THE EFFECT OF WILTING ON BUTYRIC ACID FERMENTATION IN SILAGE [1]

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Summary

A comparatively slight increase in osmotic pressure inhibits the development of butyric acid bacteria. The salt (or osmotic pressure) tolerance of butyric acid bacteria decreases with an increasing H-ion concentration in the environment. Sub-lethal salt and/or H-ion concentrations lead to a retarded commencement of clostridial development and to a reduced production of butyric acid.

It was demonstrated by means of an ensiling experiment that in wilted grass silage the osmotic pressure may play a considerable part in repressing butyric acid fermentation in the initial period of the fermentation process.

Introductory

Since the introduction of the method of ensiling wilted green fodder it has been found that the dry matter content of the material to be ensiled has a considerable effect on the fermentation processes taking place in silage. In view of the slight fermentation losses, together with the technical advantages of the wilting method, it is possible that there may be an increasing use of ensiling in the future (Kappelle et al., 1952).

Remarkably enough, there is still very little known regarding the preservative effect of wilting. To judge from the standards applicable to wet silage, wilted silages are always found to have an appreciably higher pH than might have been expected from the figures for butyric acid, ammonia and lactic acid, or in other words the correlations established for wet silage between pH on the one hand and butyric acid, ammonia and lactic acid on the other are not applicable to wilted silages. This indicates that in wilted silage the suppression of the butyric acid bacteria is not caused by the concentration of H-ions but by another factor.

The concentration of the dry matter during wilting can obviously be adduced in explanation of the preservative effect. Stolk (1956) found from experiments with pure cultures of *clostridia* that the dry matter content as such had a growth-inhibiting effect. When these experiments were repeated in this laboratory it appeared that the inhibition found by Stolk must have been partly due to oxygen adsorbed to the cellulose, since when the effect of oxygen was entirely excluded by special measures, it was found that butyric acid fermentation occurred in a medium with up to 60% dry matter (in the form of purified cellulose). But growth was inhibited when grass meal was used instead of purified cellulose (Wieringa, 1956).

In view of this result it seemed probable that the growth inhibition in wilted silages is caused by soluble compounds in silage and/or grass.

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It is of importance that during wilting there is also an increase in the concentration of carbohydrates [2]), so that wilted silage may have a higher content of lactic acid than wet silage. That this higher lactic acid content does not lead to a lower pH is due to the fact that the protein concentration also increases. RICHARD (1946) found that it was not the concentration of H-ions but the concentration of undissociated lactic acid that was responsible for the growth inhibition of the butyric acid bacteria. Although the media employed by RICHARD are not altogether comparable to silages, it may be assumed that a sufficient amount of undissociated lactic acid may occur in first-class wilted silages to afford an explanation of the good preservation, despite the high pH.

It should be remembered however, that at the time the silage is being made the lactic acid still has to be formed and that it may take a week or more to reach the necessary concentration. The great risk facing preservation is that the lactic acid decomposition caused by the *clostridia* may keep pace with production during this initial period. This fermentation trend does not occur in wilted silages, or only to a much lesser degree. So it may be concluded, that the growth inhibition of the butyric acid bacteria is caused by the soluble compounds in the grass which are concentrated during wilting. DE MAN (1957) has already indicated the possibility that the osmotic pressure may be a factor in this case. Hence the object of the investigation described here was to determine the effect of osmotic pressure on the growth of butyric acid bacteria.

METHODS

The investigation was conducted with 10 *Clostridium* strains. The basic medium used for the growth inhibition experiments had the following composition :

Yeast extract	1 %
Glucose	2 %
Sodium acetate	0,5 %
Sodium thioglycolate	0,05 %

At first the medium also contained 0,2 % of agar and the tubes were incubated aerobically at 35° C. For technical reasons the agar was omitted from the subsequent experiments and the tubes were incubated in anaerobic jars with 90 % H_2, 10 % CO_2, the pH's always being adjusted with 1 N. NaOH or 1 N. HCL.

Bacterial growth was examined macroscopically every day and in doubtful cases also microscopically after 20 days.

