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S. ORLA-JENSEN, ANNA D. ORLA-JENSEN
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I KOMMISSION HOS EJNAR MUNKSGAARD
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Together with two of my co-workers, the civil engineers Anna D. Orla-Jensen and Agnete Snog-Kjær I have made thorough bacteriological investigations to solve the problem of ensiling lucerne on a purely biological base.

It is obvious that under present conditions where the farmers are obliged to feed the cattle almost exclusively on home-grown fodder they must be more interested than ever in preventing anything from going to waste.

By the old-fashioned preparation of hay parts of it crumble into dust—especially under bad weather conditions—and some of the vitamins are lost by oxidation.

These disadvantages are overcome by the so-called ensiling, by which process the fodder is put down under anaerobic conditions. On the other hand, the fodder being here in a wet state it is necessary to take care that the proteins do not putrefy, and this is obtained by acidification.

Acidification is a natural and harmless mode of preservation, not only for plant material, but to a still higher degree for animal products. Thus sourmilk is of nearly unlimited keeping quality if protected against air and thus also against moulds. That the fresh cheese undergoes a useful ripening-process instead of a noxious putrefaction is likewise due to acidification. If only the initial pH has been lowered to 5.3, we are on safe ground. The same pH is reached in meat shortly after the butchering. However, this is not due to lactic acid bacteria, but to the proper enzymes of the meat. Nevertheless, on account of the poor sugar contents of the meat the pH is rising quickly again, but the brief acidification makes it possible to keep the meat unspoilt till it is tender, provided that the weather is not extraordinarily hot. In this connection, I cannot refrain from mentioning that acidification has a preserving effect on our food, not only before it is consumed, but also after its consumption, by preventing the contents of the large intestine from putrefaction.

Actually, the ensiling of greater crops of fodder is not the consequence of theoretical considerations, but simply of the fact that certain fodder substances contain so much water that they cannot be dried by means of the usual methods of haymaking. This especially applies to the green maize in North America, where from early times the preparation was made very rationally by cutting the plants into small pieces, and stamping them into high cylindrical silos. In more recent times it also applies to the beet-leaves, which were formerly left on the fields to decay.

From the end of the last century a vast literature is available regarding the silage problem and the bacteriology of silage; much of it, of course, of no great value to-day. But already at that time it was clearly understood that the use of silage favours the development of butyric acid bacteria in milk products, and in cheese-producing countries farmers therefore were warned against feeding the cows on silage, if the milk was to be used for cheese-making. Through dung infection the milk becomes so rich in butyric acid bacteria that the cheese, especially Emmenthal cheese, will blow up. A distinction was made between sour and sweet silage, terms which, however, had nothing to do with the pH, but only with the odour, sour smelling silage containing besides lactic acid also appreciable amounts of volatile acids, chiefly butyric acid. Instead of sour and sweet silage the terms cold and hot silage might just as well be used.

Just as in a compost-heap a rise in temperature occurs in the silage in the earliest stage. At the outset this is due to the respiration of the living plant-cells, and only after these have died, at about 45°, it must be ascribed to certain thermophilic bacteria. Often these bacteria have an optimum at 60° C., and will not stand more than 70° C. Since both respiration and the thermophilic bacteria need oxygen, it is possible in large silos to regulate the temperature by means of pressure, i. e. the higher the pressure, the lesser the increase in temperature. The water contents of the fodder also have a regulating effect on the temperature. Obviously, moisture is necessary for the biological processes, but the half-dry plant material gets warmest because heating of water requires much heat.

Rapid increase in temperature to 50° and upwards leads to sweet silage, since butyric acid bacteria do not develop so well at this temperature. Consequently, it was hoped in this way to arrive at a silage that would be well suited for dairy cattle.

Unfortunately, there is only a graded difference in quality between the sour and the sweet silage, and the sweet silage has been shown by BURRI to be just as dangerous for cheese-making as the sour silage.

Although several patents have been taken out for making sweet or hot silage, this method now has been given up for the benefit of the cold silage, because the high temperature cannot be reached without oxidising processes taking place with the effect of destroying the carotin and vitamin C of the silage.

