Role of microflora in forage conservation

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This paper attempts to update relevant information on forage microbiology provided since the 3rd General Meeting of the EGF.

Consequently it is founding rather than reporting on the reviews and monographs on this subject, published since that time. A selection is given in the references (Beck, 1978, Daeschel et al., 1987, Day, 1987, McDonald, 1981, Seale, 1986, Woolford, 1984).

The general way of handling this topic has been substantially influenced by the outstanding papers of Kandler (1983) and Condon (1987) on carbohydrate metabolism and oxygen-response of lactic acid bacteria respectively.

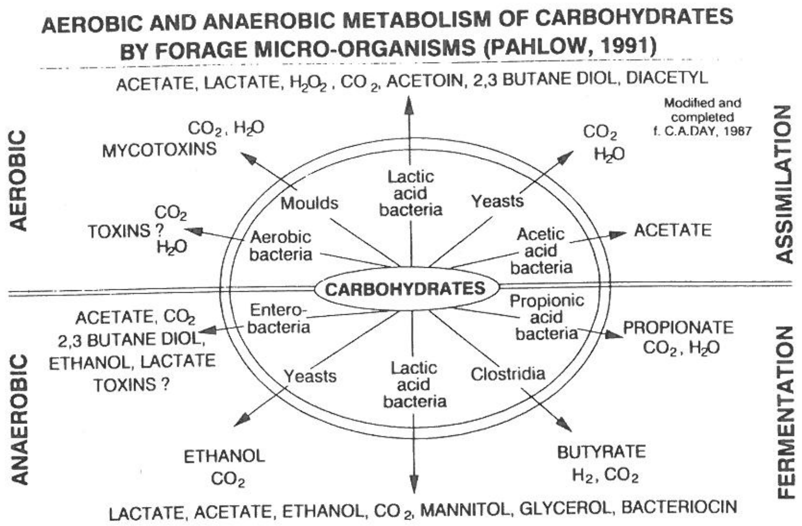


Fig. 1

Fig.1 shows how this approach has been applied to the most relevant groups of micro-organisms, which play a role during forage conservation. The scheme is centred on the chief substrates, comprising different sugars from structural or storage carbohydrates along with organic acids and alcohols. These can be either originally present in plant material and other substrates or can be produced during the conservation process. The scheme is subdivided into the hemispheres of aerobic and anaerobic metabolism of these carbon sources.

In the upper half the obligate aerobes and facultative anaerobes with their main products are given. All can grow in the presence of oxygen so long as their requirements for nutrients, water and temperature are fulfilled. Most of them catabolize organic compounds down to the end products carbon dioxide and water, often accompanied by the generation of heat. Others are confined to the production of intermediates, which then are further oxidised by the remainder of the aerobic flora. Most members are particularly acid-tolerant.

The lower half of the scheme encompasses the facultative and obligate anaerobic organisms, primarily relevant during forage conservation by ensilage.

Aerobic bacteria

This first large group comprises an omnium-gatherum of obligate aerobe and facultative anaerobe bacteria. The lactic acid bacteria (LAB) and the acetic acid bacteria (AAB) could be placed here as well; however, both will be treated separately.

Due to their particularly simple nutrient requirements the Gram-negative aerobic pseudomonads and enterobacteria often develop populations which exceed 10^7 colony forming units(cfu)/g of the fresh crop.

The pseudomonads include bacteria that are among the most metabolicly versatile organisms known. They are capable of using as many as 100 different organic compounds as sole sources of carbon and energy. The enterobacteria on the other hand can utilize the nitrogeneous constituents of organic compounds as energy-yielding respiratory substances. For anaerobic growth however, they are strictly dependent on fermentable carbohydrates (Stanier et al.,1971).