Butyric acid in the cultures was determined chromatographically with filter paper, using butanol water in an ethylamine-saturated atmosphere.

Grass silages were made in 2-litre preserving jars by a previously described method and stored at 30° C (WIERINGA, 1957).

Relative humidity figures and freezing points were determined in order to assess the osmotic value of the fluid media, and the silages. The equilibrium relative humidity (ERH) was determined both by the LiCl-cell method (MOSSEL and VAN KUYK, 1955), and the statical method. The latter method proved to be more reliable than the former, so that the ERH figures given were all determined by the statical method. The freezing points were determined in fluid media and in press juice taken from the silage samples.

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[2]) By concentrations is here only meant the percentage in the wet material.

RESULTS

1 *Effect of the increased osmotic pressure on clostridia in liquid media*

The clostridium strains used in the investigation could be classified into two groups according to their biochemical properties, following Bergey's Manual (table 1). The fermentation of lactate in the presence of acetate caused by the *Cl. butyricum* strains entirely agrees with the observations made by BHAT and BARKER (1947) and warrants the conclusion that both *Cl. butyricum* and *Cl. tyrobutyricum* in silage will participate to an equal extent in the lactic acid decomposition.

Table 1 Classification of *Clostridium* strains.

Strain	Isolated from	Name	Growth and gas in:				
			milk	lactose	saccharose	salicine	lactate + acetate
274	grass-silage	*Cl. butyricum*	+	+	+	+	+
370	,,	,,	+	+	+	+	+
OL 2	,,	,,	+	+	+	+	+
G 1	soil	,,	+	+	+	+	+
OR	grass-silage	,,	+	+	+	+	+
OO	,,	,,	+	+	+	+	+
A	steamed potato silage	,,	+	+	+	+	+
K 4 [1])	cheese	,,	+	+	+	+	+
S 46 [1])	silage	*Cl. tyrobutyricum*	–	–	–	–	+
Z	grass-silage	,,	–	–	–	–	+

[1]) These strains were kindly supplied by the State Agricultural Experimental Station at Hoorn; and the Netherlands Dairy Produce Research Institute, Ede.

As table 2 shows, the NaCl tolerance for the 10 strains differs to a fairly considerable extent. It should be noted that the results obtained were not entirely reproducible. The occurrence of small differences in salt tolerance is no doubt due to the fact that the butyric acid bacteria have less chance to grow as the osmotic pressure increases. Hence the figures in table 2 are compiled from three experiments, the tolerance given being the maximum found for each strain.

It had already been found from experiments with the semi-solid medium that the pH and an effect on the salt tolerance and the reproducibility of the results could be increased by carefully adjusting the pH of the medium. This led to the following experiment. Of six strains the salt tolerance was determined in the liquid medium at varying pH's (table 3). The investigated strains showed a decreasing salt tolerance with decreasing pH. Most strains had an optimum tolerance at about pH 6. The effect of the pH on the tolerance was determined with greater accuracy in the case of the strain which had the maximum tolerance (figure 1). The connection found between pH and salt tolerance entirely agrees with the observations made by VON SCHELHORN (1951).

Table 2 Salt tolerance of butyric acid bacteria.

Strain	NaCl in %	
	growth	no growth
274	3	3.5
370	2.5	3.0
OL 2	2.5	3.0
G 1	3.0	3.5
OR	2.5	3.0
OO	2.0	2.5
A	2.5	3
K 4	2.5	3
S 46	3.5	4
Z	4.0	4.5

Table 3 Salt tolerance in % at varying pH's.

pH	NaCl in % w/v just allowing the growth of the strains :					
	OL 2	OR	OO	K 4	S 46	Z
6.9	2.5	2.5	3.0	2.5	3.25	4.0
5.9	3.25	3.0	2.5	3.0	3.5	3.5
4.8	2.0	2.5	2.5	2.0	3.0	2.0
4.6	1.5	1.5	1.5	0	—	1.5
4.4	0.5	0	0	0	0	1.0

Von Schelhorn's studies showed that the salt or sugar tolerance of micro-organisms is highest at optimum pH. In these experiments the curve of the tolerance line has not been extended to include the basic zone as such high pH's are never encountered in silage.