It must be admitted that the value of the ensiling is completely nil, if the vitamin contents of the fodder are spoiled, and the reason why the patented method of A. I. VIRTANEN has reached such great distribution is that this method preserves the carotin-contents of the fodder in such an excellent way that we get more vitamin A by feeding with this silage instead of hay.

However, it is interesting to notice that long before the vitamins were discovered there was some empiric knowledge of these circumstances.

Already the ancient Romans regarded cabbage as a valuable medical plant, able to heal many ailments. Scurvy has been known from the time of the great voyages of discovery, and in its most pernicious forms. That cabbage was a remedy especially

for scurvy, or the marine plague as it was called at that time, was observed by the Hungarian military surgeon, HEINRICH KRAMER,[1] who had shown already in 1720 that this disease could be cured by lemon juice. As early as 1739 KRAMER recommended that all ships should carry a certain provision of "sauerkraut", of which a portion should be served every day to the whole crew. Thus he was at that time fully aware that cabbage contains an anti-scorbutic substance which is preserved by acidification.

Our earlier investigations have shown that by spontaneous souring of any product distinct different stages can be discerned. Primarily only weak acid formers such as the coli and aerogenes bacteria develop—in milk further tetracocci—subsequently the true lactic acid bacteria develop, to begin with, the spherical ones, afterwards the slowly growing, but more strongly acidifying, rod-shaped lactic acid bacteria. Then an acid reaction is reached, which promotes the development of yeast (this is of definite importance for the leaven), and if air is admitted also for the development of mould. However, in many cases the lactic acid formed is not the end product, but is fermented further by other bacteria such as propionic acid bacteria, butyric acid bacteria, and betabacteria. As shown by VAN BEYNUM and PETTE[2] the first mentioned of these bacteria do not thrive below pH 5, and as stressed by VIRTANEN[3] the butyric acid bacteria grow poorly at a pH below 4.2. Most acid-resistant are the betabacteria, *Lactobacillus pentoaceticus*, which are a sort of heterofermentative lactic acid bacteria thriving at a pH below 3.0.

The spherical lactic acid bacteria in plant materials such as beet slices, sauerkraut, and leaven are as a rule betacocci (heterofermentative lactic acid bacteria forming laevo-lactic acid or in some cases racemic lactic acid) and in animal products such as milk, cheese, and in the animal tissue as a rule streptococci (homofermentative lactic acid bacteria forming dextro-lactic acid). On the other hand, the rod-shaped lactic acid bacteria growing at room temperature in plant substances are always streptobacteria, mostly *Sbm. plantarum* forming racemic lactic acid, and in dairy products *Sbm. casei* (forming a mixture of dextro- and racemic lactic acid). At higher temperatures the streptococci are exclusively *Sc. thermophilus*, and the streptobacteria are replaced by thermobacteria, which are extremely vigorous acid formers (laevo- or racemic lactic acid).

To make good silage the first phase of the fermentation, the coli and aerogenes stage in which dry matter is lost by gas production, must be shortened as much as possible. This means that the pH must be lowered quickly below 4. By inoculating heavily with streptobacteria this can be done, these bacteria forming twice as much lactic acid as the streptococci. On the other hand, they are rather slow growing, and the dangerous butyric acid bacteria may develop. This represents the great difficulty in making silage on a purely biological base.

[1] F. EICKHOLTZ: Sauerkraut und ähnliche Gärerzeugnisse. Die Wissenschaft. Einzeldarstellungen Bd. 96. 1941.

[2] Verschlag to landbouwk. Onderzoking 45, 149, 1939.

[3] A. J. V. Systemet. Stockholm 1945.

VIRTANEN overcomes this difficulty by bringing the pH immediately below 4.2 by sprinkling with a mixture of hydrochloric and sulphuric acid during packing into the silo. The working with strong acids is very disagreeable, the workers' footwear and clothes being easily spoilt, while the silage is stamped together. Therefore in U. S. A. the less corrosive phosphoric acid is often preferred, and another advantage is obtained: that phosphates are added instead of sulphates, the former being a much more valuable addition both to fodder and dung than the latter, but being more expensive.

