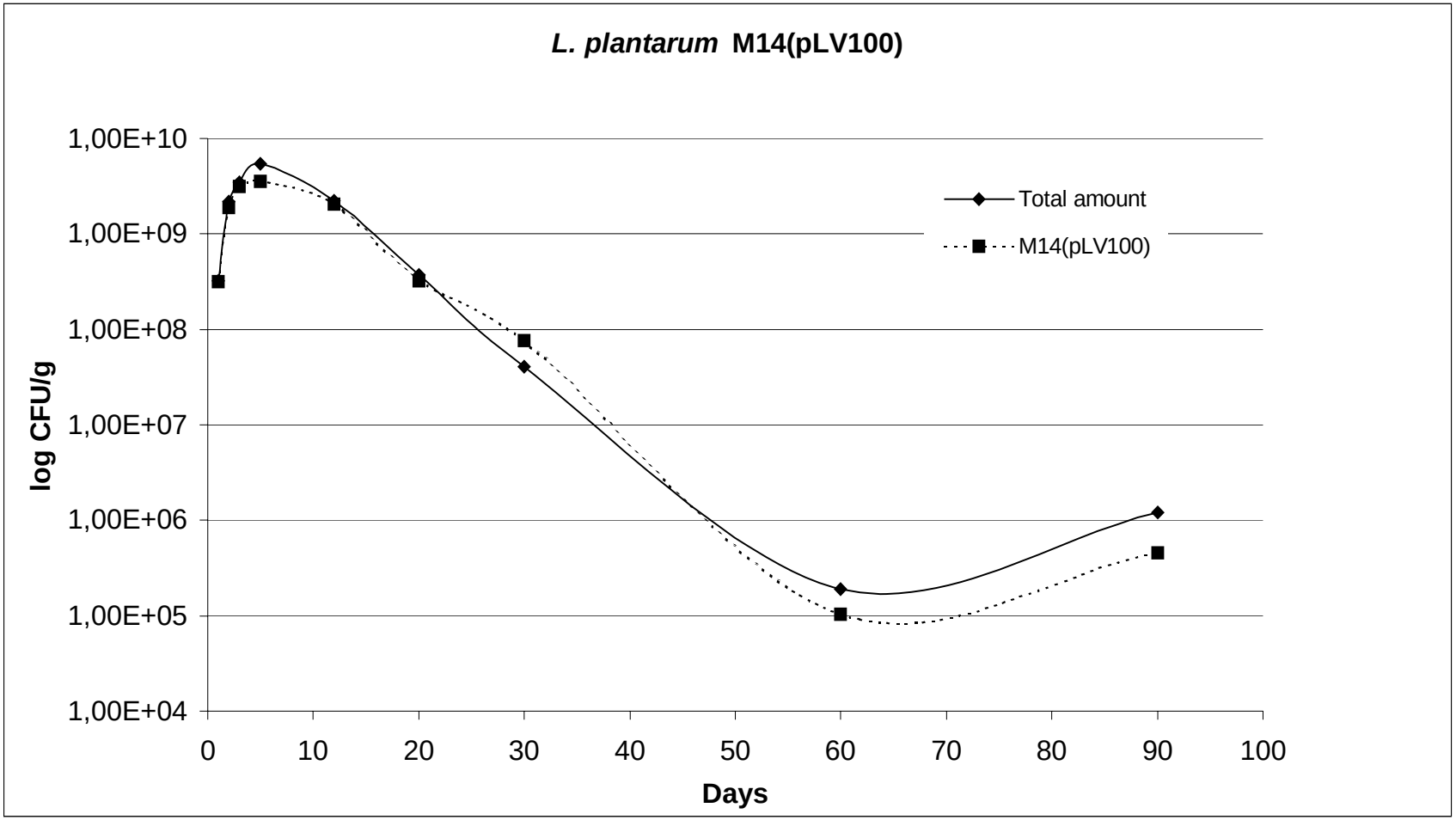
**APPENDIX 1**



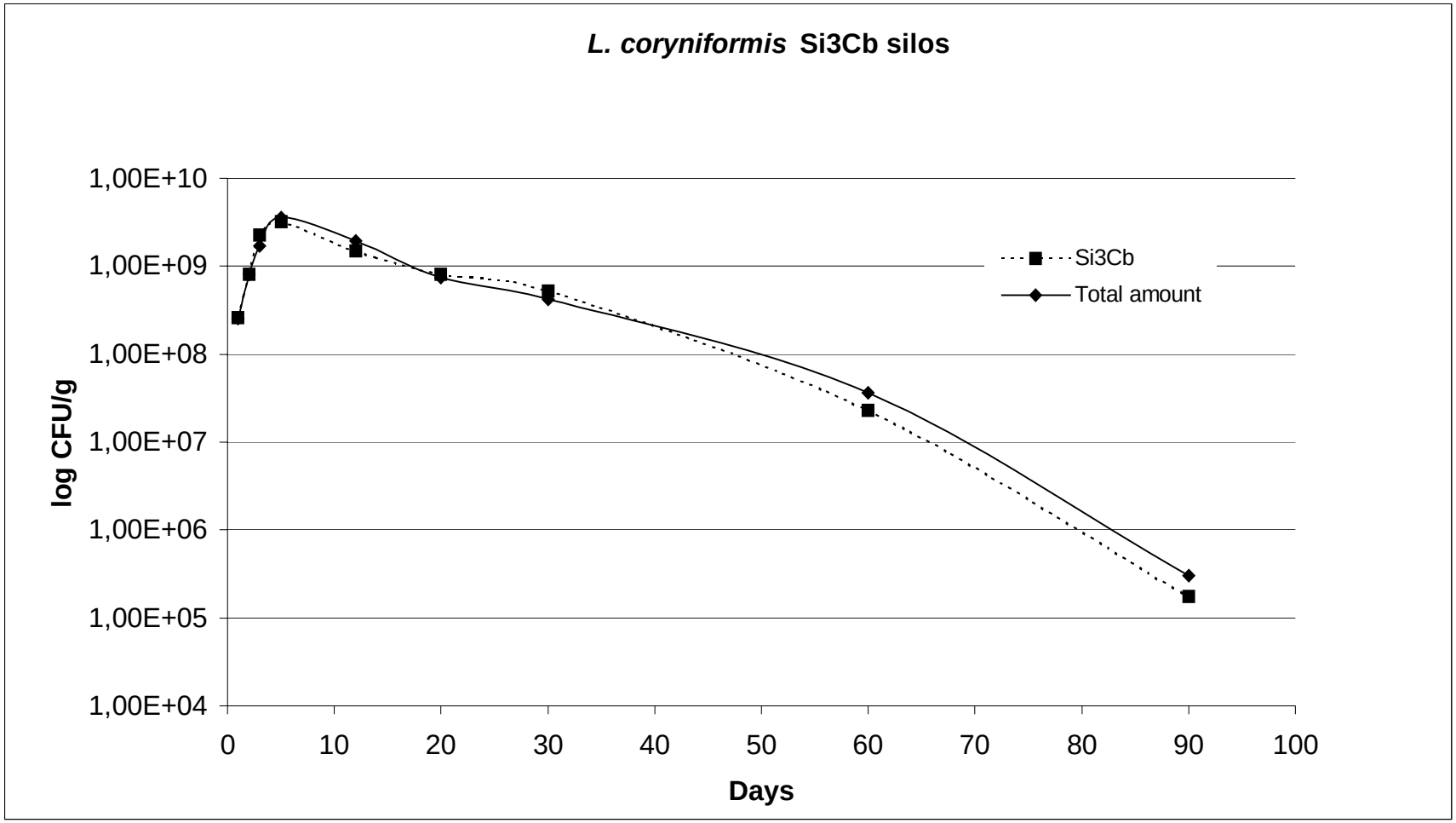

The growth in the control silos including values of the contamination (from samples from the freezer). All dotted lines are frozen except for the samples from day 90.