In order to determine whether the effect of NaCl is due to an increase in the osmotic pressure or to a specific effect of the Na-or Cl-ion, a number of strains were inoculated in the same medium in which NaCl was replaced by equivalent amounts of KCl or Na_2SO_4. It can be seen from the data given in table 4 that there is no question of a specific effect of one of the ions, but that the inhibition is to be attributed to the increased osmotic pressure.

In the above-mentioned growth experiments there was a growth deceleration which increased with an increasing salt and/or H-ion concentration. This

Table 4 NaCl, KCl en Na_2SO_4 tolerance expressed in % NaCl or equivalent amounts thereof.

Strain	NaCl	KCl	Na_2SO_4
OL 2	2.5	2.5	2.5
OR	3.0	3.0	3.0
OO	2.5	2.5	2.0
K 4	2.5	2.5	3
S 46	3.5	3.25	3.5
Z	3.5	3.5	—

tallies completely with RICHARD's observations on the effect of lactic acid. This phenomenon, which is probably due to retarded spore germination or an extended lag phase, raised the question as to whether the cells might not adapt themselves to a higher osmotic pressure.

However, a number of experiments to this effect with strains S 46 en Z led to a negative result. On inoculating from a medium with 3.5% NaCl in the same medium the lag phase was equally long. It was also found that after adaptation in 3.5% NaCl neither strain grew in a medium having a higher salt concentration.

Butyric acid determinations were carried out in all positive tubes of the in butyric acid production.

Summarising, it can be stated that an increased osmotic pressure in the experiment shown in table 3. It was found that an increase in the H-ion concentration and/or the NaCl concentration was accompanied by a decrease medium leads to growth inhibition and reduced production of butyric acid and that this effect greatly depends on the pH in the medium.

2 *Ensiling experiments with NaCl as an additive*

In order to determine whether the said effect of the osmotic pressure on the growth of the butyric acid bacteria also plays a part in the fermentation processes in grass silage, silages were made of grass with common salt added. The silages were stored for two months at 35° C.

A comparison of the freezing point of fresh grass and of the silages (table 5 above) shows that the lowering of the freezing point is increasing during the fermentation process. This may be due to the fact that during fermentation large undissociated molecules are split up into a larger number of small and partly dissociated ones.

It can be seen from figure 1 that at the pH of fresh grass (about 6.5) a decrease in the freezing point of about 3° C will be required for a complete suppression of the butyric acid bacteria. The butyric acid figures in table 5 confirm that butyric acid is only produced in those silages in which the original decrease in freezing point was considerably less than 3° C.

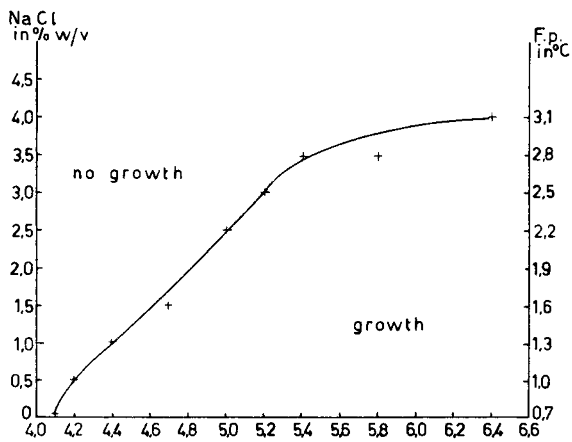

FIG. 1 INFLUENCE OF pH AND OSMOTIC PRESSURE ON THE GROWTH OF Cl TYROBUTYRICUM STRAIN Z.
+ = growth after 20 days at 35° C.
F.P. = lowering of the freezing point in °C.

Table 5 Influence of NaCl on silage quality.