If A. I. V. silage is given as sole fodder without being neutralized with soda it causes acidosis. But together with beets, which are rich in alkali, it can be given advantageously also in an unneutralized state. Most desirable it would be to acidify the silage with lactic acid, which is not only harmless to the cattle but also has some value as food. In order to bring about a lactic acid fermentation in the silage it is of great importance to make the juice come forth as quickly as possible, the juice being the nutrient of the bacteria. This is attained by adding salt—used for pickled cucumber and sauerkraut—or sugar. The addition of sugar is in many cases necessary to get a sufficient amount of lactic acid.

As shown by VAN BEYNUM and PETTE it is important to add so much liquid that this—together with the juice diffusing from the cells—fills all the spaces in the silage, so that the air is driven out. To avoid moulds and other organisms, capable of burning or neutralizing the lactic acid, conditions ought to be fully anaerobic. This is not obtained by only putting heavy weight on top of the silage, neither is it always obtained through the small amounts of liquid used in the A. I. V. method. We consider 10—20 per cent. of liquid necessary for the silage in accordance with its greater or smaller contents of water.

In Denmark whey is the only sugar-containing liquid to be proposed for ensiling. Its sugar contents can be increased by adding beet-sugar molasses. In all our experiments we have used cultures of lactic acid bacteria in whey with 5 per cent. of molasses added. If it was necessary to apply more molasses for further increasing the sugar contents of the silage, this was added to the ripe culture just before preparing the silage, the bacteria thriving badly with too much molasses.

If the molasses contains 50 per cent. of sugar there will be 7.5 per cent. of sugar in a whey with 5 per cent. molasses. Provided that the lucerne contains a little more than 80 per cent. of water and 0.7 per cent. of sugar, the juice from a lucerne with 20 per cent. molasses—whey gets about 2 per cent. of sugar. If furthermore 10—20 per cent. of molasses is added to the whey, the juice will get 3 and 4 per cent. of sugar, respectively.

By making silage—as already mentioned—two things should be obtained: (1) suppression of the putrefying process, (2) suppression of the butyric acid fermentation. As is known from cheese, putrefaction is avoided simply by lowering the pH to 5.3, the putrefying bacteria being very sensitive to acid. This is obtained by acidifying the liquid used for ensiling. Moreover, if this liquid is warm, it is possible

to get a temperature of at least 15° C. in the silage, also in the autumn, and thus the lactic acid fermentation can continue. In frosty weather it will hardly be possible to make silage on a bacteriological basis, but the cold alone will preserve it. The warmer the weather, the better, because the streptobacteria have their optimum at about 30° C.

To avoid butyric acid fermentation a pH of about 4 must be reached as quickly as possible. However, if all putrefaction—and thus all ammonia formation—is checked from the beginning, and if the air has been completely excluded so that moulds cannot develop and break down the lactic acid, there is no danger of a rise of the pH. If the temperature is too low to promote a quick further lactic acid fermentation, it will as a rule also be too low for the growth of butyric acid bacteria.

As mentioned above, to make the biological method complete with the A. I. V. method it is necessary to bring the pH below 4 as quickly as possible. Therefore we had never dared to engage on the problem in question, if we had not beforehand found an activator for the streptobacteria. In contrast to the streptococci and the thermobacteria, the streptobacteria have proved to be highly manganophile, i.e. that they are activated by a trace of manganese salts. By adding 10 mgr. $MnCl_2$, 4 H_2O to 1 litre of nutrient the rate of growth and thus the acid formation can be raised 10 times as seen from Table I, which gives the results of adding increasing amounts of $MnCl_2$, 4 H_2O to milk. In one day at 30° C. nearly the same amount of acid is formed as usually in a fortnight.

Table I.

mg. $MnCl_2$, 4 H_2O per litre added	After 1 day	After 3 days	After 14 days
0	1.8	3.2	13.5
0.1	3.6	6.5	14.9
0.2	5.0	8.3	16.2
0.5	7.0	11.5	17.1
1.0	8.6	12.4	19.4
2.5	10.8	14.4	20.0
5.0	10.8	15.3	21.2
10.0	12.2	17.1	22.1
12.5	12.2	16.9	22.1
25.0	12.2	16.9	22.1
62.5	13.5	17.3	21.8
125.0	13.1	17.3	21.8

The table shows that the optimum contents correspond to 10 mgr. $MnCl_2$, 4 H_2O per litre. Thus, we were prepared for the necessity of spending 1 kilo of manganese chloride on every 100 litres of whey. This proved unnecessary.