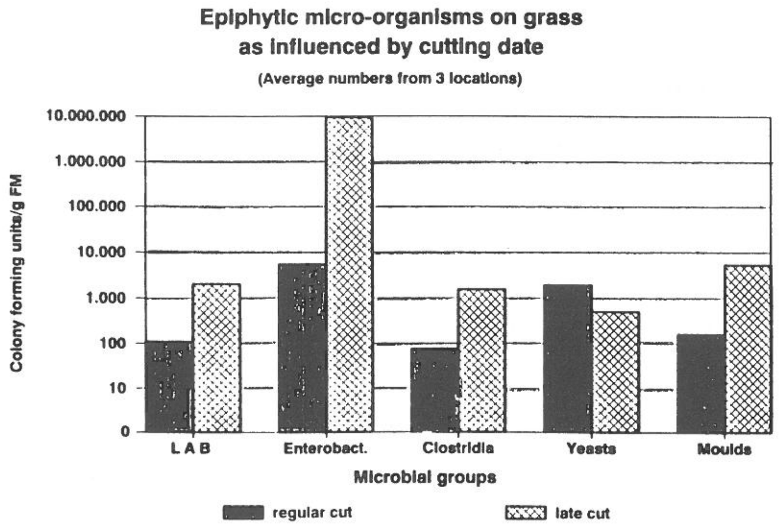


Fig. 2

On a standing crop, e.g. of grass, the latter organisms show the most dramatic increase with plant age, indicating their less fastidious nature under these conditions if compared to other relevant epiphytic microbes (Fig.2).

The metabolic activity of both the pseudomonads and the enterobacteria is readily inhibited during the conservation process either by anaerobiosis or acidification. However, it should be specifically noted that the toxic substances contained in many Gram-negative bacteria are rather stable and will remain largely unaffected over extended periods. The final concentration of these endotoxins will thus be closely related to the maximum population previously reached by these organisms and their persistence (Lindgren et al., 1987). Consequently, a negative effect on palatability and/or nutritive value of late cut forage can be expected also from these compounds in addition to the reduced digestibility of such old material. A similar increase in numbers of enterobacteria can be recorded after extended field periods during unfavourable weather conditions.

If at all, enterotoxins are still very difficult to monitor and their determination is not included in the routine analyses of feedingstuffs.

The remainder of the microbial groups, the soil-borne actinomycetes and the endospore-forming bacilli are assignable on ecological grounds to the microflora dominating the later stages of aerobic deterioration mainly of low-moisture forages. Because of their hygienic or even pathogenic relevance they are considered further in the paper given by Prof. Lindgren.

An interesting exception is the application of *Bacillus* spores as an inoculant for the preservation of high moisture alfalfa hay, as reported by Dr.Tomes in her poster presentation.

Moulds

The hyphal fungi are generally recognized to be completely undesirable spoilage organisms in conserved forage. Just two special aspects should be considered in this paper:

1. The minimum oxygen requirements of certain mould species
2. The "threshold" of visible moulding in conserved forages

The vast majority of moulds is strictly dependent on air for growth and propagation. However there is by no means a reliable "all or nothing" reaction to oxygen of these organisms, especially in farm silos, where complete anaerobiosis is impossible to achieve. Even minute quantities of oxygen are sufficient to maintain the metabolism of particular members of this group. This is indicated not only by the continously spread of their mycelium throughout the conserved material but also by the generation of conidiospores or other propagules.

Penicillium roqueforti has become a serious problem in the last few years. This mould is able to complete its conidial stage at a considerable distance from the outer surface of perfectly sealed and well compacted clamps e.g. prepared from pressed sugar beet pulp or corn cob mix, and increasingly also from whole plant maize. Tab.1 shows the results from investigating paired samples of six different silages. In all cases the fungal counts from the greenish-blue moulded, ball-shaped material, virtual pure cultures of *P. roqueforti*, have been compared to samples of corresponding silage, taken from the same cutting face, but still having a perfect appearence.

Incidence of Penicillium roqueforti in the cutting face of identical silos with and without visible moulding (cfu/g FM)

G. PAHLOW 1991

Silo number	I	II	III	IV	V	VI
moulded area	$4x10^6$	$3x10^8$	$6x10^7$	$3x10^7$	$2x10^7$	$6x10^7$
not visibly moulded area	$1x10^3$	$2x10^3$	$2x10^2$	$8x10^2$	$5x10^4$	$2x10^3$

Tab. 1

As expected, a large number of colonies/g FM developed from the visibly moulded samples. However, the invisible mycelium was already present in the rest of the apparently non-infested silage as well. The particular risk of mycotoxins, produced also some distance from the obviously spoiled material, will be subject of the special paper, given by Dr. Oldenburg.