**APPENDIX 2**



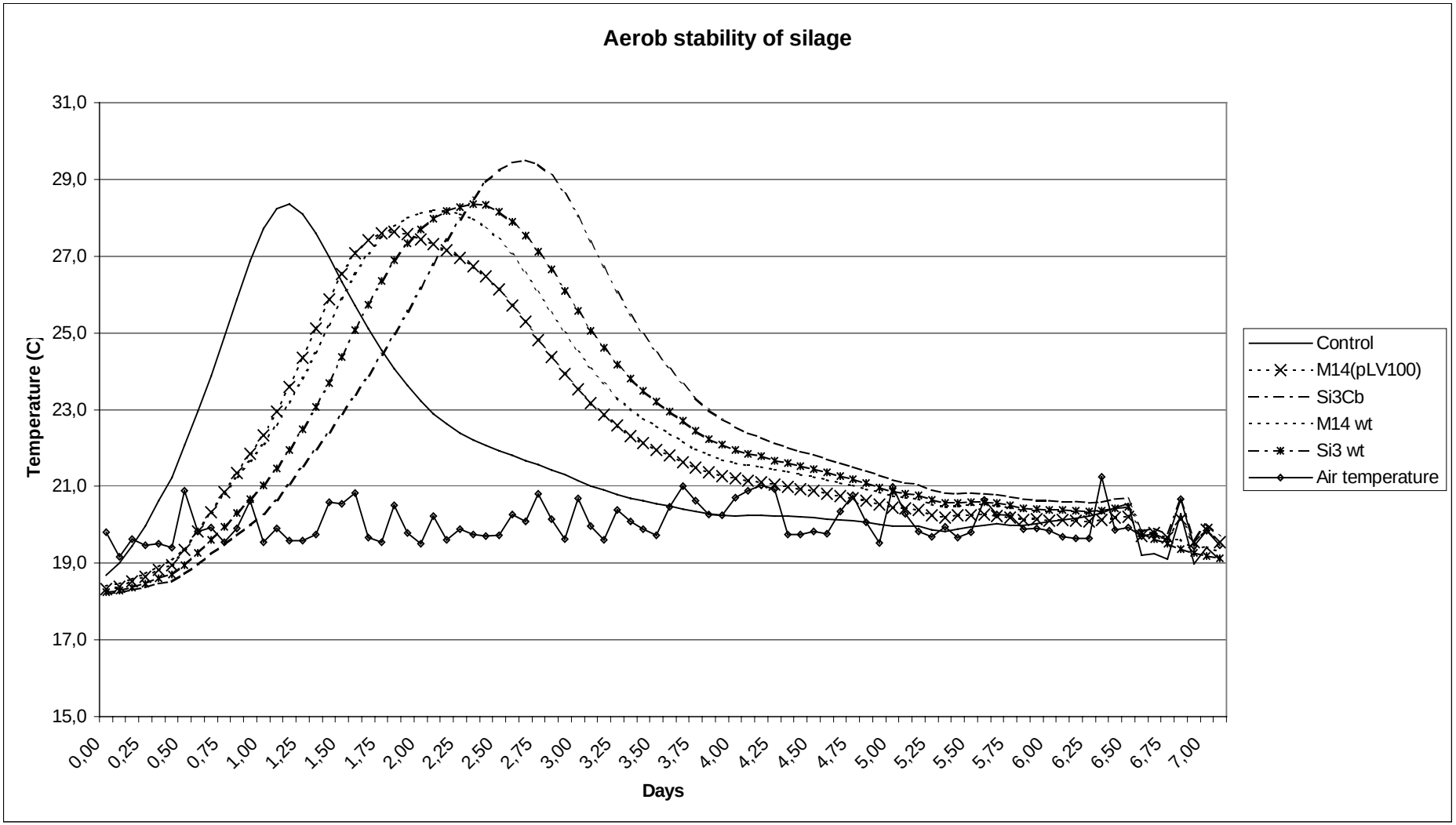

The growth of the labelled strain and total amount of LAB in the silos inoculated with *L. plantarum* M14(pLV100). The silos were opened in triplicates giving the mean values shown in the diagram.

**APPENDIX 3**




The growth of the labelled strain and total amount of LAB in the silos inoculated with *L. coryniformis* Si3Cb. The silos were opened in triplicates giving the mean values shown in the diagram.

**APPENDIX 4**




The aerobe stability of the silages, the temperature is measured every second hour for 7 days in triplicates giving mean values in the diagram.

## APPENDIX 5

Results (in µg/ml) from MIC value determination with VetMIC™ plates using either Cation Adjusted Mueller Hinton broth with 5% horse serum (upper) or LSM broth (italics). Abbreviations of antibiotics see text. For each antibiotic tested the concentration interval (µg/ml) is presented.

| Strain | Av (1-64) | Cl (0,25-32) | Cm (0,5-64) | Ct (0,06-8) | Ef (0,12-8) | Em (0,25-32) | Fu (0,06-8) | Gm (0,5-64) | Nm (1-128) | Ox (0,12-16) | Tc (0,5-64) | Pc (0,03-64) | Sm (2-256) | Trim (0,5-32) | Va (1-128) | Vi (0,25-32) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **L4** | >64 | 1 | 8-16 | >8 | >8 | <0,25 | >8 | <0,5 | <1 | 16->16 | 64->64 | >4 | 8 | <0,5 | >128 | 4 |
| | *>64* | *0,5-1* | *2-16* | *8* | *8* | *<0,25* | *>8* | *<0,5-2* | *<1* | *0,5-4* | *>64* | *0,5-0,12* | *4-16* | *<0,5* | *>128* | *2* |
| **M14** | >64 | <0,25-0,5 | 4-8 | >8 | 4->8 | <0,25 | >8 | <0,5 | <1-1 | 0,5-8 | 64 | 4 | 8 | <0,5 | >128 | 2-32 |
| | *>64* | *1-2* | *8-16* | *8* | *8* | *<0,25* | *>8* | *<0,5-1* | *<1-2* | *4-8* | *64* | *1* | *8-16* | *<0,5* | *>128* | *2* |
| **M939** | >64 | <0,25 | 8-16 | >8 | >8 | <0,25 | >8 | <0,5 | <1 | 16->16 | >64 | 4->4 | 8 | 4 | >128 | 2-8 |
| | *>64* | *0,5-32* | *8* | *8* | *>8* | *<0,25* | *>8* | *2* | *2-4* | *8* | *>64* | *2* | *32* | *0,5-32* | *>128* | *1-2* |
| **Si3** | >64 | 0,5 | 8-16 | >8 | 2 | <0,25-0,25 | >8 | <0,5 | <1 | <0,12-8 | 16-32 | 0,5-1 | 4 | <0,5 | >128 | 4-8 |
| | *>64* | *<0,25* | *4-8* | *>8* | *4->8* | *<0,25* | *>8* | *<0,5* | *<1* | *4-8* | *32* | *0,5* | *8* | *<0,5* | *>128* | *4* |

**APPENDIX 6**

Comparison between the breakpoints set by SCAN in three different documents from 2002 to 2005.

| **Antibiotic** | ***Lactobacillus***[1] | ***Lactobacillus* obligate homofermentative**[2] | ***Lactobacillus* heterofermentative**[2] | ***Lactobacillus* obligate homofermentative**[3] | ***Lactobacillus* heterofermentative**[3] | ***Lactobacillus plantarum***[3] |
|---|---|---|---|---|---|---|
| **Ampicillin** | 2 | 4 | 4 | 4 | 4 | 4 |
| **Chloramphenicol** | 16 | 4 | 4 | 4 | 4 | 8 |
| **Clindamycin** | - | 4 | 4 | 4 | 4 | 4 |
| **Erythromycin** | 4 | 4 | 4 | 4 | 4 | 4 |
| **Gentamicin** | 1 | 8 | 8 | 8 | 8 | 64 |
| **Kanamycin** | 32 | 8 | 8 | 16 | 16 | 64 |
| **Linezolid** | 4 | 4 | 4 | 4 | 4 | 4 |
| **Neomycin** | 16 | 8 | 8 | 16 | 16 | 32 |
| **Streptomycin** | 16 | 8 | 8 | 16 | 16 | 64 |
| **Tetracycline** | 16 | 8 | 8 | 8 | 8 | 32 |
| **Trimethoprim** | 32 | 8 | 8 | 8 | 8 | 8 |
| **Vancomycin** | 4 | 4 | - | 4 | - | - |
| **Virginiamycin** | 4 | 4 | 4 | 4 | 4 | 4 |

[1] Opinion of the Scientific Committee on Animal Nutrition on the criteria for assessing the safety of micro organisms resistant to antibiotics of human clinical and veterinary importance. 2002

[2] Working document of the Scientific Panel on Additives and Products or Substances used in animal feed on the updating of the criteria used in the assessment of bacteria for resistance to antibiotics of human or veterinary importance (Question Nº EFSA-Q-2004-079). 2004

[3] Opinion of the Scientific Panel on Additives and Products or Substances used in animal feed on the updating of the criteria used in the assessment of bacteria for resistance to antibiotics of human or veterinary importance (Question Nº EFSA-Q-2004-079). May 2005