Independently of the substratum used, it be milk, whey, casein, peptone, or yeast extract, or even lucerne extract, especially suited for the streptobacteria, the amount of acid was increased immensely by manganese salts. This proves that all these substrata do not contain amounts of manganese worth noticing. As soon as

only 5 per cent. beet-sugar molasses is added to the substrata, the acid formation is activated similarly, or more correctly, more vigorously than after addition of even 12.5 mgr./litre manganese salt. The activation is not further increased by addition of more Mn. This is illustrated by the following experiments (Table II), which again were performed with *Sbm. plantarum* in a casein-peptone-substratum. The consequence is that beet-sugar molasses must contain a perceptible amount of this element. An analysis also showed that the beet sugar molasses from various years contained so much manganese that an addition of 5 per cent. molasses sufficed to give the substratum the optimum contents of manganese. And, while whey and molasses solutions, separately, are poor substrata for streptobacteria (also for *Sbm. casei*), whey with 5 per cent. molasses is a good substratum. In that way we were saved the extra addition of manganese salt.

Table II.

Additions	After 2 days
None	2.9
5 per cent. molasses	10.1
Manganese	7.9
5 per cent. molasses + manganese.	9.9

However, not all sorts of silage need the addition of acid, sugar, or lactic acid bacteria.

Neither of these substances are needed for the preparation of sauerkraut, this being most likely the oldest silage known. By fine cutting of the cabbage, addition of salt, and by pressure, the juice, and thus the sugar, come out so fast that the lactic acid fermentation is started. The cabbage leaves seem to be so richly infected with the right bacteria, that nothing is gained by inoculation. This is stated both by us and in great experiments in U. S. A. For ensiling of beet leaves fine cutting of these is also said to give optimum conditions, as shown by MØLLGAARD. On the whole the ensiling of materials rich in sugar and poor in nitrogenous is not difficult. On the other hand, materials poor in sugar and rich in nitrogenous matter, and thus in buffers, are very difficult to preserve. Lucerne seems to be most difficult of all plant materials, and therefore we have worked exclusively with this, supposing that if we could overcome the difficulties here, there would be no difficulties with other plant materials.

However, considering the caloric value of the silage it will also be reasonable to replace the natural bacterial flora of the plant material by other bacteria, partly to avoid gas-production, and thus loss of dry matter, partly to get dextro-lactic acid instead of racemic or laevo-lactic acid. The fact is that the animal organism, forming in itself dextro-lactic acid, is only able to use this optic form while the laevo-lactic acid is turned out with the urine. This has been shown by PARNAS,[1] but has hitherto

[1] Biochemische Zeitschrift *38*, 53, 1912.

passed rather unnoticed. Thus, if all the lactic acid formed is dextro-lactic acid, it can be estimated that only $\frac{1}{3}$ of its caloric value is lost by the sugar fermentation. On the other hand, if racemic acid is formed $\frac{2}{3}$ is lost, and if laevo-lactic acid is formed, the whole value is lost.

Accordingly we have used a strongly acidifying *Sbm. casei*—No. 303—for all our ensiling experiments. It forms pure dextro-lactic acid. The fact that we got nearly pure dextro-lactic acid in the inoculated silage proves that it is really capable of suppressing the dominating *Sbm. plantarum* when the sugar solution used is molasses-whey.

For our laboratory experiments we got newly mowed lucerne form "Trollesminde" so quickly that it had not taken heat. 750 grs. was cut into pieces, 1—2 cm. long, and mixed with 20 per cent. liquid culture, and the necessary extra quantity of molasses. Samples were prepared both with ripe cultures (pH 3.5—3.7) and with the same media inoculated. Corresponding samples were made with the addition of bromate, and some with boiled lucerne, the latter experiment showing that part of the proteolytic action in silage is due to the enzymes of the lucerne itself. For further comparison some samples with addition of only phosphoric acid and benzoic acid were prepared.