In conclusion, one can state that the "threshold of visible moulding", often used by technical engineers to define beginning spoilage, is not suitable for a reliable judgement about the limitations of conservation techniques.

Lactic acid bacteria

Most investigations of LAB in conserved forage are focussed only on those stages after an anaerobic environment has been achieved. However, the aerobic metabolism of these bacteria is worthy of consideration as well. Pure culture studies with LAB have shown that all can grow satisfactorily in absence of oxygen; but under aerobic conditions some are inhibited partially or completely. Consequently it is quite usual to conclude that the primary ecological niche for the LAB is an anaerobic environment and metabolism in presence of oxygen, as on plant surfaces, is somewhat aberrant.

Nearly all LAB react to oxygen by generating hydrogen peroxide (H_2O_2), which is principally toxic for themselves, and it depends upon the attendant ambient conditions whether the consequences are beneficial or detrimental.

Advantages of aerobic metabolism can be e.g.:

- higher energy gain, often resulting in faster growth rates, if sugars can be converted to acetate rather than lactate
- improved antagonistic properties against other organisms by virtue of characteristic products such as acetoin and diacetyl (Lindgren and Dobrogosz, 1990)
- an increased spectrum of utilizable carbon-sources

- an improved protective system against adverse environmental conditions, developed via an inducible oxidation stress response.

These advantages for the LAB during their epiphytic stage will be realized only if a lasting accumulation of autoinhibitory or even lethal concentrations of H_2O_2 can be avoided. However, these bacteria generally lack catalase, possessed by other facultative aerobic micro-organisms, to eliminate toxic hydrogen peroxide.

Better knowledge about the response of epiphytic LAB to catalase or other defense mechanisms, however of plant origin, could contribute to explain the apparent "chopper inoculation", a phenomenon encountered also during a large-scale ecological study of epiphytic LAB on grass (Ruser, 1989). Its elucidation would be of considerable practical relevance. It would allow the more reliable estimate of LAB numbers, required, e.g., for currently developed prediction models (Muck,1990). The apparent inoculation of the crop by the harvesting machinery has been found also by some authors of posters, which are presented during this conference (Andrieu and Gouet, Lin et al.,Satter et al.).

Repeatedly, an immediate increase in numbers of LAB during the harvesting procedure has been observed as compared to a standing crop, from which no or only a small number of LAB could be recovered.

The striking difference, sometimes of a factor of 100 or more, can neither be the result of multiplication, considering the time scales given, nor of a continous contamination by the harvesting equipment, as shown by the detailed investigations of Pahlow and Ruser,(1989) and Pahlow and Müller,(1990).

However, an important difference exists between material taken prior to and after chopping. Whereas samples from the standing crop are usually cut by a scissor or scythe, leaving the material undamaged, except at the cutting site, machine-harvested forage will be heavily lacerated and covered with juice and cell contents, released from the ruptured plant tissue. Thus nutrients and other substances become instantly available for the epiphytic microflora during harvesting.

Characterization of cells in survival stages of progressive dormancy

Survival at the following stage in the continuum

Method of detection	Culturable	Recoverable	Growth responsive	Metabolically active	Dormant	Intact	Dead
Acrid. orange dir. c.	+	+	+	+	+	+	-
Anim.-pass.recovery	+	+	+	+	+	-	-
Substrate uptake	+	+	+	+	-	-	-
Direct viable count	+	+	+	-	-	-	-
Acclimation, plt. count	+	+	-	-	-	-	-
Standard plate count	+	-	-	-	-	-	-

Viable

Somnicell

ROSZAK and COLWELL (1987)

Tab. 2

Combining these facts with the available information about the detrimental effect of oxygen radicals on LAB, the following hypothesis can be created:

LAB on the outer surface of intact plants have developed a survival strategy in response to this unfavourable environment by entering into a reversible pre-step of a resting stage, called "Somnicell" (Tab.2). This term has been proposed by Roszak and Colwell,(1987), to describe bacteria in a "viable but non culturable stage, which exhibit living attributes other than the ability to reproduce in culture media". However, exactly the latter capability is essential for the recovery of the micro-organisms from plant material by the standard enumeration techniques, which have been used in nearly all studies on this subject.

