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AN *IN-VITRO* STUDY OF AEROBIC CHANGES IN SILAGES
Effects of microbial activities and impact factors

Inaugural-Dissertation
zur
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vorgelegt von
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Abbreviations

AB	antibacterial (treatment), corresponds to fungal activity
adj.	adjusted
aer.	aerobic
AM	antimycotic (treatment), corresponds to bacterial activity
BC	Buffering Capacity
CBS	Centraalbureau voor Schimmelcultures, Utrecht
corr.	corrected
cp.	counterpart
DLG	Deutsche Landwirtschafts-Gesellschaft e.V., Frankfurt a.M.
DM	dry matter
DSMZ	Deutsche Sammlung für Mikroorganismen und Zellkulturen (German Collection of Microorganisms and Cell Cultures), Braunschweig
FAL	Federal Agricultural Research Centre, Braunschweig
FM	fresh matter
fruct.	fructose
GLM	General Linear Models
gluc.	glucose
HPLC	High Performance Liquid Chromatography
ID	identification number
Iso	number of isolated micro-organism
LAB	lactic acid bacteria
MB	antimycotic + antibacterial treatment
MEA	malt extract agar
Meq	milliequivalent
MRS	medium to cultivate lactobacilli according to de MAN *et al.*, 1960
n.s.	not significant/ no significant difference
PAB	Propionic acid bacteria
suc.	sucrose
t	time
v/v	volume/volume
WSC	water soluble carbohydrates
w/v	weight/volume
(!)	highlighting an unexpected result

1 INTRODUCTION

Aerobic deterioration in silages occurs after the penetration of air, for example when damage to silo covers occurs during ensilage or after opening the silo during the feed-out phase of a well fermented silage.

Thus, aerobic spoilage of silages represents a problem for animal feeding.

Microbial metabolic activity leads to dry matter and energy losses in the silage. Dry matter losses of up to 3.5 % per day can occur in high DM silages (30 % DM) when the temperature is elevated by 15 °C above ambient (HONIG and WOOLFORD, 1980). A high proportion of DM losses can be explained by oxidation of fermentation acids (CRAWSHAW *et al.*, 1980).

Losses in energy and nutrients such as WSC and proteins reduce the nutritional value of silage for the ruminants and the accumulation of degradation products can reduce palatability and lead to feed refusals (DRIEHUIS *et al.*, 1999).

Aerobically spoiled silage can even represent a severe health risk for the animal and consequently human health if certain mycotoxins develop during mould growth (MÜLLER, 1987; ESCOULA, 1992; AUERBACH, 1996).

Thus aerobically spoiled silage is an economic problem for the farmer and for these reasons it should be prevented. Practical solutions such as attention to detail and use of appropriate ensiling technique, i.e. a high consolidation of the plant material, keeping the silo airtight and a well adapted progression rate during the feed-out phase (>2.5 m/week in summer, >1.5 m/week in winter) are the first and probably easiest areas to target. In addition, additives that prolong the aerobic stability e.g. chemical additives with the active agents sorbate and benzoate or heterofermentative inoculant lactic acid bacteria can be used to manipulate and improve the aerobic stability of silage.

Nevertheless, if progress is to be made in addressing this problem it will be necessary to have a greater understanding of all causes and species/activity interactions during the course of aerobic deterioration.

Generally, aerobic deterioration is the consequence of microbial metabolism, which is triggered when oxygen becomes available (PAHLOW *et al.*, 2003). Compounds in the readily fermented silage are converted into heat and metabolites and the effects can be monitored by measuring the temperature rise and increase of pH due to decomposition of organic acids and dry matter losses. Visually, yeast and/or mould colonies are often observed.

The objective of this research was to identify the mechanisms involved in the initial aerobic changes in silages and the micro-organisms responsible for them. Thus a model system was developed where silage was simulated and the microbial groups differentiated using antibiotic treatment.

This work was done during the employment in the EU SweetGrass Project (QLK5-CT-2001-0498) from 2002-2005. The project had the objective of investigating the benefits of feeding ensiled ryegrass (*Lolium perenne)* cultivars which were bred for high WSC content to ruminants. It was divided into four workpackages addressing agronomy, ensiling, efficiency of rumen function and livestock production. The ensiling workpackage was led by the FAL. During the years 2002-2003 observations on the "SweetGrass" silages were made which are described in *APPENDIX I*. The questions raised led to the current work which was carried out during 2004.

One assumption of the ensiling workpackage of the project was that high residual sugar contents might lead to higher aerobic instability of the silages as sugars are a nutrient source that is easily to metabolise for many micro-organisms. Thus this aspect represented an underlying working hypothesis of the thesis.

2 LITERATURE REVIEW

An awareness of the problems caused by aerobic deterioration first arose during the 1960s. This is reflected in the increasing numbers of scientific articles dealing with this subject in the 1970s.

Many of them dealt purely with the chemical and physical characterisation of aerobically deteriorating silages (e.g. OHYAMA and MASAKI, 1971; OHYAMA *et al.*, 1975; OHYAMA *et al.*, 1977; OHYAMA *et al.*, 1980; HONIG, 1975) and others with the inhibition of deterioration by chemical and biological additives (e.g. GROSS and BECK, 1970; DANIEL *et al.*, 1970; OHYAMA and McDONALD, 1975). The first workers to recognise that yeasts might play an important role in aerobic deterioration in silage were BECK and GROSS, 1964.

In this chapter those studies which concern the possible role of different microbial groups responsible for the aerobic deterioration of silages and were carried out over the last four decades are reviewed.

The prevailing view of the development of the silage spoilage flora is shown in the figure below.

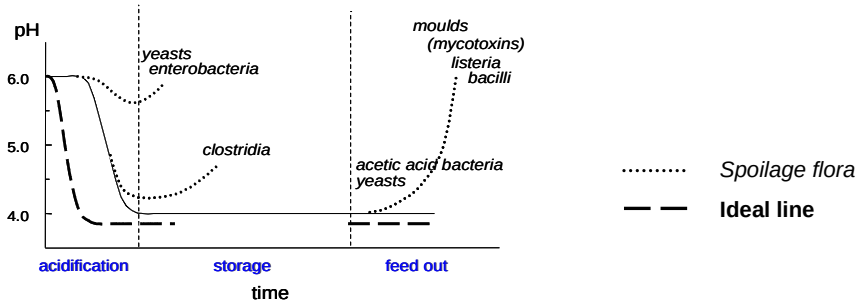


Figure 1: Silage spoilage flora (DRIEHUIS, 2002)

As one can see, in the initial feed-out phase yeasts and acetic acid bacteria are considered as the main spoilage flora followed by bacilli, listeria and moulds later on.

A relevant review of this subject entitled “The detrimental effects of air on silage” was published by WOOLFORD, 1990. Since that time knowledge of the microbiological principles of aerobic spoilage has not much expanded except that a broader range of potential additives against aerobic deterioration has been developed and in part approved by various bodies in member states of the EU.

Table 1: The identity of some of the microorganisms isolated from aerobically deteriorating silages (WOOLFORD, 1990)

Organism	Silage	Reference	Organism	Silage	Reference
Fungi (Yeasts)			**Bacteria (Proteolytic)**		
Candida krusei	Maize	BECK & GROSS (1964)	*Bacillus cereus*	Maize	WOOLFORD et al. (1978)
Hansenula anomala			*Bacillus firmus*		
Pichia fermentans			*Bacillus lentus*		
Pichia membranefaciens	Maize	HARA and OHYAMA, 1978	*Bacillus sphaericus*		
Saccharomyces bailii			*Bacillus cereus*	Grass	WOOLFORD et al. (1979)
Saccharomyces exiguus		WOOLFORD *et al.* (1978)	*Bacillus firmus*		
Candida melinii	Maize		*Bacillus laterosporus*		
Torulopsis candida			*Bacillus lentus*		
Candida krusei	Grass	WOOLFORD *et al.* (1979)	*Bacillus licheniformis*		
Candida melinii			*Bacillus pulvifacienes*		
Candida tenuis			*Bacillus sphaericus*		
Candida valida			**Bacteria (Lactic acid)**		
Hansenula anomala			*Lactobacillus buchneri*	Grass	WOOLFORD et al. (1978)
Hansenula polymorpha			*Lactobacillus bulgaricus*		
Hansenula subpelliculosa			*Lactobacillus viridescens*		
Pichia fermentans			*Pediococcus cerevisiae*	Grass	WOOLFORD et al. (1979)
Pichia media			*Lactobacillus buchneri*		
Pichia polymorpha			*Lactobacillus bulgaricus*		
Candida silvicola	Wheat & Lucerne	MOON and ELY, 1979	*Lactobacillus casei*		
Candida tenuis			*Lactobacillus coryneformis*		
Endomycopsis burtonii			*Lactobacillus helviticus*		
Endomycopsis selenospora			*Lactobacilus plantarum*		
Hansenula canadensis			*Lactobacillus salivarius*		
Candida lambica	Grass	JONSSON & PAHLOW (1984)	*Lactobacillus viridescens*		
Saccharomyces cerevisiae			**Bacteria (Acetic acid)**		
Saccharomyces exiguus			*Acetobacter* sp.	Maize	SPOELSTRA et al. (1988)
Candida holmii	Maize	MIDDEL-HOVEN & VAN BAALEN (1988)	**Bacteria (Actinomyces)**		
Candida famata			*Streptomyces* sp.	Maize	BECK (1975)
Candida milleri			*Streptomyces griseus*	Maize	LYONS *et al.*, 1975
Saccharomyces dairensis					
Fungi (Filamentous)					
Geotrichum sp.	Maize	HARA & OHYAMA (1978)			
Monascus sp.					
Mucor sp.					
Monilia sp.	Maize	WOOLFORD (unpublished)			
Penicillium notatum					
Dactylomyces thermophilus	Maize	OBERT *et al.*, 1976			
Penicillium piceum					
Thermomyces langinosus					

Fungi

Fungi are eukaryotic, heterotrophic organisms that take up nutrients by active or passive absorption.

Fungi isolated from aerobically deteriorating silages are mainly classified as yeasts and some as filamentous fungi and are summarised by WOOLFORD, 1990, in Table 1.

Yeasts

Yeasts belong to the fungal group possessing vegetative states, either as ascomycetes or basidiomycetes. They are generally characterised by budding or fission as the primary means of vegetative reproduction and have sexual states that are not enclosed in fruiting bodies (KURTZMAN and FELL, 1998). As heterotrophic fungi they obtain nutrients for their growth by secreting extracellular enzymes (proteases, lipases, amylases, cellulases) which break down complex organic molecules to simple monomers that can then be absorbed through their cell membranes (MCDONALD *et al.*, 1991).

Yeasts are one group of micro-organisms that are present at ensiling, but they hardly contribute to the lactic acid fermentation itself. They are regarded as undesirable mainly because of their leading role in aerobic spoilage and their competition for carbohydrates with lactic acid bacteria (MCDONALD *et al.*, 1991; OUDE ELFERINK *et al.*, 1999a).

Yeasts are present on fresh plant material. The majority of these are non-fermenting species of the genera *Cryptococcus, Rhodotorula, Sporobolomyces* and *Torulopsis* (MCDONALD *et al.*, 1991; MIDDELHOVEN and VAN BAALEN, 1988). At the beginning of ensiling yeasts and other micro-organisms start to grow and compete for available nutrients until oxygen is used up.

The aerobic yeasts are followed by growth of the fermentative species as anaerobic conditions prevail.

During the storage phase facultatively anaerobic yeasts can ferment sugars mainly to ethanol and CO_2 (OUDE ELFERINK *et al.*, 1999a; LENGELER *et al.*, 1999), but n-propanol, iso-pentanol, acetic, propionic, butyric, iso-butyric and lactic acid are also formed (NORD and WEISS, 1958; MCDONALD *et al.*, 1991).

Under anaerobic conditions yeasts can be suppressed by acetic acid (in combination with 1,2-propanediol), which can be produced by, for example heterofermentative LAB

(OUDE ELFERINK *et al.*, 1999b). Other inhibitors are butyric and propionic acid (OHYAMA and HARA, 1975).

If not depressed, then after opening of the silo yeasts are considered to be the main initiators of aerobic spoilage in grass silage (OUDE ELFERINK *et al.*, 1999a) which was also concluded from the studies described below. The majority of the yeasts described in literature are able to assimilate lactic acid and are *Candida* species or are strains of *Saccharomyces cerevisiae* (HOLDEN and BLACKBURN, 1987). On the other hand a dominance of *Pichia anomala* in perennial ryegrass silages was demonstrated (MARTENS and PAHLOW, 2003).

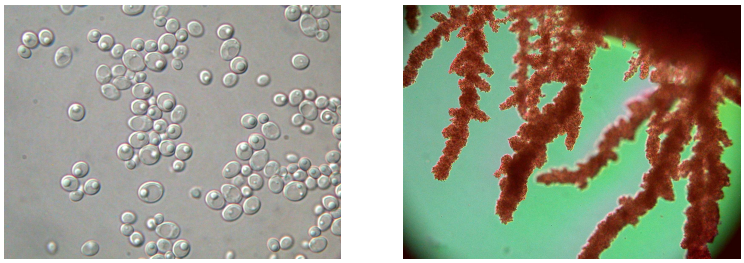


Figure 2: Pichia anomala, left: cells, 100x magnified, right: branched pseudohyphae, 10x magnified

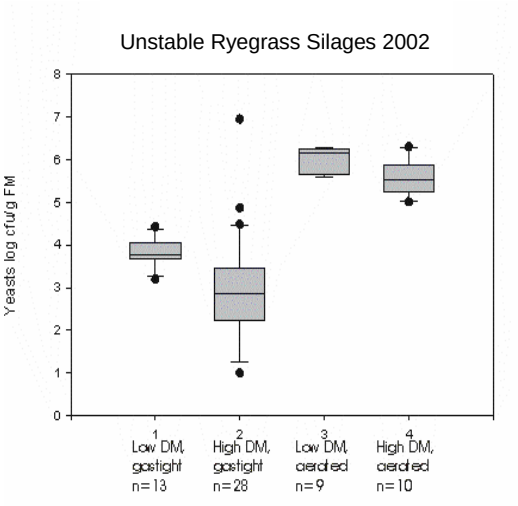
The deleterious role of yeasts in silages on exposure to air was related to their particular ability to oxidise lactic acid (BECK and GROSS, 1964). The pathway was described by ROOKE and HATFIELD, 2003.

Lactic acid (CH_3-CHOH-COOH) + O_2 ----------→ CO_2 + H_2O

Glycolytic pathway, tricarboxylic pathway

Figure 3: Oxidation of lactic acid by yeasts (ROOKE and HATFIELD, 2003)

Studies focussing on yeasts in ensiled forages then commenced (BURMEISTER and HARTMAN, 1966; BUCHER, 1970). DANIEL *et al.*, 1970, summarised the works of BECK and GROSS, 1964, WEISE, 1963, and HONIG, 1969, and a relationship between yeast numbers and aerobic stability of silages was found. Strictly anaerobic storage of silages kept yeast numbers low. However, even a yeast population size of $< 10^5$ cfu/g FM at the time of opening the silage did not necessarily mean that silage was aerobically stable (OHYAMA and McDONALD, 1975; HENDERSON *et al.*, 1979; MARTENS and PAHLOW, 2003, see Figure 4, box plot 2).



Low DM ~ 25 % DM

High DM ~ 35-40 % DM

Figure 4: Frequency distribution of yeasts in unstable laboratory grass silages (untreated control) counted on malt extract agar; box plot 1 – from low DM silages stored airtight over 90 days, box plot 2 – from high DM silages stored airtight over 90 days, box plot 3 – from low DM silages with defined air infusion over 49 days storage period, box plot 4 – from high DM silages with defined air infusion over 49 days storage period (MARTENS and PAHLOW, 2003)

This figure shows that high yeast numbers of grass silages stressed with air during the storage period were correlated to the aerobic instability of those (box plots 3 and 4). If low DM silages were aerobically unstable after a gastight storage period, yeast numbers > 3 log cfu/g FM were found. If high DM grass silages deteriorated on exposure to air after a gastight storage period yeast numbers could either be high or low.

This lack of correlation between yeast numbers and spoilage is not restricted to silages but has also been noted for wines (DEAK and REICHART, 1986).

In the 1980s JONSSON developed a synthetic plate count medium which contained lactic acid as sole carbon source to select yeasts which can utilise lactic acid aerobically (JONSSON and PAHLOW, 1984; JONSSON, 1989). However, in the own preparatory work (see *APPENDIX I*) it was found out that the medium does not necessarily exclude other yeasts, as some use the nutrients stored from the silage and might even exchange them with “non-storage” yeasts.

Studies from MIDDELHOVEN and FRANZEN, 1986, showed that the ability of the *Candida* and *Saccharomyces* strains investigated, to assimilate lactic acid only occurred or increased with decreasing pH (see also *APPENDIX I*).

Moulds

Moulds usually develop alongside or following the facultatively anaerobic yeasts as they prefer aerobic or microaerophilic conditions (MAGAN and LACEY, 1984). Their influence on initiating aerobic deterioration is considered to be low (WOOLFORD, 1990). One exception is *Penicillium roqueforti* which regularly developed on solid

medium containing lactic acid as a sole C-source and indicated that it was used as a nutrient (PAHLOW, 2005). However, moulds contribute to spoilage at the silage surface and some produce undesirable mycotoxins like zearalenone, roquefortin C and others that have the potential to have negative effects on animal health (AUERBACH, 1996; Oldenburg, 1991).

Bacteria

Bacteria are prokaryotic organisms that take up nutrients by active or passive absorption.

Bacteria isolated from aerobically deteriorating silages comprise the lactic acid bacteria, proteolytic bacilli, acetic acid bacteria and *Actinomyces* (Table 1, summarised by WOOLFORD, 1990). Furthermore, listeria and clostridia have also been found:

- *Bacillus* spp. are endospore-forming aerobic bacteria with thermotolerant or even thermophillic properties and use a wide range of carbohydrates as substrate. They probably contaminate silage mainly through soil or manure (RAMMER *et al.*, 1994, cited in OUDE ELFERINK *et al.*, 1999a). Bacilli can play a role in the later stages of aerobic deterioration (LINDGREN *et al.*, 1985).
- Acetic acid bacteria are obligate aerobic, acid-tolerant bacteria using ethanol, lactic and acetic acids as preferred substrates and have been isolated from maize silages (SPOELSTRA *et al.*, 1987). They can largely contribute to aerobic deterioration through their ability to oxidise organic acids (SPOELSTRA *et al.*, 1988).

  LENGELER et al., 1999, described the oxidative pathway.

  C_2H_5OH ---→ CH_3CHO --------…….. ---→ $C_2H_4O_2$ -------→ CO_2

  Ethanol --→ Acetaldehyde --→ Acetaldehyde hydrate ---→ Acetic acid ---→ CO_2

  Alcohol dehydrogenase *Acetaldehyde dehydrogenase*

  Figure 5: Oxidation of ethanol to acetic acid to CO_2 by Acetobacter (LENGELER et al., 1999)

- *Streptomyces* species which belong to the family *Actinomyces* are aerobic bacteria usually present in soil with the ability to degrade cellulose and other structural carbohydrates. They have been isolated from maize silage (BECK, 1975), but their role in aerobic deterioration has not been established.
- The pathogenic aerobic bacterium *Listeria monocytogenes* has often been isolated from aerobically spoiled silages (FENLON and WILSON, 1996; COAN *et al.*, 2005). It can be isolated in low numbers from soil and plants and its growth in silage

is accelerated when oxygen is available (DONALD *et al.*, 1995). Thus it has been associated with aerobic spoilage.

- An increase in spore numbers of the obligately anaerobic *Clostridium* spp. was observed in the surface layers of maize silages of opened clamp silos. Their development was attributed to growth in anaerobic niches where pH was elevated due to aerobic deterioration (DRIEHUIS and TE GRIFFEL, 2005). The pathway for their activity was described by SCHLEGEL, 1992.

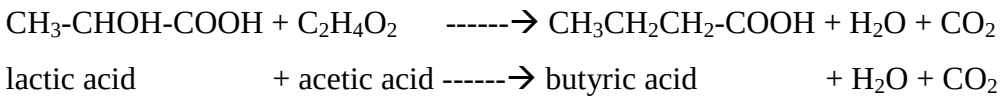


Figure 6: Butyric acid fermentation by Clostridium tyrobutyricum (SCHLEGEL, 1992)

- Lactic acid bacteria are regarded as desirable microorganisms in silage and are part of the epiphytic microflora of the plant material responsible for the lactic acid fermentation.

  However, some investigations implicating bacteria showed that *Lactobacillus* numbers can rise during the first days of spoilage (WOOLFORD and COOK, 1978;WOOLFORD *et al.*, 1978; WOOLFORD *et al.*, 1979).

  A possible metabolic pathway was found for heterofermentative LAB (i.e. *Lactobacillus buchneri* and *L. brevis*) in silages. They were able to convert lactic acid to acetic acid (BUCHER, 1969, in BECK, 1969) as shown below.

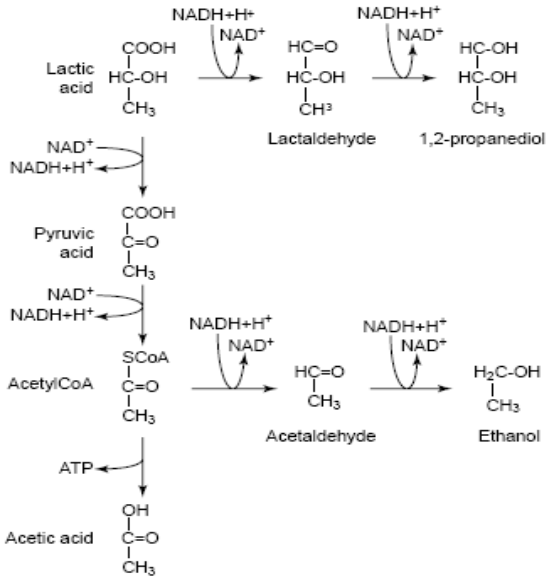


Figure 7: Anaerobic degradation pathway of lactic acid to acetic acid and 1,2-propanediol by Lactobacillus buchneri (OUDE ELFERINK et al., 1999b)

CRAWSHAW *et al.*, 1980, made a general observation based on microbial counts in grass silage on exposure to air, i.e. an increase in total bacterial numbers was reflected by a decrease in yeast numbers.

Results from other studies based on microbial counts were inconclusive (HENDERSON *et al.*, 1979). Yeast counts increased during 9 days of aeration whether DM losses were high or low and neither increasing or decreasing numbers of bacteria were related directly to susceptibility to aerobic deterioration.

There is however evidence to suggest that yeasts and lactic acid bacteria grow linearly with increasing oxygen content (REES and LOWE, 1984).

There are still many questions to be answered on the relative roles of different microbial groups during aerobic deterioration of silage and this thesis will address some of these uncertainties.

3 TASK AND AIM

The aim of the work was to further elucidate the microbiological principles of aerobic changes in silages, i.e. the relative roles of bacteria, yeasts and eventually moulds.

A review of research on spoilage by yeasts during the last 50 years including the food sector concluded that “little has changed in the knowledge of the biological processes and microbial interactions involved” (LOUREIRO and MALFEITO-FERREIRA, 2003).

Another evaluation on scientific literature on aerobic spoilage of silage by URIARTE *et al.*, 2001, concluded that yet “there is not a complete understanding of the microbiology of aerobic deterioration”.

A summary of the numerous works carried out indicates that most results have been obtained using classical plate count methods. These approaches have to be judged with caution because

1. They have a wide margin of error,
2. They do not always reflect activity (SEALE *et al.*, 1990).
3. They might underestimate the number of viable cells (ROSZAK and COLWELL, 1987).

During the Eurobac Conference in Uppsala, Sweden, in 1986 it was postulated:

“**Thus a microbiological analysis should always be accompanied by a measurement of activity** such as pH value, metabolite production or a direct measurement of microbial activity such as ATP production.” (SEALE *et al.*, 1990)

In this research project, to address this claim with using a practical and feasible approach a method was developed which simulates silage conditions in a batch culture and where the pH course and some metabolites were measured as indicators of microbial activity.

Four different types of experiments were set up sequentually.

Experiment type A:

Single yeast strains, isolated from grass silages (on lactate agar; JONSSON and PAHLOW, 1984) were inoculated into synthetic lactate medium.

Experiment type B:

The mixed microflora of silages was inoculated in synthetic lactate medium to extend the investigations on bacteria and their possible interactions. Antibiotics were used to distinguish between fungal and bacterial activity (WOOLFORD *et al.*, 1977; WOOLFORD and COOK, 1978; WOOLFORD *et al.*, 1979).

Experiment type C:

"Silage medium" with the indigenous microflora, original pH and nutrients in their given ratios was used to represent the original complex environment, together with antibiotics as in Experiment type B.

Experiment type D:

Autoclaved silage medium was inoculated with single yeasts or bacterial strains isolated from silages, to reproduce the effects of the activity of the indigenous microflora in Experiment type C.

The 4 principle working hypotheses were:

- Referring mainly to Experiment type A:

Hypothesis 1:
The pH does not influence the amount of lactate consumed by yeasts.

The hypothesis was disproved if there was a significant difference (α =0.05) in the lactate concentration after 22 h (± 2h) and 45 h (± 2h) between the treatment at pH 3.8 and pH 5.5 (or $\geq$ 4.4 in Experiment type C and D).

- Referring to the other 3 experiment types:

Hypothesis 2:
The decomposition of lactate and the rise in pH is solely caused by yeasts.

The hypothesis was disproved if lactic acid decomposition and eventually pH rise was observed in the antifungal treatment.

Hypothesis 3:
Aerobic changes in silages are dominated by yeast activity.

The hypothesis was disproved if the control differed significantly (α =0.05) from the antibacterial treatment.

Hypothesis 4:

The decomposition of lactate and the rise in pH is not influenced by other available carbon sources and compounds.

The hypothesis was disproved if the changes occuring in treatments without additional C-sources differed significantly (α =0.05) from the treatments with additional carbon sources.

4 MATERIALS AND METHODS

4.1 Shaken batch culture system

Experiments were carried out in a shaken batch culture system.

Definition: “Batch cultures describe growth occurring in a fixed volume of culture medium that is continuously being altered by the metabolism of the growing organisms until it is no longer suitable for growth. During the early stages of exponential growth in batch culture conditions may remain relatively stable, but as the cell number increases drastic changes in the chemical composition of the medium occur.” (SMITH, 2004)

Aliquots (40 ml) of the medium were transferred to sterile Erlenmeyer flasks (100 ml). The inoculated medium was covered by sterile aluminium foil. To achieve aerobic conditions the samples were shaken on orbital shakers. Two orbital shakers were used: Infors HT (Infors AG, Basel, Switzerland), KS250basic (IKA Labortechnik) as available. The speed was adjusted to about 175 rpm according to subjective visual criteria: the whole medium should be sufficiently moved so that all cells would constantly get in touch with air.

To observe mould or pellicle (BECK and GROSS, 1964) growth there was usually one not shaken sample per silage.

The samples were incubated in an air-conditioned room at 25 °C, usually for 2 days.

For inoculation yeast and LAB strains were isolated from high DM (35-40 %) grass silages and were cultured for Experiment types A and D. For Experiment types B and C a mixed microflora was used which was extracted from the silages together with the natural medium for Experiment type C (for details see 4.1.1 to 4.1.4). For Experiment type C high DM silages were used which were presumed to be prone to aerobic deterioration (characterisation of the silages see 4.1.3).

In the following the subtypes of the experiments are referred to as A, B, C, D 1, 2 et sqq..

Table 2: Experiment types

Experiment type	**A**	**B**	**C**	**D**
Synthetic medium	x	x		
Silage medium			x	
Sterile silage medium				x
Yeast isolates	x			x
LAB isolate				x
Mixed microflora		x	x	

4.1.1 Experiment type A – *Synthetic media, mono-cultures*

In this type of experiment first the requirements for lactate oxidation by yeasts were investigated, that is primarily air ingress: unshaken and shaken, lactic acid concentration and pH conditions.Within experiments the effect of temperature (20 °C and 25 °C) and length of incubation was generally tested.

In a second step a comparison was made of lactate assimilation by 4 yeast isolates at a low and high lactate concentration and a low and high pH.

Synthetic medium

The nitrogen source was based on Yeast Nitrogen Base (Difco^{TM} #233520). The only carbon source was lactic acid. There were 4 different media. The two lactic acid concentrations, 0.5 and 2.0 % v/v, were chosen to represent extremes found in silage under practical conditions on FM basis. The two pH levels were adjusted to the extremes in grass silages of 3.8 and 5.5 using 5 M NaOH.

Table 3 : Media 1-4

% lactate pH	2.0	0.5
3.8	1	3
5.5	2	4

Inoculants

Yeasts from *Lolium perenne* silages (2002) which had developed on lactate agar were isolated and identified in the own laboratory (streaking subcultures for spatial isolation, BAST, 2001; identification see *APPENDIX V*). Four yeast strains were selected: *Pichia anomala* (CBS 113 and CBS 605) as the most common yeast identified, *Issatchenkia orientalis* (CBS 1910) as another known lactate utiliser and *Saccharomyces cereviseae var. cereviseae* (CBS 1782) as a further yeast commonly present in the investigated silages. The identity of these selected isolated yeasts was later on confirmed by the

DSMZ. For inoculation they were grown in 40 ml malt extract bouillon for 24 h at 30 °C (recipe see *APPENDIX IV*) .

Experiment A 1

- 4 media as described above
- Conditions: shaken, not shaken, 20 °C and 25 °C
- Inoculant: 1 ml of a *Pichia anomala* culture (CBS 113)
- 2 replicates
- uninoculated control, 4 media, shaken, 25 °C, one replicate

Table 4: Experiment A 1

Variant	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**
Medium 1	x	x	x	x													x			
Medium 2					x	x	x	x										x		
Medium 3									x	x	x	x							x	
Medium 4													x	x	x	x				x
Agitated	x		x		x		x		x		x		x		x		x	x	x	x
Stationary		x		x		x		x		x		x		x		x				
20 °C	x	x			x	x			x	x			x	x						
25 °C			x	x			x	x			x	x			x	x	x	x	x	x
P. anomala 1	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x				
No of repl.	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	1	1	1	1

Experiment A 2

- 4 media as described above
- Conditions: shaken, 25 °C
- Inoculants: 0.1 ml of the 4 yeast strains described above
- 3 replicates

Table 5: Experiment A 2

Variant	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**
Medium 1	x	x	x	x												
Medium 2					x	x	x	x								
Medium 3									x	x	x	x				
Medium 4													x	x	x	x
P. anomala 1	x				x				x				x			
P. anomala 2		x				x				x				x		
S. cerevisiae			x				x				x				x	
I. orientalis				x				x				x				x

4.1.2 Experiment type B – *Synthetic media, mixed cultures*

In this experiment the ability of the mixed microflora of a silage to decompose lactate and possible interactions between bacteria and fungi were studied.

Synthetic medium

Media 3 (0.5 % lactic acid, pH 3.8) and 2 (2 % lactate, pH 5.5) were used (details see A) presuming a similarity to the pH and lactic acid content of the silage which the inoculant was extracted from (see below).

Inoculant

Fresh silage extract (1 ml), a 1:5 dilution was inoculated to 40 ml aliquots of lactate medium. To obtain the dilution 260 ml distilled sterile water were added to 65 g silage FM. The microorganisms were extracted during 5 min in a Stomacher. The volume of 1 ml for inoculation was chosen to provide a sufficient amount and diversity of silage micro-organisms but at the same time to minimise the transfer of nutrients from the silage to the synthetic medium.

Antibiotics

To distinguish the activity of fungi and bacteria antibiotics were used.

Antimycotic (AM): 1 ml of a 5 % Delvocid® (Natamycin) suspension, autoclaved, was added to 40 ml aliquots of medium.

Antibacterial (AB): 1 ml of a Penicillin / Streptomycin solution, filter sterilised, was added to 40 ml aliquots of medium. Stock solution: 0.3 g Penicillin-G K-salt, 0.3 g Streptomycin sulphate, 100 ml distilled water.

As a control to determine the effectiveness of the antibiotics, both AM and AB were combined (MB) as one treatment.

Experiment B 1

- 4 treatments with 3 replicates: Control (C), Antibacterial (AB), Antimycotic (AM) and MB treatment. Medium 2 (2 % lactate/ pH 5.5), microbial extract prepared from grass silage ID 15.

Experiment B 2

- 4 treatments with 3 replicates: Control (C), Antibacterial (AB) and Antimycotic (AM) treatment. Medium 3 (0.5 % lactate/ pH 3.8), microbial extract prepared from maize silage ID 16.

*Table 6: Experiment B **1** and Experiment B **2***

Treatment	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
Medium 2	x	x	x	x				
Medium 3					x	x	x	x
C	x				x			
AB		x				x		
AM			x				x	
MB				x				x
Grass silage	x	x	x	x				
Maize silage					x	x	x	x

4.1.3 Experiment type C – *Silage media, mixed cultures*

This type of experiment served to investigate in the complex microbial behaviour in an environment similar to silage.

Silage medium

Silages were extracted with sterile water in the ratio 1:5, i.e. one part of silage (g FM) and 4 parts of water (ml), for 5 min in a Stomacher. The ratio was adopted from a rapid fermentation test developed by PIEPER *et al.*, 1996, and ZIERENBERG, 2000, using minced fresh grass and water.

The chemical and microbial composition of silages used in these experiments is shown in tables 7 to 9. All silages except ID 15, 16 and 32 were taken from laboratory scale silos made in 1.5 l WECK® jars in 2004 prepared for the EU project SweetGrass (QLK5-CT-2001-0498)(method presented by PAHLOW *et al.*, 2004).

For preparing those silages herbage was wilted for approximately 24 h, cut to 15 mm length and filled into the WECK jars with a pore volume of about 4.5 l/kg DM, that is about 670-610 g FM in 1.5 l volume for a DM content of 35-40 % in 4 replicates. Silages were stored for 49 or 90 days. To simulate suboptimal conditions on the farm and to enhance undesirable yeast growth silages were challenged by defined air infusion for 8 h after 4 and 6 weeks if stored for 49 days or after 4, 8 and 12 weeks if stored for 90 days by opening two rubber seals allowing air in- and outflow.

All laboratory silages except ID 25 (inoculated with commercially available *Lactobacillus plantarum* strains) were control silages, i.e. fermented without additives.

Those high DM silages were chosen because it was presumed that they were more prone to aerobic deterioration due to lacking additive treatment and controlled air ingress during storage.

One out of four replicate silages (spare sample) was taken for the batch culture.

The other 3 replicates served for chemical analyses and together with a sample of the replicate for batch cultures for the evaluation of aerobic stability by temperature measurement according to HONIG, 1990 (*APPENDIX II*).

Silages ID 15 and 16 were taken from bunker silos of the FAL experimental station. Silage ID 32 was a laboratory silage in a 20 l glass jar similar to an desiccator. The method of HONIG was applied to samples of these silages as well.

All grass silages except ID 15 (original grass unknown) were made from *Lolium perenne*. Lucerne stands for *Medicago sativa*.

Table 7: Chemical composition of silages used for preparation of the silage medium (% DM); used in *Experiment type C*

Silage ID	Trial ID	Type of silage	Age (months)	% DM	pH	WSC * (HPLC)	Fruct.	Gluc.	Suc.	Fructan	BC [Meq/kg DM]
15	10	Grass	~ 8.0	25.8	4.5	2.3	0.4	0.0	1.9	0.6	778.9
16	11	Maize	~ 7.0	31.7	3.8	0.0	0.0	0.0	0.0	0.9	590.7
17	12	Grass	1.5	38.6	4.8	13.5	10.5	2.9	0.1	0.4	556.8
19	13	Grass	1.5	37.3	4.7	15.7	12.5	3.1	0.1	1.3	523.8
20	13	Grass/lucerne 75/25	1.5	38.3	4.6	11.7	9.4	2.2	0.0	1.2	653.6
21	13	Grass/lucerne 50/50	1.5	40.1	4.6	7.2	5.6	1.6	0.0	1.0	775.5
24	14	Grass	3.0	37.9	4.8	12.4	9.8	2.5	0.1	0.3	538.2
25	14	Grass (*L.plant.*)	3.0	38.7	4.0	10.8	8.7	2.1	0.0	0.7	706.7
26	15	Grass	3.0	37.4	4.6	18.5	14.3	4.1	0.1	0.8	544.6
27	15	Grass/lucerne 75/25	3.0	38.9	4.6	14.2	11.1	3.2	0.0	0.8	669.7
30	17	Grass	3.0	32.2	4.6	15.7	13.2	1.6	0.9	1.2	541.9
31	17	Grass/lucerne 75/25	3.0	34.9	4.6	10.7	9.1	0.8	0.8	0.8	689.7
32	19/20	Grass	3.0	39.0	4.8	11.3	9.6	1.6	0.0	0.9	603.2
34	23/24	Grass	6.0	37.4	4.7	8.5	6.9	1.6	0.0	0.6	666.6

* sum of fructose, glucose and sucrose

Table 8: Volatile fatty acids and ethanol [% of FM]; Experiment type C

Silage ID	TrialID	Type of silage	%DM(cor.)	Lactic acid	Acetic acid	Propionic acid	Butyric acid (sum) *	Ethanol
15	10	Grass	26.8	1.12	0.94	0.08	0.01	0.12
16	11	Maize	32.6	2.74	0.37	0.02	0.00	0.33
17	12	Grass	39.3	1.16	0.31	0.04	0.03	0.31
19	13	Grass	37.6	0.94	0.26	0.02	0.02	0.38
20	13	Grass/lucerne 75/25	39.0	1.44	0.33	0.01	0.03	0.29
21	13	Grass/lucerne 50/50	40.9	1.96	0.36	0.03	0.00	0.31
24	14	Grass	38.7	1.13	0.31	0.05	0.03	0.37
25	14	Grass (*L.plant.*)	39.4	2.40	0.15	0.10	0.02	0.30
26	15	Grass	38.1	1.10	0.29	0.01	0.02	0.43
27	15	Grass/lucerne 75/25	39.5	1.57	0.29	0.00	0.02	0.29
30	17	Grass	33.1	0.88	0.21	0.11	0.04	0.57
31	17	Grass/lucerne 75/25	35.6	1.20	0.32	0.10	0.04	0.23
32	19/20	Grass	38.6	1.10	0.38	0.21	0.04	0.61
34	23/24	Grass	37.4	1.17	0.69	0.18	0.08	0.11

*sum of i-butyric, n-butyric, i-valeric, n-valeric and n-hexanoic acid

Microflora

The population density in 10 g silage FM corresponds to about 46-47 ml aliquots of silage medium.

The microbial composition of the silages is shown in the Table 9 below.

Table 9: Numbers of micro-organisms used to prepare the silage medium used in Experiment type C (log cfu/g FM)

Silage ID	Yeasts	Moulds	Aer. bacteria	LAB
15	< 2	(n.d.)	8.5	-
16	5.9	n.d.	5.2	-
17	7.4	n.d.	7.0	-
19	6.4	n.d.	7.6	-
20	n.d.	6.3	6.9	-
21	n.d.	7.0	7.2	-
24	7.0	n.d.	5.2	5.3
25	5.4	4.9	4.5	< 5
26	6.8	n.d.	5.7	5.7
27	6.1	6.4	5.4	5.4
30	6.7	n.d.	6.0	4.9
31	6.8	n.d.	6.0	5.4
32	6.6	n.d.	6.9*	6.0
34	6.2	n.d.	6.8*	6.5

n.d.= not detected; - = missing value; * on plate count agar

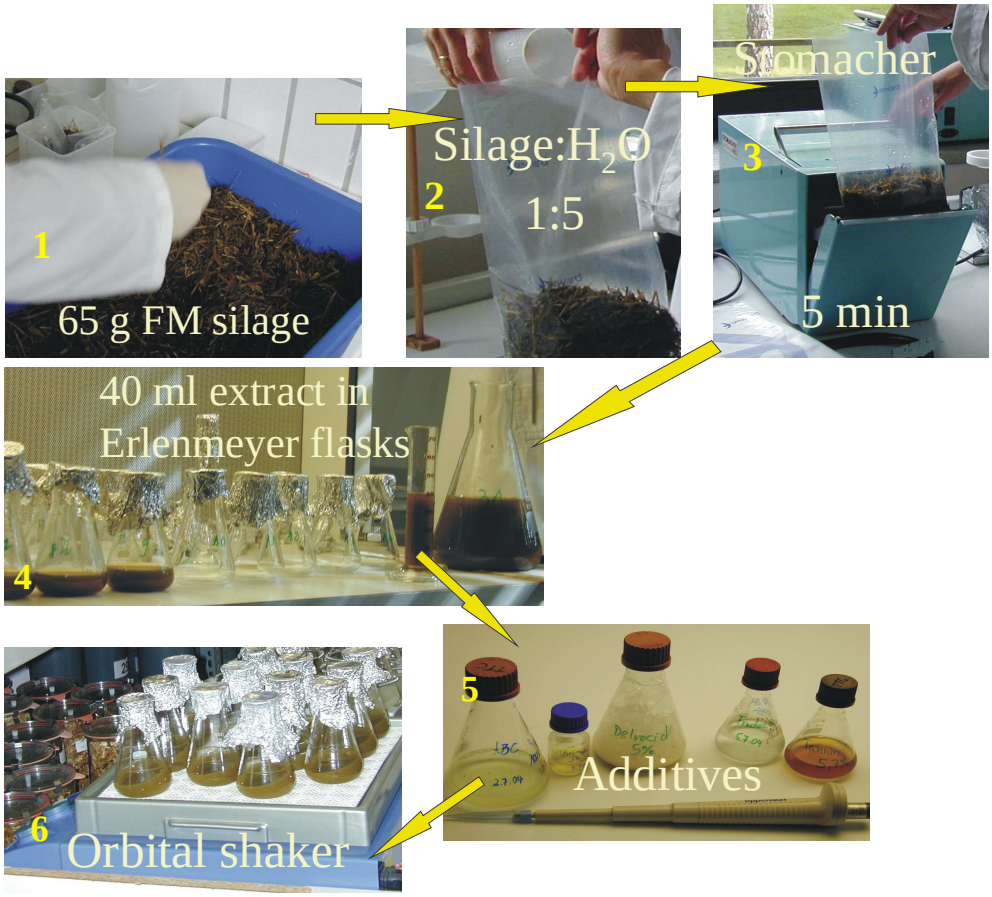
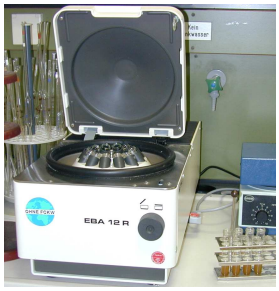


Figure 8: Sample preparation

1) pH-measurement

2) Transfusion of 2-3 ml



3) Centrifugation

4) Fill in vials

5) Measurement by HPLC

Figure 9: Sample measurement

Antibiotics

Antimycotic AM:

1 ml of a 5 % w/v Delvocid® (Natamycin) suspension, autoclaved, was added to 40 ml aliquots of medium.

Antibacterial AB:

a) 1 ml of a Penicillin / Streptomycin solution was filter sterilised and added to 40 ml aliquots of medium.

   Stock solution: 0.3 g Penicillin-G K-salt, 0.3 g Streptomycin sulphate, 100 ml distilled water.

This composition was used for the silages 15, 16 and 17. For the latter it did not prove to be sufficiently effective in the MB treatment (antibacterial + antimycotic), i.e. particularly in grass silages. Thus the composition described below was used:

b) A modified antibacterial solution (0.8 ml) described by WOOLFORD and COOK, 1978, was filter sterilised and added to 40 ml aliquots of medium.

   Stock solution (100fold): 500 mg Chlortetracycline, 500 mg Penicillin-G K-salt, 500 mg Chloramphenicol, 500 mg Streptomycin sulphate, 1250 mg Bacitracin, 100 mg Polymyxin B, 100 ml distilled water.

   The utilisation of dye rose bengal as suggested by WOOLFORD was abandoned because of the fungistatic effect sometimes observed (MARTIN, 1950; MOSSEL *et al.*, 1980).

As a control on the effectiveness of the antibiotics, both AM and AB were combined (MB) in Silages ID 15, 17, 19, 24.

Additives

Tannic acid

1 ml of a 5.7 % (w/v) tannic acid solution, filter sterilised, was added to 40 ml aliquots of silage medium. This corresponded to a tannin concentration equivalent to about 2.5 % in the DM of fresh whole plant lucerne (23 % DM) (MILTIMOR *et al.*, 1970) or of a wilted lucerne silage (~ 40 % DM) mixed with grass (60 % lucerne in FM). The origin of the tannic acid was the quebracho wood (*Aspidosperma quebracho-blanco*), a South American tree.

Fructose

To study the effect of residual WSC fructose was added at two different concentrations. Fructose was chosen as the predominant sugar left after ensiling of perennial ryegrasses (*Lolium perenne*) (own observation).

One or 2 ml resp. of a 30 % w/v filter sterilised fructose solution were added to 40 ml aliquots of silage medium. This corresponds to roughly 3 % and 6 % fructose resp. on silage FM base. Assuming a DM content of 40 % this corresponds to about 7.5 and 15 % fructose in the DM.

Potassium chloride

Potassium chloride was used to increase the osmotic pressure in the silage medium. Potassium was preferred to sodium due to its lower toxicity against yeasts (ONISHI, 1957). To adapt the medium to the osmolality of high DM grass silages a concentration of 8 % w/v KCl was used. According to WEISSBACH, 1968, this corresponds to the osmotic pressure prevailing at about 40 % DM of a silage. Silage samples (ID 17, 30, 34) measured by an osmometer were at the level of 2.3-2.7 osmol/kg.

Varying pH

To decrease the pH concentrated H_2SO_4 was used. H_2SO_4 was chosen because it is an inorganic acid with little side assumed effect, except the release of H^+ ions. Due to its high concentration the volume needed was so small that the effect on the total volume of the medium was minimised.

Oxygen

To test the influence of different aeration regimes the liquid volume to air space ratio was varied, i.e. 200 ml Erlenmeyer flasks were tested versus 100 ml flasks.

Grass silages:

Experiment C 1

- 3 treaments with 3 replicates: Control (C), Antibacterial (AB) and Antimycotic (AM) treatment. Silages ID 15, 17, 19, 24, 25, 26, 30, 32, 34. (9 silages in total).

  MB treatment was applied for silages ID 15, 16, 17, 19, 24. Aerobic stability test.

Experiment C 2

- 3 treatments with 3 replicates: C, AB and AM + 3 % fructose in FM. Silages ID 17, 19, 24, 25, 26, 30. (6 silages in total).

Experiment C 3

- 3 treatments with 3 replicates: C, AB and AM + 6 % fructose in FM. Silages ID 19, 32, 34. (3 silages in total). Aerobic stability test.

Experiment C 4

- 6 treatments with 3 replicates: C, AB and AM in 8 % KCl solution in 100 ml and in 200 ml erlenmeyer flasks. Silage ID 34.

Experiment C 5

- 2 treatments with 3 replicates: C and AB + 0.57 % tannic acid in FM. Silage ID 17.

Experiment C 6

- 6 treatments with 3 replicates: pH of the medium adjusted to 3.8, C, AB, AM without and with 6 % fructose. Silage ID 32.

*Table 10: Treatments Experiment C **1** - Experiment C **8***

Treatment	**C1**	**C2**	**C3**	**C4**	**C5**	**C6**	**C7**	**C8**
C	x	x	x	x	x	x	x	x
AB	x	x	x	x	x	x	x	x
AM	x	x	x	x		x	x	x
+ 3 % fructose		x						x
+ 6 % fructose			x			±		
+ tannic acid					x			
8 % KCl				x				
Adj. pH 3.8						x		
200 ml volume				±				
Grass silage	x	x	x	x	x	x		
Grass-lucerne sil.							x	x
Maize silage							x	
Aerobic stab. test	x		x					

Silages other than pure grass

Experiment C 7

- 3 treatments with 3 replicates: Control (C), Antibacterial (AB) and Antimycotic (AM) treatment. Maize silage ID 16, Grass-lucerne silages ID 20, 21, 27, 31. (5 silages in total).

Experiment C 8

- 3 treatments with 3 replicates: C, AB and AM + 3 % fructose in FM. Grass-lucerne silages ID 27, 31. (2 silages in total).

4.1.4 Experiment type D – *Autoclaved silage media, mono- & co-cultures*

This type of experiment served to confirm the conclusions made from the results of Experiment type C under defined conditions. On the other hand, some of results found in Experiment type D were confirmed in Experiment type C or vice versa.

In this type of experiment defined isolates were inoculated into sterile silage medium.

Silage medium

Silage medium was prepared as described in Experiment type C. It was autoclaved at 110 °C for 15 min. By plating on MEA it was ensured that no microbes had survived. One maize and one grass silage were used. The maize silage was taken from a silo bunker from the FAL experimental station. The grass silage was chosen from farm silages where the detailed composition was already known with the criteria of being a silage of > 30 % DM and judged to be a good silage according to the DLG key (ANONYMUS, 1997).

Table 11: Chemical composition of silages used for preparation of the silage media (% DM) used in Experiment type D

Type of silage	Age (months)	% DM	pH	WSC * (HPLC)	Fruct.	Gluc.	Suc.	Fructan	BC [Meq/kg DM]
Grass	10	32.6	4.4	2.4	1.7	0.7	0.0	0.4	934.7
Maize	~ 7	31.7	3.8	0.0	0.0	0.0	0.0	0.9	590.7

* sum of fructose, glucose, sucrose

Table 12: Organic acids and ethanol [% of FM] of silages used in Experiment type D

Type of silage	%DM (corr.)	Lactic acid	Acetic acid	Propionic acid	Butyric acid (sum)	Ethanol
Grass	33.5	2.72	0.50	0.00	0.05	0.19
Maize	32.6	2.74	0.37	0.02	0.00	0.33

Inoculants

2 yeast strains as described in 4.1.1 were used: *Pichia anomala* (CBS 113) and *Saccharomyces cereviseae var. cereviseae* (CBS 1782). For inoculation they were grown in 40 ml malt extract broth for 24 h at 30 °C. Aliquots (0.1 ml) were inoculated into the silage medium.

One LAB strain was isolated from 2 days batch culture from silage ID 19, found on Rogosa agar. It showed no production of CO_2 during growth on glucose and was as such revealed as a homofermentative LAB. It was identified as *Lactobacillus plantarum* by the DSMZ. For inoculation it was grown in 40 ml MRS broth for 24 h at 30 °C. Aliquots (0.1 ml) were inoculated into the silage medium.

For recipes for the broths used see *APPENDIX IV*.

Additives

Fructose

Fructose (6 % w/v on FM) base was added to study the effect of the amount of residual WSC (see Experiment type C).

Potassium chloride

An 8 % KCl (w/v) solution was made using sterile KCl and autoclaved silage medium to adapt the osmotic pressure in the medium to that prevailing in silages.

Adjustment of pH

To increase the pH of the silage medium 5 M NaOH was used. To decrease the pH concentrated H_2SO_4 was used.

Oxygen

To test the influence of different aeration regimes 200 ml Erlenmeyer flasks were tested in comparison with 100 ml flasks.

Experiment D 1

- 6 treatments with 3 replicates: *Pichia anomala*, *Lactobacillus plantarum*, *Pichia anomala* + *L. plantarum* were inoculated into grass silage or maize silage medium.

Experiment D 2

- As for D1, but + 6 % fructose on FM base.

Experiment D 3

- As for D1, but maize silage medium adjusted to the pH of the grass silage (4.4) and vice versa (3.8).

Experiment D 4

- As for D3, but + 6 % fructose on FM base.

Experiment D 5

- 3 treatments with 3 replicates: *Pichia anomala*, *Lactobacillus plantarum*, *Pichia anomala* + *L. plantarum* were inoculated into medium extracted from grass silage containing 8 % KCl.

Experiment D 6

- As for D5, but + 6 % fructose on FM base.

Experiment D 7

- 3 treatments with 3 replicates: *Pichia anomala*, *Lactobacillus plantarum*, *Pichia anomala* + *L. plantarum* were inoculated into grass silage medium. 40 ml aliquots of medium were transfused to 200 ml Erlenmeyer flasks, instead of 100 ml flasks.

*Table 13: Treatments Experiment D **1**-Experiment D **9***

Treatment	**D1**	**D2**	**D3**	**D4**	**D5**	**D6**	**D7**	**D8**	**D9**
Pichia anomala	x	x	x	x	x	x	x		
Saccharomyces cerevisiae								x	x
P. anomala + S. cerevisiae								x	x
Lactobacillus plantarum	x	x	x	x	x	x	x		
P. anomala + L. plantarum	x	x	x	x	x	x	x		
P. anomala + L. plantarum + S. cerevisiae								x	x
+ 6 % fructose		x		x		x			x
8 % KCl					x	x			
Adj. pH 3.8			x	x				±	±
Adj. pH 4.4			x	x					
200 ml volume							x		
Grass silage	x	x	x	x	x	x	x	x	x
Maize silage	x	x	x	x					

Experiment D 8

- 6 treatments with 3 replicates: *Saccharomyces cerevisiae*, *Saccharomyces cerevisiae* + *Pichia anomala*, *Saccharomyces cerevisiae* + *Pichia anomala* + *Lactobacillus plantarum* were inoculated into grass silage medium with either the natural pH of 4.4 or adapted to pH 3.8.

Experiment D 9

- As for D8, but + 6% fructose on FM base.

pH measurement

pH was measured electrometrically by a pH-meter (Microprocessor pH/ION Meter pMX2000 WTW). The pH-meter was calibrated daily using two buffers, pH 4.00 and pH 7.00 (Riedel-de Haën).

To avoid microbial contamination of the samples the electrode was rinsed with distilled water as well as with 70% ethanol (denatured) after each sample measurement.

Final measurements of pH in 100 ml Erlenmeyer flasks was generally made after 0, 22 (±1), 34 (±1), 47 (±2) h of incubation and sometimes even after 5, 10 or 12 h. In later experiments the time intervals of the initial period of incubation (5, 10 h) were missed out as it had been found that during the first 12 h there was generally no measurable pH change. For the second day the 34 h measurement replaced the 30 h measurement for the same reason.

Analysis of alcohols and volatile fatty acids

After the pH measurement at 22 and 47 h, aliquots (2-3 ml) of each sample were placed in test tubes. They were centrifuged at 4000 rpm for 10 min (EBA 12R / Hettich Zentrifugen), then transferred to vials and analysed for alcohol (ethanol), lactic acid and volatile fatty acids (acetic, propionic acid) by High Performance Liquid Chromatography (HPLC) (Kontron Instruments, column: Rezex ROH-Organic Acid H+ / Phenomenex). Further details see *APPENDIX III*.

In the results these major metabolites are presented, i.e. lactic, acetic, propionic acid and ethanol (for Experiment type A only lactic acid).

Other components that were measured in the standard HPLC analysis like butyric acid, valeric acid, partly formic acid, butanol, n-propanol, 2,3-butanediol, 1,2-propanediol were either not detected or only in traces (< 1.6 mg/ml ≈ 7.5 g/kg FM).

O_2 measurement

In one experiment (Experiment D *7*) dissolved oxygen was measured in uninoculated silage medium at 25 °C on the orbital shaker and after 2 days of incubation in the inoculated silage media. A WTW OXI 530 with the electrode WTW TriOxmatic® EO 200 was used, calibrated with distilled water in WTW PE/OXI OxiCal®, which corresponded to 101.7 % O_2 saturation or 8.23 ppm (mg/l).

Microbial counts

For recipes see APPENDIX IV.

Experiment type A + D

1 ml aliquots of the 24 h cultures were used to prepare tenfold serial dilutions using quarter strength Ringer solution.

Yeasts were spread-plated on MEA while LAB were spread-plated on MRS agar. Triplicate plates were incubated at 30 °C for 3 days and all visible colonies counted.

For method compare SEALE *et al.*, 1990.

Experiment type B + C

All aerobic silage microorganisms were counted.

Silage was suspended in sterile distilled water in the ratio 1:10, i.e. one part of silage FM and 9 parts of water, and treated in a Stomacher for 2 minutes. A logarithmic series of serial dilutions was then prepared with Ringer solution (see SEALE *et al.*, 1990).

The dilution was spread-plated on selective media as described below.

MEA was used as a complex medium for fungi as well as aerobic bacteria. To differentiate between the two microbial groups it was treated with the same antibiotics as used for the silage medium (AB and AM).

For silages ID 32 and 34 only Plate Count Agar (BUCHBINDER *et al.*, 1953, ISO 17410 Standard, 2001;ISO 4833 Standard, 2003) was used to enumerate aerobic bacteria as a commonly used medium for aerobic plate counts.

Rogosa agar (ROGOSA *et al.*, 1951) was used to enumerate LAB in silages ID 24-27, 30-32 and 34 when in the course of investigations it was found that LAB might also cause aerobic changes in the silage medium.

The plates were incubated at 30 °C for 3 days and all visible colonies counted.

Colonies of yeasts and moulds were distinguished visually. As yeasts dominated and moulds were found only in association with lucerne or additive treated silages in the results mould numbers are only given in those rare cases.

4.2 Yeast identification

For identification the current methodology at the German Collection of Microorganisms and Cell Cultures (DSMZ Braunschweig) was used (HOFFMANN, 2003).

This methodology makes use of the API50 CH test kits (Biomérieux), supplemented by a fermentation test (glucose), a nitrate utilisation test, temperature tolerance tests (if necessary) and microscopic morphology (cell shape, pseudomycelium formation, sexual reproduction). For further details see *APPENDIX V*.

4.3 Characterisation of silages

DM

The dry matter content of silages was calculated after drying at 105 °C for 48 h in a forced air oven.

Corrected DM (DMcor)

The DM was corrected for volatiles by the factors:

Maize silage: DMcor (%) = 2.22 + 0.960 * DM

Other silages: DMcor (%) = 2.08 + 0.975 * DM

according to WEISSBACH and KUHLA, 1995.

Crude Ash

Milled dry silage was ashed at 600 °C for 3 h (Weende feed analysis).

WSC fractions

WSC fractions (fructose, glucose, sucrose and fructan) were analysed by High Performance Liquid Chromatography (HPLC) (Kontron Instruments, column: Rezex RPM Monosaccharide / Phenomenex). For details see *APPENDIX VI*.

BC

The method used was that by Teagasc, Grange Research Centre, Dunsany Co. Meath, Ireland (see *APPENDIX VII*), based on PLAYNE and McDONALD, 1966;

McDONALD and HENDERSON, 1962; MUCK *et al.*, 1991.

Osmolality

Osmomat 030 – Cryoscopic Osmometer / Gonotec calibrated with 5.66 % NaCl - corresponding to 1.8 osmol/kg.

Volatile fatty acids and ethanol

Fresh silage was extracted with sulfuric acid and analysed by High Performance Liquid Chromatography (HPLC) (see *APPENDIX III*).

4.4 Statistics

Means and standard deviation were calculated using Microsoft® Excel (2002), graphs were created using SigmaPlot 8.0 (2002; 1986-2001 SPSS Inc.).

Analysis of variance was conducted using the procedure GLM (F-test, Tukey test) and regression analysis was conducted using the procedure REG provided by the software SAS 9.1 (2002-2003 by SAS Institute Inc., Cary, NC, USA).

5 RESULTS

All measured values presented in the results are also listed in *APPENDIX IX*.

Values for organic acid contents in the media are given in mg/ml as this was the concentration the micro-organisms actually encountered under the experimental conditions. It can be converted to the approximate value for g/kg FM by multiplying the figure by 4.6 or 4.7 for 40 or 35 % DM content of the original silage respectively (for calculation see *APPENDIX III*).

5.1 Experiment type A

5.1.1 Experiment A 1 – *Lactate concentration, pH, shaken – not shaken, temperature*

To verify the effect of shaking, temperature, pH and lactate concentration

- 4 media
- Conditions: shaken, not shaken, 20 °C and 25 °C
- Inoculant: 1 ml of a *Pichia anomala* culture (CBS 113)
- 2 replicates
- uninoculated control, 4 media, shaken, 25 °C, one replicate

Within individual media there were no significant differences in pH and lactic acid content between different temperatures but always a significant difference between shaken and and not shaken treatments at all time points.

The pH and lactic acid concentration in the uninoculated control treatments did not change over 53 h incubation period.

In the not shaken treatments pellicle forming was observed after 25 h.

Medium 1: 2.0 % lactate, pH 3.9

The not shaken treatments did not change significantly in pH and lactic acid content over 53 h of incubation at either temperature (Figure 10 & Figure 12).

Within the first 25 h the shaken treatments did not change in pH but lactic acid concentration decreased from 18.8 mg/ml to 15.5 mg/ml (0.6 s.d.) (Figure 11 & Figure 13). After 48 h the concentration of lactic acid had declined to 10.7 mg/ml (0.9 s.d.). The pH at that time point was 4.8 (0.4 s.d.).

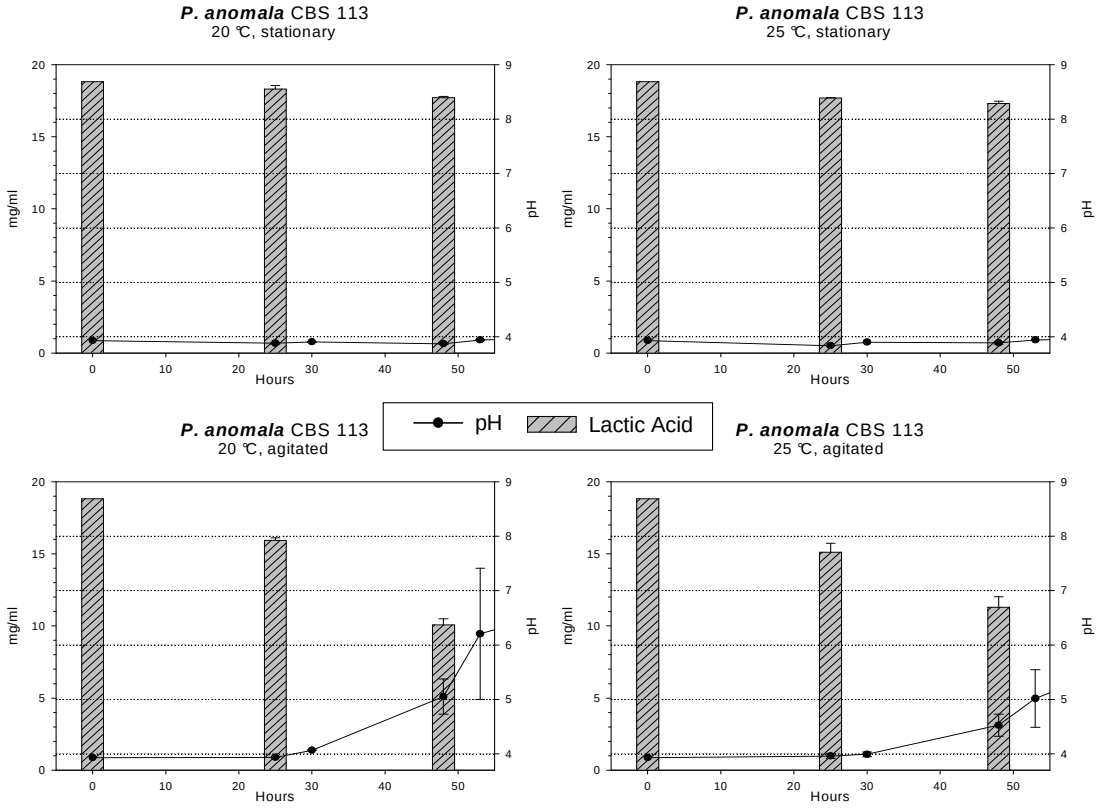


Figure 10 (top left): Changes in pH and lactic acid content over 53 h in medium 1, not shaken, at 20 °C

Figure 11 (down left): Changes in pH and lactic acid content over 53 h in medium 1, shaken, at 20 °C

Figure 12 (top right): Changes in pH and lactic acid content over 53 h in medium 1, not shaken, at 25 °C

Figure 13 (down right): Changes in pH and lactic acid content over 53 h in medium 1, shaken, at 25 °C

Medium 2: 2.0 % lactate, pH 5.5

The not shaken treatments changed very little in lactic acid content, from 18.8 to 17.5 mg/ml (0.2 s.d.) over 48 h (Figure 14 & Figure 16), but at 25 °C the final pH rose to 6.3 after 53 h (Figure 16).

The pH of the shaken treatments rose steeply over 48 h to 8.3 (0.0 s.d.) (Figure 15 & Figure 17). At the same time the lactic acid concentration decreased from 18.8 to 13.7 mg/ml (0.1 s.d.). In comparison to the same treatments with medium 1 16 % lactic acid less was decomposed.

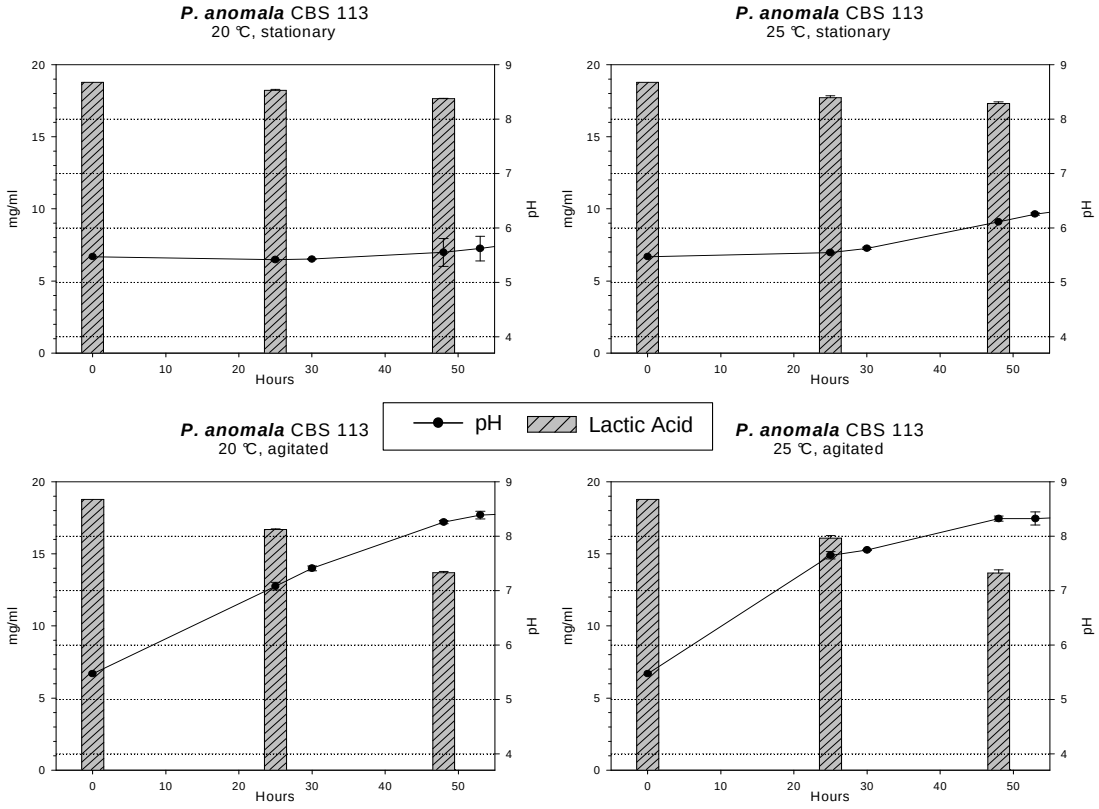


Figure 14 (top left): Changes in pH and lactic acid content over 53 h in medium 2, not shaken, at 20 °C

Figure 15 (down left): Changes in pH and lactic acid content over 53 h in medium 2, shaken, at 20 °C

Figure 16 (top right): Changes in pH and lactic acid content over 53 h in medium 2, not shaken, at 25 °C

Figure 17 (down right): Changes in pH and lactic acid content over 53 h in medium 2, shaken, at 25 °C

Medium 3: 0.5 % lactate, pH 3.8

In the not shaken treatments the lactic acid concentration decreased slightly over 48 h, from 4.7 to 3.8 mg/ml (0.4 s.d.), with no change in pH at this time (Figure 18 & Figure 20).

In the shaken treatments the lactic acid was decomposed completely within 48 h (Figure 19 & Figure 21). There was a difference (n.s.) in the pH development during the first 25 h between the treatments at 20 and 25 °C. At 20 °C the pH rose from 3.8 to 4.2 (0.1 s.d.). At 25 °C it rose to 5.9 (0.6 s.d.), but after 53 h both treatments had a pH of 8.1 (0.1 s.d.).

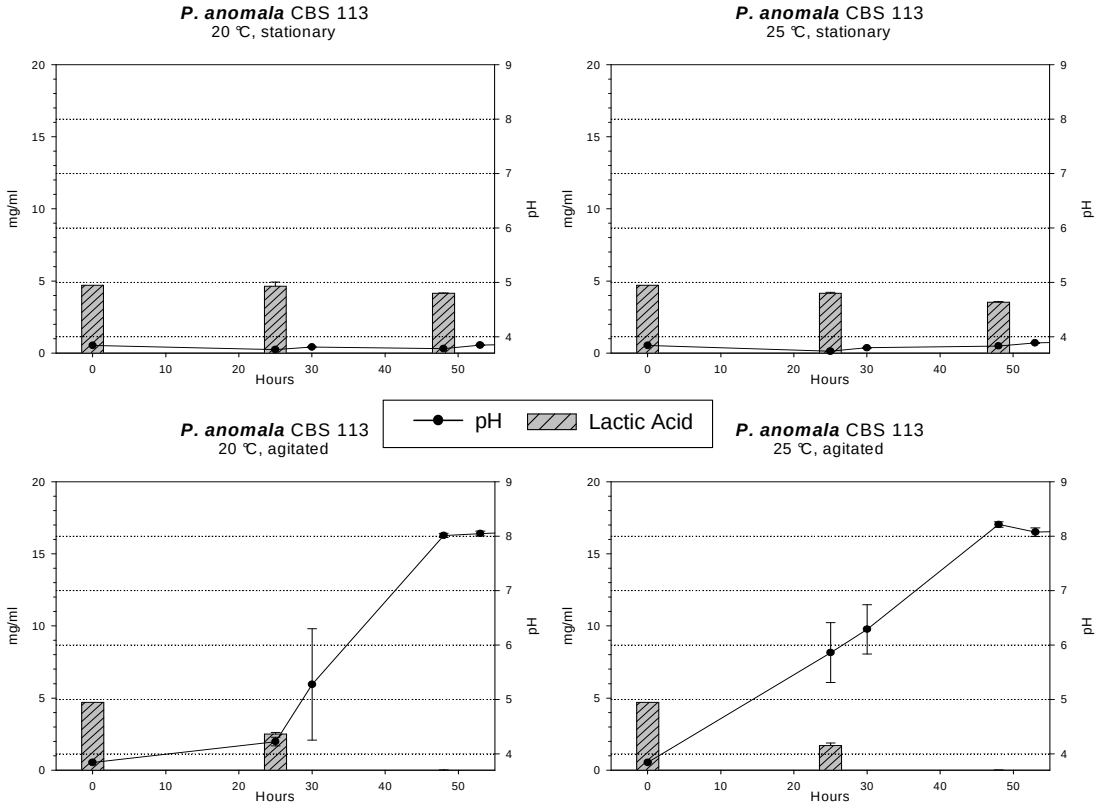


Figure 18 (top left): Changes in pH and lactic acid content over 53 h in medium 3, not shaken, at 20 °C

Figure 19 (down left): Changes in pH and lactic acid content over 53 h in medium 3, shaken, at 20 °C

Figure 20 (top right): Changes in pH and lactic acid content over 53 h in medium 3, not shaken, at 25 °C

Figure 21 (down right): Changes in pH and lactic acid content over 53 h in medium 3, shaken, at 25 °C

Medium 4: 0.5 % lactate, pH 5.5

In the not shaken treatments the lactic acid concentration declined slightly from 4.8 to 3.3 mg/ml (0.3 s.d.) within 48 h (Figure 22 & Figure 24). The pH rose faster at 25 °C and a pH of 5.9 after 25 h, compared to pH 5.3 at 20 °C. However, after 53 h the difference diminished, 6.7 resp. 6.2.

Within 48 h the pH of the shaken treatments rose towards 8.5 and the lactic acid content declined to 0.2 mg/ml at 20 °C (Figure 23) and to 0.7 mg/ml at 25 °C (Figure 25), leaving on average 9 % more lactic acid than the comparable treatments with medium 3.

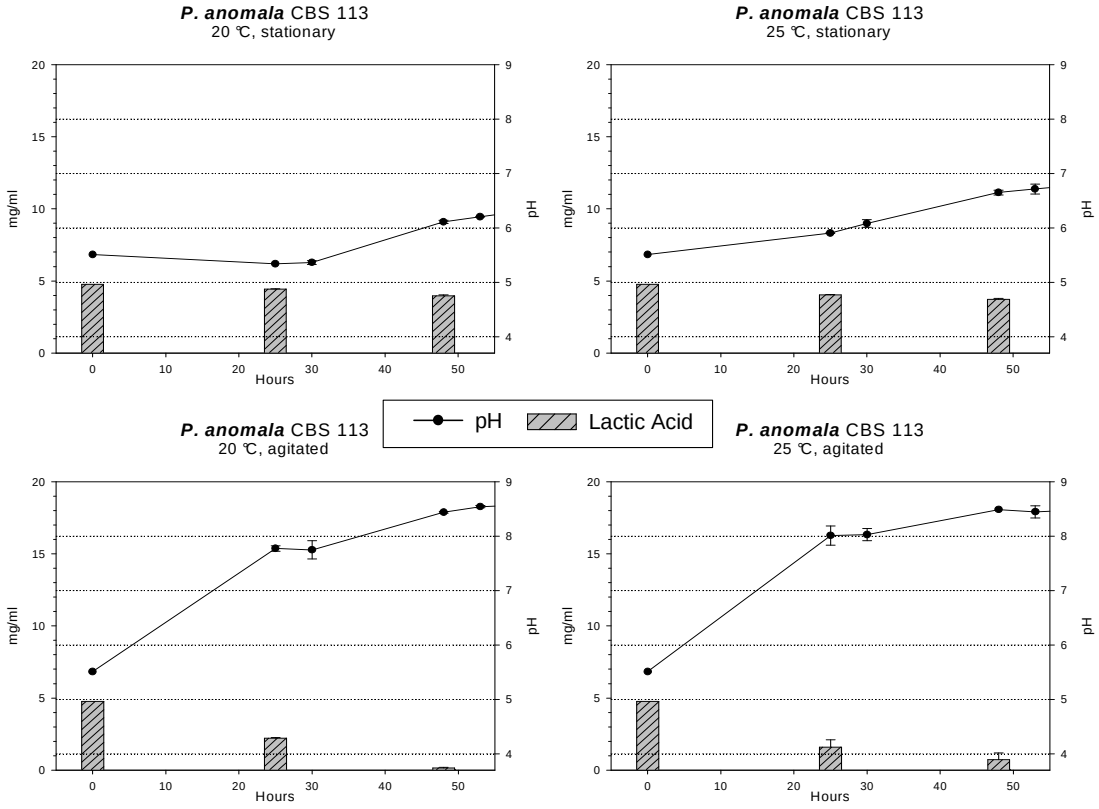


Figure 22 (top left): Changes in pH and lactic acid content over 53 h in medium 4, not shaken, at 20 °C

Figure 23 (down left): Changes in pH and lactic acid content over 53 h in medium 4, shaken, at 20 °C

Figure 24 (top right): Changes in pH and lactic acid content over 53 h in medium 4, not shaken, at 25 °C

Figure 25 (down right): Changes in pH and lactic acid content over 53 h in medium 4, shaken, at 25 °C

Summary

In all not shaken treatments only small amounts of lactic acid were decomposed within 48 h of incubation, but pH only was affected in the treatments starting with a high pH.

If considering the treatments with same initial lactic acid content, but different initial pH there was a higher decomposition rate of lactic acid in the treatments with the lower initial pH. ***The results from these experiments provide evidence to disprove Hypothesis 1*** which says that the pH does not influence the amount of lactate consumption by yeasts.

Considering the treatments with same initial pH but different lactic acid content: initial pH 3.8/3.9: there was a higher pH response in the treatments with lower lactic acid content, pH 5.5: the pH development was quite similar regardless of the lactic acid content.

There was no consistent temperature effect.

For future experiments it was concluded that shaking was needed to provide sufficient aeration of the media to enable oxidative metabolism, but that the choice of one of the two temperature regimes could be made upon practical criteria.

An incubation period of two days was proved to be enough time for a significant decrease in lactic acid concentration in the shaken treatments.

5.1.2 Experiment A 2 – *Lactate concentration, pH, 4 different yeast strains*

To compare different yeast species and strains and their ability to assimilate lactate in different concentrations and at varying pH levels

- 4 media
- Conditions: shaken, 25 °C
- Inoculants: 0.1 ml of 4 yeast strains
- 3 replicates

Medium 1: 2.0 % lactate, pH 3.9

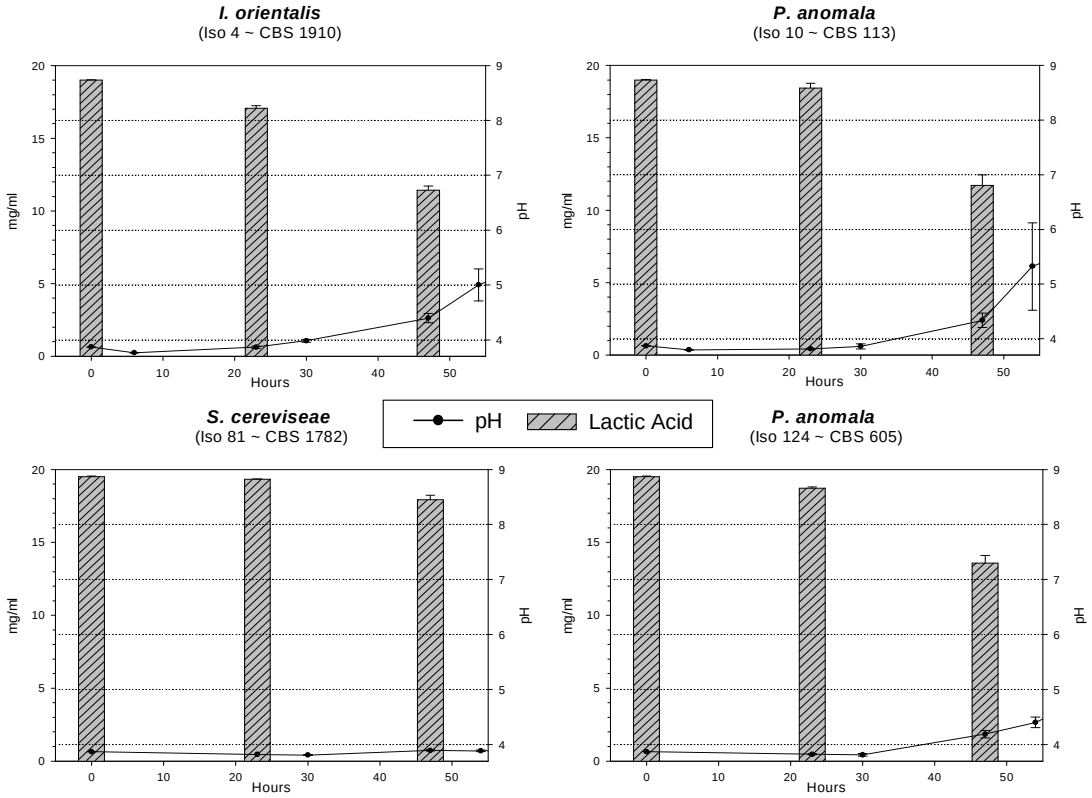
After 23 h of incubation the treatment inoculated with *Issatchenkia orientalis* differed significantly from the other treatments in pH and in lactic acid concentration (Figure 26 to Figure 29). At this time the treatment with *Saccharomyces cereviseae* also differed significantly from the other treatments in lactic acid concentration.

After 47 h the *S. cerevisiae* treatment differed significantly from all other treatments in both pH and lactic acid concentration. At that time the treatment with *Pichia anomala* (CBS 605) differed significantly from all other treatments in lactic acid concentration.

Medium 2: 2.0 % lactate, pH 5.5

After 23 h, *I. orientalis* and *S. cerevisiae* behaved as in medium 1, additionally the treatment with *P. anomala* (CBS 113) differed significantly from all other treatments in pH.

After 47 h, the same results as in medium 1 were obtained. Corresponding graphs can be viewed in *APPENDIX VIII*, figures 1-4.



Figures: Changes in pH and lactic acid concentration over 54 h, error bars = s.d.

Figure 26 (top left): Medium 1, inoculated with Issatchenkia orientalis

Figure 27 (down left): Medium 1, inoculated with Saccharomyces cerevisiae

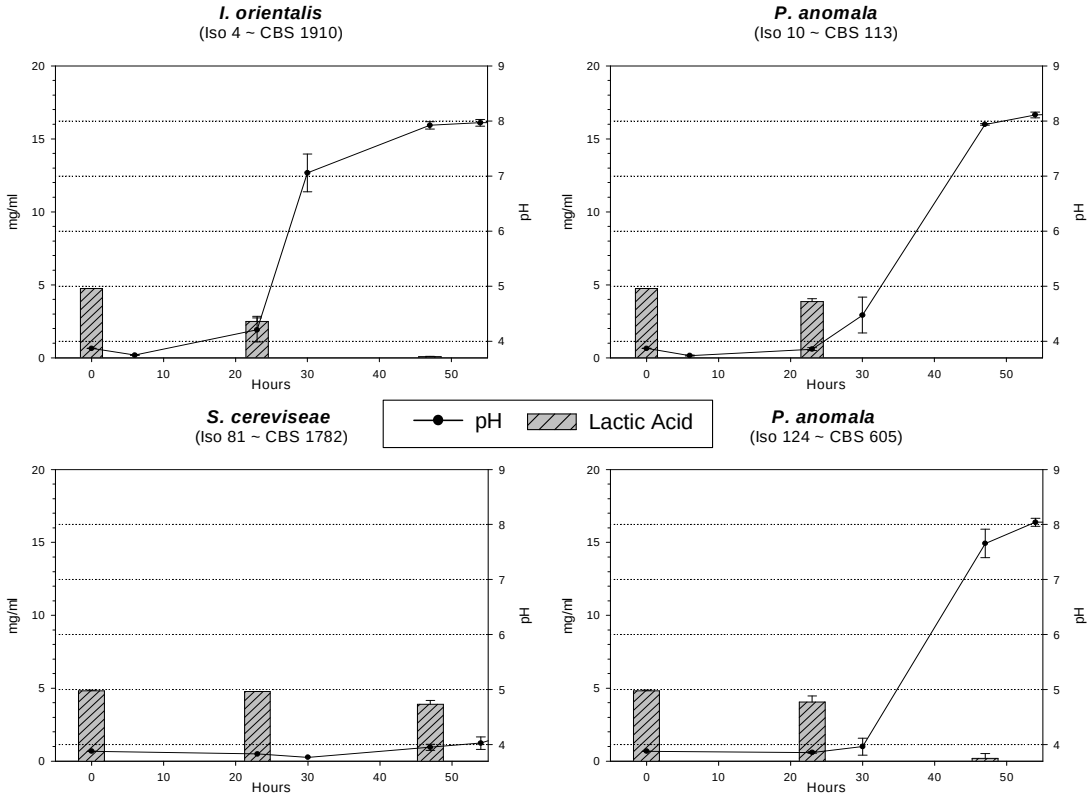
Figure 28 (top right): Medium 1, inoculated with Pichia anomala (CBS 113)

Figure 29 (down right): Medium 1, inoculated with Pichia anomala (CBS 605)

Medium 3: 0.5 % lactate, pH 3.9

After 23 h, *I. orientalis* behaved as in medium 1, additionally the treatment with *P. anomala* (CBS 113) differed significantly from *S. cerevisiae* in lactic acid content (Figure 30 to Figure 33).

After 47 h, the *S. cerevisiae* treatment differed significantly from all other treatments as well as in pH as in lactic acid content.



Figures: Changes in pH and lactic acid concentration over 54 h, error bars = s.d.

Figure 30 (top left): Medium 3, inoculated with Issatchenkia orientalis

Figure 31 (down left): Medium 3, inoculated with Saccharomyces cerevisiae

Figure 32 (top right): Medium 3, inoculated with Pichia anomala (CBS 113)

Figure 33 (down right): Medium 3, inoculated with Pichia anomala (CBS 605)

Medium 4: 0.5 % lactate, pH 5.5

After 23 h, treatments with *I. orientalis* and *S. cerevisiae* differed significantly from each other and from the two *P. anomala* treatments in pH as well as in lactic acid content.

After 47 h, same changes occurred as in medium 3, additionally *I. orientalis* differed significantly from all other treatments in lactic acid content (i.e. > *P. anomala* < *S. cerevisiae*). Corresponding graphs can be viewed in *APPENDIX VIII*, figures 5-8.

Issatchenkia orientalis

Regardless of which medium used *I. orientalis* decomposed lactic acid faster than all other inoculant species.

Pellicle formation was observed after 23 h.

During incubation for 47 h in medium 1 lactic acid concentration decreased from 19.0 to 17.1 (0.2 s.d.) at 23 h to 11.4 mg/ml (0.3 s.d.) and the pH increased to 4.4.

In medium 2 lactic acid concentration diminished from 18.6 mg/ml to 16.2 mg/ml after 23 h and to 12.2 mg/ml after 47 h. The pH rose to 8.3 (0.1 s.d.) after 47 h.

In medium 3 lactic acid declined from 4.7 mg/ml to 2.5 mg/ml (0.3 s.d.) after 23 h and to 0.1 mg/ml after 47 h. The pH increased to 7.9 (0.1 s.d.) after 47 h.

Lactic acid concentration decreased from 4.9 mg/ml to 3.1 mg/ml after 23 h and to 1.0 mg/ml after 47 h. The pH rose to 8.2 (0.0 s.d.) after 47 h.

After 47 h the highest amount of lactic acid was decomposed in the following ranking order: medium 1 (7.6 mg/ml), medium 2 (6.4 mg/ml), medium 3 (4.6 mg/ml), medium 4 (3.9 mg/ml). The higher the lactate concentration and the lower the initial pH, the more lactate was decomposed in a given time.

There was a large difference in pH change in treatments starting with a low pH and dependent on the lactate concentration, in contrast to treatments starting with a high pH.

Saccharomyces cerevisiae

Regardless of which medium was used *S. cerevisiae* showed the lowest decomposition rate of lactate compared to the other inoculant yeasts.

No pellicle was formed.

Within 23 h there was neither a change in pH nor in lactic acid concentration in all 4 media.

After 47 h the lactic acid concentration diminished by 1.6 mg/ml (medium 1), respectively 1.4 mg/ml (medium 2), 0.9 mg/ml (medium 3) and 0.6 mg/ml (medium 4).

The pH did not change significantly in medium 1 and only slightly in medium 3. It increased in the other two media to 7.0 (0.3 s.d.) (medium 2) and 6.3 (0.3 s.d.) (medium 4).