The whole, well mixed bulk of each sample was stamped together in high glass-cylinders, making a pile of about 30 cm. height. It was covered with a wooden plate over which a layer of paraffin wax was cast, and the upper parts of the cylinders were filled with gravel. To begin with the cylinders were put at 30° C., later at a lower temperature. In the first experiment the gas-formation was tested, but as it proved to be chiefly carbonic acid we gave up this complication.

In consequence of the difficulty of taking out an average sample of the silage we confined ourselves to an analysis of the press juice squeezed out in a Buchner press at 200 Atm. In the juice the following values were determined: sugar, lactic acid, volatile acids, total-, amino-, and ammonia-nitrogen, and first of all the pH. Also the titer was observed to get an idea of the total acid formed. We have pointed out above that this is nearly as significant as the pH, because it is not only the pH, but also the lactate-ion-concentration which effects the preservation, and at a certain pH this is much greater in a substance rich in buffer such as lucerne, than in a silage with lower protein contents.

For the distinction between acetic acid and butyric acid, the only volatile acids present in silage in amounts worth mentioning, we used the partition method worked out by Osburn, Wood and Werkman.[1] It is easy, quick, and exact when only two acids are concerned, and it is based on the different solubility in water and ether of the acids.

From 100 ml. press-juice 150 ml. are distilled by steam. 50 ml. of the distillate are titrated (N_2), another 50 ml. are shaken vigorously for 1 minute in a separating funnel with 50 ml. of pure neutral ether. The funnel is left quiet for 2 minutes, and the watery phase afterwards poured into a measuring flask of 50 ml., and titrated (N_1).

[1] Eng. Chemistry 8, 270, 1936.

$K = \frac{N_1}{N_2}$ is for pure acetic acid 69.3, for butyric acid 16.5. As nearly all butyric acid will be found in the first 150 ml. distilled, this is a very sharp method for detecting small amounts of butyric acid present.

From the experimental work in the laboratory only two series are to be described in detail here. Thus we may mention that in the winter 1944/45 when no fresh lucerne was to be had, we made some tests with dried powdered lucerne. It was mixed up with so much water that the water contents of fresh lucerne were obtained. Inoculation was made with a ripe culture of *Sbm. casei* 303 in whey-molasses, and the results were unusually fine both with 3 and 4 per cent. of sugar. No doubt this must be due to the fact that most of the bacteria normally present on lucerne had been killed by the drying process, so that actually a pure culture developed. After 5 weeks at 30° C. the pH was still 3.7. Practically all acid was lactic acid, and only traces of ammonia and volatile acids were found.

In the second experiment, which is to be mentioned in more detail here, we compared the results of adding so much molasses that 3 and 4 per cent. of sugar were present in the fresh prepared silage. Previous tests had shown that 2 per cent. of sugar were insufficient to keep the pH low enough. At the same time silages were prepared to find out (1) if the action of the proteolytic enzymes in the silage was due exclusively to bacteria or was originating also partly from the lucerne itself. To this end 2.4 promille of bromate was added. According to HOLGER JØRGENSEN[1] this addition checks the papainases of the plants, (2) we destroyed the proteolytic enzymes of the lucerne totally by steam-heating before the ensiling. For comparison with the two last experiments we made a silage with phosphoric acid, pH 3.5. In this case no molasses-whey was used, but the phosphoric acid was diluted to 20 per cent. of the lucerne. As usual the inoculation was made with a ripe culture of *Sbm. casei* 303. The cylinders were placed at 30° C. to begin with, and later at 20° C.

For each experiment two equal samples were prepared, and one sample was analyzed after 1 day's keeping at low temperature (5° C.), the other samples were analyzed after 5—6 months. The results appear in the following table.

The table shows that the pH of the press-juice falls from 6.3 to 5.3 by adding the acid culture, and that after five months—at least in the case of inoculated samples—it is below 4. In consequence of the fact that yeast and most of the bacteria present are destroyed by steaming of the lucerne, the amount of alcohol and volatile acids is lowest in the boiled tests. The amount of volatile acid is largest in the uninoculated tests, because the flora of bacteria beforehand present on the lucerne develops most vigorously here. In the inoculated medium they are checked by the quick formation of lactic acid. This is also to be seen from the modification of lactic acid formed, which—as appears from the data of the zinc lactate—is more inactive in the uninoculated ones, (the culture used is forming exclusively dextro-lactic acid). This is also seen from the sugar contents, which disappear more rapidly in the uninoculated

[1] Studies of the Nature of the Bromate Effect. Einar Munksgaard, Copenhagen.