Initiating factors, which can cause active cells progressively to turn to dormancy, could be drying, heat, starvation, visible or UV-radiation and, last but not least, toxic oxygen species.

The last negative factor can be counteracted very specifically by enzymes from the disintegrated plant material such as catalase and superoxide dismutase or by manganese compounds, which after accumulation in the LAB act as a defense against endogenous O_2^-.

In conclusion, the harvesting procedure exerts a specific influence on the epiphytic micro-organisms, especially the lactobacilli, substancially enhancing the recovery of a previously dormant population from forage crops by standard enumeration methods.

This view then is in keeping also with the results of Moran and his co-authors. They could observe no increase during harvest, because under the temperate conditions of Ireland there was obviously a numerous, non-dormant flora already present on the standing crop, which did not require resuscitation by detoxifying enzymes from the cell contents.

Numbers of epiphytic lactic acid bacteria on grass during the growing season

(Fehrmann/Müller 1990)

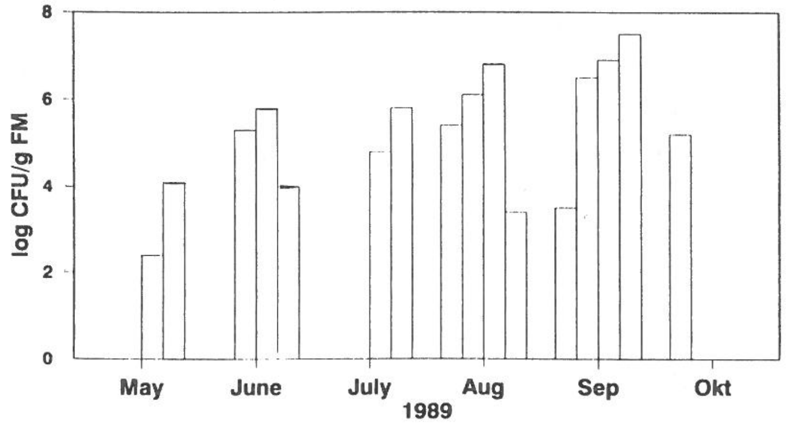


Fig. 3

Finally, a better interpretation of unexpected fluctuations of the epiphytic population will be possible with the "Somnicell"-theory in those cases where, so far, distinct changes could be explained only by overproportional multiplication or die-off rates of the micro-organisms (Fehrmann and Müller,1990, Fig.3).

Yeasts

All yeasts grow well in presence of oxygen, where energy gain as well as the spectrum of metabolizable carbohydrates is much larger than under anaerobic conditions. Their significant role in the aerobic deterioration of forages is well established and has been thorougly reviewed only recently (Woolford,1990).

However, the following table could be useful to classify this large and heterogeneous group according to the relevance of their physiological subgroups for forage conservation. Again they are characterized by their substrate requirements and response to oxygen (Tab.3).

CHARACTERISTICS OF SILAGE YEASTS

G. Pahlow (1989)

Yeast groups	- Substrates - - - - - - -		Conditions
	Carbohydrates "sugars"	Organic acids	Oxygen
Field yeasts	+	–	+
Silage yeasts Type I	+	–	+ –
Silage yeasts Type II	+		+ –
		+	+