Pichia anomala (CBS 113 and CBS 605)

The two *P. anomala* type strains behaved similarly except that in media 1-3 the lactate decomposition of CBS 113 was more effective after 47 h than that of the counterpart.

Both type strains formed pellicles.

Medium 1, 23 h, lactic acid content: CBS 113 -0.6 mg/ml, CBS 605 –0.8 mg/ml, 47h, CBS 113 -7.3 mg/ml , CBS 605 -5.9 mg/ml. The pH remained stable during the first 23 h and rose to 4.3 (0.1 s.d.) after 47 h with both inoculants.

Medium 2, 23 h, lactic acid content: CBS 113 -0.9 mg/ml, CBS 605 -1.0 mg/ml, 47h, CBS 113 -5.9 mg/ml, CBS 605 -4.7 mg/ml. The pH increased to 6.2 (CBS 113) resp. 5.7 (CBS 605) after 23 h and to 8.0 (0.1 s.d.) after 47 h with both inoculants.

Medium 3, 23 h, lactic acid content: CBS 113 -0.9 mg/ml, CBS 605 -0.8 mg/ml, 47h, CBS 113 -4.7 mg/ml, CBS 605 -4.6 mg/ml. The pH did not rise during the first 23 h, but added up to 7.9 (CBS 113) resp. 7.7 (CBS 605) after 47 h.

The reduction of lactic acid in medium 4 was quite similar to medium 3. However this had a strong effect on the pH already after 23 h, risen up to 6.6 (CBS 113) or 6.5 resp.. After 47 h a pH of 8.2-8.4 was achieved.

General conclusions are quite similar to that of *Issatchenkia orientalis.*

Summary

Issatchenkia orientalis was the most effective lactate decomposing yeast followed closely by the *Pichia anomala* strains. *Saccharomyces cereviseae* had virtually no potential to metabolise lactic acid as the sole carbon source.

The higher the concentration of lactic acid and the lower the initial pH the more lactic acid was decomposed by the lactate utilisers. The lower the lactic acid concentration and the higher the initial pH the highest pH was achieved in a given time.

The results from this experiment provide additional evidence to disprove Hypothesis 1.

SYNOPSIS Experiment type A

- Results obtained in Experiment type A provided evidence that shaking supplied sufficient aeration to the cultures to oxidise lactic acid.
- There were no significant differences between an incubation temperature of 20 or 25 °C. For practical reasons it was decided to continue with the higher temperature.
- *Pichia anomala* strains and *Issatchenkia orientalis* metabolised lactic acid as sole carbon source whereas *Saccharomyces cerevisiae* did not.
- High initial concentration of lactic acid and low initial pH enhanced lactate decomposition by *Issatchenkia orientalis* and the two *Pichia anomala* strains. ***That fact provided evidence to disprove Hypothesis 1*** which says that the pH does not influence the amount of lactate consumption by yeasts.

5.2 Experiment type B

In this type of experiment the behaviour of the mixed microflora in lactate medium was investigated.

Experiment B 1 – *Mixed culture from grass silage*

- 4 treatments with 3 replicates: Control (C), Antibacterial (AB), Antimycotic (AM) and Antibacterial & -mycotic (MB) treatment. Synthetic medium 2, inoculant: extract from grass silage ID 15.

There were no significant differences between all treatments over 47 h in pH, lactic acid and acetic acid concentration. Only ethanol concentration was significantly different between the control and the AB and MB treatments. Over 47 h pH and lactic acid content was fairly stable in all treatments. Corresponding graphs can be viewed in *APPENDIX VIII*, figures 9-12.

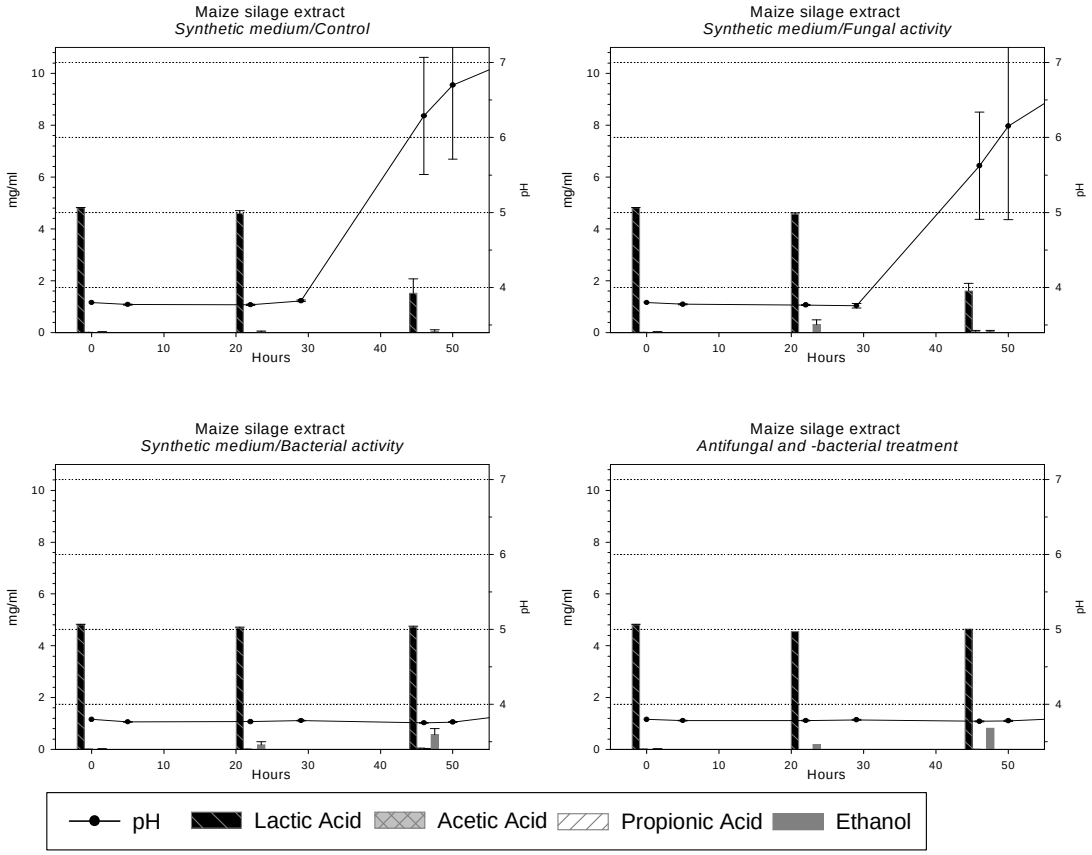
Experiment B 2 – *Mixed culture from maize silage*

- 4 treatments with 3 replicates: Control (C), Antibacterial (AB), Antimycotic (AM) and Antibacterial & -mycotic (MB) treatment. Synthetic medium 3, inoculant: extract from maize silage ID 16.

C and AB treatments were quite similar and differed significantly from AM and MB in pH at all time point (Figure 34 to Figure 37). After 46 h there was a significant difference in lactic acid content between the treatments C+AB and AM+MB.

During the first 22 h all treatments maintained a stable pH and lactic acid content. The treatments AM and MB continued this trend whereas the pH of C rose to 6.3 (0.8 s.d.) and that of AB to 5.6 (0.7 s.d.) after 46 h. At the same time the lactic acid content declined by 3.2-3.3 mg/ml in C and AB.

This finding provided evidence to confirm Hypothesis 2 saying that the decomposition of lactate and rise in pH is solely caused by yeasts.



Figures: Changes in pH, volatile fatty acids and ethanol concentrations in synthetic medium inoculated with mixed micro-flora from maize silage over 50 h, error bars = s.d.

Figure 34 (top left): Control

Figure 35 (down left): Antimycotic treatment

Figure 36 (top right): Antibacterial treatment

Figure 37 (down right): Antimycotic and –bacterial treatment

As there were very large variations in pH between the replicates for treatments C and AB after 46 and 50 h (s.d. 1.0 resp. 1.2) (in contrast to Experiment C 7 with the same silage which was done at the same time) it was decided not to continue with this type of experiment.

5.3 Experiment type C

Grass silages:

5.3.1 Experiment C 1 – *Antibiotics*

To investigate the general behaviour of silage microflora in silage extract and to differentiate between the contribution of bacteria and fungi to activity

- 3 treatments with 3 replicates: Control (C), Antibacterial (AB) and Antimycotic (AM) treatment. Silages ID 15, 17, 19, 24, 25, 26, 30, 32, 34. (9 silages in total).

  MB treatment in Silages ID 15, 17, 19, 24. Aerobic stability test.

Initially 6 silages were examined whose extracts were quite similar in initial pH, lactic acid content and yeast counts (ID 19, 24, 26, 30, 32, 34). pH ranged from 4.6-4.8 (mean 4.7, 0.1 s.d.) (Table 14), lactic acid content was in the range 2.2-2.9 mg/ml (mean 2.6, 0.3 s.d.), yeast numbers were 6.2-7.0 log cfu/g FM (mean 6.7). For those silages where numbers were enumerated (silages 24, 26, 30, 32, 34), LAB counts were in the range 4.9-6.5 log cfu/g FM (mean 6.0).

Table 14: Initial pH and concentrations of some chemical components [mg/ml] of the silage extracts; Experiment C 1

0 h	**pH**	**Lactic acid**	**Acetic acid**	**Propionic acid**	**Ethanol**
Silage 19	4.7	2.2	0.8	0.0	0.9
Silage 24	4.8	2.9	0.9	0.1	0.5
Silage 26	4.6	2.4	0.9	0.1	0.6
Silage 30	4.6	2.7	0.6	0.2	1.5
Silage 32	4.8	2.5	0.7	0.4	1.7
Silage 34	4.7	2.7	0.9	0.4	1.8

Silages 15, 17 and 25 are considered separately because of their differences (see below).

Control treatment

During the first 22 h the pH declined in all silage media by 0.1-0.5 (0.3 on average) (Table 15, column C; figures D to I in *APPENDIX VIII*). At the same time the lactic acid content rose slightly by 0.1-0.8 mg/ml except in one medium (Silage ID 30, -0.5 mg/ml). During this time acetic acid rose by 0.3 mg/ml, propionic acid increased only by 0.1 mg/ml on average and ethanol increased the most, by 2.2 mg/ml on average (1.1 s.d.).

Pellicle growth in the not shaken treatments was usually observed after 32-35 h, but in silage ID 34 only after 46 h.

At approximately 45 h after incubation the pH rose again compared to 22 h of incubation, on average it differed by -0.1 units from the initial pH. Only one medium, silage ID 32, rose above the initial value at this point of time by + 0.4. Lactic acid contents diminished by 0.0-1.4 mg/ml from the initial figure whereas acetic and propionic acid rose by 1.1 and 0.4 mg/ml on average. Ethanol content declined compared to the previous 24 h but was still 1.7 mg/ml higher than at the beginning.

Fungal activity (Antibacterial treatment)

As with the control treatment there was a small pH decline of -0.2 on average during the first 22 h of incubation (Table 15, column AB; figures D to I in *APPENDIX VIII*). In contrast to the control this was not due to an increase in lactate content which on the contrary decreased by -0.4 mg/ml from the beginning. Acetic acid content remained more or less constant. Propionic acid and ethanol contents increased on average by the same amount as the control.

After 45 h the pH rose by 0.0-1.6 units compared to the initial value, to an average value of pH 5.2. The lactic acid content diminished by -1.6 mg/ml (-1.2- -2.2) from the start of the experiment. Acetic acid rose by 0.5 mg/ml (-0.1- +1.4), propionic acid did not increase on average as well as ethanol compared to 22 h of incubation. However in single cases the ethanol content increased or decreased between 22 and 45 h by -0.7- +1.9 mg/ml.

Table 15: Measured and statistical differences in pH and some chemical components [mg/ml] between AB and C, α =0.05; **Experiment C 1**

	pH			**Lactic acid**			**Acetic acid**			**Propionic acid**			**Ethanol**		
	AB	C		AB	C		AB	C		AB	C		AB	C	
22 h															
Silage 19	4.6	4.2	*	1.8	3.0	*	0.8	1.3	*	0.1	0.2	ns	3.6	3.5	ns
Silage 24	4.6	4.5	*	2.2	3.1	*	1.1	1.3	*	0.2	0.3	ns	2.5	2.8	ns
Silage 26	4.5	4.4	*	2.0	2.6	*	0.7	1.0	*	0.1	0.2	ns	4.3	4.3	ns
Silage 30	4.3	4.2	*	2.0	2.2	*	0.7	0.9	*	0.3	0.2	ns	4.0	4.1	ns
Silage 32	4.7	4.6	*	2.0	2.7	*	0.7	1.0	*	0.5	0.5	ns	3.2	3.1	ns
Silage 34	4.6	4.6	ns	2.8	3.0	ns	0.9	1.0	ns	0.5	0.5	ns	2.6	2.4	ns
Mean	*4.6*	*4.4*		*2.1*	*2.8*		*0.8*	*1.1*		*0.3*	*0.3*		*3.4*	*3.4*	
45 h															
Silage 19	4.9	4.4	*	0.9	1.9	*	1.3	2.1	*	0.2	0.3	ns	3.8	3.3	ns
Silage 24	5.6	4.8	*	0.7	1.6	*	2.3	3.3	ns	0.2	0.9	*	1.9	1.6	ns
Silage 26	4.8	4.4	*	1.0	2.4	*	1.1	1.6	*	0.2	0.2	ns	3.9	3.7	ns
Silage 30	4.6	4.3	*	1.0	1.6	*	0.8	1.2	*	0.2	0.3	ns	4.2	3.5	ns
Silage 32	6.5	5.3	*	0.6	1.1	*	0.6	1.3	*	0.5	0.7	*	2.0	2.4	ns
Silage 34	4.7	4.5	*	1.5	2.3	ns	1.5	1.7	ns	0.6	1.1	*	4.5	2.9	ns
Mean	*5.2*	*4.6*		*1.0*	*1.8*		*1.3*	*1.9*		*0.3*	*0.6*		*3.4*	*2.9*	

Except in the case of ID 34 AB from all silages differed significantly from the control in pH, lactic acid and acetic acid contents after 22 h and 45 h (only acetic acid content in silage 24 was similar to C after 45 h) (Table 15). AB from silage ID 34 differed significantly from C in pH and propionic acid content only after 45 h.

Bacterial activity (Antimycotic treatment)

During 45 h there was a pH decline by 0.6 on average (s.d. 0.2) (Table 16, column AM; figures D to I in *APPENDIX VIII*). Lactic acid content rose during the first 22 h by 0.8 mg/ml on average (s.d. 0.6) and partly declined afterwards. Acetic, propionic acid and ethanol contents increased steadily within 45 h. Acetic acid rose by 4.4 mg/ml on average (s.d. 2.9), propionic acid by 1.7 mg/ml (s.d. 1.0) and ethanol by 2.0 mg/ml (s.d. 0.8) after 45 h.

After 22 h AM treatments from all silages differed significantly from the control in lactic and acetic acid contents (Table 16). Five out of 6 silages differed from C as well in pH and propionic acid contents. Four out of 6 silages differed from C also in ethanol contents.

After 45 h propionic acid contents of all 6 silages differed significantly from the control. Five out of 6 silages differed also significantly from C in pH and acetic acid contents. Four out of 6 still differed in lactic acid contents.

Table 16: Measured and statistical differences in pH and some chemical components [mg/ml] between AM and C, α =0.05; **Experiment C 1**

	pH			**Lactic acid**			**Acetic acid**			**Propionic acid**			**Ethanol**		
	AM	C		AM	C		AM	C		AM	C		AM	C	
22 h															
Silage 19	4.1	4.2	*	3.5	3.0	*	2.9	1.3	*	0.8	0.2	*	1.8	3.5	*
Silage 24	4.3	4.5	*	3.7	3.1	*	2.3	1.3	*	0.9	0.3	*	1.2	2.8	*
Silage 26	4.1	4.4	*	3.8	2.6	*	1.5	1.0	*	0.2	0.2	ns	1.6	4.3	*
Silage 30	4.3	4.2	ns	2.7	2.2	*	1.8	0.9	*	1.0	0.2	*	1.6	4.1	*
Silage 32	4.2	4.6	*	3.9	2.7	*	1.8	1.0	*	1.2	0.5	*	2.5	3.1	ns
Silage 34	4.6	4.6	*	2.9	3.0	ns	0.9	1.0	ns	0.5	0.5	ns	3.0	2.4	ns
Mean	*4.3*	*4.4*		*3.4*	*2.8*		*1.9*	*1.1*		*0.8*	*0.3*		*1.9*	*3.4*	
45 h															
Silage 19	3.9	4.4	*	3.2	1.9	*	5.6	2.1	*	1.4	0.3	*	3.3	3.3	ns
Silage 24	4.3	4.8	ns	2.0	1.6	ns	10.7	3.3	*	3.1	0.9	*	1.8	1.6	ns
Silage 26	4.0	4.4	*	3.8	2.4	*	3.8	1.6	*	1.0	0.2	*	2.5	3.7	ns
Silage 30	4.1	4.3	*	1.4	1.6	ns	4.6	1.2	*	2.3	0.3	*	3.2	3.5	ns
Silage 32	4.4	5.3	*	1.7	1.1	*	4.0	1.3	*	3.0	0.7	*	3.3	2.4	*
Silage 34	3.8	4.5	*	7.3	2.3	*	2.1	1.7	ns	0.8	1.1	*	5.4	2.9	*
Mean	*4.1*	*4.6*		*3.2*	*1.8*		*5.1*	*1.9*		*1.9*	*0.6*		*3.2*	*2.9*	

MB

Antibacterial + antifungal treatment (MB) was applied for silage ID 15, 17, 19, 24. Except for silage 17 it proved to maintain the initial lactic acid level quite well within the measuring period. Other components changed until 46 h, especially ethanol content rose in most cases (figures 16, 20, 36, 40 in *APPENDIX VIII*). However, as the antibiotics applied separately in AB and AM showed significant effects on the development compared to the control they were regarded as sufficiently effective to enhance the microbial group that was not suppressed.

Summary

The control treatment resulted from fungal as well as from bacterial activity. The initial increase in lactic acid content was caused by bacteria. The rise in acetic and propionic acid was also mainly due to bacterial activity whereas the initial production of ethanol was caused by yeast metabolism.

The results of these experiments provide evidence to disprove Hypothesis 3 which says that aerobic changes are dominated by yeast activity ***as the development of C and AB was not equal.***

The other three remaining silages 15, 17 and 25 are considered separately because of their difference in the initial chemical and microbial composition

Silage 15

The initial medium pH was 4.5, lactic acid and acetic acid content were nearly equal (2.6 resp. 2.5 mg/ml), yeast numbers were below the detection limit (< 2.0 log cfu/g FM), aerobic bacteria were detected at a level of 8.5 log cfu/g FM.

During the first 22 h there was no pH change in all three treatments but a reversion of the ratio of lactic and acetic acid contents (2.3 versus 2.5 mg/ml on average) (Table 17; figures J in *APPENDIX VIII*).

After 47 h the treatments differed from each other except for propionic acid and ethanol contents. Ethanol contents did not change significantly from the beginning.

The control had a mean pH of 5.6 with a high standard deviation of 1.4. The fungal treatment had an average pH of 4.9 (n.s.) whereas the pH of the bacterial treatment did not change (n.s.).

In the control the lactic acid content diminished to 1.1 mg/ml, the acetic acid content varied between 0.1-2.6 mg/ml (mean 1.6 mg/ml). In the antibacterial treatment lactic acid content decreased to 1.8 mg/ml, the acetic acid content to 1.4 mg/ml. After 22 and 47 h ***C and AB differed significantly*** in lactic acid content (Table 17). No pellicle forming was observed but mould on the surface of AB after 47 h. In the antimycotic treatment there was also a decrease in lactic acid to 1.3 mg/ml but a rise in acetic acid to 3.4 mg/ml. ***As lactic acid was also decomposed by bacteria more evidence was***

provided to disprove Hypothesis 2 which says that the decomposition of lactate is solely caused by yeasts. Acetic acid content differed from the control only after 47 h.

Table 17: Measured and statistical differences in pH and some chemical components [mg/ml] between antibiotic treatments and control, α =0.05; Silage ID 15

Silage 15	**pH**			**Lactic acid**			**Acetic acid**			**Propionic acid**			**Ethanol**		
		C			C			C			C			C	
0 h		*4.5*			*2.6*			*2.5*			*0.2*			*0.3*	
22 h		*4.5*			*2.2*			*2.5*			*0.2*			*0.4*	
AB	4.5		ns	2.4		*	2.6		ns	0.2		ns	0.4		ns
AM	4.5		ns	2.2		ns	2.4		ns	0.2		ns	0.4		ns
47 h		*5.6*			*1.1*			*1.6*			*0.4*			*0.3*	
AB	4.9		ns	1.8		*	1.4		ns	0.3		ns	0.4		ns
AM	4.5		ns	1.3		ns	3.4		*	0.3		ns	0.4		ns

Silage 17

This silage had a very high initial lactic acid content of 6.5 mg/ml, that was on average 2.5 times higher than the 7 silages described above. The initial pH was 4.8 and yeast numbers accounted for 7.4 log cfu/g FM, aerobic bacteria for 7.0 log cfu/g FM.

During the first 22 h the lactic acid content decreased significantly in all treatments.

In the control it diminished to 3.5 mg/ml, in the antibacterial treatment to 1.9 mg/ml and in the antimycotic treatment to 3.7 mg/ml (Table 18; figures K in *APPENDIX VIII*). The pH of the control and AM decreased at the same time to 4.5 or 4.2 respectively, whereas it remained relatively stable in the AB treatment (4.7). Small amounts of acetic acid were produced in the control and AB, 0.5 resp. 0.2 mg/ml, but 1.9 mg/ml in the AM treatment. The propionic acid content changed significantly in the AM treatment by + 1.1 mg/ml. The ethanol content rose in all 3 treatments to 1.5-1.9 mg/ml.

After 46 h the pH of the control rose to 5.0, of AB to 6.5 and AM remained lowest at 4.4.

The lactic acid content decreased further in all treatments: C 1.2 mg/ml, AB 0.7 mg/ml, AM 2.1 mg/ml. Acetic acid content rose in the control to 1.9 mg/ml and in AM to 5.2 mg/ml whereas it remained stable in AB at 1.1 mg/ml. Propionic acid content increased slightly in the control to 0.7 mg/ml but remarkably in AM to 3.4 mg/ml. In AB there was no change. The ethanol content was almost equal to the initial content in C and AB but rose in AM to 2.1 mg/ml.

After 22 and 46 h there were ***significant differences between control and AB*** in pH, lactic, acetic and propionic acid contents as well as between the control and AM in pH, acetic acid and propionic acid contents. After 46 h AM differed from C also in ethanol content.

These findings provided further evidence to disprove Hypothesis 3 which says that aerobic processes in silages are dominated by yeasts.

Table 18: Measured and statistical differences in pH and some chemical components [mg/ml] between antibiotic treatments and control, α =0.05; Silage ID 17

Silage 17	**pH**			**Lactic acid**			**Acetic acid**			**Propionic acid**			**Ethanol**		
		C			C			C			C			C	
0 h		*4.8*			*6.5*			*0.9*			*0.2*			*0.9*	
22 h		*4.5*			*3.5*			*1.4*			*0.3*			*1.9*	
AB	4.7		*	1.9		*	1.1		*	0.2		*	1.5		ns
AM	4.2		*	3.7		ns	2.8		*	1.3		*	1.7		ns
46 h		*5.0*			*1.2*			*1.9*			*0.7*			*0.8*	
AB	6.5		*	0.7		*	1.1		*	0.2		*	1.2		ns
AM	4.3		*	1.2		ns	5.2		*	3.4		*	2.1		*

The biggest difference between changes that occurred in this silage medium compared to the others was that the treatment ***AM decomposed lactic acid*** despite the fact that residual WSC were available. This resulted in a high production of acetic and propionic acid leading to a lower pH value than the initial one. ***Lactic acid degradation in treatment AM provides further evidence to disprove Hypothesis 2*** which says that the decomposition of lactate is solely caused by yeasts.

Silage 25

Inoculation of the grass with commercially available *Lactobacillus plantarum* (DSM 8866, 8862) before fermentation resulted in a silage with relatively low pH of 4.0 and high lactic acid content of 6.2 mg/ml in the medium. Beside yeasts (5.4 log cfu/g FM) there were also moulds (4.9 log cfu/g FM) in contrast to the preceding silages. LAB numbers were below 5.0 log cfu/g FM, aerobic bacteria numbers were 4.5 log cfu/g FM.

There was virtually no bacterial activity (AM treatment) except some ethanol production up to 2.3 mg/ml after 44 h (figure 42 in *APPENDIX VIII*). Control and AM differed significantly in pH and lactic acid content (Table 19).

The development of control and AB looked quite similar (figures 41 and 43 in *APPENDIX VIII*). There was no significant difference between these treatments in any variable at both measurement times. Under the conditions imposed by the extract from this type of silage (i.e. low pH and high lactate concentration) ***Hypothesis 2 and Hypothesis 3 were supported*** saying that the aerobic changes including decomposition of lactate and rise in pH are solely caused by yeasts. After 21 h the pH value was still the same but lactic acid content diminished to 5.5 resp. 5.3 mg/ml (C, AB). The ethanol content rose to 2.2 or 2.1 mg/ml respectively whereas the acetic acid content remained at 0.4 mg/ml. Propionic acid content increased at most by 0.1 mg/ml.

Pellicle growth was observed after 34 h in the not shaken treatment.

After 44 h the pH rose to 4.8 in both treatments. The lactic acid content of the control diminished to 3.4 mg/ml and of the antibacterial treatment to 3.0 mg/ml. The acetic acid content increased to 0.9 resp. 1.0 mg/ml. Propionic acid content was 0.1 mg/ml in both

treatments. Ethanol content was lower than after the first 22 h and varied from 0.2-1.5 mg/ml (mean of C and AB 1.0 mg/ml).

Table 19: Measured and statistical differences in pH and some chemical components [mg/ml] between antibiotic treatments and control, α =0.05; Silage ID 25

Silage 25	**pH**			**Lactic acid**			**Acetic acid**			**Propionic acid**			**Ethanol**		
		C			C			C			C			C	
0 h		*4.0*			*6.2*			*0.4*			*0.0*			*0.2*	
21 h		*4.0*			*5.5*			*0.4*			*0.0*			*2.2*	
AB	4.0		ns	5.3		ns	0.4		ns	0.1		ns	2.1		ns
AM	4.0		ns	6.4		*	0.3		ns	0.0		ns	1.8		ns
44 h		*4.8*			*3.4*			*0.9*			*0.1*			*0.9*	
AB	4.8		ns	3.0		ns	1.0		ns	0.1		ns	1.1		ns
AM	4.0		*	6.3		*	0.5		ns	0.1		ns	2.3		ns

In contrast to silage 17 which had a similar initial lactic acid content there was virtually no bacterial activity. Within 44 h the lactic acid content increased by 0.2 mg/ml, acetic and propionic acid content by 0.1 mg/ml and ethanol by 2.1 mg/ml. AM differed significantly from the control in lactic acid content and after 44 h also in pH.

A comparison of the results for batch culture with those obtained using the aerobic stability test (HONIG, 1990)

The temperature changes during up to 8 days at an ambient temperature of 20 °C of the 9 silages used in experiment C 2 are considered. Initial and final pH of the silages as well as organic acid contents were determined in an extract of the final silage diluted as in the batch culture.

The results can be divided in two groups: 7 silages became unstable (ID 17, 19, 24, 25, 26, 30, 32) (example see Figure 38), i.e. rose > 3 °C above ambient, within 96 h, 2 silages (ID 15, 34) remained stable during this time.

If the unstable silages are considered first, the temperature of 5 of them (ID 17, 24, 26, 30, 32) rose > 3 °C above ambient within 48 h and in silage 19 and 25 the temperature rose within 72 h.

The final pH of all those silages was above 7.0. The lactic acid content declined to below 0.4 mg/ml.

Silage ID 34 did not become unstable during the measurement period of 96 h nor changed in pH, but increased in lactic acid content from 2.7 to 3.3 mg/ml. In the batch culture of the same silage there were only slight pH changes within 46 hours and the highest lactic acid production was shown by the bacterial treatment when compared to the other silages.

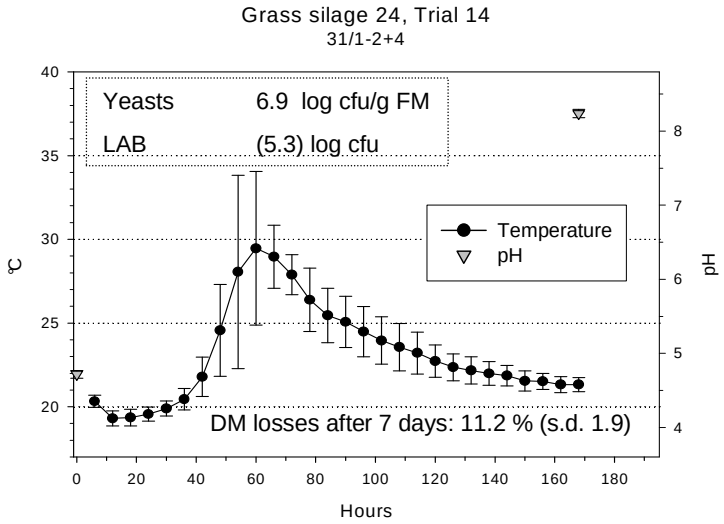


Figure 38: Temperature changes over 7 days and initial and final pH of silage ID24, error bars = s.d.

Silage ID 15 became unstable only after 144 h, the pH did not rise after 8 days. Acetic acid content was slightly higher (+ 0.3 mg/ml) then than lactic acid content which accounted for 2.4 mg/ml.

Those two silages had no or only late pellicle growth as batch culture.

Along with monitoring the temperature, in two silages, ***ID 32 and 34***, in addition, the changes in pH and organic acid and ethanol concentrations were checked daily over 4 days. Two replicate samples were taken daily.

In ***silage 32***, from day 0 to day 1, a slight rise in lactic acid content to 2.8 mg/ml (+0.3 mg/ml, s.d. 0.4) was observed and a rise in ethanol content by 0.6 mg/ml (s.d. 0.4) to 2.4 mg/ml. Acetic and propionic acid changed slightly by -0.1 resp. -0.2 mg/ml. pH rose only by 0.1 to 5.0.

From day 2 on all measured components diminished (compared to the initial values) whereas the pH increased, day 2: lactic acid -0.5 mg/ml, ethanol -1.0 mg/ml, acetic acid -0.6 mg/ml, propionic acid -0.3 mg/ml, pH 6.1, day 3: lactic acid -1.8 mg/ml, ethanol -1.2 mg/ml, acetic acid -0.5 mg/ml, propionic acid -0.4 mg/ml, pH 7.1. On day 4 there was only 0.4 mg/ml lactic acid left and none of the other components, pH 8.3.

As in the temperature test ***silage ID 34*** remained stable within 4 days the development was different to silage ID 32.

The pH fluctuated only by +0.1 units maximum and could be de facto considered as constant.

Nevertheless lactic acid contents varied every day. On day 1, lactic acid content diminished from 2.6 to 2.1 mg/ml and increased again the following days to: 2.5, 2.8, 3.3 mg/ml on day 2, 3, and 4. The content on day 4 was significantly higher compared to day 1.

Ethanol contents diminished by 0.5-0.7 mg/ml from day 1 to 4. Acetic acid contents changed from 0.9 to 0.7 mg/ml on day 1 and rose again to 1.0 mg/ml on day 4. Propionic acid contents varied by ± 0.1 mg/ml.

5.3.2 Experiment C 2 – *+3 % fructose*

To investigate the effect of increasing residual WSC levels on fungi and bacteria in comparison to results obtained in experiment C 1

- 3 treatments with 3 replicates: C, AB and AM + 3 % fructose in FM. Silage ID 17, 19, 24, 25, 26, 30. (6 silages in total).

Five silages are considered together whose media were quite similar in initial pH and yeast counts (ID 17, 19, 24, 26, 30). pH varied from 4.6-4.8 (Table 20), yeast numbers were 6.4-7.4 log cfu/g FM.

Table 20: Initial pH and concentrations of some chemical components [mg/ml] of the silage extracts; Experiment C 2

0 h	**pH**	**Lactic acid**	**Acetic acid**	**Propionic acid**	**Ethanol**
Silage 17	4.8	6.5	0.9	0.2	0.9
Silage 19	4.7	2.2	0.8	0.0	0.9
Silage 24	4.8	2.9	0.9	0.1	0.5
Silage 26	4.6	2.4	0.9	0.1	0.6
Silage 30	4.6	2.7	0.6	0.2	1.5

The pH development in these silages was similar. In all treatments there was a pH decline after 22 h. Only silage ID 25 which started with a low pH is considered separately.

Control treatment

The pH declined from 4.7 to 4.3 on average (Table 21, column C*; figures M to Q in *APPENDIX VIII*). There was a further slight decrease up to 45 h by 0.1 units. The development of lactic acid was not consistent and ranged from -3.3 to +0.7 mg/ml (-0.7 mg/ml on average) during the first 22h and further -0.2 mg/ml after 45 h to 2.4 mg/ml on average.

Acetic acid, propionic acid and ethanol contents rose within 45 h compared to the initial value: acetic acid 1.3 mg/ml (+0.5 mg/ml) after 22 h, 3.1 mg/ml (+2.3 mg/ml) after 45 h, propionic acid 0.3 mg/ml (22 h), 0.5 mg/ml (45 h), ethanol 5.7 mg/ml (+4.8 mg/ml) after 22 h and 4.5 mg/ml after 45 h.

Compared to the control without added fructose (Experiment *C **1***) after 22 h 4 out of 5 silages differed significantly from its sugar supplemented counterpart in pH and ethanol contents (Table 21). After 45 h there were significant differences in all silages in pH and acetic acid contents. Treatments of 3 out of 5 silages also differed significantly in lactic acid and ethanol contents.

Table 21: Measured and statistical differences in pH and some chemical components [mg/ml] between C with additional fructose and C without, α =0.05;* **Experiment C 2**

C	**pH**			**Lactic acid**			**Acetic acid**			**Propionic acid**			**Ethanol**		
	C*	C		C*	C		C*	C		C*	C		C*	C	
22 h															
Silage 17	4.3	4.5	*	3.2	3.5	ns	1.5	1.4	ns	0.4	0.3	*	2.4	1.9	ns
Silage 19	4.2	4.2	ns	2.9	3.0	ns	1.5	1.3	ns	0.2	0.2	ns	6.4	3.5	*
Silage 24	4.5	4.5	*	2.7	3.1	ns	1.3	1.3	ns	0.2	0.3	ns	4.7	2.8	*
Silage 26	4.3	4.4	*	2.5	2.6	ns	1.2	1.0	ns	0.3	0.2	*	7.6	4.3	*
Silage 30	4.2	4.2	*	2.1	2.2	ns	1.1	0.9	*	0.3	0.2	ns	7.2	4.1	*
Mean	*4.3*	*4.4*		*2.7*	*2.9*		*1.3*	*1.2*		*0.3*	*0.2*		*5.7*	*3.3*	
45 h															
Silage 17	4.2	5	*	2.8	1.2	*	2.8	1.9	*	0.6	0.7	ns	2.4	0.8	*
Silage 19	4.1	4.4	*	2.4	1.9	*	3.3	2.1	*	0.4	0.3	ns	5.8	3.3	*
Silage 24	4.2	4.8	*	2.6	1.6	*	5.5	3.3	*	0.8	0.9	ns	2.9	1.6	ns
Silage 26	4.1	4.4	*	2.6	2.4	ns	2.4	1.6	*	0.3	0.2	ns	6.5	3.7	*
Silage 30	4.1	4.3	*	1.8	1.6	ns	1.8	1.2	*	0.3	0.3	ns	4.9	3.5	ns
Mean	*4.2*	*4.6*		*2.4*	*1.7*		*3.1*	*2.0*		*0.5*	*0.5*		*4.5*	*2.6*	

Fungal activity (Antibacterial treatment)

The pH decreased from 4.7 to 4.4 on average after 22 h (Table 22, column AB; figures M to Q in *APPENDIX VIII*). In contrast to the AB treatment without added fructose where the pH increased, in this treatment the pH did not change between 22 and 45 h of incubation.

Within 22 h lactic acid was decomposed by 1.5 mg/ml to 1.9 mg/ml and declined further to 1.1 mg/ml after 45 h. Acetic acid contents increased especially during the second day to 2.4 mg/ml after 45 h, that is an increase of 1.6 mg/ml compared to the starting concentration. Propionic acid contents increased only by 0.1 mg/ml to 0.2 mg/ml until 45 h. However, ethanol was produced to a large extent during the first 22 h: + 4.6 mg/ml to 5.5 mg/ml in total. It slightly decreased to 5.2 mg/ml towards 45 h.

Compared to the control after 45 h there was significantly less lactic acid in all AB treatments (Table 22). In 3 out of 5 silages there were also significant differences in pH, acetic and propionic acid contents. ***Again this provides evidence to disprove Hypothesis 3.***

After 22 and 45 h the pH and acetic acid concentrations differed significantly between the AB without and with additional fructose. In 4 out of 5 silages also the ethanol content differed significantly.

Table 22: Measured and statistical differences in some chemical components [mg/ml] between AB and C, both with added fructose, α =0.05; **Experiment C 2**

+3% fructose	**pH**			**Lactic acid**			**Acetic acid**			**Propionic acid**			**Ethanol**		
	C	AB		C	AB		C	AB		C	AB		C	AB	
22 h															
Silage 17	4.3	4.5	*	3.2	1.5	*	1.5	1.3	*	0.4	0.2	ns	2.4	2.4	*
Silage 19	4.2	4.4	*	2.9	1.8	*	1.5	1.0	*	0.2	0.2	ns	6.4	6.9	ns
Silage 24	4.5	4.5	ns	2.7	2.1	*	1.3	1.2	ns	0.2	0.2	ns	4.7	4.8	ns
Silage 26	4.3	4.3	ns	2.5	2.0	*	1.2	1.0	ns	0.3	0.1	ns	7.6	7.3	*
Silage 30	4.2	4.2	ns	2.1	2.0	ns	1.1	1.0	*	0.3	0.2	ns	7.2	6.0	ns
Mean	*4.3*	*4.4*		*2.7*	*1.9*		*1.3*	*1.1*		*0.3*	*0.2*		*5.7*	*5.5*	
45 h															
Silage 17	4.2	4.6	ns	2.8	1.1	*	2.8	2.1	*	0.6	0.2	ns	2.4	2.7	*
Silage 19	4.1	4.4	*	2.4	1.1	*	3.3	2.4	*	0.4	0.2	ns	5.8	7.0	*
Silage 24	4.2	4.6	ns	2.6	1.2	*	5.5	4.0	ns	0.8	0.3	ns	2.9	4.3	*
Silage 26	4.1	4.4	*	2.6	1.1	*	2.4	2.0	ns	0.3	0.2	ns	6.5	6.7	ns
Silage 30	4.1	4.3	*	1.8	1.1	*	1.8	1.5	*	0.3	0.2	ns	4.9	5.4	ns
Mean	*4.2*	*4.4*		*2.4*	*1.1*		*3.1*	*2.4*		*0.5*	*0.2*		*4.5*	*5.2*	

Table 23: Measured and statistical differences in some chemical components [mg/ml] between AB with added fructose and AB without, α =0.05;* **Experiment C 2**

AB	**pH**			**Lactic acid**			**Acetic acid**			**Propionic acid**			**Ethanol**		
	AB*	AB		AB*	AB		AB*	AB		AB*	AB		AB*	AB	
22 h															
Silage 17	4.5	4.7	*	1.5	1.9	ns	1.3	1.1	*	0.2	0.2	ns	2.4	1.5	ns
Silage 19	4.4	4.6	*	1.8	1.8	ns	1.0	0.8	*	0.2	0.1	ns	6.9	3.6	*
Silage 24	4.5	4.6	*	2.1	2.2	ns	1.2	1.1	*	0.2	0.2	ns	4.8	2.5	*
Silage 26	4.3	4.5	*	2.0	2.0	ns	1.0	0.7	*	0.1	0.1	ns	7.3	4.3	*
Silage 30	4.2	4.3	*	2.0	2.0	ns	1.0	0.7	*	0.2	0.3	ns	6.0	4.0	*
Mean	*4.4*	*4.5*		*1.9*	*2.0*		*1.1*	*0.9*		*0.2*	*0.2*		*5.5*	*3.2*	
45 h															
Silage 17	4.6	6.5	*	1.1	0.7	*	2.1	1.1	*	0.2	0.2	ns	2.7	1.2	*
Silage 19	4.4	4.9	*	1.1	0.9	ns	2.4	1.3	*	0.2	0.2	ns	7.0	3.8	*
Silage 24	4.6	5.6	*	1.2	0.7	ns	4.0	2.3	*	0.3	0.2	ns	4.3	1.9	*
Silage 26	4.4	4.8	*	1.1	1.0	ns	2.0	1.1	*	0.2	0.2	ns	6.7	3.9	*
Silage 30	4.3	4.6	*	1.1	1.0	ns	1.5	0.8	*	0.2	0.2	ns	5.4	4.2	ns
Mean	*4.4*	*5.3*		*1.1*	*0.9*		*2.4*	*1.3*		*0.2*	*0.2*		*5.2*	*3.0*	

Concerning the pH but not the lactic acid decomposition the results provide evidence to disprove Hypothesis 4 saying that the decomposition of lactate and the rise in pH is not influenced by other available carbon sources.

Bacterial activity (Antimycotic treatment)

After 22 h the pH dropped from 4.7 to 4.2 on average and further to 4.1 after 45 h (Table 24, colum AM; figures M to Q in *APPENDIX VIII*), the lowest value obtained for the three treatments. At the same time (22 h) the lactic acid content differed between -2.8 to +1.3 mg/ml (mean 0.1 mg/ml) from the initial value. Acetic acid content increased by 1.4 mg/ml on average, propionic acid increased by 0.7 mg/ml and ethanol concentration by 0.8 mg/ml.

Fortyfive hours after incubation the lactic acid content decreased to 2.5 mg/ml (-0.9 mg/ml). On the other hand acetic acid content increased to 5.9 mg/ml with an increase of 5.3 mg/ml from the start of the incubation. Propionic acid content increased to 2.9 mg/ml (+2.8 mg/ml) and ethanol content to 2.8 mg/ml (+1.9 mg/ml).

The observed lactate decomposition by bacteria provides evidence to disprove Hypothesis 2 which says that lactate degradation is solely caused by yeasts**.**

Compared to the control treatment after 22 and 45 h there were significant differences in acetic acid and propionic acid contents in all treatments (Table 24). Partly there were also differences in ethanol and lactic acid contents and pH.

Table 24: Measured and statistical differences in some chemical components [mg/ml] between AM and C, both with added fructose, α =0.05; **Experiment C 2**

+3% fructose	**pH**			**Lactic acid**			**Acetic acid**			**Propionic acid**			**Ethanol**		
	C	AM		C	AM		C	AM		C	AM		C	AM	
22 h															
Silage 17	4.3	4.2	*	3.2	3.7	ns	1.5	2.6	*	0.4	1.2	ns	2.4	2.0	*
Silage 19	4.2	4.2	ns	2.9	3.1	ns	1.5	2.8	*	0.2	1.0	*	6.4	1.7	*
Silage 24	4.5	4.3	*	2.7	4.0	*	1.3	2.3	*	0.2	0.8	*	4.7	1.7	*
Silage 26	4.3	4.1	*	2.5	3.7	*	1.2	1.6	*	0.3	0.2	*	7.6	1.5	*
Silage 30	4.2	4.3	*	2.1	2.5	*	1.1	1.6	*	0.3	0.9	*	7.2	1.3	*
Mean	*4.3*	*4.2*		*2.7*	*3.4*		*1.3*	*2.2*		*0.3*	*0.8*		*5.7*	*1.6*	
45 h															
Silage 17	4.2	4.2	ns	2.8	2.0	*	2.8	5.5	*	0.6	5.2	ns	2.4	2.5	*
Silage 19	4.1	4.0	*	2.4	2.5	ns	3.3	5.8	*	0.4	1.8	*	5.8	3.6	*
Silage 24	4.2	4.2	ns	2.6	2.6	ns	5.5	9.8	*	0.8	4.1	ns	2.9	1.9	*
Silage 26	4.1	3.9	*	2.6	3.7	*	2.4	3.8	*	0.3	1.1	*	6.5	2.6	*
Silage 30	4.1	4.1	ns	1.8	1.4	ns	1.8	4.7	*	0.3	2.4	*	4.9	3.2	*
Mean	*4.2*	*4.1*		*2.4*	*2.5*		*3.1*	*5.9*		*0.5*	*2.9*		*4.5*	*2.8*	

Compared to the AM treatment without additional fructose after 45 h, 3 out of 5 silages had significant differences in lactic and propionic acid contents (Table 25).

Table 25: Measured and statistical differences in pH and some chemical components [mg/ml] between AM with additional fructose and AM without, α =0.05;* **Experiment C 2**

AM	**pH**			**Lactic acid**			**Acetic acid**			**Propionic acid**			**Ethanol**		
	AM*	AM		AM*	AM		AM*	AM		AM*	AM		AM*	AM	
22 h															
Silage 17	4.2		ns	3.7		ns	2.6		*	1.2		*	2.0		ns
Silage 19	4.2	4.1	ns	3.1	3.5	*	2.8	2.9	ns	1.0	0.8	*	1.7	1.8	ns
Silage 24	4.3	4.3	ns	4.0	3.7	ns	2.3	2.3	ns	0.8	0.9	ns	1.7	1.2	ns
Silage 26	4.1	4.1	ns	3.7	3.8	ns	1.6	1.5	ns	0.2	0.2	ns	1.5	1.6	ns
Silage 30	4.3	4.3	ns	2.5	2.7	ns	1.6	1.8	*	0.9	1.0	*	1.3	1.6	ns
Mean	*4.2*	*4.2*		*3.4*	*3.4*		*2.2*	*2.1*		*0.8*	*0.7*		*1.6*	*1.5*	
45 h															
Silage 17	4.2		ns	2		*	5.5		*	5.2		*	2.5		ns
Silage 19	4.0	3.9	ns	2.5	3.2	*	5.8	5.6	ns	1.8	1.4	*	3.6	3.3	ns
Silage 24	4.2	4.3	ns	2.6	2.0	*	9.8	10.7	ns	4.1	3.1	*	1.9	1.8	ns
Silage 26	3.9	4.0	ns	3.7	3.8	ns	3.8	3.8	ns	1.1	1.0	ns	2.6	2.5	ns
Silage 30	4.1	4.1	ns	1.4	1.4	ns	4.7	4.6	ns	2.4	2.3	ns	3.2	3.2	ns
Mean	*4.1*	*4.1*		*2.5*	*2.6*		*5.9*	*6.2*		*2.9*	*2.0*		*2.8*	*2.7*	

Silage ID 25

Corresponding graphs can be viewed in *APPENDIX VIII*, figures R.

Control treatment

Until up to 21 h of incubation the pH remained stable at 4.0 whereas the lactic acid content decreased from 6.2 to 5.0 mg/ml (Table 26, column Fr3, row C) which was significantly lower than its counterpart without additional fructose. Acetic acid and propionic acid contents rose only by 0.1 mg/ml, but ethanol contents increased most by 3.0 mg/ml from an initial concentration of 0.2 mg/ml.

After 44 h the pH increased to 4.3, that was 0.5 units less than the control without additional fructose (Table 26), lactic acid content decreased to 3.4 mg/ml, that was the same amount as the control without additional fructose. Acetic acid content increased by 1.8 mg/ml to 2.2 mg/ml, propionic acid increased from 0.0 to 0.2 mg/ml and ethanol content to 3.1 mg/ml which was significantly higher than the counterpart without additional fructose.

Fungal activity (Antibacterial treatment)

During the first 21 h the pH remained stable at 4.0 (Table 26, column Fr3, row AB). The lactic acid content was reduced by 1.5 mg/ml, acetic acid content increased by 0.1 mg/ml, propionic acid content changed very little and 1.3 mg/ml ethanol was produced.

After 44 h the pH reached 4.4, lactic acid content was reduced by 2.7 mg/ml, acetic acid content increased to 1.9 mg/ml, propionic acid to 0.2 mg/ml and ethanol to 2.9 mg/ml.

Compared to the control treatment there was a significant difference in ethanol content after 21 h only.

Compared to the AB treatment without additional fructose, like in the control there was only a significant difference in lactic acid content after 21 h and in ethanol content after 44 h (Table 26).

Bacterial activity (Antimycotic treatment)

There was very little change in pH and lactic acid and propionic acid content within 44 h, similar to the AM treatment without additional fructose (Table 26).

Acetic acid content raised by 0.2 mg/ml after 21 h which was significantly higher than in the counterpart without additional fructose, but decreased after 44 h to the initial 0.4 mg/ml. Ethanol content increased to 1.3 mg/ml after 21 h and to 1.8 mg/ml after 44 h.

Compared to the control there were significant differences in lactic acid contents after 21 and 44 h. After 21 h additionally there were significant differences in pH and ethanol content. After 44 h the treatments differed from the control in lactic and acetic acid contents.

There were no significant differences compared to the AM treatment without additional fructose except for the acetic acid content after 21 h.

Because of the different results within the two measurement times ***there was no evidence to disprove nor to confirm Hypotheses 2, 3 and 4.***

Table 26: Measured and statistical differences in pH and some chemical components [mg/ml] between treatments with (Fr3) and without (Fr0) additional fructose, α =0.05; Silage ID 25

Silage 25	**pH**			**Lactic acid**			**Acetic acid**			**Propionic acid**			**Ethanol**		
	Fr3	Fr0		Fr3	Fr0		Fr3	Fr0		Fr3	Fr0		Fr3	Fr0	
0 h		*4.0*			*6.2*			*0.4*			*0*			*0.2*	
21 h															
C	4.0	4.0	ns	5.0	5.5	*	0.5	0.4	ns	0.0	0.0	ns	3.2	2.2	ns
AB	4.0	4.0	ns	4.7	5.3	*	0.5	0.4	ns	0.0	0.1	ns	1.5	2.1	ns
AM	4.0	4.0	ns	6.2	6.4	ns	0.6	0.3	*	0.0	0.0	ns	1.3	1.8	ns
44 h															
C	4.3	4.8	ns	3.4	3.4	ns	2.2	0.9	ns	0.1	0.1	ns	3.1	0.9	*
AB	4.4	4.8	ns	3.5	3.0	ns	1.9	1.0	ns	0.1	0.1	ns	3.1	1.1	*
AM	4.0	4.0	ns	6.0	6.3	ns	0.4	0.5	ns	0.1	0.1	ns	1.7	2.3	ns

5.3.3 Experiment C 3 – *+6 % fructose*

To investigate the effect of increasing residual WSC levels on fungi and bacteria in comparison to results obtained in Experiment C 1 and C 2

- 3 treatments with 3 replicates: C, AB and AM + 6 % fructose in FM. Silage ID 19, 32, 34. (3 silages in total). Aerobic stability test.

Table 27: Initial pH and concentrations of some chemical components [mg/ml] of the silage extracts; Experiment C 3

0 h	**pH**	**Lactic acid**	**Acetic acid**	**Propionic acid**	**Ethanol**
Silage 19	4.7	2.2	0.8	0.0	0.9
Silage 32	4.8	2.5	0.7	0.4	1.7
Silage 34	4.7	2.7	0.9	0.4	1.8

Control treatment

After 22 h the pH decreased by 0.4 units on average (Table 28, column C*; figures S-U in *APPENDIX VIII*). Lactic acid was produced, 0.3 mg/ml on average, acetic and propionic acid as well, 0.7 and 0.2 mg/ml respectively. Ethanol production varied from 1.6 to 7.8 mg/ml (3.3 mg/ml on average).

There was a further drop in pH after 45 h by 0.7 units from the beginning to 4.1. All other parameters increased: lactic acid content rose further by 0.6 mg/ml to 3.0 mg/ml, acetic acid by 2.6 mg/ml to 3.4 mg/ml, propionic acid by 0.4 to 0.7 mg/ml and ethanol by 7.0 mg/ml to 8.5 mg/ml on average.

Table 28: Measured and statistical differences in pH and some chemical components [mg/ml] between C with additional fructose and C without, α =0.05;* **Experiment C 3**

C	**pH**			**Lactic acid**			**Acetic acid**			**Propionic acid**			**Ethanol**		
	C*	C		C*	C		C*	C		C*	C		C*	C	
22 h															
Silage 19	4.2	4.2	*	2.8	3.0	ns	2.2	1.3	*	0.3	0.2	*	8.7	3.5	*
Silage 32	4.3	4.6	*	2.5	2.7	*	1.2	1.0	*	0.6	0.5	ns	9.6	3.1	*
Silage 34	4.5	4.6	*	2.9	3.0	ns	0.9	1.0	ns	0.5	0.5	ns	3.4	2.4	ns
Mean	*4.3*	*4.5*		*2.8*	*2.9*		*1.4*	*1.1*		*0.5*	*0.4*		*7.2*	*3.0*	
45 h															
Silage 19	4.0	4.4	*	2.5	1.9	*	3.9	2.1	*	0.4	0.3	ns	7.8	3.3	*
Silage 32	4.2	5.3	*	1.9	1.1	*	2.5	1.3	*	0.7	0.7	ns	7.9	2.4	*
Silage 34	4.0	4.5	*	4.6	2.3	*	3.7	1.7	*	1.0	1.1	ns	9.8	2.9	*
Mean	*4.1*	*4.7*		*3.0*	*1.7*		*3.4*	*1.7*		*0.7*	*0.7*		*8.5*	*2.9*	

Compared to the control without additional fructose after 45 h there were significant differences in pH, lactic and acetic acid contents and in ethanol contents in all 3 silages (Table 28).

Fungal activity (Antibacterial treatment)

Within 22 h the pH dropped by 0.3 units on average (Table 29, column AB; figures S-U in *APPENDIX VIII*). Lactic acid concentration fell by 0.2 mg/ml, whereas acetic acid and propionic acid were produced (0.3 and 0.1 mg/ml resp.). Ethanol content rose by 5.8 to 7.2 mg/ml on average.

After 45 h the pH further decreased to 4.3 and the lactic acid content to 1.6 mg/ml (-0.9 mg/ml). At the same time acetic acid content increased by 1.8 mg/ml to 2.6 mg/ml, propionic acid to 0.4 mg/ml, ethanol by 7.9 mg/ml to 9.3 mg/ml.

Compared to the control treatment after 45 h there were significant differences in lactic, acetic and propionic acid concentrations (Table 29). For silages 19 and 34 the pH differed significantly from the control.

Table 29: Measured and statistical differences in pH and some chemical components [mg/ml] between AB and C with additional fructose, α =0.05; **Experiment C 3**

+6% fructose	**pH**			**Lactic acid**			**Acetic acid**			**Propionic acid**			**Ethanol**		
	C	AB		C	AB		C	AB		C	AB		C	AB	
22 h															
Silage 19	4.2	4.3	*	2.8	2.0	*	2.2	1.3	*	0.3	0.2	*	8.7	9.0	ns
Silage 32	4.3	4.3	ns	2.5	2.2	*	1.2	1.1	*	0.6	0.5	ns	9.6	9.1	ns
Silage 34	4.5	4.6	*	2.9	2.7	ns	0.9	0.9	ns	0.5	0.4	ns	3.4	3.7	ns
Mean	*4.3*	*4.4*		*2.8*	*2.3*		*1.4*	*1.1*		*0.5*	*0.4*		*7.2*	*7.2*	
45 h															
Silage 19	4.0	4.2	*	2.5	1.2	*	3.9	2.9	*	0.4	0.2	*	7.8	9.8	ns
Silage 32	4.2	4.4	ns	1.9	1.3	*	2.5	2.1	*	0.7	0.5	*	7.9	8.2	ns
Silage 34	4.0	4.2	*	4.6	2.2	*	3.7	2.8	*	1.0	0.5	*	9.8	10.0	ns
Mean	*4.1*	*4.3*		*3.0*	*1.6*		*3.4*	*2.6*		*0.7*	*0.4*		*8.5*	*9.3*	

In comparison to the AB treatment without additional fructose there were significant differences in pH, acetic acid and ethanol contents (Table 30).

Concerning the pH but not the lactic acid decomposition Hypothesis 4 was disproved saying that changes in both parameters are not influenced by other available carbon sources.

Table 30: Measured and statistical differences in pH and some chemical components [mg/ml] between AB with additional fructose and AB without, α =0.05;* **Experiment C 3**

AB	**pH**			**Lactic acid**			**Acetic acid**			**Propionic acid**			**Ethanol**		
	AB*	AB		AB*	AB		AB*	AB		AB*	AB		AB*	AB	
22 h															
Silage 19	4.3	4.6	*	2.0	1.8	ns	1.3	0.8	*	0.2	0.1	ns	9.0	3.6	*
Silage 32	4.3	4.7	*	2.2	2.0	*	1.1	0.7	*	0.5	0.5	ns	9.1	3.2	*
Silage 34	4.6	4.6	*	2.7	2.8	ns	0.9	0.9	ns	0.4	0.5	ns	3.7	2.6	ns
Mean	*4.4*	*4.6*		*2.3*	*2.2*		*1.1*	*0.8*		*0.4*	*0.4*		*7.2*	*3.1*	
45 h															
Silage 19	4.2	4.9	*	1.2	0.9	ns	2.9	1.3	*	0.2	0.2	ns	9.8	3.8	*
Silage 32	4.4	6.5	*	1.3	0.6	*	2.1	0.6	*	0.5	0.5	ns	8.2	2.0	*
Silage 34	4.2	4.7	*	2.2	1.5	ns	2.8	1.5	*	0.5	0.6	ns	10.0	4.5	*
Mean	*4.3*	*5.4*		*1.6*	*1.0*		*2.6*	*1.1*		*0.4*	*0.4*		*9.3*	*3.4*	

Bacterial activity (Antimycotic treatment)

After 22 h the pH dropped by 0.4 to 4.4 (Table 31, column AM; figures S-U in *APPENDIX VIII*). Lactic acid content rose by 0.7 mg/ml to 3.2 mg/ml, acetic acid content rose by 1.0 mg/ml to 1.8 mg/ml, propionic acid concentration rose by 0.5 mg/ml and ethanol content by 1.1 mg/ml.

Table 31: Measured and statistical differences in pH and some chemical components [mg/ml] between AM and C with additional fructose, α =0.05; **Experiment C 3**

+6% fructose	**pH**			**Lactic acid**			**Acetic acid**			**Propionic acid**			**Ethanol**		
	C	AM		C	AM		C	AM		C	AM		C	AM	
22 h															
Silage 19	4.2	4.1	ns	2.8	3.2	ns	2.2	2.8	*	0.3	1.0	*	8.7	1.9	*
Silage 32	4.3	4.3	ns	2.5	3.4	*	1.2	1.5	*	0.6	0.9	*	9.6	2.1	*
Silage 34	4.5	4.7	*	2.9	3.0	ns	0.9	0.9	ns	0.5	0.4	ns	3.4	3.6	ns
Mean	*4.3*	*4.4*		*2.8*	*3.2*		*1.4*	*1.8*		*0.5*	*0.8*		*7.2*	*2.5*	
45 h															
Silage 19	4.0	4.0	ns	2.5	2.3	ns	3.9	5.9	*	0.4	2.0	*	7.8	3.7	*
Silage 32	4.2	4.1	ns	1.9	2.9	*	2.5	4.0	*	0.7	4.2	*	7.9	2.9	*
Silage 34	4.0	3.8	*	4.6	8.2	*	3.7	2.2	*	1.0	0.6	*	9.8	6.0	*
Mean	*4.1*	*4.0*		*3.0*	*4.5*		*3.4*	*4.0*		*0.7*	*2.3*		*8.5*	*4.2*	

After 45 h the pH dropped further to 4.0, additional lactic acid was produced to a content of 4.5 mg/ml. Acetic acid content increased to 4.0 mg/ml, propionic acid rose by 2.0 mg/ml to 2.3 mg/ml, ethanol content increased by 2.7 mg/ml to 4.2 mg/ml on average.

After 45 h the acetic acid, propionic acid and ethanol concentrations of all 3 silages differed significantly from the control (Table 31). In two out of 3 silages the lactic acid contents were also significantly different.

AM treatments of silages 19 and 32 with and without additional fructose differed significantly in lactic acid and propionic acid contents after 45 h (Table 32). Silage 34 had no significant differences within the measurement period.

Table 32: Measured and statistical differences in pH and some chemical components [mg/ml] between AM with additional fructose and AM without, α =0.05;* **Experiment C 3**

AM	**pH**			**Lactic acid**			**Acetic acid**			**Propionic acid**			**Ethanol**		
	AM*	AM		AM*	AM		AM*	AM		AM*	AM		AM*	AM	
22 h															
Silage 19	4.1	4.1	ns	3.2	3.5	ns	2.8	2.9	ns	1.0	0.8	*	1.9	1.8	ns
Silage 32	4.3	4.2	*	3.4	3.9	*	1.5	1.8	*	0.9	1.2	*	2.1	2.5	ns
Silage 34	4.7	4.6	ns	3.0	2.9	ns	0.9	0.9	ns	0.4	0.5	ns	3.6	3.0	ns
Mean	*4.4*	*4.3*		*3.2*	*3.4*		*1.8*	*1.9*		*0.8*	*0.8*		*2.5*	*2.4*	
45 h															
Silage 19	4.0	3.9	ns	2.3	3.2	*	5.9	5.6	ns	2.0	1.4	*	3.7	3.3	ns
Silage 32	4.1	4.4	ns	2.9	1.7	*	4.0	4.0	ns	4.2	3.0	*	2.9	3.3	ns
Silage 34	3.8	3.8	ns	8.2	7.3	ns	2.2	2.1	ns	0.6	0.8	ns	6.0	5.4	ns
Mean	*4.0*	*4.0*		*4.5*	*4.1*		*4.0*	*3.9*		*2.3*	*1.7*		*4.2*	*4.0*	

<u>Comparison of the batch culture technique to temperature development in the aerobic stability test</u> (HONIG, 1990)

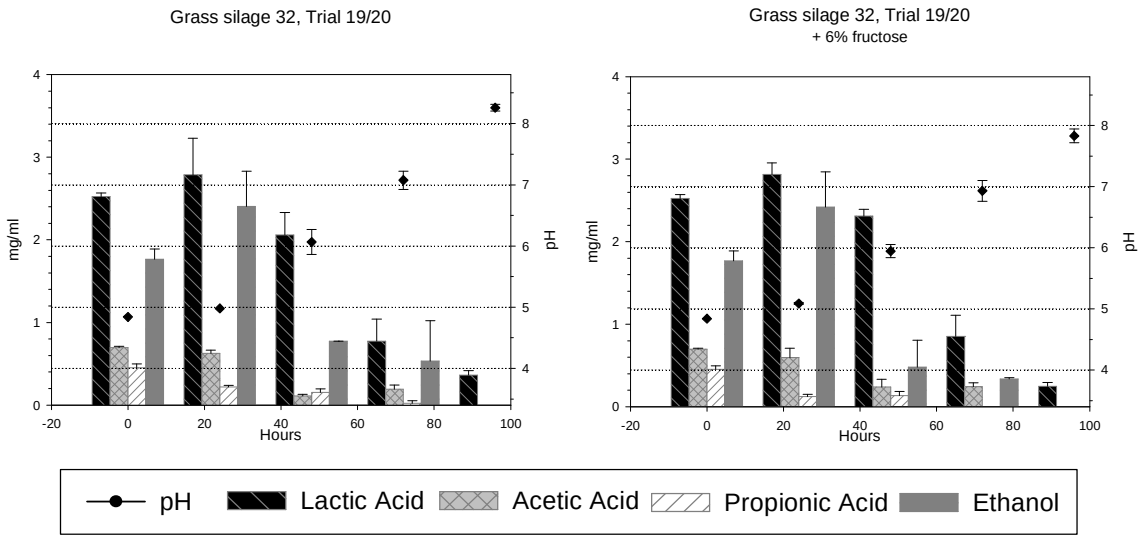
In the aerobic stability test a concentrated fructose solution was added to silages 32 and 34 in two replicates, resulting in additional 6 % fructose. Temperature development, pH and organic acids and ethanol were recorded for 4 days.

Silage 32 became unstable after 24 h, i.e. rose > 3 °C above ambient, whereas silage 34 remained stable within 96 h.

The calculated DM losses in silage 32 of the treatment with added fructose were 2.5 times higher (21.5 %) than the counterpart without fructose added. In the stable silage (ID 34) there were no differences.

Silage 32

Regarding silage 32 the pH development was similar to the control but ended with a significantly lower pH value of 7.8 compared to 8.3 after 96 h (Figure 39 and Figure 40). The fructose treatment did not differ significantly from the control in any parameter on any day during the measurement period, except for pH after one and 4 days.



Figures: Daily changes in pH and some chemical components [mg/ml] in the temperature test over 4 days, error bars = s.d.

Figure 39 (left): Untreated control

Figure 40 (right): With addition of 6 % fructose on FM base

The corresponding batch culture had a much higher ethanol production and acetic acid was produced instead of consumed, in contrast to the actual silage in the HONIG aerobic stability test. In addition, the pH dropped instead of rising. However the lactic acid content was comparable within the same measurement period.

At the end of the incubation period over 4 days on exposure to air the silage replicate with the lower pH (7.8) of the treatment with fructose addition and the one with the higher pH (8.3) of the control were investigated microbiologically as the daily visual scoring for yeasts and moulds showed a slower (visible) growth of yeast in the fructose treatment compared to the control. The yeast counts of the fructose treatment were 9.5 log cfu/g FM and LAB 8.2 log cfu/g FM, whereas in the control yeast counts were 11.4 log cfu/g FM and LAB 7.0 log cfu/g FM, i.e. the control had nearly a 100-fold higher amount of yeasts compared to the fructose added treatment, but in contrast the latter had an approximately 20-fold higher amount of LAB.

Silage 34

The pH, organic acid and ethanol concentrations remained more or less stable over 4 days. Only the ethanol content of the fructose treatment decreased significantly within the first 24 h.

During the first two days of incubation there was no significant difference between the fructose treatment and the control. On day 3 and 4 the control had a significantly higher

ethanol content and on day 4 the acetic acid content was also significantly higher in the control compared to the fructose treatment.

The corresponding batch culture increased in all variables (lactic, acetic, propionic acid and ethanol contents), whereas the actual silage in the HONIG aerobic stability test remained more or less stable in the acids and ethanol was decomposed.

Summary: Influence of WSC content on changes occurring in batch cultures

General observations (Experiment C 1-Experiment C 3)

Control treatment

The higher the WSC content of the medium the higher the level of ethanol production and the later and smoother the pH rise.

There was a trend towards a lower final pH as the WSC content increased. Arranging the treatments according to their total WSC content (sum of fructose, glucose, sucrose) after 53 h of incubation (including grass-lucerne silages (Experiment C 7 and Experiment C 8), silage 25 excluded):

Least significant difference 0.21.
Means with the same letter in the Tukey grouping are not significantly different.

Table 33: Tukey grouping of pH values after 53 h of incubation in relation to the corresponding initial WSC content in FM

Tukey grouping			pH mean	n	% WSC
	A		5.55	3	4.4
	B		5.13	3	3.7
	C		4.89	3	4.7
	D		4.64	3	3.2
	D		4.56	3	5.5
	D		4.54	3	6.9
	D		4.51	3	5.1
E	D		4.45	3	5.9
E	D		4.44	3	6.7
E	F		4.26	3	10.4
E	F		4.26	3	7.7
G	F		4.16	3	9.9
G	F		4.14	3	8.1
G	F	H	4.10	3	8.5
G	F	H	4.09	3	8.9
G	H		4.01	3	9.2
	H		3.92	3	11.9

Three way correlations between pH, time and WSC content

For grass silages (ID 15, 17, 19, 24, 26, 30, 32, 34):
$pH(t)=5.09-0.01*t[h]+0.0002*t^2[h]-0.08*WSC[\%\ FM]$, $r^2=0.51$, n=174.

For grass silages (ID 15, 17, 19, 24, 26, 30, 32, 34) plus grass-lucerne silages (ID 27, 31) (Experiment C 7 and Experiment C 8):
$pH(t)= 5.08-0.01*t[h]+0.0002*t^2[h]-0.08*WSC[\%\ FM]$, $r^2=0.53$, n=228.

Correlation between pH, time and (WSC*BC):
$pH(t)=4{,}99-0.01*t[h]+0.0002*t^2[h]-0.0003*WSC[\%\ FM]*BC[Meq\ in\ FM]$, $r^2=0.43$, n=228.

Fungal activity (Antibacterial treatment)

The higher the WSC content of the medium the higher the level of ethanol production and the later and smoother the pH rise.

Correlation between pH, time and WSC content

For grass silages (ID 15, 17, 19, 24, 26, 30, 32, 34):
$pH(t)=5.3-0.005*t[h]+0.0002*t^2[h]-0.12*WSC[\%\ FM]$, $r^2=0.39$, n=174.

For grass silages (ID 15, 17, 19, 24, 26, 30, 32, 34) plus grass-lucerne silages (ID 27, 31) (Experiment C 7 and Experiment C 8):
$pH(t)=5.28-0.005*t[h]+0.0003*t^2[h]-0.12*WSC[\%\ FM]$, $r^2=0.44$, n=228.

Correlation between pH, time and (WSC*BC):
$pH(t)=5.13-0.005*t[h]+0.0003*t^2[h]-0.0005*WSC[\%\ FM]*BC[Meq\ in\ FM]$, $r^2=0.35$, n=228.

Bacterial activity (Antimycotic treatment)

There was no significant influence of WSC concentration of the medium on the development of pH, organic acid or ethanol concentrations.

Correlation between pH, time and WSC content

For grass silages (ID 15, 17, 19, 24, 26, 30, 32, 34):
$pH(t)=4.78-0.022*t[h]+0.0002*t^2[h]-0.01*WSC[\%\ FM]$, $r^2=0.68$, n=174

For grass silages (ID 15, 17, 19, 24, 26, 30, 32, 34) plus grass-lucerne silages (ID 27, 31) (Experiment C 7 and Experiment C 8):

$pH(t)=4.82-0.02*t[h]+0.0001*t^2[h]-0.02*WSC[\%\ FM]$, $r^2=0.58$, n=228

Correlation between pH, time and (WSC*BC):

$pH(t)=4.78-0.02*t[h]+0.0001*t^2[h]-0.0001*WSC[\%\ FM]*BC[Meq\ in\ FM]$, $r^2=0.57$, n=228.

The correlation equations show that the influence of WSC was highest in the AB treatment and lowest in the AM treatment. This relationship also holds true for the product WSC*BC.

5.3.4 Experiment C 4 – *Potassium chloride, 100 ml – 200 ml Erlenmeyer flasks*

To study the influence of osmotic pressure adjusted to conditions in the actual silage, on the microbial activity

- 6 treatments with 3 replicates: C, AB and AM in 8 % (w/v) KCl solution in 100 ml and in 200 ml erlenmeyer flasks. Silage ID 34.

Yeast numbers in the silage were 6.2 log cfu/g FM and LAB numbers 6.5 log cfu/g FM.

Within 22 h all treatments behaved similarly compared to the counterparts without KCl addition. pH and organic acid values remained relatively stable from the beginning. Only ethanol was produced.

The treatments in 200 ml erlenmeyer flasks (figures V in *APPENDIX VIII*) resembled the counterparts in 100 ml volume.

Control treatment

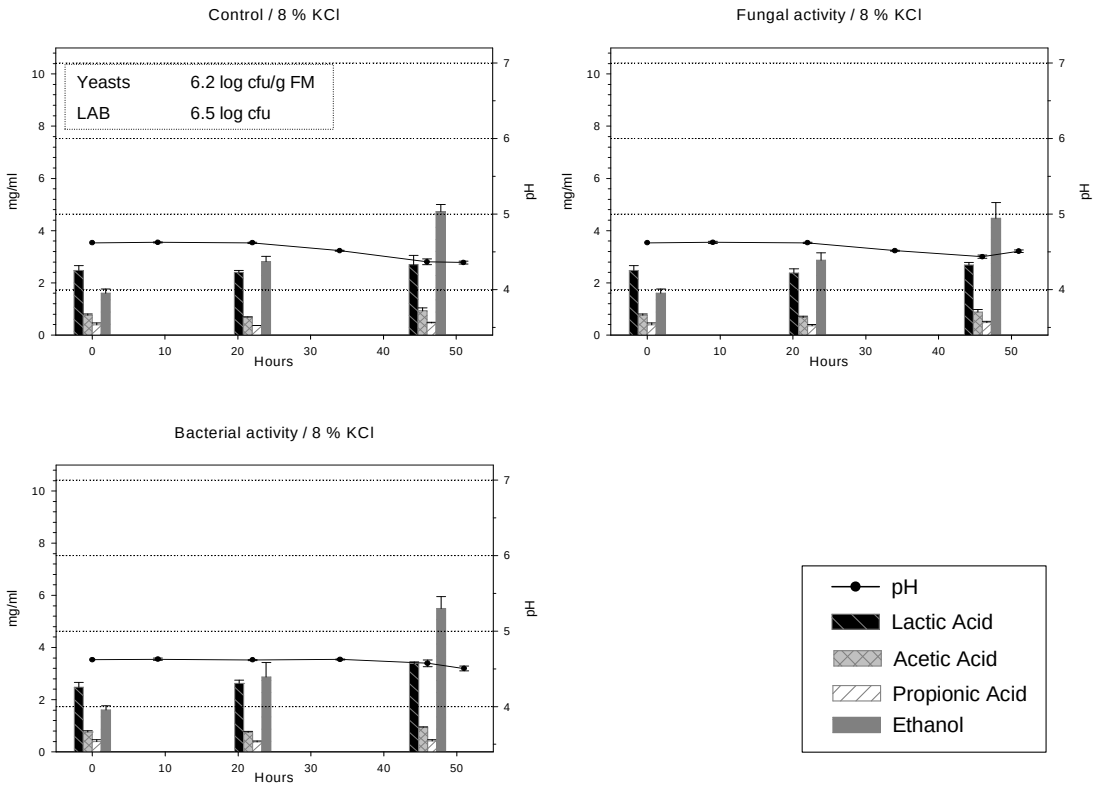
After 46 h of incubation the pH decreased from 4.6 to 4.4 (Figure 41). Lactic acid content increased from 2.5 to 2.7 mg/ml. Acetic acid content rose from 0.8 to 0.9 mg/ml, propionic acid content from 0.4 to 0.5 mg/ml. Ethanol content increased from the initial concentration of 1.6 mg/ml to 4.7 mg/ml.

Compared to the control without KCl (figure 30 in *APPENDIX VIII*) there were no significant differences after 22 h but after 46 h pH, acetic acid and propionic acid contents differed significantly.

Fungal activity (Antibacterial treatment)

Neither after 22 nor after 46 h there were significant differences between AB and C in any variable (Figure 43).

Compared to AB without KCl (figure 32 in *APPENDIX VIII*) there were significant differences after 46 h in pH, lactic and acetic acid contents. pH and acetic acid content were significantly lower with KCl whereas lactic acid content was significantly higher.



Figures: Changes in pH and some chemical components in grass silage extracts with 8 % KCl (silage ID 34) in 100 ml Erlenmeyer flasks over 51 h

Figure 41 (top left): Control

Figure 42 (down left): Antimycotic treatment

Figure 43 (top right): Antibacterial treatment

Bacterial activity (Antimycotic treatment)

There were no significant differences between C and AM after 22 and 46 h except that the pH of AM was significantly higher after 46 h (+0.2 units) (Figure 43).

Only after 46 h AM with and without KCl (figure 31 in *APPENDIX VIII*) differed significantly in pH, lactic, acetic and propionic acid contents. The organic acid contents were significantly higher without KCl and pH was significantly lower (-0.8 units).

5.3.5 Experiment C 5 – *Tannic acid*

To investigate the effect of tannin as a characteristic ingredient of legumes on aerobic changes

- 2 treatments with 3 replicates: C and AB + 0.57 % tannic acid in FM. Silage ID 17.

Yeast numbers accounted for 7.4 log cfu/g FM and aerobic bacteria numbers for 7.0 log cfu/g FM.

Control treatment

After 22 h the pH rose to 5.0 (Table 34, column C*, figure 74 in *APPENDIX VIII*). That is 0.5 units higher than the control without additive. Lactic acid concentration decreased from 6.5 to 3.5 mg/ml. Acetic acid concentration declined from 0.9 to 0.5 mg/ml in contrast to the untreated control which rose by 0.5 mg/ml. Propionic acid content was slightly reduced (-0.1 mg/ml) in contrast to the counterpart. Ethanol content increased to 4.4 mg/ml (without tannin 1.9 mg/ml).

There were significant differences in pH (+0.5 units), lactic (-0.7 mg/ml), acetic (-0.9 mg/ml) and propionic acid (-0.2 mg/ml) concentrations compared to the control without tannin after 22 h.

After 46 h the pH reached 6.6, that is 1.6 units more than the counterpart. Lactic acid concentration decreased to 1.2 mg/ml, acetic acid to 0.2 mg/ml. Propionic acid content increased to 0.3 mg/ml, ethanol content to 4.9 mg/ml.

At that point of time there were significant differences in pH, acetic and propionic acid and ethanol concentrations compared to the control without tannin (Table 34). On average, with tannin acetic acid content was only a tenth compared to the control without tannin and ethanol content was 6.5-fold higher.

Table 34: Measured and statistical differences in some chemical components [mg/ml] between C with tannin and C without, α =0.05; Experiment C5*

Tannic acid	**pH**			**Lactic acid**			**Acetic acid**			**Propionic acid**			**Ethanol**		
	C*	C		C*	C		C*	C		C*	C		C*	C	
0 h	*4.8*			*6.5*			*0.9*			*0.2*			*0.9*		
22 h	5.0	4.5	*	2.8	3.5	*	0.5	1.4	*	0.1	0.3	ns	4.4	1.9	*
46 h	6.6	5.0	*	1.2	1.2	ns	0.2	1.9	*	0.3	0.7	*	4.9	0.8	*

Fungal activity (Antibacterial treatment)

Within 22 h the pH rose to 4.9 (counterpart 4.7) (Table 35). The lactic acid content diminished from 6.5 mg/ml to 2.8 mg/ml (cp. 1.9 mg/ml). Acetic and propionic acid behaved like the control. Ethanol content amounted for 2.1 mg/ml (cp. 1.2 mg/ml).

AB with tannin differed significantly from AB without tannin in pH (-0.2 units), lactic (+ 0.9 mg/ml) and acetic acid contents (-0.6 mg/ml).

After 46 h the pH increased to 5.7 (cp. 6.5). Lactic acid content declined to 1.2 mg/ml (cp. 0.7 mg/ml), acetic acid content to 0.6 mg/ml (cp. 1.1 mg/ml). Propionic acid content remained stable. Ethanol amounted for 2.1 mg/ml (cp. 1.2 mg/ml).

There were significant differences in the same variables as after 22 h.

Table 35: Measured and statistical differences in some chemical components [mg/ml] between AB with tannin and AB without, α =0.05; Experiment C5*

Tannic acid	**pH**			**Lactic acid**			**Acetic acid**			**Propionic acid**			**Ethanol**		
	AB*	AB		AB*	AB		AB*	AB		AB*	AB		AB*	AB	
0 h	*4.8*			*6.5*			*0.9*			*0.2*			*0.9*		
22 h	4.9	4.7	*	2.8	1.9	*	0.5	1.1	*	0.1	0.2	ns	2.8	1.5	ns
46 h	5.7	6.5	*	1.2	0.7	*	0.6	1.1	*	0.2	0.2	ns	2.1	1.2	ns

Compared to the control there were significant differences only after 46 h in acetic acid content which was higher in AB (+0.4 mg/ml) and in ethanol content which was less than half of the control (2.1 vs. 4.9 mg/ml) (Table 36).

Table 36: Measured and statistical differences in some chemical components [mg/ml] between AB and C, both with tannin, α =0.05; Experiment C5

Tannic acid	**pH**			**Lactic acid**			**Acetic acid**			**Propionic acid**			**Ethanol**		
	C	AB		C	AB		C	AB		C	AB		C	AB	
0 h	*4.8*			*6.5*			*0.9*			*0.2*			*0.9*		
22 h	5.0	4.9	ns	2.8	2.8	ns	0.5	0.5	ns	0.1	0.1	ns	4.4	2.8	ns
46 h	6.6	5.7	*	1.2	1.2	ns	0.2	0.6	*	0.3	0.2	*	4.9	2.1	ns

Altogether tannic acid addition varied the development from the treatments without additive.

This finding provides further evidence to disprove Hypothesis 4.

5.3.6 Experiment C 6 – *pH 3.8 (+ fructose)*

To investigate the effect of initial pH on aerobic changes without or in combination with additional WSC

- 6 treatments with 3 replicates: pH of the medium adapted to 3.8, C, AB, AM without and with 6 % fructose. Silage ID 32.

Yeast numbers accounted for 6.6 log cfu/g FM and LAB numbers for 6.0 log cfu/g FM.

In general, the statistical differences given relate to the differences between the treatments with and without added fructose.

Corresponding graphs see figures X and Y in *APPENDIX VIII*.

Control treatment

The initially adjusted pH of 3.8 dropped to 3.6, and 3.4 (!) resp. with added fructose after 21 h (significant difference) (Table 37) which was the lowest pH achieved within all experiments. Lactic acid contents declined from 2.4 mg/ml to 1.9 mg/ml in both treatments (n.s.). Acetic acid decreased by 0.2 mg/ml to 0.6 mg/ml without additional fructose and increased by the same amount to 1.0 mg/ml with additional fructose (significant difference). Propionic acid contents remained stable within 45 h in both treatments. Ethanol content increased from 1.9 mg/ml to 3.3 mg/ml, 7.1 mg/ml respectively (significant difference).

After 45 h the pH added up to 3.9 or 3.5 respectively (significant difference). Lactic acid further decreased to 1.0 mg/ml, 1.2 mg/ml respectively (significant difference). Acetic acid accounted for 0.3 mg/ml without additional fructose and 1.6 mg/ml with fructose addition, that is more than 5 times higher (significant difference). Ethanol was decomposed or volatilised to 1.4 mg/ml or 7.0 mg/ml respectively (significant difference).

Within 45 h the treatment with additional fructose differs significantly from the treatment without additional fructose in pH, lactic acid, acetic acid and ethanol contents (Table 37).

Table 37: Measured and statistical differences in some chemical components [mg/ml] between C without and C with additional fructose at pH 3.8, α =0.05; Experiment C6*

pH 3.8	**pH**			**Lactic acid**			**Acetic acid**			**Propionic acid**			**Ethanol**		
	C	C*		C	C*		C	C*		C	C*		C	C*	
0 h	*3.8*			*2.4*			*0.7*			*0.4*			*1.9*		
21 h	3.6	3.4	*	1.9	1.9	ns	0.6	1.0	*	0.5	0.5	ns	3.3	8.9	*
45 h	3.9	3.5	*	1.0	1.2	*	0.3	1.6	*	0.5	0.4	ns	1.4	7.0	*

Comparison to the treatments at natural pH

After 21 h the control without additional fructose at low pH had a significantly lower lactic acid content than the counterpart at natural pH (1.9 vs. 2.7 mg/ml) (Table 38). The acetic acid content too, was significantly lower (0.6 vs. 1.0 mg/ml).

After 45 h acetic acid and propionic acid content were significantly lower at low pH, 0.3 vs. 1.3 mg/ml resp. 0.5 vs. 0.7 mg/ml.

Table 38: Measured and statistical differences in some chemical components [mg/ml] between C at pH 3.8 and C at natural pH (without fructose), α =0.05; Experiment C6*

pH 3.8	**pH**			**Lactic acid**			**Acetic acid**			**Propionic acid**			**Ethanol**		
	C*	C		C*	C		C*	C		C*	C		C*	C	
0 h	*3.8*	*4.8*		*2.4*	*2.5*		*0.7*	*0.7*		*0.4*	*0.4*		*1.9*	*1.7*	
21 h	3.6	4.6		1.9	2.7	*	0.6	1.0	*	0.5	0.5	ns	3.3	3.1	ns
45 h	3.9	5.3		1.0	1.1	ns	0.3	1.3	*	0.5	0.7	*	1.4	2.4	*

The treatment with additional fructose at low pH had a significantly lower lactic acid content than the counterpart at natural pH (1.9 vs. 2.5 mg/ml) corresponding to the treatments without additional fructose after 21 h, the same was acetic acid content (1.0 vs. 1.2 mg/ml) (Table 39). After 45 h lactic acid content was still significantly lower at low pH (1.2 vs. 1.9 mg/ml), the same as acetic acid content (1.6 vs. 2.5 mg/ml) and then propionic acid content (0.4 vs. 0.7 mg/ml).

Table 39: Measured and statistical differences in some chemical components [mg/ml] between C at pH 3.8 and C at natural pH (with fructose), α =0.05; Experiment C6*

pH 3.8	**pH**			**Lactic acid**			**Acetic acid**			**Propionic acid**			**Ethanol**		
	C*	C		C*	C		C*	C		C*	C		C*	C	
0 h	*3.8*	*4.8*		*2.4*	*2.5*		*0.7*	*0.7*		*0.4*	*0.4*		*1.9*	*1.7*	
21 h	3.4	4.3		1.9	2.5	*	1.0	1.2	*	0.5	0.6	ns	8.9	9.6	ns
45 h	3.5	4.2		1.2	1.9	*	1.6	2.5	*	0.4	0.7	*	7.0	7.9	ns

<u>Fungal activity (Antibacterial treatment)</u>

After 21 h the pH changed like in the control. Lactic acid concentration diminished to 1.8 mg/ml, 1.9 mg/ml respectively with additional fructose (n.s.) (Table 40). Acetic acid concentration declined from 0.8 to 0.5 mg/ml without additional fructose and rose to 0.9 mg/ml with fructose added (significant difference). Propionic acid contents remained stable within 45 h in both treatments. Ethanol content increased from 1.9 mg/ml to 3.0 mg/ml, 8.7 mg/ml respectively (significant difference).

After 45 h the pH corresponded to the control (n.s.). Lactic acid content diminished to 0.8 and 1.1 mg/ml respectively (significant difference). Acetic acid accounted for 0.6 mg/ml or 1.4 mg/ml respectively (significant difference). Ethanol content was at the level 2.1 and 7.0 mg/ml (significant difference).

At both measurement points AB did not differ significantly from C except a slightly lower acetic acid content after 21 h (Table 41 and Table 42).

Table 40: Measured and statistical differences in some chemical components [mg/ml] between AB without and AB with additional fructose at pH 3.8, α =0.05; Experiment C6*

pH 3.8	**pH**			**Lactic acid**			**Acetic acid**			**Propionic acid**			**Ethanol**		
	AB	AB*		AB	AB*		AB	AB*		AB	AB*		AB	AB*	
0 h	*3.8*			*2.4*			*0.7*			*0.4*			*1.9*		
21 h	3.6	3.4	*	1.8	1.9	ns	0.5	0.9	*	0.5	0.5	ns	3.0	8.7	*
45 h	3.9	3.5	*	0.8	1.1	*	0.1	1.4	*	0.5	0.5	ns	2.1	7.0	*

Table 41: Measured and statistical differences in some chemical components [mg/ml] between AB and C (without additional fructose) at pH 3.8, α =0.05; Experiment C6

pH 3.8	**pH**			**Lactic acid**			**Acetic acid**			**Propionic acid**			**Ethanol**		
	AB	C		AB	C		AB	C		AB	C		AB	C	
0 h	*3.8*			*2.4*			*0.7*			*0.4*			*1.9*		
21 h	3.6	3.6	ns	1.8	1.9	ns	0.5	0.6	*	0.5	0.5	ns	3.0	3.3	ns
45 h	3.9	3.9	ns	0.8	1.0	ns	0.1	0.3	ns	0.5	0.5	ns	2.1	1.4	ns

Table 42: Measured and statistical differences in some chemical components [mg/ml] between AB and C (with additional fructose) at pH 3.8, α =0.05; Experiment C6

pH 3.8	**pH**			**Lactic acid**			**Acetic acid**			**Propionic acid**			**Ethanol**		
	AB	C		AB	C		AB	C		AB	C		AB	C	
0 h	*3.8*			*2.4*			*0.7*			*0.4*			*1.9*		
21 h	3.4	3.4	ns	1.9	1.9	ns	0.9	1.0	ns	0.5	0.5	ns	8.7	8.9	ns
45 h	3.5	3.5	ns	1.1	1.2	ns	1.4	1.6	ns	0.5	0.4	ns	7.0	7.0	ns

Comparison to the treatments at natural pH

After 21 h lactic acid contents were significantly lower at low pH: 1.8 vs. 2.0 mg/ml without and 1.9 vs. 2.2 mg/ml with additional fructose, the same was acetic acid content: 0.5 vs. 0.7 mg/ml and 0.9 vs. 1.1 mg/ml (Table 43). After 45 h significant differences in lactic and acetic acid contents remained but ratios changed in the variable lactic acid without additional fructose. There the content at low pH was higher than at natural pH (0.8 vs. 0.6 mg/ml) (Table 44).

Whether with or without fructose addition, after the first 21 h less lactic acid was left at low pH than at natural pH which provides evidence to disprove ***Hypothesis 1*** saying that the pH does not influence the lactate consumption***.*** *However, after 45 h the results in lactic acid content and differences between the treatments at low and natural pH* ***were contradictory.***

Table 43: Measured and statistical differences in some chemical components [mg/ml] between AB at pH 3.8 and AB at natural pH (without fructose), α =0.05; Experiment C6*

pH 3.8	**pH**			**Lactic acid**			**Acetic acid**			**Propionic acid**			**Ethanol**		
	AB*	AB		AB*	AB		AB*	AB		AB*	AB		AB*	AB	
0 h	*3.8*	*4.8*		*2.4*	*2.5*		*0.7*	*0.7*		*0.4*	*0.4*		*1.9*	*1.7*	
21 h	3.6	4.7		1.8	2.0	*	0.5	0.7	*	0.5	0.5	ns	3.0	3.2	ns
45 h	3.9	6.5		0.8	0.6	*	0.1	0.6	*	0.5	0.5	ns	2.1	2.0	ns

Table 44: Measured and statistical differences in some chemical components [mg/ml] between AB at pH 3.8 and AB at natural pH (with fructose), α =0.05; Experiment C6*

pH 3.8	**pH**			**Lactic acid**			**Acetic acid**			**Propionic acid**			**Ethanol**		
	AB*	AB		AB*	AB		AB*	AB		AB*	AB		AB*	AB	
0 h	*3.8*	*4.8*		*2.4*	*2.5*		*0.7*	*0.7*		*0.4*	*0.4*		*1.9*	*1.7*	
21 h	3.4	4.3		1.9	2.2	*	0.9	1.1	*	0.5	0.5	ns	8.7	9.1	ns
45 h	3.5	4.4		1.1	1.3	*	1.4	2.1	*	0.5	0.5	ns	7.0	8.2	ns

Bacterial activity (Antimycotic treatment)

AM remained rather stable in pH and organic acid contents. There were no significant differences between AM with and without additional fructose at both measurement points (Table 45).

Until 45 h lactic acid concentration increased by 0.2 mg/ml on average, acetic acid content dropped by 0.2 mg/ml. Propionic acid content did not change on average. Ethanol content increased from 1.9 to 3.3 mg/ml on average within 45 h.

Table 45: Measured and statistical differences in some chemical components [mg/ml] between AM without and AM with additional fructose at pH 3.8, α =0.05; Experiment C6*

pH 3.8	**pH**			**Lactic acid**			**Acetic acid**			**Propionic acid**			**Ethanol**		
	AM	AM*		AM	AM*		AM	AM*		AM	AM*		AM	AM*	
0 h	*3.8*			*2.4*			*0.7*			*0.4*			*1.9*		
21 h	3.8	3.8	ns	2.5	2.4	ns	0.6	0.6	ns	0.5	0.4	ns	2.3	2.4	ns
45 h	3.8	3.7	ns	2.7	2.6	ns	0.5	0.5	ns	0.4	0.3	ns	3.2	3.4	ns

After 21 h AM treatments were significantly higher in pH (!), lactic acid and ethanol concentrations than the control treatments (Table 46). In the fructose treatments acetic acid and propionic acid contents were significantly lower at low pH (0.6 vs. 1.0 mg/ml and 0.4 vs. 0.5 mg/ml) (Table 47).

After 45 h the AM treatment without fructose was significantly lower in lactic acid and ethanol concentrations than the control. In contrast, the AM treatment with additional

fructose had significantly higher ethanol and acetic acid contents than the control with fructose.

Table 46: Measured and statistical differences in some chemical components [mg/ml] between AM and C (without additional fructose) at pH 3.8, α =0.05; Experiment C6

pH 3.8	**pH**			**Lactic acid**			**Acetic acid**			**Propionic acid**			**Ethanol**		
	AM	C		AM	C		AM	C		AM	C		AM	C	
0 h	*3.8*			*2.4*			*0.7*			*0.4*			*1.9*		
21 h	3.8	3.6	*	2.5	1.9	*	0.6	0.6	ns	0.5	0.5	ns	2.3	3.3	*
45 h	3.8	3.9	ns	2.7	1.0	*	0.5	0.3	ns	0.4	0.5	ns	3.2	1.4	*

Table 47: Measured and statistical differences in some chemical components [mg/ml] between AM and C (with additional fructose) at pH 3.8, α =0.05; Experiment C6

pH 3.8	**pH**			**Lactic acid**			**Acetic acid**			**Propionic acid**			**Ethanol**		
	AM	C		AM	C		AM	C		AM	C		AM	C	
0 h	*3.8*			*2.4*			*0.7*			*0.4*			*1.9*		
21 h	3.8	3.4	*	2.4	1.9	*	0.6	1.0	*	0.4	0.5	*	2.4	8.9	*
45 h	3.7	3.5	ns	2.6	1.2	*	0.5	1.6	*	0.3	0.4	ns	3.4	7.0	*

Comparison to the treatments at natural pH

After 21 h lactic acid contents of AM treatments at low pH were significantly lower than at natural pH (2.5 vs. 3.9 mg/ml without and 2.4 vs. 3.4 mg/ml with fructose addition) (Table 48 and Table 49), same were acetic acid and propionic acid contents (0.6 vs. 1.8 mg/ml and 0.6 vs. 1.5 mg/ml for acetic acid resp. 0.5 vs. 1.2 mg/ml and 0.4 vs. 0.9 mg/ml for propionic acid).

After 45 h there were still significant differences in lactic, acetic and propionic acid contents. However, with lactic acid ratios changed when no fructose was added. At low pH lactic acid content was higher than at natural pH (2.7 vs. 1.7 mg/ml).

Table 48: Measured and statistical differences in some chemical components [mg/ml] between AM at pH 3.8 and AM at natural pH (without fructose), α =0.05; Experiment C6*

pH 3.8	**pH**			**Lactic acid**			**Acetic acid**			**Propionic acid**			**Ethanol**		
	AM*	AM		AM*	AM		AM*	AM		AM*	AM		AM*	AM	
0 h	*3.8*	*4.8*		*2.4*	*2.5*		*0.7*	*0.7*		*0.4*	*0.4*		*1.9*	*1.7*	
21 h	3.8	4.2		2.5	3.9	*	0.6	1.8	*	0.5	1.2	*	2.3	2.5	ns
45 h	3.8	4.4		2.7	1.7	*	0.5	4.0	*	0.4	3.0	*	3.2	3.3	ns

Table 49: Measured and statistical differences in some chemical components [mg/ml] between AM at pH 3.8 and AM at natural pH (with fructose), α =0.05; Experiment C6*

pH 3.8	**pH**			**Lactic acid**			**Acetic acid**			**Propionic acid**			**Ethanol**		
	AM*	AM		AM*	AM		AM*	AM		AM*	AM		AM*	AM	
0 h	*3.8*	*4.8*		*2.4*	*2.5*		*0.7*	*0.7*		*0.4*	*0.4*		*1.9*	*1.7*	
21 h	3.8	4.3		2.4	3.4	*	0.6	1.5	*	0.4	0.9	*	2.4	2.1	ns
45 h	3.7	4.1		2.6	2.9	*	0.5	4.0	*	0.3	4.2	*	3.4	2.9	ns

Experiments with silages other than pure grass

5.3.7 Experiment C 7 – *Maize, grass-lucerne*

To study aerobic changes in other silages compared to grass silages

- One or 3 treatments with 3 replicates: Control (C), (Antibacterial (AB) and Antimycotic (AM) treatment). Maize silage ID 16, Grass-lucerne silage ID 20, 21, 27, 31. (5 silages in total).

5.3.7.1 Maize

The maize silage was characterised by a pH of 3.8, 4.9 mg/ml lactic acid, no residual WSC, yeast numbers of 5.9 log cfu/g FM and aerobic bacteria numbers of 5.2 log cfu/g FM.

Control treatment

During 22 h the pH rose to 4.0 (Figure 44). Lactic acid content decreased by 1.1 mg/ml, acetic acid disappeared, propionic acid was absent and ethanol content remained constant.

After 46 h the pH increased to 5.8. Lactic acid content diminished from the initial 4.9 to 1.9 mg/ml, ethanol content by 0.7 to 0.1 mg/ml.

Pellicle was formed in the not shaken treatment only after 3 days.

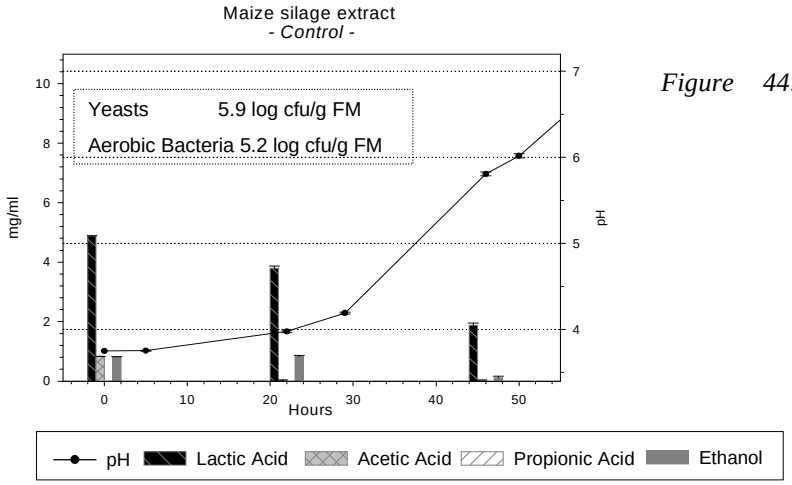


Figure 44: Changes in pH, organic acids and ethanol in maize silage extract, control, over 50 h

Fungal activity (Antibacterial treatment)

The development of pH and organic acid contents and ethanol was like the control. Within 46 h there were no significant differences between AB and C. ***With maize silage results provide evidence to confirm Hypothesis 2*** saying that the decomposition of lactate and the rise in pH is solely caused by yeasts**.**

Bacterial activity (Antimycotic treatment)

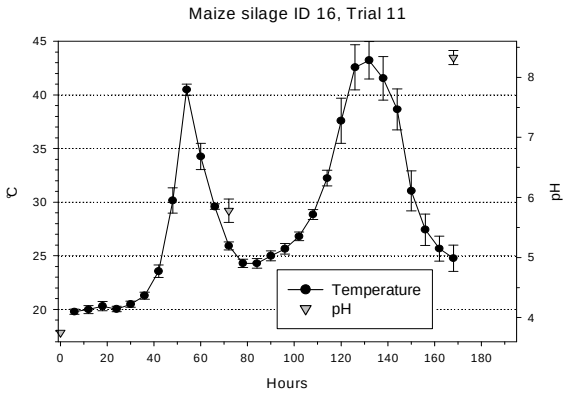
Within 46 h the pH remained constant (figure 83 in *APPENDIX VIII*). After 46 h the relation of lactic and acetic acid contents changed: lactic acid content dropped from the initial 4.9 mg/ml to 4.2 mg/ml whereas acetic acid content rose from 0.8 to 1.8 mg/ml. Ethanol content changed slightly from initial 0.8 mg/ml to 0.9 mg/ml after 22 h and 0.4 mg/ml after 46 h.

AM differed significantly from C in pH and organic acids in both measurement points.

Comparing this maize silage cultures to grass silage cultures with similar initial conditions (ID 25) (Experiment C 1) the development of pH and acids were similar but the decomposition of lactic acid and pH increase was much more rapid in C and AB of the maize silage.

Comparison of the batch culture technique to temperature development in the aerobic stability test (HONIG, 1990)

In the aerobic stability test the maize silage became unstable after 42 h. There were two temperature peaks, the first after 54 h, the second after 126 h (Figure 45) indicating that two different microbial groups might have alternated. The pH rose from 3.8 to 5.8 after 71 h and 8.3 after 168 h. Lactic acid content diminished from 4.9 to 2.4 mg/ml after 71 h and was totally decomposed after 168 h (Figure 46). Acetic acid content decreased



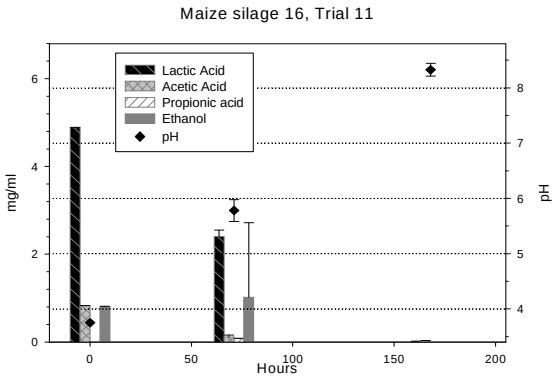


Figure 45 (left): Temperature changes in maize silage in the aerobic stability test over 7 days

Figure 46 (right): Changes in pH, volatile fatty acids and ethanol contentsin maize silage in the temperature test after 0, 3 and 7 days

from 0.8 to 0.1 mg/ml after 71 h and was totally decomposed after 168 h. Ethanol content rose during the first 71 h from 0.8 to 3.0 mg/ml but disappeared after 168 h.

In the corresponding batch culture the change in pH (5.8), lactic acid (1.9 mg/ml) and acetic acid concentration (0.1 mg/ml) within the first 46 h was similar to that of the silage within 71 h. However, in contrast to the batch culture where the ethanol content decreased to 0.1 mg/ml within 46 h it increased 3.8 fold during the first 71 h in the aerobic stability test in the silage on exposure to air and disappeared only after 168 h.

5.3.7.2 Grass-lucerne

Two of the grass-lucerne silages (***ID 20 and 21***) were solely colonised by moulds (6.3 resp. 7.0 log cfu/g FM) and had a pH of 4.6. Aerobic bacteria numbers ranged from 6.9-7.2 log cfu/g FM. Only the control treatments were observed.

In the batch cultures, the silage containing 25 % lucerne in FM (***ID 20***) remained stable in pH the first 21 h despite a high lactic acid accumulation of 6.9 mg/ml compared to the initial 3.6 mg/ml (Figure 47). At the same time acetic acid content rose from 0.9 to 1.3 mg/ml. Propionic acid (0.1 mg/ml) was produced and ethanol content rose from 0.5 to 2.0 mg/ml.

After 35 h mould growth was observed on the surface.

Until 45 h the pH dropped to 4.1 while the lactic acid content dropped as well from 6.9 to 5.8 mg/ml. Acetic acid content remained rather stable at 1.2 mg/ml, propionic acid content increased further to 0.6 mg/ml and ethanol content to 4.1 mg/ml.

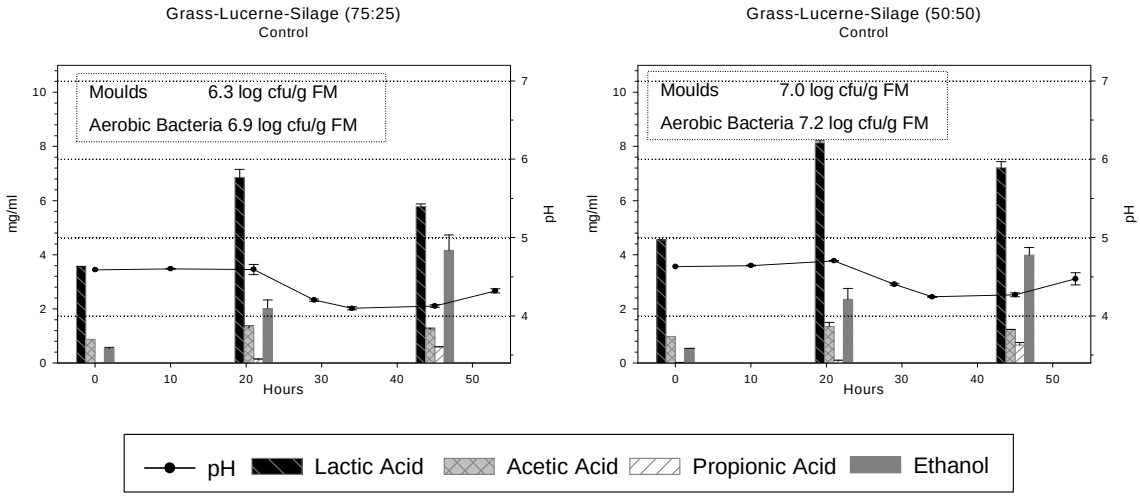


Figure 47 (left): Changes in pH and some chemical components in extract of grass-lucerne silage (ID 20) with 25 % lucerne on FM base over 53 h

Figure 48 (right): Changes in pH and some chemical components in extract of grass-lucerne silage (ID 21) with 50 % lucerne on FM base over 53 h

The silage containing 50 % lucerne (***ID 21***) had a comparable development except that the lactic acid production after 21 h was even higher (increasing from 4.5 to 8.1 mg/ml) and even a rise of pH within that time by 0.1 units (Figure 48). After 45 h the pH

dropped to 4.3, lactic acid content accounted for 7.2 mg/ml. At that time mould growth was observed on the surface.

After 21 h cultures of silages 21 and 20 differed significantly in pH (4.7 vs. 4.6) and lactic acid content (8.1 vs.6.9 mg/ml). After 45 h they differed in the same variables, pH 4.3 vs. 4.1, lactic acid content 7.2 vs. 5.8 mg/ml.

Silage 27 with a lucerne fraction of 25 % was occupied as well from yeasts as from moulds (6.1 resp. 6.4 log cfu/g FM). LAB accounted for 5.4 log cfu/g FM. It started with a pH of 4.6 and a lactic acid content of 3.2 mg/ml. All 3 treatments (C, AB, AM) were carried through.

Control treatment

Within 22 h the pH dropped from 4.6 to 4.5 (Figure 49). Lactic acid content increased from 3.2 mg/ml to 3.6 mg/ml. Acetic acid content diminished by 0.3 mg/ml to 0.5 mg/ml, propionic acid content remained at 0.1 mg/ml. Ethanol content rose from 0.5 to 3.6 mg/ml.

Mould growth was observed after 35 h in the unshaken treatment.

After 46 h the pH dropped by another 0.1 unit. Lactic acid content increased to 3.9 mg/ml, acetic acid content rose again to 1.3 mg/ml. Propionic acid content remained constant and ethanol content increased to 3.7 mg/ml.

Fungal activity (Antibacterial treatment)

In contrast to the control the pH remained constant during the first 22 h and rose by 0.4 to 5.0 after 46 h (Figure 51). At the same time the lactic acid content dropped from 3.2 mg/ml to 3.0 mg/ml after 22 h and to 2.0 mg/ml after 46 h. Acetic acid content decreased from 0.9 to 0.4 mg/ml within 22 h and rose again slightly to 0.8 mg/ml after 46 h. Propionic acid content remained constant at 0.1 mg/ml and ethanol content rose to 3.2 and 3.8 mg/ml resp.after 22 and 46 h.

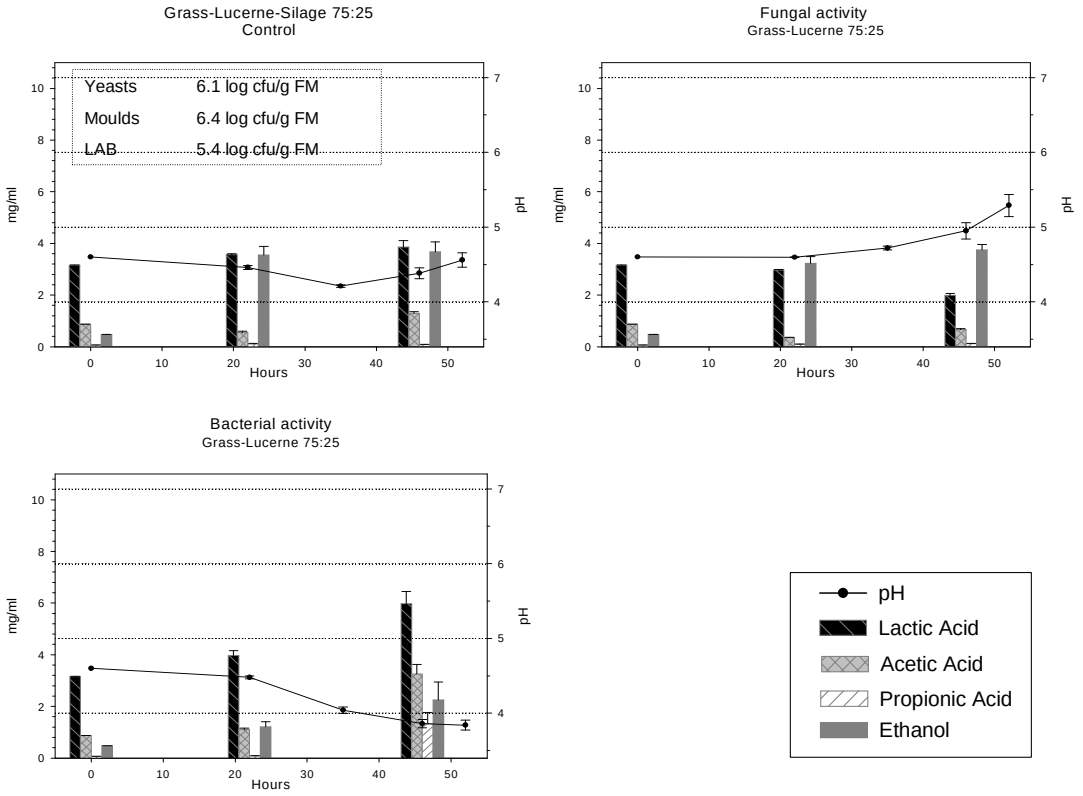
AB differed significantly from C in pH and lactic acid content in both measurement points. Additionally, after 46 h AB was significantly lower in acetic acid content (0.7 vs. 1.3 mg/ml). ***The results provide evidence to disprove Hypothesis 3*** saying that aerobic changes are dominated by yeasts activity.

Bacterial activity (Antimycotic treatment)

Within the first 22 h the pH dropped by 0.1 units equal to the control (Figure 50). However at the same time lactic acid and acetic acid content increased to 4.0 and 1.1 mg/ml resp.. There was no change in propionic acid content (0.1 mg/ml). Ethanol content increased from 0.5 mg/ml to 1.2 mg/ml.

After 46 h the pH dropped further to 3.9. Lactic acid was further produced to final 6.0 mg/ml. Acetic and propionic acid content increased as well to 3.3 mg/ml and 1.3 mg/ml resp.. Ethanol content rose to 2.3 mg/ml.

Within 22 h AM was significantly higher in acetic acid than C (1.1 vs. 0.5 mg/ml) and significantly lower in ethanol content (1.2 vs. 3.5 mg/ml). Additionally, after 46 h the treatments differ in pH and lactic acid content (AM vs. C pH 3.9 vs. 4.4 and 6.0 vs. 3.9 mg/ml lactic acid).



Figures: Changes in pH and some chemical components [mg/ml] in grass-lucerne silage extracts (silage ID 27) over 52 h, error bars = s.d.

Figure 49 (top left): Control

Figure 50 (down left): Antimycotic treatment

Figure 51 (top right): Antibacterial treatment

Silage 31 with 25 % lucerne contained 6.8 log cfu/g FM yeasts and no moulds. LAB were present with 5.4 log cfu/g FM. The silage had a pH of 4.6 and lactic acid accounted for 3.6 mg/ml.

Control treatment

After 22 h the pH diminished to 4.5, lactic acid content decreased by 0.6 mg/ml to 3.0 mg/ml (Figure 52). Acetic acid content decreased from 0.9 to 0.7 mg/ml, propionic acid content remained constant at 0.2 mg/ml. Ethanol content increased from 0.7 to 2.6 mg/ml.

Pellicle growth was observed after 33 h in the unshaken treatment.

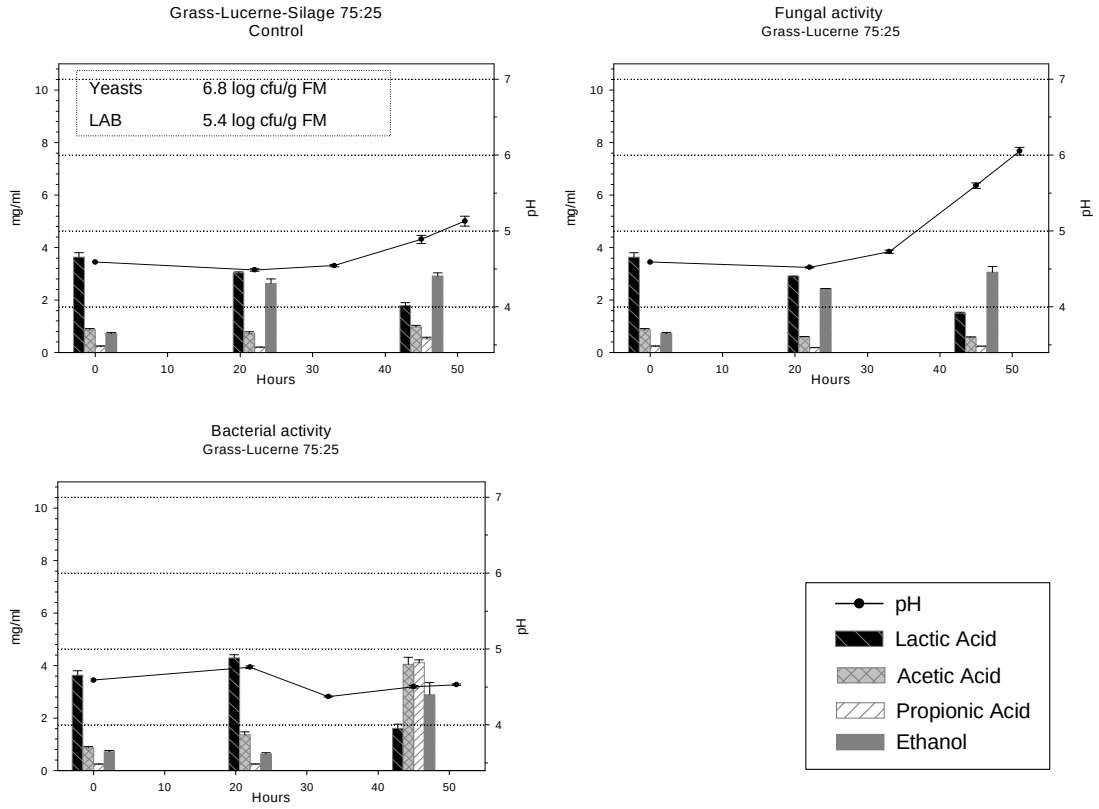
After 45 h the pH rose to 4.9. Lactic acid content further diminished to 1.8 mg/ml, acetic acid content increased to 1.0 mg/ml, propionic acid content to 0.5 mg/ml and ethanol content to 2.9 mg/ml.

Fungal activity (Antibacterial treatment)

The development during the first 22 h is nearly equal to C treatment (n.s.).

After 45 h pH rose up to 5.6 (Figure 54), that is 0.7 units above the control. Lactic acid content diminished to 1.5 mg/ml, i.e. 0.3 mg/ml less compared to the control. Acetic acid content did not change from 22 to 45 h (0.6 mg/ml). Propionic acid content remained constantly at 0.2 mg/ml and ethanol content increased to 3.1 mg/ml.

Only after 45 h the pH, acetic and propionic acid content differed significantly from C, pH 5.6 vs. 4.9, acetic acid 0.6 vs. 1.0 mg/ml, propionic acid 0.2 vs. 0.5 mg/ml.



Figures: Changes in pH and some chemical components [mg/ml] in grass-lucerne silage extracts (silage ID 31) over 51 h, error bars = s.d.

Figure 52 (top left): Control

Figure 53 (down left): Antimycotic treatment

Figure 54 (top right): Antibacterial treatment

Bacterial activity (Antimycotic treatment)

In contrast to C and AB pH increased (!) during the first 22 h by 0.2 despite a lactic acid production of 0.6 mg/ml (Figure 53). Besides acetic acid content increased from 0.9 to

1.4 mg/ml, propionic acid content did not change (0.2 mg/ml) and ethanol content even diminished by 0.1 mg/ml.

After 45 h the pH dropped to 4.5 whereas the lactic acid content decreased also to 1.6 mg/ml, comparable to AB. Acetic and propionic acid reached an exceptionally high concentration of 4.1 mg/ml each. Ethanol content increased to 2.9 mg/ml.

After 22 h AM and C differed significantly in all variables except for the ethanol content. After 45 h they still differed in pH, acetic and propionic acid contents.

Comparison of the batch culture technique to temperature development in the aerobic stability test (HONIG, 1990)

Silage 20 became unstable after 82 h, *silage 21* after 96 h.

Within 168 h pH of *silage 20* rose from 4.6 to 8.2, lactic acid content diminished from 3.6 to 0.6 mg/ml, acetic acid content from 0.9 to 0.3 mg/ml. There was no propionic acid and ethanol was completely depleted from 0.5 mg/ml initial content.

The pH of silage 21 rose from 4.7 to 7.7 within 168 h. At the same time lactic acid content decreased from 4.5 mg/ml to 2.1 mg/ml and acetic acid content from 1.0 to 0.2 mg/ml. Ethanol disappeared.

Compared to the batch cultures it was similar that finally more lactic acid was left in the *silage 21* with the higher ratio of lucerne.

Silage 27 became unstable only after 120 h. This corresponded to the batch culture where the lactic acid content even increased within 46 h and pH dropped. After 168 h the silage pH rose to 7.2, lactic acid content diminished to 1.2 mg/ml, acetic acid content to 0.2 mg/ml. Ethanol and propionic acid disappeared.

Silage 31 became unstable already within 6 h. pH rose from 4.6 to 7.7 after 168 h. Organic acids and ethanol were completely decomposed except a small amount of propionic acid (0.1 mg/ml). In the batch culture, within 45 h the lactic acid concentration halved and the pH rose to 4.9.

5.3.8 Experiment C 8 – *grass-lucerne +3 % fructose*

To investigate the effect of increased WSC level in grass-lucerne silages

- 3 treatments with 3 replicates: C, AB and AM + 3 % fructose in FM. Grass-lucerne silages ID 27, 31. (2 silages in total).

Control treatment

In both measurement points comparing the treatments with and without additional fructose of silage 27 the pH differed significantly, in the fructose treatment being lowest after 46 h with 4.0 (Figure 55). Besides the ethanol content with added fructose was

significantly higher, highest after 22 h with 6.0 mg/ml (without additional fructose 3.6 mg/ml). After 46 h also lactic acid and acetic acid content were significantly higher with additional fructose (4.9 vs. 3.9 mg/ml and 1.8 vs. 1.3 mg/ml).

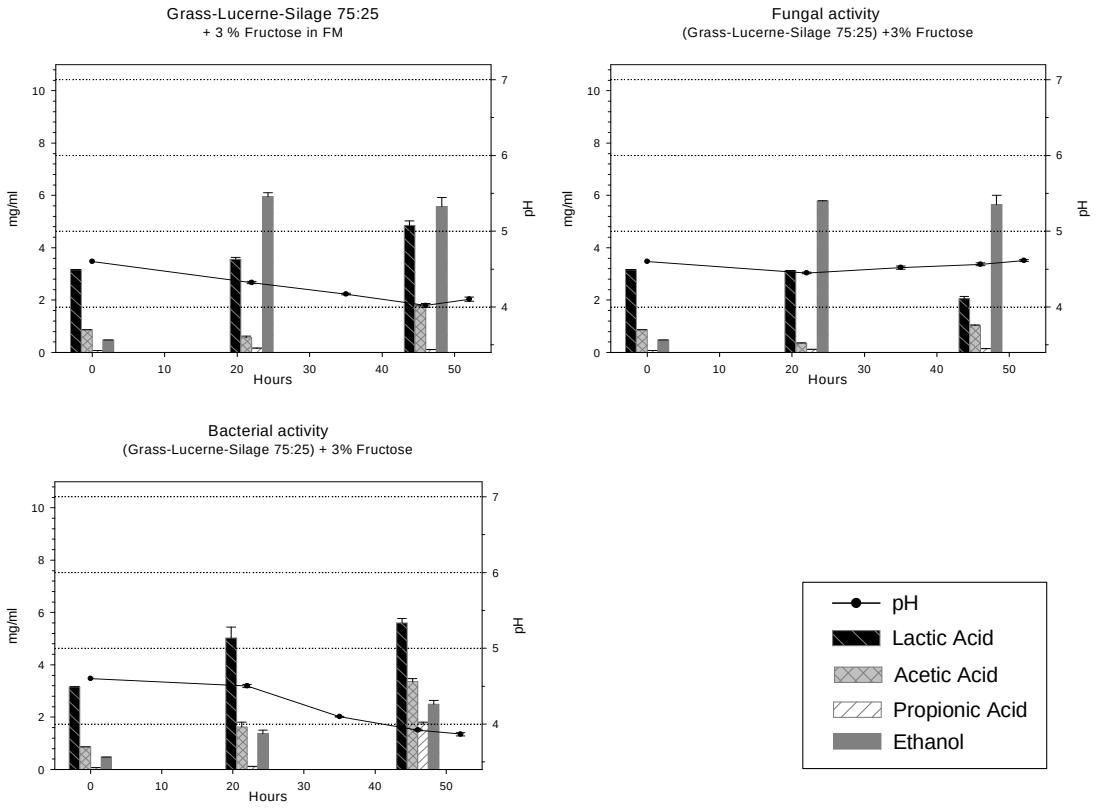
In silage 31 there were similar significant differences in pH and ethanol contents, but acetic acid content differed already after 22 h (Figure 58).

Fungal activity (Antibacterial treatment)

Silage 27

In both measurement points between AB with and without additional fructose there were significant differences in pH which remained rather constant with additional fructose (Figure 57), and in ethanol content which was as high as in the control with additional fructose in both silages.

After 22 h AB differed significantly from C in pH and acetic acid contents. After 46 h there were significant differences in pH, acetic acid content and lactic acid content which was significantly lower.



Figures: Changes in pH and some chemical components [mg/ml] in grass-lucerne silage extracts with added fructose (silage ID 27) over 52 h, error bars = s.d.

Figure 55 (top left): Control

Figure 56 (down left): Antimycotic treatment

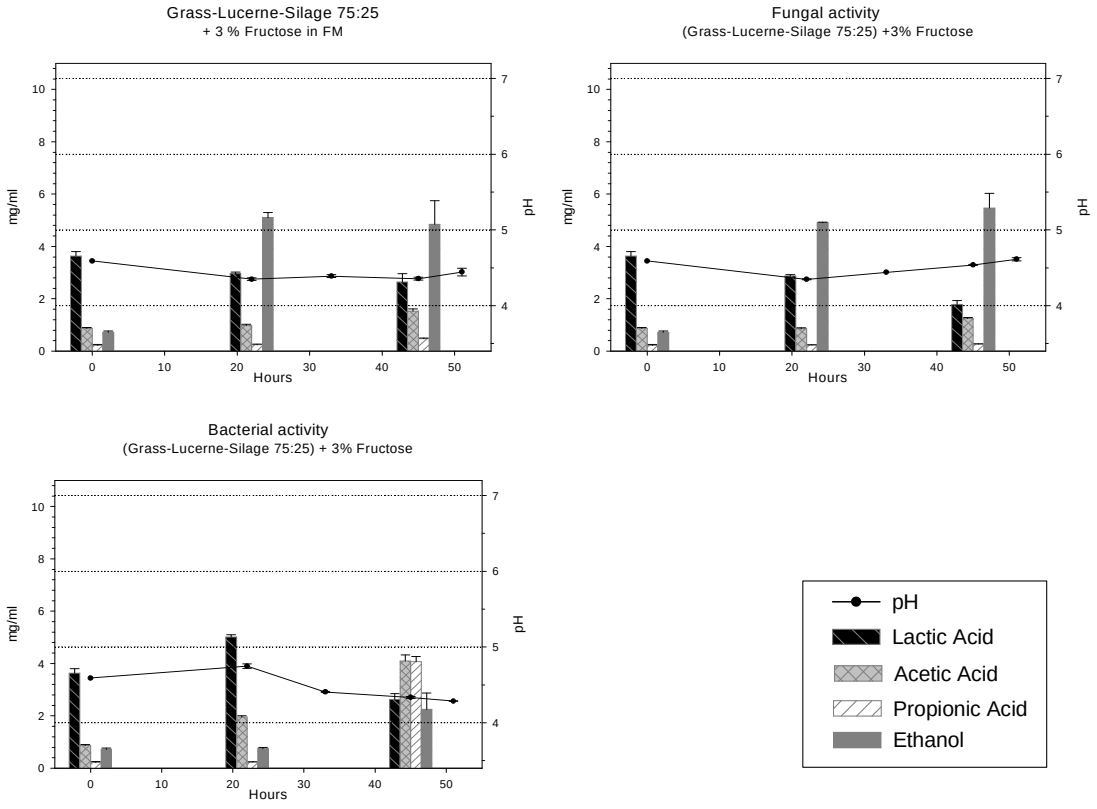
Figure 57 (top right): Antibacterial treatment

Silage 31

After 22 and 45 h pH, acetic acid and ethanol contents differed significantly from the counterpart without additional fructose.

After 22 h there were no significant differences between AB and C. After 45 h pH was significantly higher in AB (4.5 vs. 4.4) and lactic acid content was significantly lower (1.8 vs. 2.6 mg/ml) (Figure 60) than in C.

The findings provide evidence to disprove Hypothesis 3 which says that aerobic changes in silages are dominated by yeast activity.



Figures: Changes in pH and some chemical components [mg/ml] in grass-lucerne silage extracts with added fructose (silage ID 31) over 52 h, error bars = s.d.

Figure 58 (top left): Control

Figure 59 (down left): Antimycotic treatment

Figure 60 (top right): Antibacterial treatment

<u>Bacterial activity (Antimycotic treatment)</u>

Silage 27

After 22 h lactic and acetic acid contents were significantly higher than in AM without additional fructose. After 46 h only propionic acid content was significantly higher with fructose addition (Figure 56).

Comparing treatment AM to C after 22 h pH (!), lactic and acetic acid contents were significantly higher in AM. At the same time propionic acid and ethanol content were significantly lower. After 46 h there were also significant differences in all parameters, but pH of AM was lower and propionic acid content higher than in C.

Silage 31

After 22 h lactic and acetic acid content of AM with additional fructose were significantly higher than without additional fructose (5.0 vs. 4.3 mg/ml resp. 2.0 vs. 1.4 mg/ml) (Figure 59).

After 45 h pH was significantly lower and lactic acid content significantly higher with additional fructose compared to without.

Comparing AM to C they were significantly different in all variables in both measurement points except propionic acid content after 22 h.

SYNOPSIS Experiment type C

- Aerobic changes were observed in the fungal as well as in the bacterial variant.
- In the antibacterial treatment lactic acid was decomposed and pH rose.
- In the antifungal treatment in general, lactic acid was formed during the first day and decomposed during the second day of incubation, while pH dropped. This demonstrated that lactate oxidation can be also caused by bacteria, which provides evidence ***to disprove Hypothesis 2***.
- The control treatment neither corresponded totally to the antibacterial treatment nor to the antifungal treatment.
- ***These findings provide evidence to disprove Hypothesis 3*** which says that aerobic changes in silages are dominated by yeast activity.
- Only when the initial pH was ≤ 4.0 were developments in control and AB equal.
- Increased WSC level increased ethanol production by yeasts and reduced the pH rise.
- Tannin addition reduced the lactate consumption of fungi but enhanced it in the control with the combined micro-flora. Ethanol production was increased with both treatments.
- ***Those findings provide evidence to disprove Hypothesis 4*** which says that the decomposition of lactate and the rise in pH is not influenced by other available carbon sources or compounds.
- Potassium chloride (KCl) reduced both fungal and bacterial activity.
- An interesting finding was that in the antibacterial treatment where the medium had been adjusted to pH 3.8, the pH dropped further during the first day of incubation and even more extremely when fructose was added, despite a reduction in lactic acid content.
- With maize, yeast activity was similar to the control.
- In grass silage with admixed lucerne the pH reacted rather slowly to changes in acid contents.
- Results of the aerobic stability test were not clearly related to the batch culture results, but proved that lactic acid and ethanol can be formed in silages on exposure to air.

5.4 Experiment type D

In Experiment type C it was demonstrated that yeasts and/or LAB can play a role in the aerobic changes of silage. Experiment type D was carried out to verify the relative roles of these groups of micro-organisms in a silage-based medium, but in absence of a natural microbial population. Sterile silage medium was inoculated with either a yeast, a lactic acid bacterium or a mixture of both micro-organisms.

The treatments inoculated with yeasts and LAB can be compared to the control (C) of *Experiment type C*, treatments with yeasts can be compared to AB and treatments with LAB are comparable to AM.

Propionic acid contents played a minor role in these samples (0.0-0.5 mg/ml) and remained at a relatively stable level. It is therefore not generally mentioned in the following.

The inoculation rates were similar, but only equal if the experiments were carried out on the same date.

5.4.1 Experiment D 1 – *grass, maize*

To investigate the activity of yeast and LAB isolates in grass and maize silage medium

- 6 treatments with 3 replicates: *Pichia anomala*, *Lactobacillus plantarum*, *Pichia anomala* + *L. plantarum* were inoculated in grass silage medium as well as in maize silage medium. Compare to *Experiment **C 1*** and *Experiment **C 7***.

The initial lactic acid contents of grass and maize silage media in this experiment were 7.4 mg/ml and 6.0 mg/ml respectively at pH levels of 4.4 and 3.8 respectively.

Grass and maize silage media were inoculated with 6.7 log cfu/40 ml silage extract *Pichia anomala* (CBS 113) and 8.3 log cfu/40 ml silage extract *Lactobacillus plantarum.* This is a higher level of LAB relative to yeasts compared to the natural population density of the silages investigated in Experiment type C where yeast numbers were equal to LAB numbers or up to 1.8 log units lower.

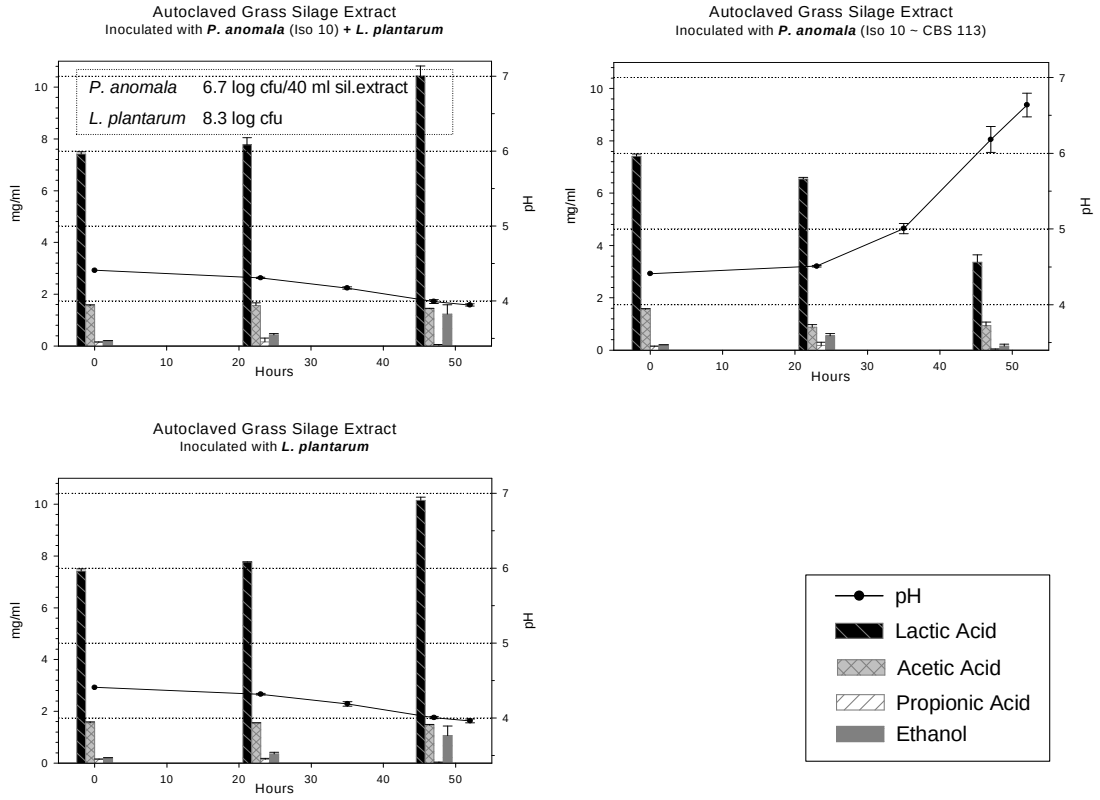
Compared to the average population density of the investigated grass silages in *Experiment **C 1*** in Experiment type D there were around one log unit lower numbers of yeasts (that is 10 times) but 1.3 log units more LAB.

Grass

Yeasts

Within 23 h pH rose from 4.4 to 4.5 (Figure 63). Lactic acid content declined from 7.4 to 6.5 mg/ml, acetic acid content from 1.6 to 0.9 mg/ml, propionic acid content remained constant at 0.2 mg/ml and ethanol content slightly increased from 0.2 to 0.6 mg/ml.

After 47 h the pH reached 6.2. Lactic acid content further decreased to 3.4 mg/ml, that is less than half of the initial amount. Acetic acid content remained at 0.9 mg/ml, propionic acid disappeared and ethanol content diminished to 0.2 mg/ml which was the initial value.



Figures: Changes in pH and some chemical components [mg/ml] in autoclaved grass silage extract with inoculants over 52 h

Figure 61 (top left): Pichia anomala + Lactobacillus plantarum

Figure 62 (down left): Lactobacillus plantarum

Figure 63 (top right): Pichia anomala

LAB

In contrast to the yeasts treatment pH dropped and lactic acid content increased during the measurement period (Figure 62). After 23 h pH decreased from 4.4 to 4.3, lactic

acid content increased from 7.4 to 7.8 mg/ml. Acetic and propionic acid content remained constant and ethanol content increased by 0.2 mg/ml.

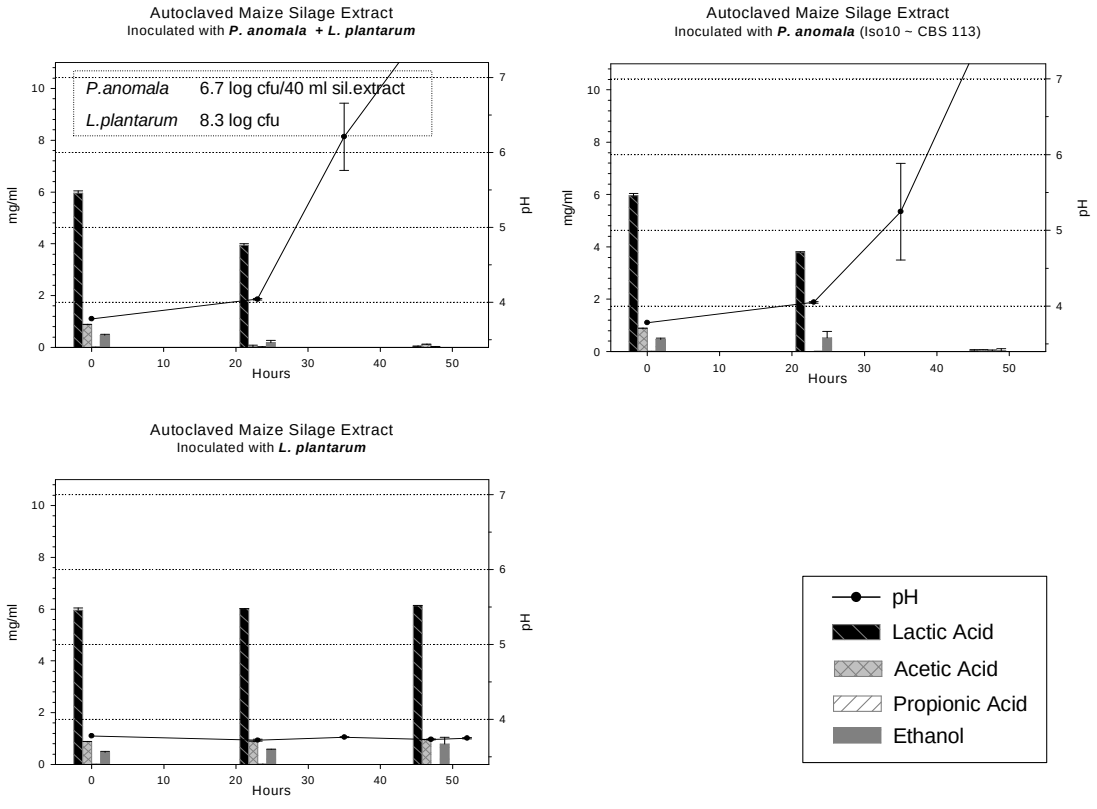
After 47 h pH was at 4.0, lactic acid amounted for 10.2 mg/ml. Acetic acid and propionic acid content differed from the initial value by -0.1 mg/ml. Ethanol content increased to 1.1 mg/ml.

Treatment LAB differed significantly from treatment Yeasts in pH, lactic and acetic acid contents at both measurement points.

Yeasts + LAB

This treatment (Figure 61) had an equal development as the LAB treatment. There were no significant differences to the LAB treatment. The yeasts treatment differed significantly in pH, lactic and acetic acid content from the other treatments at all time points.

Maize



Figures: Changes in pH and some chemical components [mg/ml] in autoclaved maize silage extract with inoculants over 52 h

Figure 64 (top left): Pichia anomala + Lactobacillus plantarum

Figure 65 (down left): Lactobacillus plantarum

Figure 66 (top right): Pichia anomala

Yeasts

Within 23 h the pH increased from 3.8 to 4.1 (Figure 66). Lactic acid content diminished from 6.0 to 3.8 mg/ml. Acetic acid was totally decomposed (-0.9 mg/ml). Propionic acid was absent. Ethanol content remained constant at 0.5 mg/ml.

After 47 h pH increased further to 7.7. All other components were nearly completely decomposed. There were 0.1 mg/ml lactic and acetic acid remaining.

LAB

There was nearly no change in any parameter during 47 h (Figure 65).

After 47 h pH decreased by 0.1 units, 0.2 mg/ml lactic acid was produced, 0.1 mg/ml acetic acid was formed and ethanol content increased to 0.8 mg/ml.

Yeasts + LAB

This treatment (Figure 64) was comparable to the yeasts treatment. There was no significant difference between the both in any parameter at both measurement points. However, it differed significantly from the LAB treatment in pH, lactic and acetic acid content. Final pH was 7.8.

5.4.2 Experiment D 2 – *grass, maize +6 % fructose*

To study the effect of increased residual WSC level on yeast and LAB

- As for Experiment ***D 1*** (*Pichia anomala, Lactobacillus plantarum, Pichia anomala + L. plantarum* inoculated in grass and maize silage medium) but + 6 % fructose on FM base. Compare to *Experiment **C 3***.

Inoculation rate was as in *Experiment **D 1***.

Grass

Yeasts

Compared to the treatment without added fructose there were significant differences in pH, acetic acid and ethanol contents.

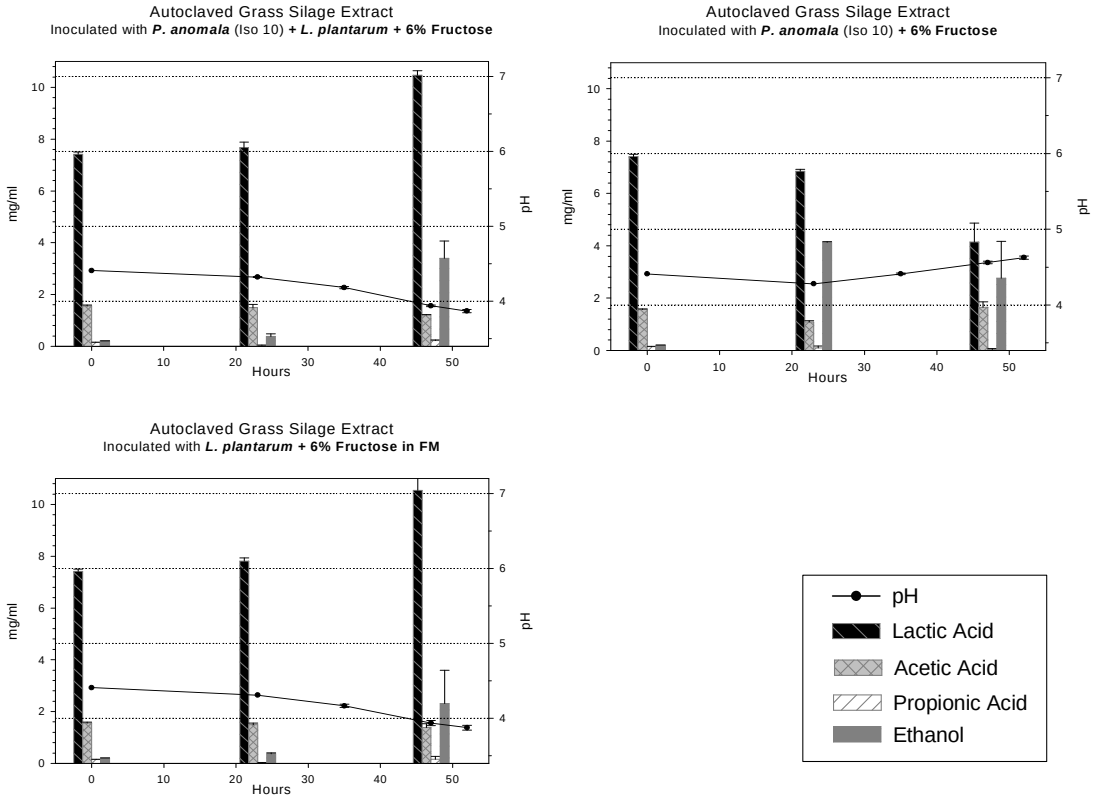
The pH curve was much more shallow and pH increased only to 4.6 after 47 h, i.e. a plus of 0.2 units (Figure 69). Final lactic acid content amounted to 4.2 mg/ml, that is 0.8 mg/ml more than in the treatment without additional fructose (n.s.). ***These findings provide evidence to disprove Hypothesis 4***. Final acetic acid content exceeded the counterpart by 0.8 mg/ml (significant). Ethanol production was highest after 23 h with a content of 4.1 mg/ml (cp. 0.6 mg/ml, significant difference).

LAB

There were no significant differences in any parameter between the LAB treatment with and without fructose addition at both measurement points except for propionic acid which was significantly higher after 47 h (0.2 mg/ml vs. 0.0 mg/ml) (Figure 68). Also after 47 h there is a higher ethanol content on average in the fructose treatment (2.3 mg/ml), that is 1.2 mg/ml higher than of the counterpart (n.s.).

Yeasts + LAB

This treatment (Figure 67) again corresponds to the LAB treatment. Only the final ethanol content is higher with 3.4 mg/ml instead of 2.3 mg/ml without additional fructose (n.s.).



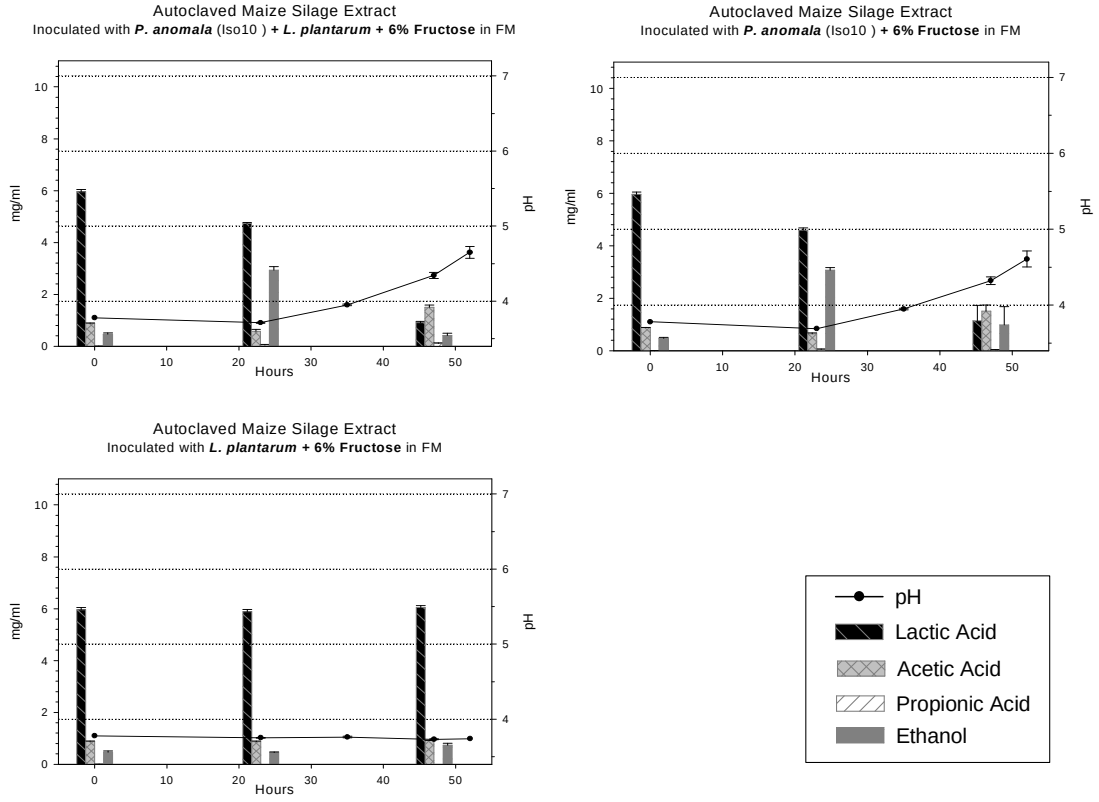
Figures: Changes in pH and some chemical components [mg/ml] in autoclaved grass silage extract with inoculants and additional fructose over 52 h

Figure 67 (top left): Pichia anomala + Lactobacillus plantarum

Figure 68 (down left): Lactobacillus plantarum

Figure 69 (top right): Pichia anomala

Maize



Figures: Changes in pH and some chemical components [mg/ml] in autoclaved maize silage extract with inoculants and additional fructose over 52 h

Figure 70 (top left): Pichia anomala + Lactobacillus plantarum

Figure 71 (down left): Lactobacillus plantarum

Figure 72 (top right): Pichia anomala

Yeasts

There are significant differences in pH, lactic and acetic acid content between the treatments with and without additional fructose in both measurement points.

During the first 23 h pH decreased (!) by 0.1 units even though the lactic acid content diminished by 1.4 mg/ml (Figure 72), i.e. a significant lesser decomposition compared to the treatment without fructose. Acetic acid content decreased by 33 % and ethanol content increased to 6 times higher (3.1 mg/ml) than the initial content. Both differed significantly from the counterpart.

After 47 h the pH rose to 4.3. Lactic acid content diminished to 1.1 mg/ml, which was significantly higher than the counterpart without fructose. The same was true for acetic acid content which increased to 1.5 mg/ml. Ethanol was decomposed again to 1.0 mg/ml on average (n.s.).

The findings provide evidence to disprove Hypothesis 4 saying that lactate decomposition and pH rise are not influenced by other available carbon sources.

LAB

There were no significant differences between the treatment with and without additional fructose in any variable at both measurement points. All parameters remained relatively stable within 47 h of incubation (Figure 71).

Yeasts + LAB

There were no significant differences between this treatment and the yeasts treatment with fructose in any parameter at both measurement points (Figure 70).

5.4.3 Experiment D 3 – *grass, maize with reversed pH*

To assess the influence of pH and fodder crop type on aerobic changes

- As for *Experiment **D 1*** (*Pichia anomala*, *Lactobacillus plantarum*, *Pichia anomala* + *L. plantarum* inoculated in grass and maize silage medium), but maize silage medium adjusted to the pH of the grass silage (4.4) and vice versa (3.8). Compare to *Experiment **C 6***.

Inoculation rate was comparable to *Experiment **D 1***: *Pichia anomala* 6.7 log cfu/g FM, *Lactobacillus plantarum* 8.5 log cfu/g FM.

Grass

Yeasts

The adjusted pH of 3.8 remained constant during the first 23 h (Figure 75). At the same time the lactic acid content diminished from 7.0 to 5.9 mg/ml, that means significantly more lactic acid was decomposed than at pH 4.4 (+0.3 mg/ml on average). Acetic acid content decreased from 1.5 to 0.9 mg/ml. Propionic acid content remained stable at 0.1 mg/ml. Ethanol content increased to 1.2 mg/ml, that is about twice as much as the treatment at pH 4.4 (significant difference).

After 47 h the pH increased to 4.1. Lactic acid content decreased to 3.8 mg/ml, that was a 0.4 mg/ml higher content than of the counterpart at the natural pH (n.s.). Acetic acid content further diminished to 0.2 mg/ml which was significantly lower than the counterpart at pH 4.4 and ethanol content rose to 1.8 mg/ml (cp. 0.2 mg/ml, n.s.).

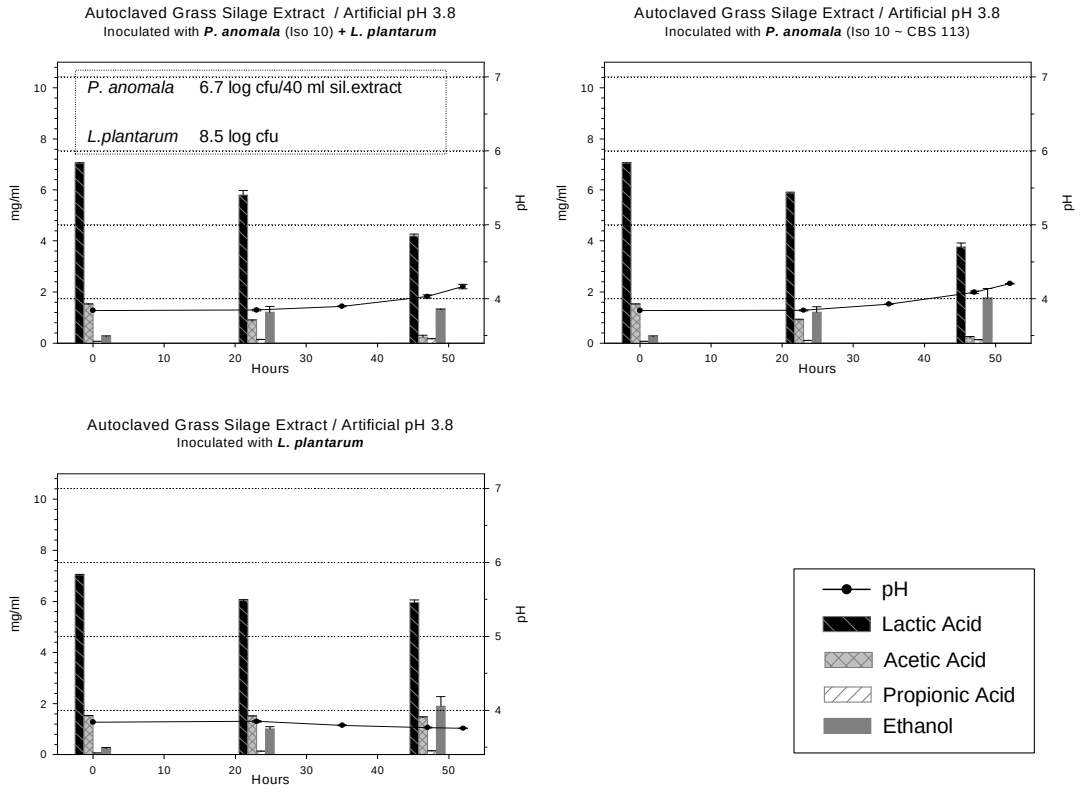
LAB

The pH did not change within 47 h (Figure 74). However lactic acid content diminished from 7.0 to 6.0 mg/ml within 23 h and remained constant until 47 h. It differed significantly from the LAB treatment at pH 4.4 which increased.

Acetic acid content did not change within 47 h. Propionic acid content increased slightly by 0.1 mg/ml and ethanol content rose steadily from 0.2 to 1.0 mg/ml after 23 h (significantly different from LAB treatment pH 4.4) to 1.9 mg/ml after 47 h.

Yeasts + LAB

In contrast to the counterpart at pH 4.4 which resembled the LAB treatment (Experiment D 1) this treatment resembled the yeasts treatment (Figure 73). There were no significant differences in any parameter at any point of time.



Figures: Changes in pH and some chemical components [mg/ml] in autoclaved grass silage extract with inoculants with an initial pH of 3.8 over 52 h

Figure 73 (top left): Pichia anomala + Lactobacillus plantarum

Figure 74 (down left): Lactobacillus plantarum

Figure 75 (top right): Pichia anomala

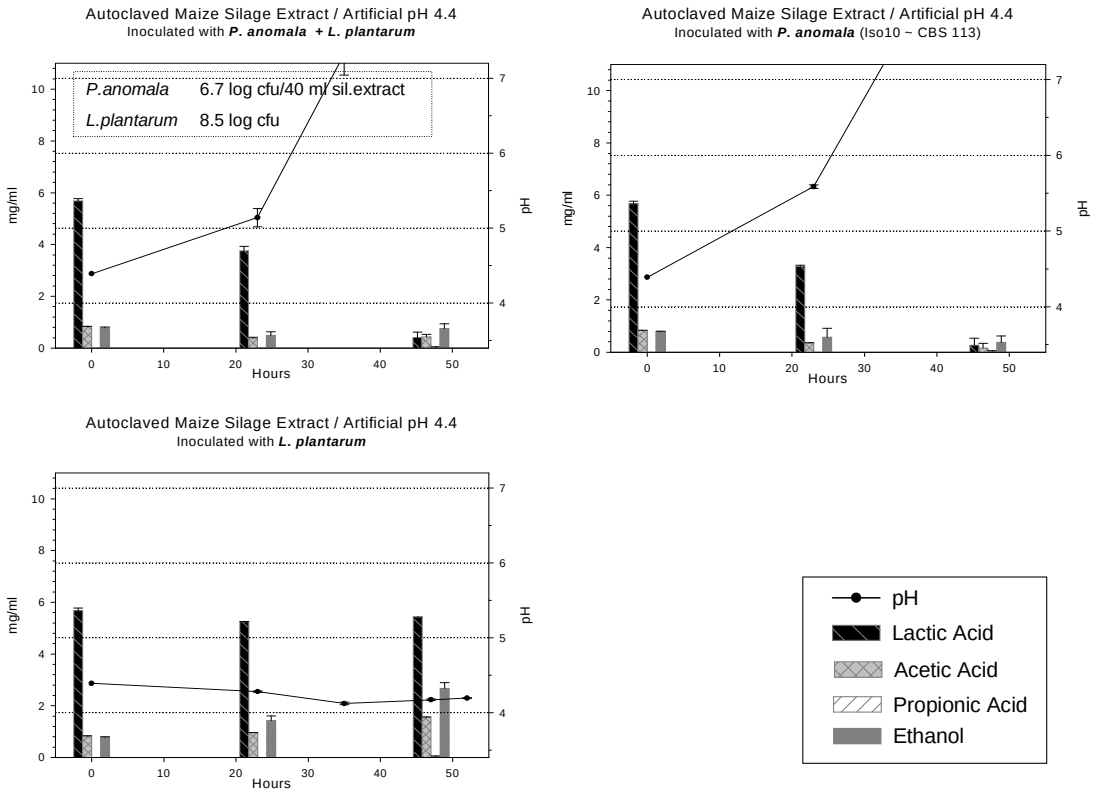
Maize

Yeasts

At the adjusted initial pH of 4.4 the pH rose to 5.6 after 23 h (Figure 78). Lactic acid was decomposed from 5.7 mg/ml to 3.3 mg/ml, that is a significantly higher decomposition rate (by 0.5 mg/ml) than of the counterpart at the natural pH level. Acetic acid content decreased from 0.8 to 0.4 mg/ml whereas in the counterpart it was depleted completely within 23 h (significant difference). Ethanol content decreased from 0.8 to 0.6 mg/ml which was similar to the counterpart at pH 3.8 (n.s.).

After 47 h the pH rose to 8.2. Lactic acid content diminished to 0.3 mg/ml, acetic acid content to 0.2 mg/ml and ethanol content to 0.4 mg/ml. At this point of time organic acid contents did not differ significantly from the counterpart at pH 3.8.

Compared to the grass silage at pH 4.4 the lactic acid decomposition in maize silage extract was faster, -5.4 mg/ml in contrast to -4.1 mg/ml within 47 h.



Figures: Changes in pH and some chemical components [mg/ml] in autoclaved maize silage extract with inoculants with an initial pH of 4.4 over 52 h

Figure 76 (top left): Pichia anomala + Lactobacillus plantarum

Figure 77 (down left): Lactobacillus plantarum

Figure 78 (top right): Pichia anomala

LAB

After 23 h the pH dropped from 4.4 to 4.3 (Figure 77). Lactic acid content decreased from 5.7 to 5.2 mg/ml which was in contrast to the LAB treatment with the initial pH of 3.8 which was constant. Acetic acid content did not differ significantly from the counterpart. Ethanol content increased to 1.4 mg/ml (significantly higher than in LAB treatment at pH 3.8).

After 47 h the pH decreased further to 4.2. Lactic acid content accounted for 5.4 mg/ml (significantly lower than cp. at pH 3.8 with 6.1 mg/ml). Acetic acid content rose to 1.6 mg/ml which was significantly higher than the counterpart at pH 3.8. Ethanol content increased to 2.7 mg/ml which again was significantly more than in the counterpart.

The trend of decomposing instead of producing lactic acid was opposing the grass silage treatment at pH 4.4 (Experiment D 1).

Yeasts + LAB

There were no significant differences in the variables between this treatment (Figure 76) and the yeasts treatment at both measurement points, but for pH and lactic acid content after 23 h. pH accounted for 5.1 then which was significantly lower than the yeasts treatment (5.6). Lactic acid content dropped to 3.8 mg/ml which was 0.5 mg/ml higher than in the yeasts treatment.

5.4.4 Experiment D 4 – *grass, maize with reversed pH +6 % fructose*

To assess the influence of pH and fodder crop type on aerobic changes providing residual WSC

- As for *Experiment **D 3*** (*Pichia anomala, Lactobacillus plantarum, Pichia anomala + L. plantarum* inoculated in grass and maize silage medium at adjusted pH 3.8 and 4.4 resp.) but + 6 % fructose on FM base. Compare to *Experiment **C 6***.

Inoculation rate was as in *Experiment **D 3***.

Grass

Yeasts

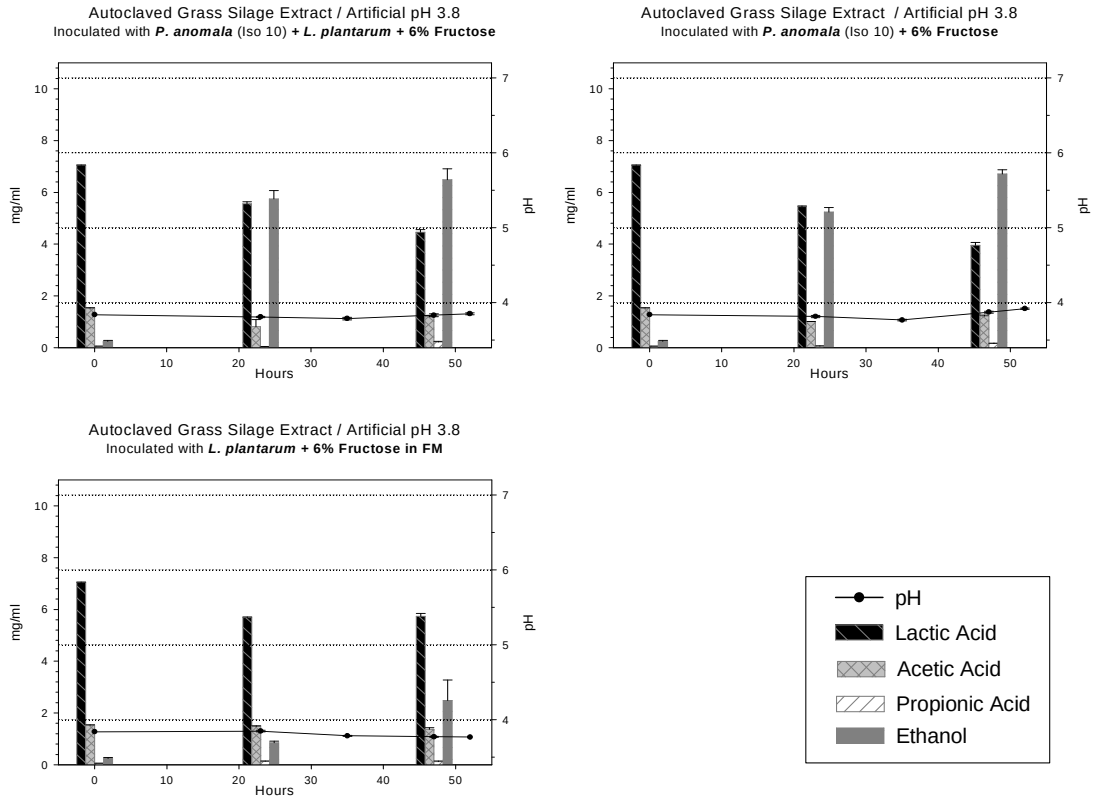
Within 47 h the pH remained stable whereas the lactic acid content decreased from 7.0 to 5.9 mg/ml after 23 h (significantly higher content than in the counterpart without additional fructose) (Figure 81). Acetic acid content was not significantly different to

the counterpart without additional fructose. However within 23 h ethanol content increased to 5.2 mg/ml, that was 4 times higher than without additional fructose (significant difference) (Experiment D 3).

After 47 h lactic acid content decreased to 4.0 mg/ml which did not differ significantly from the treatment without additional fructose. Finally 1.2 mg/ml acetic acid were contained, a 6 times higher amount than without additional fructose (significant difference). Ethanol content rose further to 6.7 mg/ml, that was 3.7 times higher than without additional fructose (significant difference).

Comparing this treatment to the fructose treatment at the natural pH significantly less lactic acid was decomposed during the first 23 h in the latter (difference of 1.0 mg/ml). At the lower pH the ethanol production was significantly higher (+ 1.1 mg/ml) after 23 h.

After 47 h there was no more significant difference in lactic acid content, but in acetic acid content which was higher at the higher pH level. Ethanol content was significantly higher at the lower pH (2.4 times).



Figures: Changes in pH and some chemical components [mg/ml] in autoclaved grass silage extract with inoculants with an initial pH of 3.8 and additional fructose over 52 h

Figure 79 (top left): Pichia anomala + Lactobacillus plantarum

Figure 80 (down left): Lactobacillus plantarum

Figure 81 (top right): Pichia anomala

LAB

After 23 h the lactic acid content in the fructose added treatment (Figure 80) was significantly lower than in the counterpart without additional fructose (5.7 vs. 6.1 mg/ml). Acetic acid and ethanol content did not differ significantly from each other in both treatments.

After 47 h there were no significant differences between both.

Comparing this treatment to the counterpart with fructose at the natural pH (Experiment D 2) there were big differences in lactic acid content which increased at the high pH and which decreased at the low pH level during 47 h. Acetic acid content did not differ significantly after 23 h but ethanol content was significantly higher at the low pH by 0.5 mg/ml. This difference diminished after 47 h and was no more significant.

Yeasts + LAB

At both measurement points this treatment (Figure 79) did not differ significantly from the yeasts treatment in any parameter.

Maize

Yeasts

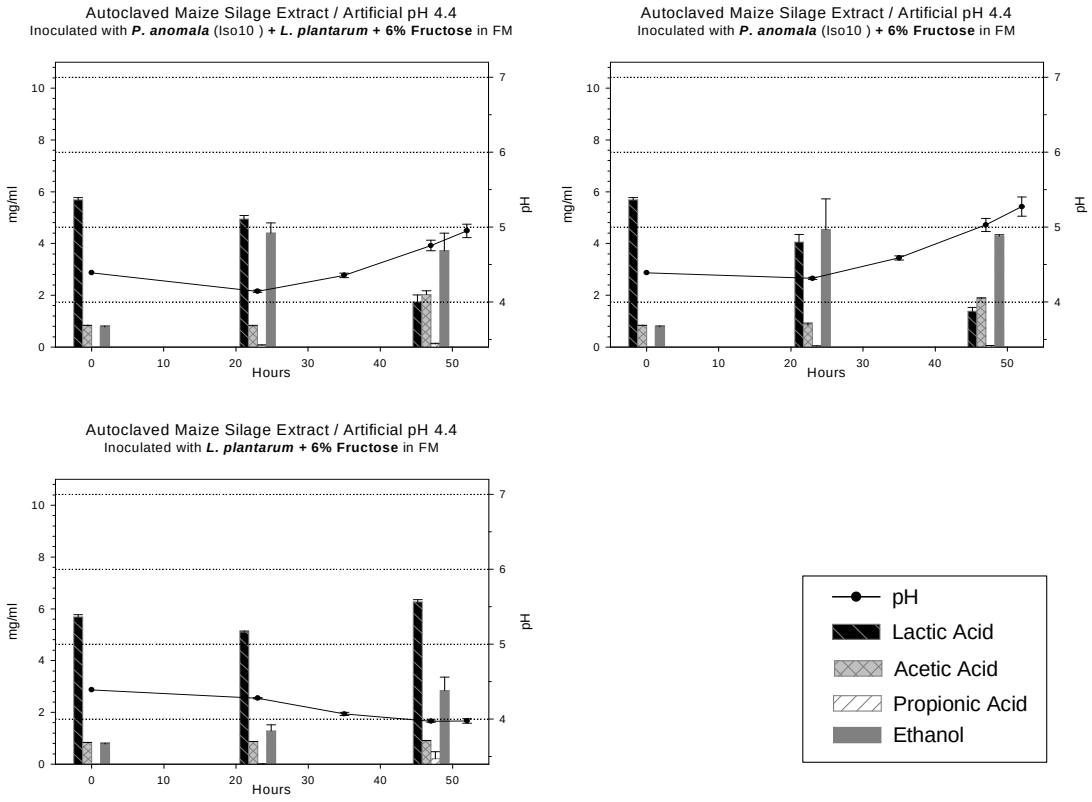
During the first 23 h the pH declined from 4.4 to 4.3 (Figure 84). The counterpart without fructose rose to 5.6 (Experiment D 3). Lactic acid content diminished from 5.7 to 4.1 mg/ml (cp. 3.3 mg/ml, significant difference). In contrast to the counterpart acetic acid content was not reduced after 23 h (significant difference). Ethanol content rose to 4.5 mg/ml whereas it was reduced to 0.6 mg/ml in the treatment without fructose (significant difference).

After 47 h pH rose to 5.3 (cp. 8.2). Lactic acid content was reduced to 1.4 mg/ml which was significantly less reduction compared to the counterpart without additional fructose.

These results again provide evidence to disprove Hypothesis 4. Acetic acid content increased to 1.9 mg/ml whereas it diminished to 0.2 mg/ml in the counterpart without fructose (significant difference). Ethanol content finally accounted for 4.3 mg/ml which was significantly higher than in the counterpart.

Compared to the yeasts treatment with additional fructose at the natural pH there were significant differences in lactic and acetic acid but not in ethanol content after 23 h. Lactic acid content was higher at the low natural pH whereas acetic acid and ethanol content were higher at the higher pH. After 47 h lactic and acetic acid content were similar but ethanol content was significantly higher at the high pH.

The lactic acid decomposition was higher in the maize silage extract at pH 4.4 (-4.3 mg/ml within 47 h) than in the grass silage extract at pH 3.8 (- 3.0 mg/ml). Ethanol production was also higher in the maize silage extract.



Figures: Changes in pH and some chemical components [mg/ml] in autoclaved maize silage extract with inoculants with an initial pH of 4.4 and additional fructose over 52 h

Figure 82 (top left): Pichia anomala + Lactobacillus plantarum

Figure 83 (down left): Lactobacillus plantarum

Figure 84 (top right): Pichia anomala

LAB

The development of the LAB treatment with fructose looks very similar to “without fructose”. There were slight but significant differences at the single measurement points in lactic and acetic acid contents.

After 23 h the pH dropped from 4.4 to 4.3 (Figure 83). Lactic acid content diminished from 5.7 to 5.1 mg/ml, acetic acid content remained constant and ethanol content increased from 0.8 to 1.3 mg/ml.

After 47 h the pH dropped further to 4.0 (cp. without fructose 4.2). Lactic acid was then produced and increased to 6.3 mg/ml (cp. 5.4 mg/ml). Acetic acid content remained stable at 0.9 mg/ml whereas it increased to 1.6 mg/ml without fructose. Ethanol content increased to 2.9 mg/ml.

This treatment differed significantly from the counterpart with fructose at the natural pH (Experiment D 2) in lactic acid and ethanol contents in both measurement points as all values remained constant at the low pH.

Compared to the treatment with grass silage extract at pH 4.4 there is a similar pH trend. However the lactic acid production in maize silage extract is much lower compared to grass silage: final lactic acid content in maize silage extract with fructose 6.3 mg/ml (+ 0.6 mg/ml), in grass silage extract with fructose 10.5 mg/ml (+ 3.1 mg/ml), without additional fructose 10.2 mg/ml (+ 2.7 mg/ml).

Yeasts + LAB

There are no significant differences between this treatment and the yeasts treatment at both measurement points except for pH and lactic acid content after 23 h. pH dropped to 4.1 at that time whereas in the yeasts treatment it dropped only to 4.3 (Figure 82). Lactic acid content decreased to 5.0 mg/ml in contrast to 4.1 mg/ml with only yeasts.

5.4.5 Experiment D 5 – *Potassium chloride*

To study the influence of osmotic pressure adjusted to the conditions in the actual silage

- 3 treatments with 3 replicates: *Pichia anomala*, *Lactobacillus plantarum*, *Pichia anomala* + *L. plantarum* were inoculated in 8 % KCl medium from grass silage. Compare to *Experiment **C 4***.

Inoculation rate was 7.7 log cfu/ 40 ml silage extract *Pichia anomala* and 9.7 log cfu/ 40 ml silage extract *Lactobacillus plantarum*.

Lactic acid contents were not compared directly to the treatments without KCl by the Tukey test because of lower initial contents due to the KCl addition.

Yeasts

The addition of KCl lead to a relative stability of pH and organic acid contents (Figure 87).

After 23 h pH remained at 4.4. Lactic acid content rose slightly from 5.2 to 5.6 mg/ml. Acetic acid content dropped from 1.2 to 1.1 mg/ml. Ethanol content increased from 0.2 to 0.6 mg/ml.

After 47 h the pH rose to 4.6. Lactic acid content resembled the initial content. Acetic acid content further diminished to 0.5 mg/ml. Ethanol content increased to 1.5 mg/ml.

This treatment varied conspicuously from the counterpart without KCl (*Experiment **D 1***) which rose clearly in pH (6.2 after 47 h) and only decomposed lactic acid (-4.1 mg/ml within 47 h resp. -55 %). The final ethanol content of the KCl treatment was higher than without KCl (1.5 mg/ml vs 0.2 mg/ml on average, n.s.).

LAB

KCl restricted the bacterial activity but still lactic acid was formed.

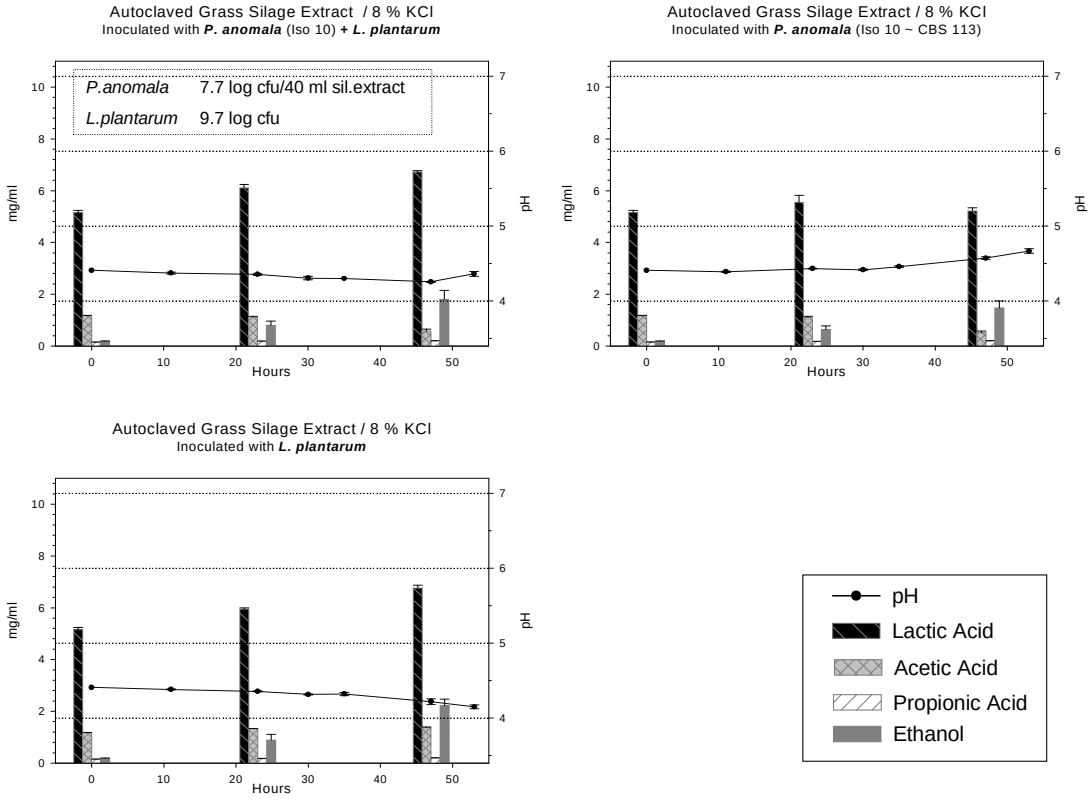
Within 23 h the pH remained constant at 4.4 (Figure 86). Lactic acid content rose from 5.2 to 6.0 mg/ml. Acetic acid content increased from 1.2 to 1.3 mg/ml, ethanol content from 0.2 to 0.9 mg/ml.

After 47 h the pH dropped to 4.2. Lactic acid content increased to 6.8 mg/ml, that was a production of 1.6 mg/ml (+ 31 %) within 47 h in contrast to 2.7 mg/ml (+ 37 %) without KCl. Acetic acid content rose to 1.4 mg/ml which was similar to the treatment without KCl. Ethanol content increased to 2.2 mg/ml which was more than twice as much as without KCl (n.s.).

After 23 h the LAB treatment differed significantly from the yeasts treatment in pH and lactic acid content. After 47 h lactic acid content of both treatments differed significantly (6.7 mg/ml in LAB vs. 5.2 mg/ml) and acetic acid content (1.4 mg/ml vs. 0.5 mg/ml).

Yeasts + LAB

This treatment (Figure 85) resembled the LAB treatment. There were no significant differences at both measurement points except for acetic acid content after 47 h. Then the yeasts + LAB treatment was significantly lower in acetic acid content compared to the LAB treatment (0.6 to 1.4 mg/ml).



Figures: Changes in pH and some chemical components [mg/ml] in autoclaved grass silage extract with inoculants with 8 % KCl (w/v) over 52 h

Figure 85 (top left): Pichia anomala + Lactobacillus plantarum

Figure 86 (down left): Lactobacillus plantarum

Figure 87 (top right): Pichia anomala

5.4.6 Experiment D 6 – *Potassium chloride +6 % fructose*

To study the influence of osmotic pressure adjusted to the conditions in the actual silage with added residual WSC

- As for *Experiment **D 5*** (*Pichia anomala, Lactobacillus plantarum, Pichia anomala + L. plantarum* inoculated in 8 % KCl medium from grass silage) but + 6 % fructose on FM base.

Inoculation rate was as in *Experiment **D 5***.

Yeasts

After 23 h there was no significant difference to the KCl treatment without additional fructose (Experiment D 5).

After 47 h acetic acid content was significantly higher with fructose than without (1.1 vs. 0.5 mg/ml), same was the ethanol content (5.2 vs. 1.5 mg/ml).

LAB

After 23 and 47 h there were no significant differences to the KCl treatment without additional fructose (Experiment D 5).

Yeasts + LAB

After 23 h lactic acid content was significantly lower than with KCl without additional fructose (5.7 vs. 6.1 mg/ml).

After 47 h the acetic acid content was significantly higher than in the counterpart without additional fructose. Ethanol content was significantly higher with fructose addition: 4.6 vs. 1.8 mg/ml.

5.4.7 Experiment D 7 *– 200 ml Erlenmeyer flasks*

To study the effect of increased air influx

- 3 treatments with 3 replicates: *Pichia anomala*, *Lactobacillus plantarum*, *Pichia anomala* + *L. plantarum* were inoculated in grass silage medium. 40 ml aliquots of medium were transfused to 200 ml Erlenmeyer flasks instead of 100 ml flasks.

Inoculation rate as *Experiment **D 5***.

Yeasts

Within the first 23 h the pH rose from 4.5 to 4.7 while lactic acid content diminished from 5.9 to 5.6 mg/ml (Figure 90). Acetic acid content of initially 1.2 mg/ml halved. Ethanol content increased from 0.2 to 0.8 mg/ml.

After 47 h the pH increased to 6.8. Lactic acid content decreased to 2.1 mg/ml, that is 36 % of the initial content (-3.8 mg/ml within 47 h). In 100 ml flasks lactic acid decreased to 45 % of the initial content (-4.1 mg/ml within 47 h). Acetic acid as well as ethanol concentration in 200 ml flasks accounted for 0.7 mg/ml and did not differ significantly from the treatment in 100 ml flasks.

LAB

After 23 h the pH decreased from 4.5 to 4.4 (Figure 89). Lactic acid content increased from 5.9 to 6.6 mg/ml. Acetic acid content remained stable at 1.2 mg/ml and ethanol content rose from 0.2 to 1.0 mg/ml.

After 47 h the pH dropped to 4.2. Meanwhile the lactic acid content further increased to 7.5 mg/ml. That was an increase of 1.6 mg/ml within 47 h (+27 %), compared to 2.7 mg/ml (+37 %) in 100 ml flasks (Experiment D 1). Acetic acid content rose to 1.3 mg/ml and ethanol content to 2.1 mg/ml. Ethanol production was higher in the 200 ml flasks compared to the 100 ml flasks (+1.0 mg/ml, n.s.).

Yeasts + LAB

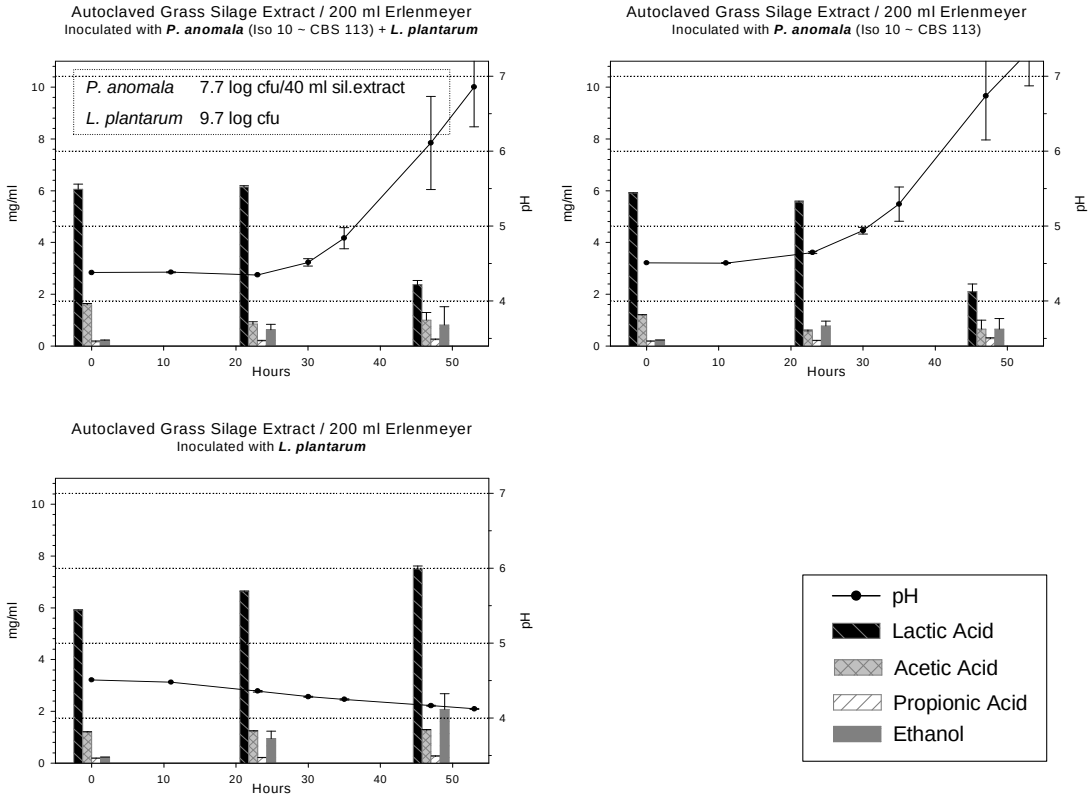
In 200 ml flasks the development after 47 h resembled rather the yeasts treatment in contrast to the 100 ml flasks where it resembled the LAB treatment (Experiment D 1).

Within 23 h the pH remained constant at 4.4 (Figure 88). Same did the lactic acid content. Within the same time in the yeasts treatment 0.4 mg/ml lactic acid were decomposed and in the LAB treatment 0.7 mg/ml lactic acid were produced. Acetic acid content was halved from initially 1.6 mg/ml. Ethanol content increased from 0.2 to 0.6 mg/ml.

After 47 h the pH rose to 6.1 (yeasts treatment 6.7). Lactic acid content diminished to 2.4 mg/ml (yeasts treatment 2.1 mg/ml, n.s.). Acetic acid content accounted for 1.0 mg/ml and ethanol content for 0.8 mg/ml. Ethanol content was significantly lower than in the LAB treatment, but was nearly equal to the yeast treatment (n.s.).

Oxygen measurement

The initial oxygen saturation of the uninoculated silage medium was about 95 %. At the end of the two days of incubation oxygen was nearly used up in the treatments that contained yeasts (< 2 %) but with LAB only the oxygen saturation was still above 90 % and could even resemble the initial content.



Figures: Changes in pH and some chemical components [mg/ml] in autoclaved grass silage extract with inoculants in 200 ml Erlenmeyer flasks over 52 h

Figure 88 (top left): Pichia anomala + Lactobacillus plantarum

Figure 89 (down left): Lactobacillus plantarum

Figure 90 (top right): Pichia anomala

5.4.8 Experiment D 8 – + *Saccharomyces cerevisiae*

To study the behaviour of the fermentative yeast S. cerevisiae alone and in co-culture

In Experiment C 6 the adjusted pH of 3.8 still dropped over 21 h of incubation in the antibacterial treatment. Experiment D 8 was done to verify whether this rather unusual finding might be due to the activity of Saccharomyces cerevisiae and also to study the difference of a yeast specie cultured alone or in co-culture with another yeast specie.

- 6 treatments with 3 replicates: *Saccharomyces cerevisiae, Saccharomyces cerevisiae + Pichia anomala, Saccharomyces cerevisiae + Pichia anomala + Lactobacillus plantarum* were inoculated in grass silage medium either with the natural pH of 4.4 or adjusted to pH 3.8. Compare to *Experiment **C 1*** and *Experiment **C 6***.

Inoculation rates: *Saccharomyces cerevisiae* 7.4 log cfu/ 40 ml silage extract, *Pichia anomala* 7.4 log cfu/ 40 ml silage extract, gave together 7.7 log cfu/ 40 ml silage extract, *Lactobacillus plantarum* 9.6 log cfu/ 40 ml silage extract.

Yeasts

Saccharomyces cerevisiae

- At natural pH

Even though *Saccharomyces cerevisiae* could nearly not metabolise lactic acid when offered as sole carbon source (see Experiment **A 2**) the yeast decomposed lactic acid in the complex silage medium very effectively. ***That provides evidence to disprove Hypothesis 4*** saying that the decomposition of lactate and the rise in pH is not influenced by other available carbon sources.

Within 23 h the pH rose from 4.4 to 4.6 (Figure 92). Lactic acid content diminished from 6.2 to 5.5 mg/ml. Acetic acid content decreased from 1.5 to 0.7 mg/ml. Ethanol content increased from 0.2 to 1.7 mg/ml.

After 47 h the pH reached 7.5. Lactic acid content dropped to 2.1 mg/ml, that was a decrease of 4.1 mg/ml or 66 %. Absolutely that was the same amount as was decomposed by *Pichia anomala* (Experiment **D 1**), relative to the initial content *Pichia anomala* metabolised only 55 % of the available lactic acid. Acetic acid content diminished to 0.1 mg/ml, propionic acid content rose to 0.2 mg/ml. Ethanol content accounted finally for 2.3 mg/ml which was more than with *Pichia anomala* (1.1 mg/ml, n.s.).

- At adjusted pH 3.8

There was no significant difference to the treatment at natural pH in any variable (except pH) at both measurement points except that lactic acid content was slightly but significantly higher at the lower pH (5.6 vs. 5.5 mg/ml) after 23 h. The pH rose from 3.8 to 3.9 after 23 h and to 6.0 after 47 h (Figure 86 in *APPENDIX VIII*).

The finding in Experiment **C 6** of dropping pH could not be repeated with this experiment.

Saccharomyces cerevisiae + Pichia anomala

- At natural pH

Within 23 h the pH rose to 4.6 as with *Saccharomyces cerevisiae* alone (Figure 93). Lactic acid content decreased to 5.3 mg/ml which was a slightly but significantly higher decrease than with *Saccharomyces cerevisiae* alone (-0.1 mg/ml difference). Acetic acid

content dropped from 1.5 to 0.8 mg/ml (*Saccharomyces cerevisiae* 0.7 mg/ml, significant difference). Ethanol content increased to 1.4 mg/ml (n.s.).

After 47 h the pH increased to 6.7 which was significantly lower than with only *Saccharomyces cerevisiae* (7.5). Corresponding to that lactic acid was less decomposed to final 2.7 mg/ml (significant difference). Acetic acid content decreased to 0.6 mg/ml which was significantly higher than with *Saccharomyces cerevisiae* alone. Propionic acid rose to 0.2 mg/ml. Ethanol content remained at 1.4 mg/ml which was significantly lower than in the *Saccharomyces cerevisiae* treatment.

Inoculated with both yeasts lactic acid decomposition and pH rise were not intensified but slightly lessened after 47 h.

- At adjusted pH 3.8

The development of organic acid and ethanol contents was similar to the treatment at natural pH. After 23 h there were slight but significant differences in lactic acid content which was 0.2 mg/ml higher at the lower pH and in acetic acid content which was 0.1 mg/ml lower at the lower pH (figure 86 in *APPENDIX VIII*).

After 47 h there were significant differences in acetic acid contents accounting for only 0.1 mg/ml at the low pH versus 0.6 mg/ml.

Yeasts + LAB

- At natural pH

Within the first 23 h pH and lactic acid content remained constant (Figure 91). However acetic acid content decreased to 0.9 mg/ml and ethanol content increased to 1.4 mg/ml (n.s. compared to the two yeasts treatment).

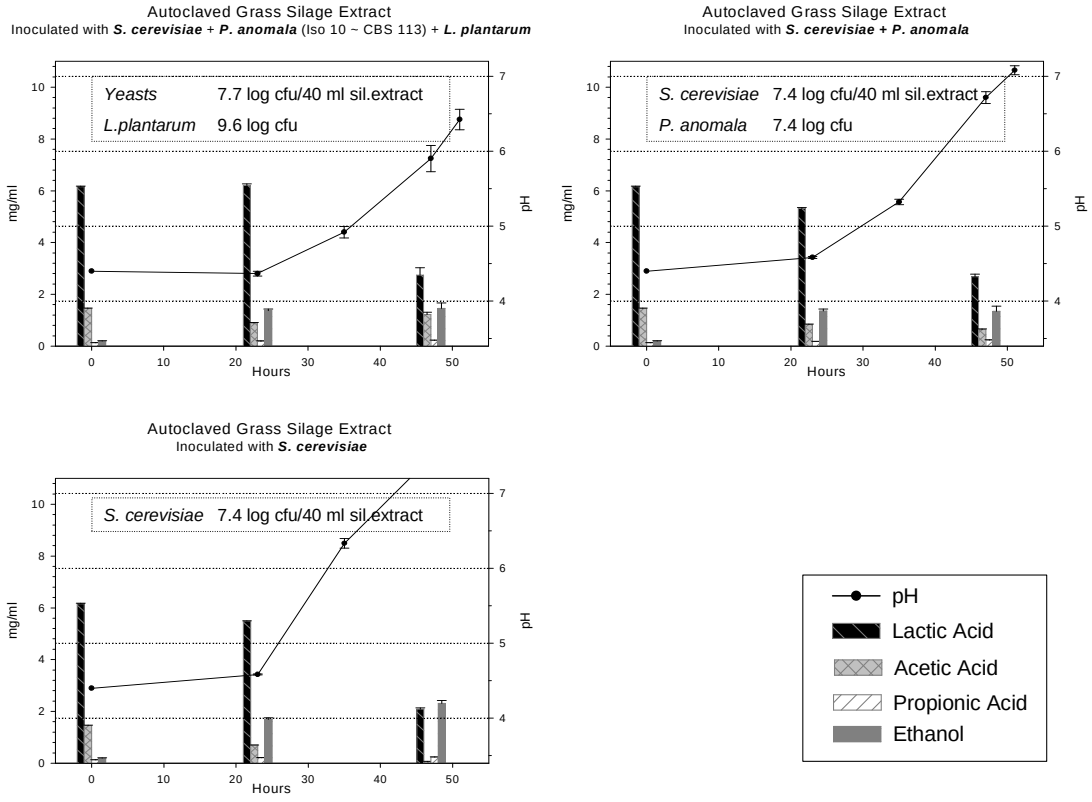
After 47 h the pH rose to 5.9 which was still significantly lower than in the yeasts treatments. Lactic acid content diminished to 2.8 mg/ml (n.s. compared to the two yeasts treatment). Acetic acid content finally accounted for 1.2 mg/ml which was significantly higher than in the yeasts treatments. Ethanol concentration increased to 1.5 mg/ml, comparable to the two yeasts treatment (n.s.).

In contrast to the treatment with only *Pichia anomala* and *Lactobacillus plantarum* (*Experiment **D 1***) whose development resembled the LAB's treatment this treatment resembled much more the yeasts treatment.

- At adjusted pH 3.8

After 23 h the lactic acid content was significantly lower than at natural pH (5.6 vs. 6.2 mg/ml), the same was acetic acid content (0.7 vs 0.9 mg/ml) (figure 85 in *APPENDIX VIII*).

After 47 h the lactic acid content was as high as in the two yeasts treatment (3.0 mg/ml), but was not significantly higher than at the natural pH (2.7 mg/ml). Acetic acid content was significantly lower than at natural pH (0.3 vs. 1.2 mg/ml) and significantly higher than the two yeasts treatment at pH 3.8 (0.1 mg/ml).



Figures: Changes in pH and some chemical components [mg/ml] in autoclaved grass silage extract with inoculants at natural pH over 52 h

Figure 91 (top left): Saccharomyces cerevisiae + Pichia anomala + Lactobacillus plantarum

Figure 92 (down left): Saccharomyces cerevisiae

Figure 93 (top right): Saccharomyces cerevisiae + Pichia anomala

5.4.9 Experiment D 9 – + *Saccharomyces cerevisiae +6 % fructose*

To study the behaviour of the fermentative yeast S. cerevisiae alone and in co-culture with WSC supply

- Like *Experiment **D 8*** (*Saccharomyces cerevisiae, Saccharomyces cerevisiae + Pichia anomala, Saccharomyces cerevisiae + Pichia anomala + Lactobacillus plantarum* were inoculated in grass silage medium either with the natural pH of 4.4 or adjusted to pH 3.8) + 6% fructose. Compare to *Experiment **C 3*** and *Experiment **C 6***.

Inoculation rate was as in *Experiment **D 8***.

Yeasts

Saccharomyces cerevisiae

- At natural pH

After 23 h the pH dropped to 4.3 (figure 89 in *APPENDIX VIII*) and was significantly lower than without additional fructose (Experiment D 8). Lactic acid content diminished to 5.7 mg/ml which was a significantly higher content than without additional fructose (5.5 mg/ml). Acetic acid content dropped to 0.7 mg/ml which differed significantly from the treatment without additional fructose by -0.1 mg/ml. Propionic acid content rose to 0.5 mg/ml which was significantly higher than without fructose (0.2 mg/ml). Ethanol content was 3.4 times higher (5.8 mg/ml on average, significant difference).

After 47 h the pH increased to 7.2 (7.5 without additional fructose, n.s.). Lactic acid content diminished to 2.8 mg/ml (2.1 mg/ml without additional fructose, significant difference). The acetic acid content of 0.1 mg/ml resembled the treatment without additional fructose. Final propionic acid content was still significantly higher than the counterpart (0.5 vs. 0.2 mg/ml). Ethanol content increased to 6.8 mg/ml (nearly 3 times higher than the cp.).

- At adjusted pH 3.8

There was no significant difference to the treatment at natural pH in any variable (except pH) at both measurement points except that lactic acid content was slightly but significantly lower at the lower pH (5.5 vs. 5.7 mg/ml) after 23 h (Figure 95). Propionic acid increased from 0.2 to 0.6 mg/ml during the first 23 h whereas without additional fructose it rose only to 0.3 mg/ml at the same time. The pH decreased very slightly over the first 23 h of incubation from 3.8 to 3.7 and increased to 4.5 after 47 h.

This pH drop was at least an approach to the finding in *Experiment C 6*.

Saccharomyces cerevisiae + Pichia anomala

- At natural pH

Within 23 h the pH remained constant (figure 90 in *APPENDIX VIII*). Lactic acid content diminished from 6.2 to 5.5 mg/ml. Acetic acid content decreased from 1.5 to 1.2 mg/ml. Ethanol content increased to 5.4 mg/ml. Figures of all variables were significantly different to the counterpart without additional fructose and to the treatment with only *Saccharomyces cerevisiae.* Propionic acid content was significantly lower than with only *Saccharomyces cerevisiae.*

After 47 h pH rose to 5.0. Lactic acid content decreased to 3.2 mg/ml. Acetic acid content accounted for 1.3 mg/ml. Ethanol content rose to 5.5 mg/ml. These figures were significantly different from the treatments without additional fructose or with only *Saccharomyces cerevisiae.*

Comparison *Saccharomyces cerevisiae + Pichia anomala* to only *Pichia anomala* with fructose (*Experiment **D 2***) after 47 h: pH 5.0 versus 4.6 (-0.4 units, n.s.), lactic acid decomposition 2.9 mg/ml (-48 % of the initial content) versus 3.3 mg/ml (-44 %), acetic acid changes -0.1 versus +0.1 mg/ml, final propionic acid content 0.3 versus 0.0 mg/ml (significant difference), final ethanol amount 5.5 versus 2.8 mg/ml (significant difference).

- At adjusted pH 3.8

After 23 h lactic acid was decomposed from 6.2 to 5.2 mg/ml (Figure 96). That was the significantly highest degradation rate of lactate within 23 h of all *Experiment **D 8*** and *Experiment **D 9*** treatments. Only the rate of *Saccharomyces cerevisiae + Pichia anomala* without additional fructose at natural pH was comparable (n.s.). Acetic and propionic acid content decrease resembled the counterpart at natural pH. The increase in ethanol content to 5.9 mg/ml was highest in *Experiment **D 9*** within 23 h and significantly higher than at natural pH.

After 47 h lactic acid content decreased to 3.4 mg/ml which was comparable to the treatment at natural pH (n.s.). Acetic acid content diminished further to 0.7 mg/ml which was significantly lower than at natural pH but again comparable to the treatment without additional fructose at natural pH. Propionic acid content (0.3 mg/ml) resembled the counterpart at natural pH, but was lower than with *Saccharomyces cerevisiae* alone with fructose (0.5 mg/ml). Ethanol content (5.8 mg/ml) was not significantly higher than at natural pH.

Comparison *Saccharomyces cerevisiae + Pichia anomala* to only *Pichia anomala* with fructose at pH 3.8 (*Experiment **D 4***) after 47 h: pH 4.1 versus 3.9 (+0.2 units), lactic acid decomposition 2.8 mg/ml (46 % of the initial content) versus 3.1 mg/ml (44 % of

the initial content), acetic acid degradation 0.7 versus 0.3 mg/ml, final propionic acid content 0.3 versus 0.2 mg/ml (significant difference), final ethanol content 5.9 versus 6.7 mg/ml (n.s.).

Yeasts + LAB

- At natural pH

After 23 h the pH decreased to 4.2 which was significantly lower than without additional fructose. Lactic acid content even increased from 6.2 to 6.4 mg/ml (figure 88 see *APPENDIX VIII*). This increase was similar to the same treatment but with only *Pichia anomala* as yeast (*Experiment **D 2***) (+0.3 mg/ml). Acetic acid content decreased from 1.5 to 1.2 mg/ml which was significantly higher than without additional fructose. Propionic acid content increased to 0.3 mg/ml (significantly higher than without additional fructose). Ethanol content rose to 5.1 mg/ml. That was significantly higher than without additional fructose and in the same treatment but with only *Pichia anomala* as yeast (+4.7 mg/ml) (*Experiment **D 2***).

After 47 h the pH rose to 4.8 whereas in the counterpart with only *Pichia anomala* as yeast dropped further to 3.9. Lactic acid content diminished to 4.0 mg/ml which was still the highest content in *Experiment **D 9*** but significantly lower compared to the counterpart with only *Pichia anomala* which further increased to 10.5 mg/ml. Acetic acid content increased to 1.7 mg/ml and was therewith highest in *Experiment **D 9*** and significantly higher than the counterpart with only *Pichia anomala* (1.2 mg/ml). Propionic acid content accounted for 0.3 mg/ml and was significantly higher than without additional fructose. Ethanol content rose to 5.3 mg/ml which was similar to the treatment without LAB.

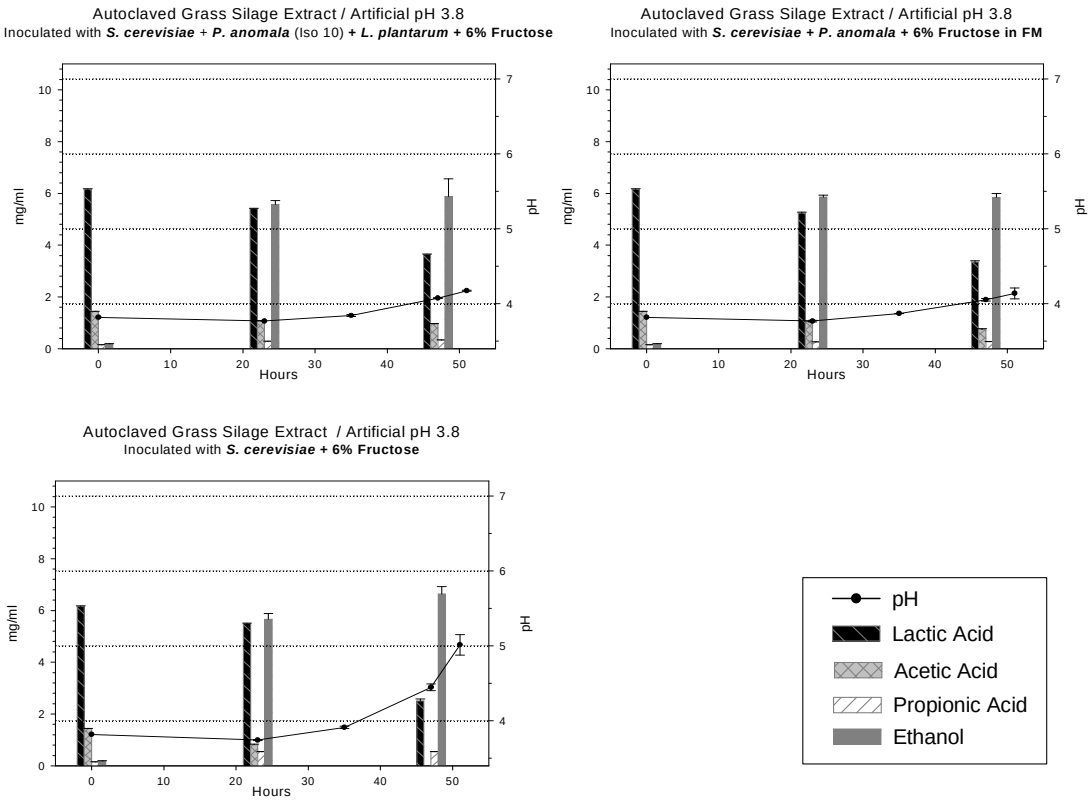
- At adjusted pH 3.8

After 23 h compared to the treatment at natural pH: the lactic acid content was significantly lower (5.4 mg/ml vs. 6.4 mg/ml), acetic and propionic acid content were similar, ethanol content was significantly higher (5.6 mg/ml vs. 5.1 mg/ml). The lactic acid degradation was comparable to the counterpart with only *Pichia anomala* as yeast (Experiment D 4).

After 47 h lactic acid content was not significantly lower than at natural pH but acetic acid content was (Figure 94). Propionic acid and ethanol contents were similar to those at natural pH.

Comparing this treatment to the counterpart with only *Pichia anomala* as yeast after 47 h: pH 4.1 vs. 3.8 (n.s.), lactic acid decomposition of 2.5 mg/ml (-41 % of the initial content) vs. 2.6 mg/ml (-37 % of the initial content), final acetic acid content 1.0 mg/ml

vs. 1.2 mg/ml, propionic acid content 0.3 mg/ml vs. 0.2 mg/ml, ethanol content 5.9 vs. 6.5 mg/ml (n.s.).



Figures: Changes in pH and some chemical components [mg/ml] in autoclaved grass silage extract with inoculants with an initial pH of 3.8 and additional fructose over 52 h

Figure 94 (top left): Saccharomyces cerevisiae + Pichia anomala + Lactobacillus plantarum

Figure 95 (down left): Saccharomyces cerevisiae

Figure 96 (top right): Saccharomyces cerevisiae + Pichia anomala

SYNOPSIS Experiment type D

- Results of Experiment type D confirmed the findings of Experiment type C and the micro-organisms identified, that is yeasts and LAB, which were responsible for aerobic changes in silage extracts.
- Pure *Lactobacillus plantarum* at a high inoculation rate dominated the whole process in grass silage extract at a natural pH of 4.4 in co-culture with *Pichia anomala.*
- Higher air ingress and low pH (3.8) reduced the competitiveness of *Lactobacillus plantarum* significantly.
- An answer to the question of whether lactate assimilation by yeasts was enhanced at low pH or not (*Hypothesis 1*) remained undecided.
- Fructose addition decelerated pH increase in the yeast treatment as it was shown previously in the antibacterial treatment.
- Despite the high inoculation rate *Lactobacillus plantarum* was not competitive in maize silage medium, even at the adjusted pH of 4.4 and with fructose added.
- *Saccharomyces cerevisiae* oxidised lactate as well as *Pichia anomala,* in contrast to the finding in Experiment type A.
- Inoculation of both yeast species in co-culture did not intensify lactate decomposition. However, in that case they dominated over the *Lactobacillus plantarum* activity.
- Propionic acid was not found to be a by-product of *Lactobacillus plantarum.* In the antimycotic treatment in Experiment type C unidentified bacteria produced propionic acid.
- The *Saccharomyces cereviseae* strain did not decrease the pH to the minimum found in the antibacterial treatment in Experiment type C with unidentified yeasts.

6 DISCUSSION

6.1 Microbial activities under "normal" occurring conditions

To discuss the results, in the context of this thesis, there is a need to define what is considered "normal" conditions.

"Normal" conditions means the standard preparation and incubation of batch cultures as described under 4.1 as well as the usual pH conditions for high DM grass and grass-lucerne silages (30-40 % DM) which were in the range 4.4-4.7.

Referring to Experiments *C 1, C 7, D 1, D 8.*

6.1.1 Fungal metabolism

Yeasts

Refers to Experiment type A and Experiments *C 1, C 7, D 1, D 8*.

- Lactic acid assimilation

The indigenous yeast flora and the inoculated yeast strains of all silages generally assimilated lactate, leading to a considerable decrease in lactic acid content within two days of incubation. The only exception was *Saccharomyces cereviseae* which rarely consumed lactic acid when it was present as the sole carbon source, but when cultured in a complex medium it metabolised lactic acid as effectively as *Pichia anomala*.

This ability of some yeasts to assimilate lactic acid is well documented and has long been used for taxonomic differentiation (WICKERHAM and BURTON, 1948). Good growth was observed with *Pichia anomala* (= *Hansenula anomala*), but lactate assimilation capacity can vary within the same strains (MILIGY *et al.*, 1975).
In a recent taxonomic study (BOEKHOUT *et al.*, 2002), the ability of *Saccharomyces cerevisiae* to assimilate DL-lactate was described as variable (+-), which agreed with the findings of KURTZMAN and FELL, 1998. WILES, 1953 (cited in MORRIS, 1958) described a positive effect on lactic acid as did SPENCER *et al.*, 1997. DELFINI *et al.*, 2002, found that 100 % of the 48 *Saccharomyces* strains investigated could degrade L(+)-lactic acid whereas only 40 % could degrade D(-)-lactic acid.

D(-)-lactic acid was found to be the predominant isomer produced in silage (CAI *et al.*, 1998; SCHAADT and JOHNSON, 1968) which means that the *Saccharomyces cerevisiae* strain used probably belonged to the minority of the *Saccharomyces* strains which is able to assimilate this lactate isomer.

The mechanism for the L-lactate, D-lactate, acetate, pyruvate and propionate transport in *Saccharomyces cereviseae* is electroneutral proton/anion symport (WALKER, 1998; CASSIO *et al.*, 1987).

In silage research it has generally been assumed that *Saccharomyces cereviseae* does not belong to the yeasts that decompose lactate, but rather consume residual WSC (JONSSON and PAHLOW, 1984). As shown in the current experiments, this yeast species metabolises lactic acid as well as sugars in a complex silage medium.

High lactate concentrations had no inhibiting effect on yeast metabolism, on the contrary it stimulated metabolic activity as shown in *Experiment type A*. In addition the lactate concentration of the grass silage medium used in *Experiment type D* which was on average 2.6 times higher than most grass silages of *Experiment type C* did not show an inhibiting effect. MILIGY *et al.*, 1975, found that growth rate decreased with increasing lactic acid concentration. However, limited growth is not correlated with decreased metabolic activity (MOON, 1983; THOMAS and DAVENPORT, 1985, cited in SAVARD *et al.*, 2002). The current findings confirm the results of SAVARD *et al.*, 2002, who found that lactic acid alone (0.7 % v/v at pH 3.74) did not prevent the growth of yeasts.

- Acetic acid production and degradation

There were only slight changes in acetic acid contents in the complex media in the present work. Acetic acid concentrations either increased (by up to 1.4 mg/ml) or decreased (by up to 1.1 mg/ml) within 45 h.

Acetic acid is regarded as minor fermentation product during the course of alcoholic fermentation. Acetate can be oxidised by the glyoxylate cycle enzymes (WALKER, 1998).

Also, it was observed that acetic acid can have inhibitory effects on yeast growth (SAVARD *et al.*, 2002; WOLTHUSEN *et al.*, 1989, “content of effective acetic acid”) although this was not examined in the present study. This mechanism is used to inhibit the growth of spoilage yeasts when heterofermentative LAB strains are inoculated onto forage before ensiling (OUDE ELFERINK *et al.*, 1999b).

- Propionic acid production and degradation

Propionic acid was neither produced nor degraded except in the case of *Saccharomyces cerevisiae* when inoculated alone and in the presented cases generally, it was not metabolised by the indigenous yeast micro-flora or the inoculated ones.

There is evidence of the inhibitory effect of propionic acid on yeast activity (OHYAMA and HARA, 1975) and it is used as an additive in silage making (GROSS and BECK, 1970) to prevent aerobic spoilage.

In this context SAVARD *et al.*, 2002, observed that *Saccharomyces* strains were only completely inhibited with a mixture of lactic, acetic and propionic acids of 0.7, 0.3 and 0.2% (v/v), respectively at pH 3.74.

- Ethanol production and degradation

The highest increase in ethanol content usually occurred during the first day of incubation and often decreased again until the end of the second day. *Saccharomyces cerevisiae* produced more ethanol than *Pichia anomala*.

This is not surprising as *Saccharomyces cerevisiae* is a facultative aerobic organism and ferments hexoses to alcohol under anaerobic as well as aerobic conditions, the latter by respiro-fermentative metabolism (FLIKWEERT, 1999; WALKER, 1998; ALEXANDER and JEFFRIES, 1990; HANEGRAAF *et al.*, 2000; MOLLER *et al.*, 2002).

Indeed *Pichia anomala* is also known to produce ethanol but at a lower level, if aerated. This suggests a role for this organism in low-alcohol wine production, with a positive side effect of an esteric and fruity flavour (ERTEN and CAMPBELL, 2001). This ester odour also occurred in the grass silage media in the present experiments.

A partial ethanol depletion in the medium after a rise in ethanol content was observed. This might be explained by the mechanism of passive diffusion into the yeast cell (WALKER, 1998). Another explanation could be oxidation of ethanol to acetate by *Pichia anomala* following WSC depletion as oxygen availability decreased (FREDLUND *et al.*, 2004). An example for this pathway is Experiment D 2 (maize silage extract with fructose addition).

- pH effect

The pH response to lactic acid consumption was not as rapid as anticipated. The pH could even decrease slightly despite the fact that organic acid concentrations diminished. This contradictory finding could not totally be explained by the buffering capacity of the silages (Table 7).

6.1.2 Bacterial metabolism

As lactic acid was produced in most experiments, it was previously referred to as LAB activity in the results section. Here, this activity is considered more generally as LAB metabolism.

The results of earlier investigations to elucidate whether LAB might be involved in aerobic activity in silages: BUCHER (1969) (cited in BECK, 1969), clearly demonstrated that heterofermentative LAB could metabolise lactic acid to acetic acid and therefore had potential for causing aerobic spoilage.

In aerobically deteriorating maize silage, both WOOLFORD and COOK, 1978, and WOOLFORD *et al.*, 1978, observed a substantial rise in LAB numbers within 7 days of exposure to air. A similar observation has been made in grass silage, the highest increase in LAB numbers (within 4 days of opening) being found in high dry matter silages (49 % DM) (WOOLFORD *et al.*, 1979).

- Lactic acid production and assimilation

When the mixed microflora was used lactic acid was produced within the first day of incubation, but decomposed further during the second day. However, when *Lactobacillus plantarum* alone was inoculated the concentration increased until the end of the incubation period.

In batch cultures from silages 15 and 17 lactic acid was decomposed from the start of the incubation (Experiment C 1).

In support of this there is a body of published information that illustrates advantages of aerobic metabolism for LAB in silage. A number of pathways exist for the production of lactate, acetate, ethanol and other volatile fatty acids under aerobic conditions, such as the Embden-Meyerhoff-Parnas pathway, lactate dehydrogenase, pyruvate formate-lyase pathway and others (CONDON, 1987).

CONDON, 1987, listed several advantages of aerobic metabolism for heterolactic as well as for homolactic acid bacteria which included faster growth rates and higher yield of biomass during aeration. Indeed, some substrates such as lactate can only be utilised in the presence of O_2.

For *Lactobacillus plantarum* it was found that if grown in high glucose concentrations under aerobic conditions the end-products were D- and L-lactate only. However, when glucose was exhausted lactate was converted to acetate. MURPHY and CONDON, 1984b proposed that “The most likely mechanism consists of the O_2 inducible pyruvate oxidase and an acetate kinase which converts acetyl phosphate to acetate with the

synthesis of ATP". This allows *Lactobacillus plantarum* to continue growing when O_2 is available (MURPHY and CONDON, 1984b, MURPHY and CONDON, 1984a, BROSNAN, 1984). For further discussion of this topic see 6.2.5.

The latter finding would explain why lactic acid decomposed from the start of the incubation with silage 15 extract, as there was virtually no sugar available from this silage. A conversion of lactate to acetate during air infiltration in silages was also found by PAHLOW, 1982.

The findings in the above mentioned references explain why evidence was provided to disprove Hypothesis 2 saying that the decomposition of lactate in silage is solely caused by yeasts.

The lactate concentration of the grass silage medium used in *Experiment type D* which was on average 2.6 times higher than most of the grass silages of *Experiment type C* did not show an inhibiting effect on LAB metabolism.

- Acetic acid production and degradation

With a mixed microflora acetic acid content rose steadily during the two days of incubation from 0.9 up to 9.8 mg/ml maximum. *Lactobacillus plantarum* when inoculated alone hardly produced any acetic acid.

Aerobic acetic acid production by LAB under aerobic conditions is related to lactate and sugar metabolism as described above.

- Propionic acid production and degradation

Propionic acid concentration increased over two days, with values ranging from +0.3 to 3.0 mg/ml, highest with silage 17 with an increase of 3.4 mg/ml. With *Lactobacillus plantarum* alone as inoculum there was virtually no change in propionic acid content.

Lactobacillus diolivorans can degrade 1,2-propanediol to propionic acid anaerobically:

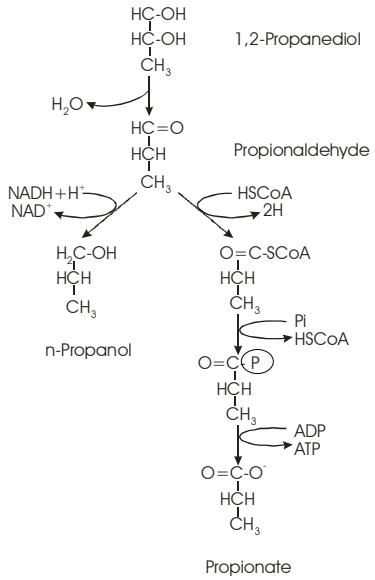


Figure 97: Anaerobic degradation pathway of 1,2-propanediol to propionic acid and n-propanol by Lactobacillus diolivorans (KROONEMAN et al., 2002)

Still, this cannot explain the high amounts of propionic acid produced in the AM treatments after 45 h incubation in Experiment C 1 with silage 30, C 2 with silage 17 and 30 and C 3 with silage 32 as there was either no or a maximum of 0.5 mg/ml 1,2-propanediol detected initially and after 22 h.

Propionate is a minor fermentation product of many bacteria (SCHLEGEL, 1992).

However, there are some bacteria that are able to form higher amounts of propionate like *Propionibacterium* spp., *Veillonella alcalescens (=Micrococcus lactilyticus), Clostridium propionicum, Selenomonas* and *Micromonospora* spp.(SCHLEGEL, 1992).

Despite difficulties that have been encountered some propionic acid forming bacteria have been isolated from silages (MERRY and DAVIES, 1999). These belong to the genus *Propionibacterium* (PAB) and *Veillonella alcalescens* (ROSENBERGER, 1956; de MAN, 1957; WOOLFORD, 1975). The PAB are counted as anaerobic bacteria but are micro-aerotolerant (SCHLEGEL, 1992) and even aerotolerant in some cases (MCDONALD *et al.*, 1991). Under anaerobic conditions they metabolise lactate to propionate and acetate via the Methylmalonyl-CoA- (or "randomising") pathway (SCHLEGEL, 1992;LENGELER *et al.*, 1999) and prefer lactate over sugar as substrate. The pathway from lactate to propionate was described by SCHLEGEL, 1992:

$3\ CH_3\text{-}CHOH\text{-}COOH \dashrightarrow 2\ CH_3\text{-}CH_2\text{-}COOH + CH_3\text{-}COOH + CO_2 + H_2O$

3 lactate ---→ 2 propionate + acetate + carbon dioxide + water

Figure 98: The formation of propionate from lactate via the Methylmlonyl-CoA pathway (SCHLEGEL, 1992)

This pathway could partly explain the lactic acid degradation due to bacterial activity right from the beginning in silage 17, Experiment C 1, associated with high acetic acid and propionic acid production. However, lactate was not preferred over fructose there, as with additional fructose less lactic acid was decomposed and more propionic acid was formed (Experiment C 2) (formation of propionate from hexoses see 6.2.3).

Literature describing an aerobic pathway was not found. Under controlled aeration PAB have a much higher cell yield than if grown under strictly anaerobic conditions (SCHLEGEL, 1992).

In silages inoculated with PAB even higher propionic acid concentrations were found during ensiling when air stress was applied (PAHLOW and HONIG, 1994;WYSS *et al.*, 1994). Concerning the limited tolerance of PAB to low pH WOOLFORD, 1975, stated that “propionibacteria could survive the ensiling process even when the pH dropped to below 4”.

The complex interaction between LAB and propionic acid bacteria has been studied, for example in hard cheeses (KERJEAN *et al.*, 2000; PIVETEAU *et al.*, 1995). In this process LAB prepare the cheese for the propionic acid fermentation through production of the lactate substrate. In addition, a large study with 42 LAB/PAB co-cultures showed that LAB could not only activate propionic acid bacteria or destroy inhibitors, but could also interact as antagonists (KERJEAN *et al.*, 2000). On the other hand a mixed culture of *Propionibacterium jensenii* and *Lactobacillus paracasei* was found to inhibit food spoilage yeasts not only by their fermentation products but by their cells *per se* (SCHWENNINGER and MEILE, 2004).

Clostridium propionicum is a propionic acid producing bacterium but can be excluded in the present work as it is obligately anaerobic (JANSSEN, 1991) and no butyric acid was detected.

Propionic acid production in some of the present experiments together with the knowledge provided by the cited literature provides evidence that *Propionibacteria* were present and active in the aerobic processes in some silage extracts.

For ruminant nutrition there are also health and nutritional benefits for propionic acid producing bacteria if they convert lactic acid to propionate in the rumen and thus reduce the incidence of lactic acidosis (ELSDEN, 1945) or raise protein:fat ratios (ABZUL-RAZZAQ and BICKERSTAFFE, 1989).

- Ethanol

Ethanol content increased continuously over two days.

Ethanol can occur as a by-product of aerobic lactate production (KANDLER, 1983). This is not only true for heterofermentative LAB (PLIHON *et al.*, 1996), but also for the homofermentative species (CONDON, 1987).

- pH effect

Even though there was no further increase and often a decrease in lactic acid concentration after 45 h of incubation, in many cases the pH still dropped compared to that observed at 22 h, or remained constant. Though acetic and propionic acid contents rose lactate is the strongest acid with the highest effect on pH (pK_a 3.86; acetic acid 4.76, propionic acid 4.87; RAUSCHER *et al.*, 1965) and it is difficult to explain these observations.

6.1.3 Co-culture

In most cases pH and lactic acid content were intermediate to values for the antibacterial and antifungal treatments as a result of the opposing metabolic pathways of yeasts and LAB, except in *Experiment **D 1*** where LAB dominated the process in all respects. The ethanol concentrations observed mainly corresponded to yeast activity and the acetic and propionic acid contents ranged between those for yeast and bacterial activity only.

A number of published observations may help to explain ***the outcome of the present experiments which provide evidence to disprove Hypothesis 3***, that aerobic changes are dominated by yeast activity. Recently more specific studies have been reported on the interaction of yeasts and lactic acid bacteria in mixed cultures in foods and beverages. For example, symbiotic associations were described for decreasing wine acidity (ELISEEVA *et al.*, 2000). GOBETTI *et al.*, 1994b, describe the metabolism of carbohydrates in sourdough and in these experiments *Saccharomyces cerevisiae* and *Lactobacillus plantarum* competed for fructose and the yeast had a detrimental effect on bacterial growth. *Saccharomyces cerevisiae* even increased ethanol production in co-culture with LAB compared to the corresponding mono-culture (GOBETTI *et al.*, 1994b; DAMIANI *et al.*, 1996). In other studies LAB growth suppressed the ethanol production by yeasts (NARENDRANATH *et al.*, 1997). On the other hand yeasts can stimulate bacterial growth (GOBETTI *et al.*, 1994a).

Other investigations dealing with cheese surfaces showed that yeasts can liberate growth factors such as vitamins or amino acids for bacterial utilisation (CORSETTI *et al.*, 2001). In kefiran production *Saccharomyces cerevisiae* was shown to enhance the growth of *Lactobacillus kefiranofaciens* (CHEIRSILP *et al.*, 2003).

LINDGREN and DOBROGOSZ, 1990, overviewed the subject of antagonistic substances derived from LAB such as the metabolic end products organic acids, 2,3-butanediol, CO_2 and others. They also discussed the role of bacteriocins which might be able to suppress yeast activity. However, SEALE, 1986, described the antibiosis of LAB against yeasts and moulds as weak.

6.2 Factors influencing microbial metabolism

6.2.1 pH

In Experiment type A it was found that a low pH enhanced lactate assimilation by oxidative yeasts and provided evidence to disprove ***Hypothesis 1,*** but for grass silage extracts in Experiments C 6, D 3 and D 4 the finding was only true for the first 23 h of incubation and not for maize silage.

The findings of MIDDELHOVEN and FRANZEN, 1986, that a low pH enabled or enhanced lactate assimilation of yeasts could not be generalised.

With reference to *Experiments C 1, C 6, D 1 to D 4, D 8 and D 9.*

There was a clear effect of pH on bacterial activity. A pH of 4.0 and below largely inhibited the metabolic activity of LAB and allowed the yeasts to control the whole aerobic process.

This finding was confirmed by NARENDRANATH and POWER, 2005, who observed a reduction in specific growth rate of LAB and a corresponding decrease in lactic acid produced when the pH was lowered from 5.5 to 4.0.

Numerous yeasts are capable of growth over a wide pH range reported to be 2-8 by WALKER, 1998. *Pichia anomala* has been found to grow even at pH values between 2.0 and 12.4 (FREDLUND *et al.*, 2002).

On the other hand most LAB grow optimally at a pH of > 5.0 (HUTKINS and NANNEN, 1992; BECK, 1969). BECK, 1969, described a pH of 4.5-5.0 as optimum for growth of *Lactobacillus plantarum*, and assumed that there was still 90 % growth at pH 4.0 compared to the optimum. In the present batch culture experiments growth was not measured directly but metabolic activity was almost completely inhibited at a pH $\leq$ 4.0.

pH regulation in lactic acid and other bacteria has been reviewed by HUTKINS and NANNEN, 1992; TSAU *et al.*, 1992; CHADWICK and GARDEW, 1999.

A model describing the effect of medium parameters such as pH on the growth of isolates of *Candida milleri* and *Lactobacillus sanfranciscensis* from sourdough, confirmed the above observations showing an optimum pH between 5 and 6 for the LAB (GAENZLE *et al.*, 1998).

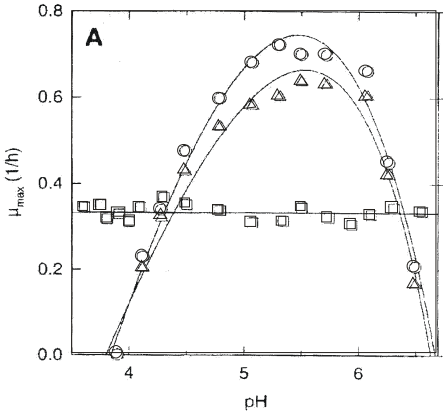


Figure 99: Effect of pH on maximum growth rates (μ_{max}) for Lactobacillus sanfranciscensis 1 (○) and 2 (Δ) and Candida milleri (□) (GAENZLE et al., 1998)

This pH effect on bacterial and fungal activity may explain why inoculated silages are more prone to aerobic deterioration (KUNG *et al.*, 1991; PHILLIP and FELLNER, 1992; SANDERSON, 1993; UMANA *et al.*, 1991; WARDYNSKI *et al.*, 1993; WYSS, 1993) as they mostly achieve a lower pH than uninoculated silages.

OHYAMA *et al.*, 1975, also found that silages with a pH value of < 4.0 deteriorated more often than those where the pH was > 4.0. Another consideration might be the "self-sterilisation" which leads to lower LAB numbers in mature inoculated silages (PAHLOW, 1982) if the LAB : fungi ratio is lowered.

6.2.2 Fodder crop

Grass and maize

With reference to *Experiments C 1, C 7, D 1, D 2, D 3, D 4.*

To determine whether the differences in activities between grass and maize silages were only due to differences in pH it was reversed in the two silage media used. Fructose was added to the maize silage medium where it was actually lacking.

The high lactic acid production which occurred when grass silage medium (pH 4.4) was inoculated with the *Lactobacillus plantarum* was not achieved in the maize silage medium adjusted to pH 4.4 regardless of whether fructose was added.

The combination of *Lactobacillus plantarum* with *Pichia anomala* in the pH adjusted maize silage medium increased lactic acid production only slightly compared to *Pichia anomala* alone, whereas in the grass silage medium (pH 4.4) about the same amount of lactic acid was produced in co-culture as with *Lactobacillus plantarum* alone.

Only when the pH of the grass silage medium was adjusted to 3.8 (corresponding to that of maize silage medium) was the lactate oxidising activity of *Pichia anomala* dominant in the co-culture. *Lactobacillus plantarum* alone in the adjusted grass silage medium also degraded lactic acid, whereas in the maize silage medium at pH 3.8 it remained inactive.

These experiments demonstrated a principal difference in the behaviour of the same defined microorganisms (inoculants) in silage media depending on the type of crop fermented.

6.2.3 Carbon sources and compounds

- WSC

With reference to *Experiments C 2, C 3, D 2, D 4, D 6, D 9.*

Fructose addition enhanced ethanol production in the yeast treatments. In addition it increased acetic acid formation and decelerated the increase in pH.

In the bacterial treatments the effect was not as clear as for the yeasts, but often more lactic and propionic acid was produced when fructose was added.

This can be explained in terms of the stoichiometry of the process. *Saccharomyces cerevisiae* for example produces 2 CO_2 molecules + 2 ethanol (C_2H_5OH) molecules from 1 glucose ($C_6H_{12}O_6$) molecule (which is a hexose like fructose) by respiro-fermentation (FLIKWEERT, 1999).

In the sugar metabolism of LAB 1 glucose ($C_6H_{12}O_6$) molecule yields 2 pyruvate molecules which can be converted into 2 lactic acid (CH_3-CHOH-COOH) molecules. However, the conversion from pyruvate to lactate depends on several factors and it might be converted to ethanol, acetic acid, formate or acetoin and 2,3-butanediol as well (CONDON, 1987).

The generation of propionate from hexoses might be explained by the following equation:

$$3\ C_6H_{12}O_6 \rightarrow 2\ CH_3\text{-}COOH^- + 2\ CO_2 + 4\ CH_3\text{-}CH_2\text{-}COOH^- + 6\ H^+$$

$$3\ \text{Glucose} \rightarrow 2\ \text{acetate}^- + 2\ \text{carbondioxide} + 4\ \text{propionate}^- + 6\ H^+$$

Figure 100: The formation of propionate from glucose via the random pathway (LENGELER et al., 1999)

In the EU-Sweetgrass project it was found that optimal ensiling technique improved aerobic stability most regardless whether residual sugar contents were high or low because it suppressed the yeast growth during the storage phase successfully (PAHLOW *et al.*, 2005).

- Tannic acid

With reference to *Experiment C 5*.

The addition of tannic acid ($C_{76}H_{52}O_{46}$) reduced lactic acid consumption and limited pH rise in the fungal treatment. It also enhanced ethanol production.

However, in the control tannic acid accelerated lactic acid degradation and pH rise. Acetic and propionic acid production were reduced in favour of ethanol production. This was probably due to bacterial activity, although this was not investigated separately on that occasion due to a lack of silage material.

Several workers have observed that legumes such as lucerne (*Medicago sativa*) have a stabilising effect on silages exposed to air, due to an inhibition of yeast growth (O´KIELY and MUCK, 1987;MUCK and O´KIELY, 1992;O´KIELY and MUCK, 1992; PAHLOW *et al.*, 2000). As it is not yet clear which is the active compound in these plants, tannin which is a characteristic component of lucerne was tested for its antimicrobial effect in this trial.

In general, the antimicrobial activities of tannins (glucose esters of gallic acid) are well documented (CHUNG *et al.*, 1998b).

WAUTERS *et al.*, 2001, reported that tannic acid inhibited the growth of *Saccharomyces cerevisiae* due to iron deprivation. JACOB and PIGNAL, 1975, found that the effect of tannic acid and hydrolysing capacities of yeasts depended on its concentration and partly on the pH of the medium.

AYED and HAMDI, 2002, investigated the production of tannase (an enzyme that degrades tannin) by *Lactobacillus plantarum*. Intestinal LAB were not inhibited by tannic acid up to a concentration of 0.5 mg/ml. This resistance was probably due to the fact that they do not require iron for growth (which is bound by tannic acid) (CHUNG *et al.*, 1998a).

In the present trial tannic acid seemed to inhibit the yeasts and alter the metabolic pathway, but enhanced bacterial lactate oxidation. Thus the stabilising effect of legumes observed in silages does not appear to be exclusively due to tannin. O´KIELY and MUCK, 1987, suggested that lucerne ony had a stabilising effect after fermentation had occurred.

The findings on WSC and tannic acid addition provide evidence to disprove Hypothesis 4 indicating that the decomposition of lactate and rise in pH is not influenced by other available carbon sources.

6.2.4 Osmotic pressure / Halotolerance

With reference to *Experiments C 4, D 5, D 6*.

KCl reduced the metabolic activity of yeasts as well as bacteria, and in the concentration used it did not favour the dominance of the one or the other group of micro-organisms. Yeasts were virtually unable to oxidise lactate in the presence of KCl. With *Pichia anomala* alone small amounts of lactate were formed. With LAB organic acid production was clearly reduced.

The osmolality of wilted grass silage amounts to about 1.8-2.5 osmol/kg or even higher (HOEDTKE, 2004). The osmolality of the silage medium was close to zero, about 0.2 osmol/kg. To adapt it to the osmolality of high DM grass silages a concentration of 8 % KCl was used. As mentioned earlier, according to WEISSBACH, 1968, this corresponds to the osmotic pressure prevailing in a silage of about 40 % DM. The osmolality of silage samples measured using an osmometer had values of 2.2-2.3 osmol/kg.

As the osmotic pressure was increased by adding salt it cannot be excluded that the inhibiting effect on microbial activity was due to a lack of halotolerance. This seems to be true, at least for the yeasts, where lactate assimilation ceased.

When salt- and sugar tolerance of yeasts were compared under the same osmotic pressure (ONISHI, 1957) differences were observed that indicated limiting factors other than osmotic pressure. Temperature and pH can also affect the salt tolerance of microorganisms (ONISHI, 1963).

In his studies *Saccharomyces rouxii* fermented glucose to increasing amounts of glycerol in parallel with rising NaCl or KCl concentrations, suggesting that these salts affect yeast metabolism and alter the fermentation pathway.

This might explain why *Pichia anomala* produced lactate in the presence of KCl.

Lactic acid formation in small amounts during yeast fermentation was also observed by NORD and WEISS, 1958. During alcoholic fermentation, a concentration of 0.3-0.7 mg/ml lactic acid was generally found (DELFINI *et al.*, 2002), but some wine yeasts were even able to raise the concentration to more than 20 mg/ml lactic acid (SUAREZ LEPE, 1999).

In future work an alternative way to alter the osmotic pressure in the medium is required for making such comparisons.

6.2.5 Oxygen

With reference to *Experiments C 4* and *D 7*.

The use of 200 ml Erlenmeyer flasks, rather than 100 ml to promote greater aeration of the cultures stimulated the yeast dominance in the combined microflora.

The 200 ml cultures with KCl showed no significant differences to their counterparts in 100 ml flasks.

A very interesting finding was that there was virtually no O_2 consumption in the treatments with LAB only, but a nearly complete depletion of O_2 within 2 days when yeasts were inoculated.

For yeasts, oxygen is described as essential nutrient (SPENCER *et al.*, 1997). They can grow under microaerophilic conditions, but there are no strict anaerobes among the yeasts. Oxygen is the terminal electron acceptor at the end of the chain through which energy is released from carbohydrates taken up by the cell (SPENCER *et al.*, 1997).

LAB usually grow anaerobically but many of them are aerotolerant to a degree, or facultative anaerobes, depending on the substrate (CONDON, 1983).

The rate of O_2 uptake by *Lactobacillus plantarum*, which precedes H_2O_2 accumulation depends on the substrate and was recorded to be less with lactate added to a complex medium (MURPHY and CONDON, 1984b). The accumulation of H_2O_2 depends on the inoculation rate and the concentration of dissolved oxygen and is faster and higher with increasing rate and concentration (MURPHY and CONDON, 1984b).

Due to a high intracellular level of Mn^{2+} which can scavenge O_2^- *Lactobacillus plantarum* is able to grow under aerobic conditions (ARCHIBALD and FRIDOVICH, 1981; KANDLER, 1983; ARCHIBALD, 1986). There are also other mechanisms to help bacteria to tolerate reactive O_2 species which can be formed in the metabolic pathway (O_2^-, $OH^\cdot$, H_2O_2) among LAB, for example superoxide dismutase (SOD), NADH oxidase/NADH peroxidase, adaptation (CONDON, 1987; GOETZ *et al.*, 1980a;GOETZ *et al.*, 1980b; KANDLER, 1983; HIGUCHI *et al.*, 2000) and Recombinase A activity (gene/proteine RecA) or Cytochrome d oxidase (gene/proteine cydA) (MIYOSHI *et al.*, 2003).

The presence of O_2 “allows (the LAB) a wider range of substrates to be used for ATP generation and also the utilisation of pathways which are dormant in anaerobic cultures” (CONDON, 1987).

The findings suggest that LAB compete better with yeasts in the inner layers of silage where reduced concentrations of oxygen are available. This confirmed the observations made by PAHLOW, 1985, who studied different and defined levels of oxygen infusion throughout an ensiling period of 90 days. He found that the lowest oxygen infusion level of 100 mg/kg DM daily which corresponds to about 100 cm penetration depth in concrete silos, triggered LAB activity and thereby controlled the yeasts.

6.3 Comparison of agitated batch culture system with the aerobic stability test according to HONIG, 1990

With reference to *Experiments C 1* and *C 2*.

Temperature rise in the original fresh material during the aerobic stability test was not clearly related to pH rise in the batch cultures as temperature release is an indication for microbial metabolism *per se* whereas pH rise indicates the degradation of organic acids which is not necessarily the first metabolic activity to occur.

The only rule that was observed was: corresponding batch cultures of those silages (ID 15 and 34) that proved to be relatively stable in the aerobic stability test (> 4 days) did not change in their composition over the first 24 h of incubation.

Another interesting finding was the visualisation and microbial counts of silage ID 32 during the aerobic stability test. Even though the treatment without and with additional fructose did not differ significantly in pH and organic acid contents, there was an indication that the LAB were involved and competed for nutrients under aerobic conditions: higher final LAB numbers and slower yeast growth (visual observation) combined with lower final yeast numbers were observed in the fructose treatment in contrast to the control treatment (without additional fructose).

One of the main differences of both approaches (HONIG test and batch culture) is the physical condition (solid and liquid). In a liquid medium all nutrients are freely available and equal distribution of micro-organisms is ensured. Another factor is the temperature which rises continuously in the insulated cans in the HONIG test once the spoilage process has started, whereas it remains very constant in the shaken batch cultures at ambient temperature.

However, when investigating the changes of chemical composition in the aerobic stability test, it was proved that lactic acid (and ethanol) production can indeed take place in silages on exposure to air, as found in the batch cultures (see for example Experiment C 1, Silage ID 34).

This specific investigation during the temperature test which is usually carried out over 7 days was much more labour intensive and required a higher number of samples than the measurement of the batch cultures.

6.4 Evaluation of the *in-vitro* method

The model *in-vitro* system in this work was developed as a tool to use alongside current microbiological approaches, that are often very time consuming and labour intensive and although of value do not always truly reflect the activities occurring in real time during the spoilage process.

For example consumables required for the **standard plate count method** in silage microbiology are:

- sterile Petri dishes,
- agar, various nutrient compounds (at least a carbon and a nitrogen source) and antibiotics for the solid medium which has to be specific for each group of target micro-organisms
- sterile Ringer solution for the dilution series and
- sterile pipette tips for diluting and dispensing samples during plating out.

The solid medium has to be prepared at least 2 days before the plating and can be stored up to 4 weeks at 4 °C. Autoclaved Ringer solution must be at ambient temperature before usage. Samples are diluted in decimal series taking several steps. The diluted samples are plated in triplicates, usually at least in two different logarithmic dilutions which have to be anticipated correctly. A count of colonies grown on the plates can be made after 3 days of incubation at 30 °C.

By counts on the malt extract agar and lactate agar plates it can be calculated how many yeasts and moulds with the potential to assimilate lactate were present in about one gram of silage fresh matter at the time of opening. If samples were plated on Rogosa or MRS agar (in the correct dilution) they reveal the numbers of lactic acid bacteria. For the total count of aerobic bacteria the use of plate count agar is advisable. In this type of approach counts cannot directly indicate metabolic activity and interactions and are also subject to underestimation of both numbers and groups, due to non-culturability of some micro-organisms in laboratory media under the conditions employed.

This contrasts markedly with the approach taken in the current work (see below) where the metabolic activity, rather than viable microbial counts was used as an indicator of different microbial activities.

For **Experiment types A to D** different media were used. The same processing was performed with an incubation period of 2 days, measurements by HPLC at day 0, 1 and 2 and pH measurements, usually one at day 0 and 2 at each of the following days.

The liquid lactate medium utilised in **Experiment type A and type B** requires lactic acid and Yeast Nitrogen Base as ingredients. It has to be prepared and pH adjusted one day before inoculation. The autoclaved medium can be stored for several months at 4 °C if kept in portions in sterile containers.

Experiment type A revealed the performance of naturally-occurring yeasts isolated from silages to oxidise lactate in an artificial environment where lactic acid was the sole carbon source at different pH values. As revealed in Experiment type D the performance found under these conditions did not necessarily correspond to that in a complex silage medium (example *Saccharomyces cerevisiae*).

Experiment type B showed the ability of the whole mixed microflora of a fresh silage to decompose lactate. Bacterial and fungal activities were differentiated by antibiotics. Probably due to the high dilution of the mixed inoculant results were not quite consistent (high standard deviation). This approach was therefore given up in favour of the following:

For the silage medium in **Experiment type C** sterile distilled water together with stomacher bags were required. The original indigenous microflora of the silage was included in the medium and its fungal and bacterial activity differentiated by antibiotics.

This type of experiment revealed the actual effects of microbial activities and interactions in silage on exposure to air. The antibiotics used in this work coupled with the metabolites formed enabled the identification of the responsible groups of micro-organisms.

In order for the autoclaved silage medium in **Experiment type D** to reproduce results obtained in Experiment type C larger quantities of the same silage were required to provide an amount of homogeneous silage medium and perform a proof of principle experiment.

With this type of experiment, which evolved over the duration of the project, the activity of known silage micro-organisms in a defined medium which had very close chemical composition to the silage was possible.

Alltogether the four types of experiments allowed the observation of changes in silage composition during exposure to air as reflected the changes in organic acid and ethanol

concentrations over time. In turn this indicated which type of micro-organism was responsible for the respective changes.

The current *in vitro* approach coupled with unlimited variations in experimental design offers a practical opportunity for investigating microbial activity during aerobic spoilage of silage. Past plate count method can only give evidence of the microbial *status quo* at one point of time and only the potential for deterioration can be estimated.

The model system developed during these studies has allowed a detailed exploration of the relative roles of different microbial groups in the aerobic deterioration process in silage and also some factors which influences their activity. Its simplicity and convenience make it attractive for use in further in-depth studies of the aerobic spoilage process.

7 CONCLUSIONS

1. This study confirmed the lactate assimilating activity of yeasts in silages upon exposure to air.
2. Additionally it demonstrated a co-activity between lactic acid bacteria in grass and grass-legume silage media with a pH of $\geq$ 4.4 at silo opening, but not in maize silage medium in the initial aerobic phase. This activity was revealed by the detection of lactic and acetic acid produced during the first day and frequently the degradation of lactic acid during the second day of incubation.

   The major participation of LAB in aerobic processes is not aberrant as these mainly aerotolerant micro-organisms account for the dominant bacterial group in silages and it also explains the growing numbers of LAB colonies during exposure to air as described in the literature.
3. Activity and competitiveness of LAB depends on their species composition and abundance and that of their competitors. In the presence of yeasts in most of the cases they maintain or increase the lactic acid content, at least during the first day of incubation.
4. Increased air ingress enhances yeast dominance.
5. Increased osmotic pressure by KCl (8 % w/v) inhibits the metabolism of yeasts and LAB likewise.
6. The level of WSC content influences yeast metabolism in particular. The lower the content the faster the pH rise.
7. A second bacterial group that can occur in silage are the propionic acid producers which were only identified by propionic acid formation.
8. Results of the batch culture studies are not directly transferable to the fresh silages. However, the method is a useful tool for elucidating the microbial dynamics and influential factors during exposure of silage to air.

   It shows that even slight changes in growth conditions might alter the end result.
9. The activity and competitiveness of lactic acid bacteria depends very much on the amount of oxygen they were exposed to. Thus many of the results may not refer to surface conditions but to the inner layers of the opened silo.
10. The findings suggest one of the reasons why some silages change more rapidly than others under aerobic conditions, despite containing similar numbers of yeasts:

Aerobic stability of grass silages is a matter of interaction and competitiveness of lactic acid bacteria and yeasts and in some cases propionic acid producing bacteria.

11. The presented *in-vitro* method of Experiment types C and D offers a feasible and systematic opportunity to study aerobic changes and processes in silages under controlled conditions.

Future opportunities

- The opportunities for future investigations by varying the presented method are nearly unlimited.
- The influence of different concentrations of organic acids other than lactate (mainly acetic and propionic acid, WOLTHUSEN *et al.*, 1989) if initially present, especially at low pH, might be investigated, as in preparing the silage medium silage is diluted 1:5.
- A further attempt to increase the osmotic pressure of the medium might be the use of potassium phosphate as suggested by MIDDELHOVEN and FRANZEN, 1986, although salt-intolerance versus osmotolerance might still present the same problem.
- The possible role of bacilli might be studied if the ambient temperature was raised during the incubation period. This would inhibit the less thermotolerant yeasts.
- The role of propionibacteria is yet to be elucidated. Inoculation of different strains of propionibacteria into autoclaved silage medium in individual or co-culture offers one possibility.
- Another very interesting subject is to find out if homofermentative lactic acid bacteria (at least *Lactobacillus plantarum*) are generally more competitive in grass silage medium than in maize silage medium and if so why.

Exploiting the knowledge of microbial interrelationships in silage during exposure to air offers the opportunity to better control aerobic changes in the future.

One of the strengths of this study was that it did not rely exclusively on colony counts, but examined metabolic processes and activity. Thus it provides a focus for future studies on interrelationships between different micro-organisms and factors influencing their growth and activity.

New requirements for future practical silage inoculant research in the industry and for silage management based on the current findings:

- **LAB inoculants for grass silages should be competitive under aerobic conditions.**
- Thus aerotolerant LAB with the ability to metabolise residual sugars into lactic acid under air stress are required.
- High residual sugar contents in grass silages should be ensured to prevent the LAB from decomposing lactate after opening the silo.
- The inoculant should only acidify the grass down to a pH of about 4.4, but be acid tolerant.
- The competitiveness of LAB against yeasts should be enhanced in the feed-out phase by limiting the oxygen concentration in the silo.
- More specific knowledge on PAB in silages is requested. This might require an improvement in culture and screening techniques to enable selection of competitive, aerotolerant and acid tolerant strains.

8 SUMMARY

8.1 Summary

Aerobically spoiled silage is still a problem in animal feeding.

During the last four decades of research yeasts were identified as main initiators of aerobic deterioration of grass silages due to their ability to decompose lactic acid which represents the main silage preservative agent.

Yet the microbial dynamics in silages on exposure to air is still not fully understood.

The aim of the work was to elucidate the role of yeasts under different growth conditions and to identify the role of other micro-organisms possibly involved in aerobic changes of silages.

Therefore silages on exposure to air were simulated in a shaken batch culture system which included the indigenous microflora of a silage and used either a synthetic lactate medium or diluted silage extract as the complex medium. Bacterial and fungal activity was differentiated by the use of antibiotics. Bacterial activity referred to antimycotic treatment, fungal activity to antibacterial treatment. In the control treatment the activity of the mixed microflora was observed. Incubation period was two days at 25 °C ambient temperature. Effects of microbial activity were determined at least 5 times during the incubation period by measuring pH and organic acid and ethanol contents (by HPLC). An attempt was made to compare the results obtained to a standard aerobic stability test.

Generally, naturally fermented high dry matter grass silages (30-40 % DM) with a pH of 4.4-4.8 were investigated.

The following results were obtained:

1. Yeasts oxidised lactic acid as expected, which confirmed earlier studies from literature.
2. In the bacterial treatment, activity of lactic acid bacteria was identified due to formation of lactic and acetic acid.
3. This production counteracted the assimilation of lactic acid in the control where all microbial groups were present resulting in an intermediate level of lactic acid after the first day of incubation.
4. During the second day of incubation lactic acid bacteria often started to oxidise lactic acid, possibly due to the depletion of available sugars.
5. Additional fructose decelerated the pH increase in the antibacterial treatment, but hardly had an effect on the bacterial activity.

6. Tannic acid hampered yeast activity but favoured pH increase by bacteria.
7. The use of KCl as a means of increasing the osmotic pressure to be comparable to silage conditions constrained both bacterial and fungal activity.
8. Increased air ingress and low pH (≤ 4.0) favoured the growth of yeasts over lactic acid bacteria.
9. A high propionic acid production which sometimes occurred in the bacterial treatment could not be explained by LAB metabolism, but was probably due to propionic acid producing bacteria e.g. propionibacteria.
10. This *in-vitro* study revealed co-activity of lactic acid bacteria and yeasts under lightly aerobic conditions which might explain the stability of grass silages that sometimes occurrs despite high yeast numbers.
11. The *in-vitro* method using silage extract as medium was suitable to monitor aerobic metabolic processes in silages and thus indicate the microbial groups responsible for them.

The results suggest that aerobic stability is a matter of microbial interactions and competitiveness.

For further studies on aerobic deterioration the dynamic approach of the method was proved to be extremely useful.

8.2 Zusammenfassung

Aerob verdorbene Silage stellt nach wie vor ein Problem in der Tierfütterung dar.

Im Laufe der letzten vier Jahrzehnte wurden in der Forschung Hefen als Hauptverursacher des Beginns von aerobem Verderb von Grassilagen erkannt, da sie Milchsäure abbauen können, die als das hauptsächliche Konservierungsmittel natürlicherweise in Silage vorkommt.

Trotzdem herrscht noch kein umfassendes Verständnis der mikrobiellen Prozesse in Silage unter Lufteinfluß.

Das Ziel dieser Arbeit war es, die Rolle von Hefen unter verschiedenen Wachstumsbedingungen näher zu beleuchten und die mögliche Rolle anderer Mikroorganismen, die an aeroben Umwandlungen von Silage beteiligt sind, zu bestimmen.

Dazu wurde Silage unter Lufteinfluß in geschüttelten Batchkulturen simuliert. Dabei wurde der epiphytische Besatz der Silage einem synthetischen Milchsäuremedium zugesetzt oder in verdünntem Silageextrakt, der als komplexes Medium diente, genutzt. Bakterielle und pilzliche Tätigkeit wurde durch den Einsatz von Antibiotika unterschieden. Die antimykotische Behandlung beschrieb die bakterielle Aktivität, während die antibakterielle Behandlung zur Beschreibung der pilzlichen Aktivität diente. Die unbehandelte Kontrolle umfasste die Tätigkeit der gesamten Mischflora. Die Bebrütungsdauer betrug zwei Tage bei einer Umgebungstemperatur von 25 °C. Die Auswirkungen mikrobieller Stoffwechseltätigkeit wurden meist zu 5 Zeitpunkten während der gesamten Inkubationsdauer anhand von pH und dem Gehalt an organischen Säuren und Ethanol gemessen (über HPLC). Die Ergebnisse wurden teilweise mit dem Standardverfahren zur Bestimmung aerober Stabilität verglichen.

Im allgemeinen wurden natürlicherweise vergorene hoch angewelkte Grassilagen mit einem Trockenmassegehalt von 30-40 % und einem pH von 4,4-4,8 untersucht.

Dabei wurden die folgenden Ergebnisse gewonnen:

1. Hefen oxidierten Milchsäure wie erwartet. Dies bestätigte frühere Untersuchungen aus der Literatur.
2. In der Bakterienvariante wurde die Stoffwechseltätigkeit von Milchsäurebakterien identifiziert aufgrund der Bildung von Milchsäure und Essigsäure.
3. Diese Säureproduktion wirkte dem Laktatabbau in der Kontrollvariante entgegen, in der alle Mikroorganismengruppen vertreten waren. Das führte nach dem ersten Tag der Bebrütung zu einem mittleren Milchsäuregehalt, der zwischen dem der Bakterien- und dem der Hefevariante lag.
4. Im Laufe des zweiten Inkubationstages begannen die Milchsäurebakterien oftmals, Milchsäure zu oxidieren, was möglicherweise auf die Erschöpfung an verfügbaren Zuckern zurückzuführen ist.
5. Zugesetzte Fructose verlangsamte den pH-Anstieg in der Hefevariante, hatte aber kaum eine Auswirkung auf die Bakterienvariante.
6. Tannin schränkte die Hefeaktivität ein, aber förderte den Milchsäureabbau durch Bakterien.
7. KCl, das dazu eingesetzt wurde, den osmotischen Druck im Medium dem der Silage anzupassen, schränkte sowohl Bakterien als auch Hefen in ihrer Aktivität ein.
8. Erhöhte Luftzufuhr führte zu einer Dominanz der Hefeaktivität über die der Milchsäurebakterien. Einen ähnlichen Effekt hatte ein niedriger pH-Wert von $\leq 4{,}0$.

9. Die starke Propionsäurebildung, die teilweise in der bakteriellen Variante auftrat, konnte nicht durch Stoffwechsel von Milchsäurebakterien erklärt werden, sondern ist auf die Tätigkeit von Propionsäure bildenden Bakterien wie die Propionibakterien zurückzuführen.
10. Diese *In-vitro*-Untersuchung zeigte eine gleichzeitige Aktivität von Milchsäurebakterien zu Hefen unter eingeschränkt aeroben Bedingungen auf. Diese kann erklären, warum manche Grassilagen trotz hoher Hefekeimzahlen stabil bleiben.
11. Die *in-vitro*-Methode, bei der Silageextrakt als Medium benutzt wurde, erwies sich als geeignet, um aerobe Stoffwechselprozesse in Silagen zu beobachten und lieferte damit einen Hinweis auf die dafür verantwortlichen Mikroorganismengruppen.

Die Ergebnisse legen nahe, dass aerobe Stabilität eine Frage von mikrobieller Interaktion und Konkurrenzfähigkeit ist.

Für weitere Untersuchungen zum aeroben Verderb hat sich dieser dynamische Versuchsansatz als besonders geeignet erwiesen.

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Erklärung

Hiermit erkläre ich, dass ich die eingereichte Dissertation selbständig und ohne fremde Hilfe verfasst, nur die von mir angegebenen Quellen und Hilfsmittel genutzt und die den verwendeten Werken wörtlich oder inhaltlich entnommenen Stellen als solche kenntlich gemacht habe.

Rostock, Oktober 2005

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THESES

"AN *IN-VITRO* STUDY OF AEROBIC CHANGES IN SILAGES – Effects of microbial activities and impact factors"

submitted by Siriwan Martens

The aerobic deterioration of silages represents a major problem for animal feeding.

Microbial metabolic activity leads to dry matter and energy losses in the silage accompanied by reductions in feed value and palatability. If mycotoxins develop they can directly affect the health of animal and humans.

To avoid aerobic spoilage of silages it is necessary to understand its basic principles.

Aim of the work

1. Many investigations showed that yeasts are responsible for the initiation of aerobic deterioration of silages, especially of grass silages. This is due to the ability of many yeasts to assimilate lactic acid which represents the principle preservative agent of silages.

   On the other hand, it has been observed that low numbers of yeasts at the time of opening do not necessarily reduce the chances of aerobic instability, nor do high numbers of yeasts always indicate that rapid deterioration will definitely occur.

2. Moulds were found occasionally, but in general their activity was not closely related to the decomposition of organic acids. Different bacteria were also isolated from deteriorating silages such as proteolytic bacteria (bacilli), lactic acid bacteria, acetic acid bacteria, actinomyces, clostridia and listeria. Among those, the deleterious role of acetic acid bacteria was clearly identified in the initiation of aerobic spoilage in maize silages. After the oxidation of ethanol to acetic acid the acetic acid bacteria grow at the expense of complete oxidation of acetate to CO_2. The thermotolerant bacilli develop during the later stages of deterioration. Clostridia can occur in anaerobic niches of deteriorating silages. Under certain conditions listeria are accompanying micro-organisms. Lactic acid bacteria are generally the predominant bacterial micro-flora at the time of opening as they have desirable activities in terms of preservation and are commonly added as inoculants to improve the lactic acid fermentation.

3. The above mentioned findings are mainly based on plate count methods. Those do not always reflect activity. To gain new perceptions a novel method was used.

4. The present investigations elucidated the roles of bacteria and fungi respectively in aerobic changes of silages.

5. An *in-vitro* method was developed which simulates the silage environment in shaken batch cultures. pH and some metabolites such as lactic, acetic and propionic acid and ethanol were measured as indicators of microbial activity in a time course experiments. To differentiate bacterial and fungal action antimycotic and antibacterial agents were used in a medium containing the indigenous microflora from silage.

6. The studies comprised three principle types of experiments:
   a) Yeast inoculants (4 type strains) inoculated into synthetic lactate medium
   b) Silage extract as a complex medium plus the indigenous microflora – 9 grass silages, 4 grass-lucerne silages and 1 maize silage were used to provide the extract
   c) Autoclaved silage extract from one grass and one maize silage with yeast (2) or/and lactic acid bacteria (1) inoculants

7. Principle variations in experiment types 2 and 3:
   - Addition of fructose (3 and 6 % of FM w/v) to investigate the effect of increased levels of residual sugars
   - Adjusting the pH to 3.8 or 4.4 to investigate the pH effect
   - Changing the liquid volume to air space ratio to investigate the influence of different oxygen ingress (100 ml vs 200 ml Erlenmeyer flasks)
   - Use of 8 % KCl (w/v) to adjust the osmotic pressure to normal silage conditions

Main conclusions of the work

8. The lactate oxidising activity of yeasts was confirmed.

9. A co-activity of lactic acid bacteria was revealed. They produce lactic acid in grass and grass-legume silage media with an initial pH of ≥ 4.4 during the first day of incubation, but not in maize silage medium.

10. This activity counteracts the lactate decomposition by yeasts in the initial phase of exposure to air and thus maintains or even increases the total concentration of lactic acid. During the second day of incubation lactic acid bacteria often contribute to the lactate degradation.

11. A high level of residual water soluble carbohydrates hampers the pH rise by yeasts which consume organic acids and produce increased levels of ethanol by respiro-fermentation.

12. Yeasts are more competitive at low pH (≤ 4.0) or with increased air ingress.

13. Propionic acid production occurred occasionally. It can be attributed to bacteria whose major fermentation product is propionic acid such as Propionibacteria.

Scientific evaluation of the results

14. The results confirm earlier investigations that yeasts represent the main spoilage microflora in grass silage. A new insight is provided, in that lactic acid bacteria compete with yeasts under aerobic (but oxygen limited) conditions and are able to slow down the onset of aerobic deterioration which is characterised by the decomposition of the preserving lactic acid.

15. Another new finding is that propionic acid producing bacteria are active along with (facultatively) aerobic micro-flora of some grass silages.

16. The *in-vitro* method applied was suitable to investigate the metabolic activity of silage microflora under defined conditions.

17. Suggestions for further investigations are:
    - to gradually increase the ambient temperature during the course of incubation as it occurs in practice during deterioration,
    - to reduce the dilution rate of the silage extract to see if there is a possible effect of concentration
    - apply other means to raise the osmotic pressure to avoid the problem of salt-intolerance

- to find out if homofermentative lactic acid bacteria are generally less competitive in maize silage medium and why
- to elucidate the role of Propionibacteria.

Common importance of the results

18. Recommendations to control aerobic deterioration:

    The most general task is to support the competitiveness of lactic acid bacteria in silages upon exposure to air.

    To meet this claim:

    - High residual sugar contents should be ensured to prevent the lactic acid bacteria from decomposing lactate at an early stage after opening the silo.
    - Oxygen ingress in the silage should be limited during the feed-out phase to enhance the competitiveness of lactic acid bacteria against yeasts.
    - Whenever possible, grass should only be acidified down to a pH of about 4.4 in the ensiling phase.
    - If inoculants are applied they should be aerotolerant and acid tolerant - criteria which are already postulated for the ensiling phase.

APPENDIX INDEX

Own Preparatory Work

The preliminary aim of the work was to investigate the role of yeasts in aerobic deterioration of grass silage. In the literature there are numerous reports that yeasts are the initiators of aerobic instability in grass and other silages (see LITERATURE REVIEW), especially yeasts with the ability of assimilating lactate (JONSSON and PAHLOW, 1984; JONSSON, 1989).

JONSSON and PAHLOW, 1984, stated that a population of > 5 log cfu/g silage FM at the time of opening causes fast aerobic deterioration if the yeasts can utilise lactic acid aerobically.

In order to investigate whether those observations can be generalised, first own observations were made in small scale laboratory silages in 2002/2003: if ensiled with defined air infusion (2*24 h over a 49 d storage period) (comparable to practical farm conditions) all unstable silages contained at least 5 log cfu/g silage FM. On the other hand, if ensiled strictly anaerobic low dry matter grass silages (~ 25 % DM) were unstable with yeast numbers between 3-4 log cfu/g FM on malt extract agar and similarly on lactate agar which was introduced by JONSSON and PAHLOW, 1984, whereas in unstable high DM silages (35-40 % DM) yeast numbers varied widely from below the detection limit up to 5 log cfu/g FM (Figure 4 in the LITERATURE REVIEW; MARTENS and PAHLOW, 2003).

The above mentioned lactate agar for the enumeration of lactate assimilating yeasts offering lactate as sole carbon source has a pH of 3.4-3.8.

The question arose whether this medium would allow the development of all lactate assimilating yeasts grown under different conditions in silages. The two main differences between low and high dry matter grass silages were seen in pH and osmotic pressure, which are both higher in high DM silages. The pH of the latter can vary between 4.0 and 5.5 or even higher.

MIDDELHOVEN and FRANZEN, 1986, investigated the ability of 6 yeast species (15 type strains) to assimilate lactate by growing them at pH 5.8 and pH 4.0 in liquid cultures. They stated that most of the strains were able to grow with lactate as carbon source at the lower pH even when they did not grow at all at pH 5.8.

Thus, to answer the above mentioned question, in the own work, the pH of the lactate agar was varied between 3.8 to 6.0 in 5 steps. In a second treatment the osmotic pressure was raised by 8.3 % KCl in the different pH levels respectively.

Mixed silage flora was plated on the original lactate agar and on another one with a pH adjusted to the original pH of the silage.

Yeast isolates from grass silages were plated on 5 pH levels and the medium supplemented with KCl.

No distinct effect could be observed except an inhibition by KCl.

As even a yeast identified as *Saccharomyces cereviseae* whose abilities of lactate assimilation are inconsistent for taxonomical purposes grew on all modifications of the lactate medium it was doubted that the only offered carbon source was used.

Growth tests on only Yeast Nitrogen Base agar and on pure agar without external C-source were positive. That led to the assumption that some yeasts can either a) utilise nutrients from agar or b) carry over nutrients in their cells from the former growth medium. Additionally in a mixed yeast flora even an exchange of nutrients on the agar plate is possible, so that yeasts which cannot grow on the plates by their own metabolism can survive by others (HOFFMANN, 2004). DAVENPORT, 1980, also emphasizes that a medium is only part of an environmental system which includes interactions and carry-over. LOUREIRO and MALFEITO-FERREIRA, 2003, describe the general obstacles associated with selective media.

The conclusion was that the plate count method was not specific enough to study the role of yeasts in high DM grass silage deterioration and the factors influencing their growth and activity.