Table III.

The press juice of lucerne	Silage		pH	In 100 cc 0.1 n.				Zinc-lactate		Per cent. N as				Percentage
Inoculation	Sugar content per cent.	Additions		Titer	Alcohol oxidized to acetic acid	Volatile acid	K	α O	Water per cent.	Total	Protein	Amino acids	Ammonia	Sugar found
After 25 hours at 5° C.														
Uninoculated	3	0	6.3	44		6.0				0.29	0.10	0.19	0.01	3.1
Uninoculated	4	0	6.5	48		6.1		— 6.0	12.9	0.31	0.10	0.22	0.01	4.0
Inoculated	3	0	5.3	56		9.7				0.24	0.06	0.18	0.01	
Uninoculated	0.7	phosphoric acid	3.5	395		0.6				0.19	0.05	0.14	0.00	0.72
After 5 months														
Uninoculated	3	0	3.8	323	27.6	120.5	68	+ 0.2	16.7	0.60	0.07	0.50	0.09	0.00
Inoculated	3	0	3.7	305	9.0	95,0	68	— 3.5	14.3	0.53	0.06	0.45	0.06	0.07
Inoculated	3	bromate	3.5	270	14.3	67.3	69	— 3.9	14.6	0.44	0.06	0.37	0.04	0.44
Boiled and inoculated	3	0	3.8	225	4.8	31.1	68	— 6.7	13.3	0.22	0.04	0.18	0.01	0.60
Uninoculated	4	0	4.1	356	18.7	94.0	69	0	17.8	0.64		0.61	0.07	0.14
Inoculated	4	0	3.8	365	16.8	29.5	65	— 5.6	13.9	0.57	0.06	0.41	0.06	0.68
Inoculated	4	bromate	3.8	345	19.3	30.3	69	—10.5	13.5	0.45		0.41	0.03	0.95
Boiled and inoculated	4	0	3.7	300	6.8	28.1	69			0.27		0.25	0.01	0.85
Uninoculated	0.7	phosphoric acid	3.1	430	47.1	20.2		— 0.3	16.9	0.30		0.29	0.00	0.05

tests than in the inoculated ones, and thus the sugar is no more able—as the source of acid it is—to protect against neutralization from the top downwards. Therefore also some more soluble N and NH_3 is found in the uninoculated than in the inoculated tests. A little less volatile acid was found with 4 per cent. of sugar than with 3 per cent., but the quality of the inoculated lucerne was in this experiment about the same in both cases. This means that provided good protection against air and a not too long keeping 3 per cent. of sugar will suffice, i. e. an addition of 2.3 per cent., because the young lucerne itself contains about 0.7 per cent. The constant K shows that no butyric acid has been formed anywhere in this series.

Just as in previous experiments, steaming of the lucerne has checked the breaking down of the protein matter. The addition of bromate has had a similar effect. Nevertheless the quantity of amino acids does not seem to be altered by this addition. The addition of phosphoric acid to pH 3.5 checks the breaking down of the proteins in the same way as the steaming, but is unable completely to check the lactic acid bacteria fermentation. pH has decreased to 3.1 and in the juice many lactic acid bacteria are present—mostly betabacteria. The lactic acid formed was nearly inactive just as in the other uninoculated tests, nor is the A. I. V. method able to check the lactic acid fermentation, as has been observed by other workers.

The surface of the small samples of silage used for the experiments was always covered to the best of our ability, nevertheless, the noxious influence of the air could

not be completely avoided. Therefore the best result was obtained only by carrying through the experiment on a somewhat larger scale at "Virumgaard", the plant experiment station of the Danish government at Lyngby. By courtesy of the director, Mr. LUNDEN, two research silos were placed at our disposal. Their diameter was 96 cm., their height 200 cm. each. 400 kilos were treated in one silo according to the A. I. V. method, and in the other silo 400 kilos of the same batch of lucerne were treated according to our method. The surface was covered with a paper bag, and on top of this a 40 cm. layer of earth was placed. For the A. I. V. silage it took 16 days to settle completely from a height of 182 cm to 102 cm. On the other hand, the whey-molasses-silage settled down from 156 cm. to 100 cm. in one day, probably on account of the plentiful water contents. The air was never altogether expelled from the A. I. V. silage, while it was expelled practically immediately from the whey-molasses-silage. The silage was prepared on the 3rd July, when the air temperature was 23° C. Further, the silage in both silos, heated itself to 28° C., and the temperature afterwards decreased little by little to 16° on the 10th September, when the first samples were drawn. Owing to the fermentation the temperature of the molasses-silage rose one further degree during the first day, and afterwards fell more than that of the A. I. V. silage. After two days the pH of the molasses silage fell below 4. In these experiments the lucerne was not thoroughly cut, but only treated on a "rougher" (a kind of masticator).

The A. I. V. silage in this experiment smelling rather badly and containing black putrefied spots, there is no reason for referring to its analyses. It was only made as a control for the whey-molasses-silage, which was in an excellent state.

The reason why the A. I. V. silage was spoilt we cannot explain. Maybe the weather was too hot for that method, or only 7.5 per cent. of acid liquid was added, while 9 per cent. is usually supposed to be necessary.

This experiment proves that the silage can be produced from lucerne on a purely biological base. A necessary condition is that a cheese factory in the neighbourhood will take care of the pasteurizing and acidifying of the molasses-whey, and of its transportation; where this cannot be arranged one has to be content with the A. I. V. method. During the summer whey is available in sufficient quantities. In Denmark about one third of the cheese-whey goes into the sewage, and poisons the water courses. On the other hand, in the autumn, when the beet-top is to be ensiled, there is no more whey available than the farmers themselves want to use. Beet-tops, however, can also—as already stated—be ensiled only by fine cutting. The small sugar-containing plate which follows the leaves is enough for acidification. Nevertheless the result is better when only as much A. I. V. acid is added as to bring the pH down to 5.3.

The molasses in itself presents a danger because it contains spores of aerobic and anaerobic bacteria. Nevertheless the lactic acid bacteria seem to be able to keep them down.

The addition of molasses-whey to the silage does not cause any dilution of the silage, because it contains just as much dry matter as the silage itself, and the two materials link very well together.

Before closing this paper a few words about the bacteria spontaneously developed in the silage of lucerne may be added.

While at the beginning of the ensiling of cabbage and all sorts of beets great numbers of betacocci (leuconostocs) are found, these are only rarely found on lucerne. Here *Sbm. plantarum* develops very quickly, and also *Sbm. casei* may develop, but later on the latter are superseded by a hetero-fermenting bacterium, which shows great preference for pentoses, especially arabinose, and forming noticeable amounts of acid only from pentoses, the acid formed being equal parts of inactive lactic acid and acetic acid. Originally I found this bacterium in kefir-grains, and therefore it was called *Bbm. caucasicum*, but already in 1921 FRED and PETERSON showed that it was predominating in corn-silage.[1] They described it in detail, and were of opinion that it played a principal part in the corn-silage. They have given it the very significant name of *Lactobacillus pentoaceticus*, which at any rate ought to be corrected to *Bbm. pentoaceticum*.

In the following table we give the results of some tests of the fermenting properties of these bacteria. The figures denote acid in terms of per thousand lactic acid.

In a freshly isolated state *Sbm. plantarum* acidifies milk very little, while *Sbm. casei* always acidifies strongly in the course of 3—5 days at 30° C. It also attacks casein if the pH rises. That is why it plays a part in the ripening of many cheeses. It appears from the table that *Sbm. plantarum*—unlike *Sbm. casei*—ferments arabinose (and sometimes xylose), raffinose and melibiose but not inulin. On the other hand, the strains of *Sbm. casei* present in silage always seem to ferment this last sugar.

When *Bbm. pentoaceticum* finally gains the upper hand it must be ascribed—in the first place—to its great resistance to acid, but the question is: from where does it acquire the amount of arabinose necessary for its growth? Really, the only possibility is that it is released during the ensiling from pentose-containing substances in the lucerne, such as pectin, hemicellulose, and other pentosans.

A determination of pentose in our press-cakes before and after the ensiling, however, showed that in all cases great amounts could not be involved, because almost an increase of pentosans in the dry matter seemed to have taken place during the ensiling. But this might have been caused by a dissolution of nitrogenous matter and salts. As a sure basis the contents of raw cellulose can be used here, because it is known not to dissolve by the high acidity of the silage. The humifaction and the formation of peat is based on this fact. Therefore the deciding factor must be the proportion between raw cellulose and pentosans in the dry matter of the press-cakes, and this explains the question. In some cases the pentosans have risen from 12.7 to 14.5 per cent. during the ensiling, while in the same case the raw cellulose has risen from 23.6 to 48 per cent. and the proportion of cellulose to pentosan has thus risen from 2 to 3. The mellowing process, which the lucerne endures more or

[1] Biol. Chemistry *41*, 181, 431, 1920.
Journal of Biol. Chemistry *42*, 175, 1920.
Journal of Biol. Chemistry *42*, 273, 1920.
Journal of Biol. Chemistry *46*, 319, 1921.

Table IV.

No.	Bacterium isolated from	Rotatory power of the lactic acid	Xylose	Arabinose	Sorbitol	Mannitol	Glucose	Mannose	Saccharose	Melibiose	Lactose	Raffinose	Inulin	Salicin	Milk: Time of curdling	Milk: Amount of acid
	Streptobacterium casei	d														
11	Emmenthal cheese		0	0.2	0.2	3.6	13.3	10.1	5.2	0.5	11.9	0.1	0.1	11.0	3	15.5
303	Lucerne silage uninoculated		1.1	1.1	3.4	3.4	10.7	5.6	10.1	0.6	9.6	0	12.4	6.8	5	12.4
390			1.1	1.7	4.5	5.1	8.5	7.3	7.3	0	6.8	0	5.1	4.5	4	12.1
381			1.1	1.1	4.5	4.5	9.0	7.7	6.8	0	6.8	0	7.9	5.1	4	12.1
407			0	0.6	4.5	4.5	8.5	8.5	10.9	0	8.5	0	10.1	5.6	4.5	13.8
493			1.1	1.1	3.4	3.4	9.6	5.6	9.0	0	8.4	0.5	10.7	6.8	3	14.2
	Streptobacterium plantarum	i														
24	Soured potatoes		12.6	12.8	2.9	2.9	8.8	8.8	9.2	7.0	8.6	8.6	0	7.9		2.9
333	Lucerne silage uninoculated		5.6	13.0	4.0	4.0	7.3	4.5	4.0	5.5	6.8	5.6	0	4.5		3.2
343			2.8	8.5	1.1	4.0	4.0	4.0	3.4	6.0	5.1	5.6	0	4.0		2.2
444			2.4	14.1	0	0	10.1	9.0	9.0	6.0	0.6	6.5	0	7.3		0.9
465			1.1	10.7	4.0	4.0	6.2	5.6	5.6	5.6	7.9	6.8	0.6	7.9		2.0
526			1.1	10.7	3.4	3.4	7.3	5.6	9.0	5.6	7.3	5.6	0	6.8		2.5
	Betabacterium pentoaceticum	i														
2	Kefir grains		0.2	16.0	0	0	2.7	0	0	0	0.2	0	0	0		0
300	Corn silage		8.7	9.7	0	0	3.8	0	0.5	0	4.5	0	0	0		2
379	Lucerne silage uninoculated		6.2	5.1	0	0	4.5	0	0	0	0	0	0	0		0
447			5.6	13.5	0	0	2.8	0	0	0	0	0	0	0		0
490			7.9	10.7	0	0.6	4.5	0	0	0	0	0	0	0		0
510			7.3	15.2	0	0	4.0	0	6.2	0	0	0	0	0		0
515			8.5	14.1	0	0	4.0	0	5.1	0	0	0.6	0	0		0

less by ensiling, must partly be due to a real retting process, by which *Bbm. pentoaceticum* is supplied with pentoses, and from this we understand why this bacterium, as well as the other lactic acid bacteria by preference living on plant material, preferably ferment arabinose, a quality which is really met with only within this group of lactic acid bacteria.

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