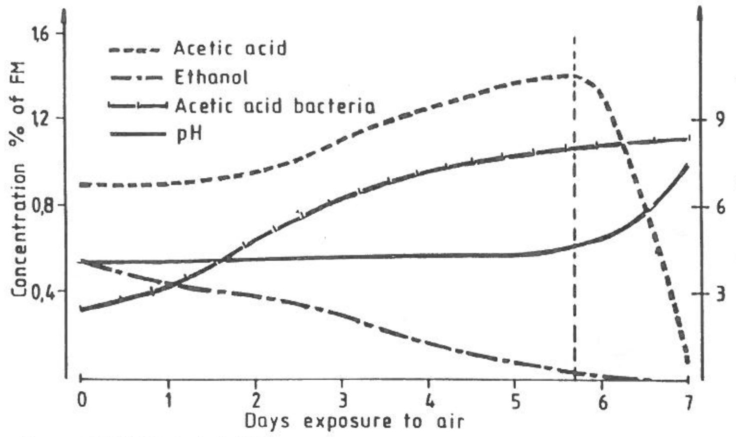
Tab. 3

The so-called field yeasts, adapted to the plant habitat, can utilize sugars only in presence of oxygen. They don't survive without air. The next subgroup develops also anaerobically as long as they can successfully compete for fermentable carbohydrates. In forages with any access of oxygen this type is rapidly superseded by the third subgroup, able to utilize organic acids. These organisms usually represent up to 100 % of the yeast flora in farm silages after extended storage.

Acetic acid bacteria

High-energy silages often contain elevated levels of ethanol, which can be oxidised by the AAB to acetic acid and in a further step even towards CO_2 and water. Dr. Spoelstra and his group have identified these organisms as initiating aerobic deterioration of maize silage.(Fig.4). According to Bergey's Manual of Systematic Bacteriology, the AAB are strictly aerobic organisms. So it is not clear, how they can survive in perfectly sealed silos. However, as early as 1932 Ruschmann has provided evidence that the "true acetic acid bacteria", which were carefully separated from the acetate-producing Coli-Aerogenes-Group, could grow anaerobically by a yeast-like fermentation of sugars to ethanol and CO_2. It will be a challenge for the immediate future to study this aspect with several strains isolated from silage.

Acetic acid bacteria and silage parameters during unloading



Source: SPOELSTRA et al. 1988

Fig. 4

Entering the anaerobic hemisphere of silage fermentation (Fig.1), the Enterobacteria (EB) have to be considered once more. As mentioned already, these undesirable and loss-generating competitors of the LAB can be suppressed by acidification. A superior LAB flora (Fig.5), as established in one of the silages by inoculation, can inhibit the EB very fast and keep their chance of continuing the production of enterotoxins to a minimum.

Suppression of Enterobacteria by different LAB-flora

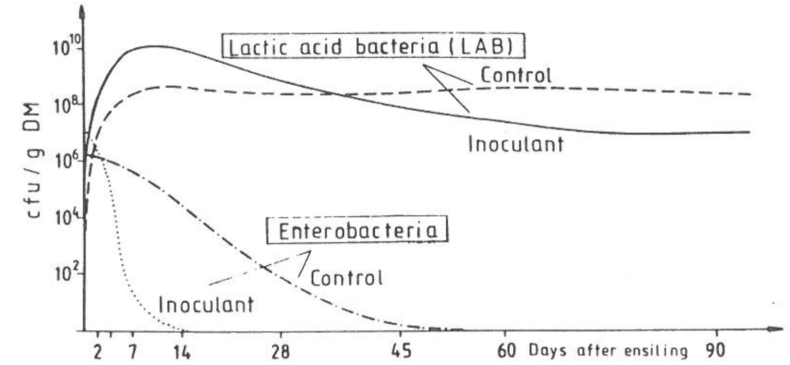


Fig. 5

The major role EB play in the degradation of nitrate in silage via the anticlostridial intermediate nitrite has been intensively studied by Spoelstra (1987). Members of *Escherichia*, *Hafnia* and *Klebsiella* were predominant in his investigations. Which possible role *Rahnella aquatilis*, frequently isolated from grass in Braunschweig and found also by Heron et al.(1990), could play among this microbial group remains to be further investigated. Generally, a flora of EB, persisting in silage, effectively counteracting acidification, prolongs the phase during fermentation, until they are eliminated from the conserved forage.

Yeasts

Yeasts can also cause considerable losses in dry matter during anaerobic growth, while the energy losses remain negligible, because the high energy compound ethanol is their main product. Without oxygen most silage yeasts can use only a few carbohydrates, including those which are homolactically fermented by certain LAB. Selected LAB-strains, able to compete successfully for the same sugars, have a real chance, of keeping the yeasts in grass silage well below the critical level of 10^5 cfu/g of forage, known to result in an aerobically unstable product on exposure to oxygen. In maize silage the situation is different, as it is unlikely that fermentable sugars will be exhausted to a similar extent as in grass silage.