Description	Number	Dry matter		Freezing point decrease		ERH silage	pH	Butyric acid
		grass	silage	grass	silage			
Grass	3	25.1	23.7	1.50	3.10	98.5	4.45	0.4
Grass + 1% NaCl .	3	25.1[1]	25.0	2.2[2]	3.83	97.0	4.55	0.1
Grass + 2% NaCl .	3	25.1[1]	25.7	2.9[2]	4.48	96.5	4.55	0.0
Grass + 3% NaCl .	3	25.1[1]	26.1	3.5[2]	5.17	95.5	4.50	0.0
Grass	1	16.2	12.6	–	–	98.5	5.9	2.4
Wilted grass	1	26.4	25.4	–	–	97.0	4.6	0.2
Wilted grass	2	35.9	35.9	–	–	95.5	4.7	0.0

1) Exclusively NaCl.
2) Calculated.

Only small amounts of butyric acid were formed, because the pH decreased and there was a further lowering of the freezing point (due to osmotic pressure) when lactic acid production proceeded.

No butyric acid was found in the silages with 2% and 3% NaCl in which, owing to the lowering of the freezing point, it could be assumed that the osmotic pressure in the initial period would be too great to permit the growth of butyric acid bacteria. This was entirely in accordance with expectations.

The equilibrium relative humidity (ERH) values were determined in addition to the decreases in the freezing point. Judged by the butyric acid figures, the ERH figures found in the experiment with common salt showed a good agreement with the results of an earlier experiment (table 5 below). From this it may be concluded that an ERH of about 96% is the limiting value below which further production of butyric acid comes to an end.

In order to ascertain whether the butyric acid found in silages with 0 and 1% NaCl might have been formed before sufficient lactic acid had been produced, a number of silages were made of grass with and without molasses. These silages were stored at 30° C and analysed after 1, 2 and 4 days.

Table 6 Rate of butyric acid production in new made silage.

Silage	% butyric acid and pH after					
	1 day		2 days		4 days	
	B.a	pH	B.a	pH	B.a	pH
Grass	0.0	5.0	0.2	5.4	0.5	5.2
Do. + 2% molasses	0.0	4.9	0.2	4.9	0.3	4.6
Lacerated grass	0.0	5.0	0.2	5.0	0.2	4.7

It can be inferred from the results shown in table 6 that considerable amounts of butyric acid can be formed in a short period.

Discussion

In the interplay of factors determining the course of fermentation during ensiling, the struggle between the lactic acid bacteria and butyric acid bacteria assumes an important role. Many precautions can be taken in order to stimulate the formation of lactic acid as compared to the formation of butyric acid. Since many measures for promoting the lactic acid bacteria also assist the growth of butyric acid bacteria (e.g. addition of sugar or anaerobiosis) the best ensiling methods are those which inhibit the growth of butyric acid bacteria. The most important of the factors which are more harmful to the clostridia than to the lactic acid bacteria are:

1 low temperature (maximum 20–25° C);
2 low pH (maximum 4.2);
3 high lactic acid content;
4 high osmotic pressure.

According to recent temperature measurements in silos, temperatures in the range 30° to 40° C for three weeks are normal in practice (Kappelle, unpublished). The temperature can only be expected to remain low when gas-tight silos are employed.

In a biological acidification, the factors referred to under 2 and 3 will gradually gain strenght, so that during the first days subsequent to ensiling, the butyric acid bacteria will have an excellent opportunity to develop (table 6).

The fact that this development does not take place in wilted silage may be attributed to the increase in the osmotic pressure in the environment as shown by the bacteriological experiments (table 2). That this osmotic pressure may play a part in silage is also shown by the fact that increasing the salt content in the experimental silages led to a marked inhibition of clostridia (table 5).

When only slight wilting is applied, viz. when there is dried to a sub-lethal osmotic pressure, spore germination and butyric acid production are retarded. In particular, it is the time-saving effected by this retarded growth that enables the lactic acid bacteria to improve an unsatisfactory conservation. Fortunately a positive correlation can be shown between the effect of pH and osmotic pressure on growth inhibition of *Cl. butyricum* and *Cl. tyrobutyricum* (table 3, figure 1).

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