For the last three groups only a few aspects have been identified, which are not up to date according to the premises of this paper. Most concern recent developments in the field of microbial additives.

Lactic acid bacteria

In silage inoculants, consisting of LAB in combination with an enzyme preparation, the effect of the latter component has usually not been very satisfactory. For a rapid, sufficient release of fermentable sugars from structural carbohydrates the activity of the enzymes was often far below the needs. Only recently a silage inoculant has become available, claimed to comprise LAB which are genetically equipped with exogenous DNA, coding for an appropriate enzyme.

Convincing data about the capabilities of this newest type of inoculant are not yet available. Its suitability will mainly depend upon the amount of saccharolytic enzymes produced during bacterial growth, the enzyme activity as influenced by pH and temperature and the homolactic fermentability of the sugars released for the corresponding LAB.

Saccharolytic clostridia

The indisputable negative role, which clostridia play during anaerobic spoilage and the subsequent problems they cause in cheese production by virtue of their heat resistant spores in milk, are well documented. Their economic importance has stimulated efforts, to control them efficiently by supplementing silage inoculants with viruses, which specifically act only on closely related vegetative cells of clostridia. (In line with the term "bacteriophages", they have been named "clostridiophages").

The high degree of homology required for an infection turned out to be a limitation for the general applicability of this principally very logical combination. It will be nearly impossible to provide in one single inoculant sufficient phages against all strains of clostridia which may be encountered in European silages. Another limitation arises from the fact that only active cells but not the spores can be destroyed, and that this attack must occur before the pH in silage has decreased below 5, where the clostridiophages lose their activity (Ogata and Hongo, 1979).

Propionic acid bacteria

This last group has been investigated by Prof. Lindgren's group in 1983 among other inoculants for its performance in silage with little success, as no propionic acid fermentation was initiated.

However, the requirements of the PAB obviously match well with the conditions in good silage such as low pH, anaerobiosis, fermentable sugars as well as organic acids. Consequently, with respect to their main product propionic acid (PA), it seemed worthwile trying to establish these bacteria in a silage, it being acceptable for a small quantity of lactate to be fermented to PA in favour of improved aerobic stability.

In a preliminary study with grass silage in Völkenrode a rather high population of PAB was achieved, which produced the equivalent of about 6 l of PA/t FM and considerably increased the aerobic stability of the resulting silage (cf.the paper of Dr. Honig, reporting on the same experiment).

Conclusion

In spite of our gain in knowledge about the role of micro-organisms in forage conservation which has been achieved during the last two decades, more new questions have been raised than we were able to answer. However, a rather optimistic perspective seems to be justified, as more people than ever have recognized the relevance of forage-microbiology for future progress in this applied field of research.

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BIOCHEMISTRY IN FORAGE CONSERVATION

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The biochemical processes which come into play immediately after a plant is cut and which continue throughout the storage period are many and varied, especially if the crop is conserved when the dry matter content is low. Evidence of this is to be found in the odours emitted from silages and in a comparison of gas chromatograms of volatile components in extracts of steam distillates from grass with those from high moisture silages (Kibe and Kasuya, 1979). Details of the pathways followed in many of the reactions which occur are given by McDonald *et al* (1991) and Woolford (1984). If controlled, these reactions will contribute to the efficient conservation of crops as fodder for livestock. Many, however, have yet to be identified and some of these may hold the key to an explanation of why some silages are more palatable than others or are used more efficiently for production.

Biochemical processes occurring during conservation result from the continuing metabolism of the plant cells, from the enzymes of the dead tissue and from the microorganisms on the plant (Table 1).

TABLE 1. **Factors influencing the conservation of crops**

Enzymes	**Microorganisms**
Respiratory	Lactic acid bacteria
Proteolytic	Enterobacteria
Polysaccharide-degrading	Clostridia
	Fungi
	Yeasts
	Moulds
	Bacillus
	Listeria
	Acetic acid bacteria
	Propionic acid bacteria

ENZYMES

Respiration

Plants obtain energy and reducing power not only from light reactions of photosynthesis but also from the degradation or respiration of products of photosynthetic carbon dioxide fixation (Duffus and Duffus, 1984). The overall reaction for respiration is generally represented as the complete oxidation of a molecule of glucose: