Citation:

Beijerinck, M.W., On Lactic acid fermentation in milk., in:
KNAW, Proceedings, 10 I, 1907, Amsterdam, 1907, pp. 17-34

birds, I refer to the academical thesis of Mr. SONIES, which is now going through the press and will soon be published, also in "Petrus Camper". I will only mention that the small polar cartilage, discovered by NOORDENBOS in mammals and which also appears in Selachians, was found by SONIES also in birds.

Microbiology. — *"On Lactic acid fermentation in milk".* By Professor Dr. M. W. BEIJERINCK.

(Communicated in the meeting of April 26, 1907).

In milk left to itself, which in consequence of spontaneous infection, contains the more generally distributed germs, with certain regularity some special floras are observed, whose composition is chiefly controlled by two factors: temperature and oxygen pressure. If the latter is very slight, that is, if the microbes of the milk are reduced to more or less anaërobic conditions, the floras become simple of composition and produce certain fermentations. The three principal of these are the Aërobacter-, the Butyric acid- and the Lactic acid fermentations, of which the two first are always characterised by the evolution of hydrogen and carbonic acid, whilst in the lactic acid fermentations, which may occur under different forms, beside the lactic acid, no gas at all, or carbonic acid only is formed. Sometimes this fermentation is accompanied by a vigorous slime formation, which slime consists of the swollen cell walls of the inferred lactic acid ferments.

For domestic purposes the lactic acid fermentation should be considered as useful; both the others as noxious.

The fermentation experiment the dairy industry applies to judge of the purity of milk has for its object to determine the commonness or the rarity of the germs of Aërobacter and of the butyric acid ferment. To this end a high standing glass is filled with milk, placed in a water bath of 40° C. and it is observed whether any fermentation gas is evolved, and if so, after how much time. In good milk this production of gas does not occur because then the lactic acid ferments develop so quickly that the other microbes are expelled. Artificially the Aërobacter fermentation is easily obtained by infecting non-acidified milk with faeces, soil or canal water and cultivating at about 37° to 40° C. After 6 to 12 hours production of gas is observed originating from *Aërobacter coli* or more rarely from *A. aërogenes.* The nature of the thereby obtained varieties changes with the temperature.

At temperatures beneath 40° the Aërobacter fermentation, after lasting some hours, is replaced by a butyric acid fermentation which again, after some time is succeeded by a lactic acid fermentation. Externally the Aërobacter and the butyric acid fermentations cannot be distinguished, but this can be done easily with the microscope.

If 3 to 5 % chalk is added to a culture in a stoppered bottle at 35° to 40° C., the butyric acid fermentation can go on longer, and by early transplanting, likewise in milk with chalk and with exclusion of air, check the development of lactic acid ferments, without, however, quite dispelling them.

Microscopically the butyric acid fermentation may be recognised by the long, thin, at neutral reaction highly motile rods, sometimes mixed with elongated or more rounded clostridia, colouring blue by iodium, all belonging to the species *Granulobacter saccharobutyricum*.

To accumulate from such a crude butyric acid fermentation in milk the lactic acid ferments, which hardly ever lack there, it will suffice to transplant some drops into milk without chalk, and, if necessary, to repeat this after the butyric acid fermentation, which always sets in at first, is finished. Whether this be done in open or closed bottles or tubes, at 37° to 40° C., lactic acid rods of the genus *Lactobacillus* will be seen to appear, which by repeated transplantations completely dispel the butyric acid ferments.

If in these experiments instead of using fresh, unheated infection material, the soil, water, or faeces are previously heated to 80° or 95°C., by which only spore-forming microbes can develop in the milk, the fermentations of *Aërobacter* and the lactic acid ferments do not arise, their germs producing no spores, but a butyric acid fermentation is obtained, from which the aërobic spore-formers may be dispelled by repeated transplantation at exclusion of air.

1. *Properties of the active lactic acid ferments.*

As many bacteria of the most different groups can produce lactic acid it seems not superfluous to indicate what are the characteristics of the lactic acid ferments proper.

The active forms of dairy industries, yeast manufactories, distilleries, tanneries, and breweries, although joined by transitions, may be practically classified into the physiological genera *Lactococcus, Lactobacillus* and *Lactosarcina*, of which the two first only occur in the dairy products[1]).

[1]) In the chief floras of milk- and dairy products occur, to my knowledge, no species of *Lactosarcina*. When EMMERLING asserts to have found a yellow *Sarcina* in Armenian mazun (Centralbl. f. Bacteriologie, 2t. Abt. Bd. 4, p. 418, 1898), this can only have been a common infection from without. Also in butter sarcine species may accidentally occur but they do not belong to the chief flora, which consists of lactic acid ferments and lipophili.

They are always immotile, no-spore forming bacteria, which bear drying very well and which, by heating to 65° or 75° C., in which they just remain alive, while these temperatures are deadly to most other non sporeproducers, may be separated from these ("lacticisation"). They require for nitrogenfood peptones, such as are found in milk, malt extract, or other juices of plant- or animal origin, and for carbon food certain sugars, which may differ for different species. They do not peptonise proteids and, thus, do not liquefy gelatine; the secreted lactic acid can dissolve a certain quantity of caseine, but chemically this substance remains unchanged. These circumstances regulate their distribution in nature, where they are by no means general, but may rapidly multiply, especially under the influence of man. They are, however, found in the soil and can, by methods mentioned below, be accumulated and cultivated in a condition of pureness.

They are always more or less distinctly microaërophilous, some species or varieties can, however, grow very well at the air; other forms cannot and behave as real anaërobics. Access or absence of air is commonly of no consequence to the acid formation, but in the yeast industry a species is used, which at full atmospheric pressure produces no acid, and in the dairy industry are also forms which display the same property.

Always, even on good nutrient media, to which belong in particular maltextract agar, and milk- or whey-agar, the growth of the colonies remains limited, especially if the air and the produced acid can act simultaneously. If the acid is neutralised by chalk the growth of the colonies at the air may also become important. Yet, in most cases, the recognition of these ferments may repose on the smallness of their colonies compared with those of other bacteria.

Catalase is constantly absent, and hereupon an excellent diagnosis can be based, for which it is only necessary that a culture plate, on which all kinds of bacteria may occur, be flowed with strongly diluted hydrogensuperoxyd which is by all microbic species, except the lactic acid ferments, indifferently whether they belong to *Lactococcus*, *Lactobacillus* or *Lactosarcina*, changed into a scum of little oxygen bubbles. .

Even the lately described [1]) large celled *Sarcina*, which in consequence of continued research I now consider as identic with the stomach sarcine (*Sarcina ventriculi*), and whose acid producing power is very slight, — i. e. 3 c.c. of normal acid per 100 c.c. of

[1]) These Proceedings 25 Februari 1905. Archives Néerlandaises T. 1 and 2. T. 11, p. 200, 1906.

2*

maltextract or glucose broth, — does not at all decompose hydrogen superoxyd.

If we consider how generally catalase is met with in the animal and vegetable kingdom, as also in the microbes, its very absence in the lactic ferments appears in a peculiar light.

All active lactic acid ferments from milk invert sugar (invertase reaction) and can more or less easily decompose esculine and indican (emulsine reaction). The reaction on esculine is demonstrated by introducing, for example, 0.1°/₀ of this substance and a few drops of ferricitrate solution into whey agar or whey gelatin. Streaks drawn on it of species which decompose esculine produce intensely brown or black diffusion fields of esculetiniron, brown at more alcaline, black at more acid reaction, so that the lactic acid ferments become recognisable by the black fields in the midst of which their colonies are placed [1]). So long as esculine is present it is recognised by the magnificent blue fluorescence of the whole plate at feeble alcaline reaction. Indican may be used in a corresponding way but then no iron salt is wanted as the indoxyl produced from the glucosid oxidises of itself at the air to indigo blue. The lactic acid ferments decompose these two glucosides, slowly indeed, yet these reactions are very characteristic and useful. Amygdaline is not decomposed by the lactic acid ferments. [2])

To the most remarkable properties of the lactic acid ferments belongs their power of reducing levulose to mannite, [3]) which latter substance may even in concentrated nutrient solutions be recognised by its ready cristallisation at evaporation. A single drop dried on the object glass, commonly gives at microscopical investigation full certainty as to the existence of this reaction.

The lactic acid ferments thereby strongly contrast with the so nearly allied vinegar bacteria, in as much as the latter do just the reverse, i. e. they change by oxidation mannite into levulose.

Like so many other bacteria the lactic acid ferments possess, also with regard to various pigments, a strongly reducing power,

---

1) The knowledge of this extremely sensitive reaction, which has been applied for years in my laboratory, I owe to my colleague Mr. H. TER MEULEN.

2) Amygdalin is decomposed with much more difficulty by the action of microbes in general than the other glucosides named in the text. Moulds mostly decompose it into amygdalinate of amonium; beer yeast into amygdalonitril glucosid and glucose. Splitting under production of bitter almond oil, hydrocyanic acid and glucose I detected hitherto only with *Saccharomyces apiculatus* and with the anaërobic ferment of butyric acid fermentation, *Granulobacter saccharobutyricum.*

3) Ferments lactiques de l'industrie. Archives Néerlandaises 1901. KAYSER, Fermentation lactique. Annales de l'Institut agronomique 1904.

as is easily shown by inoculation into deep test-tubes of boiled milk coloured with litmus. The red litmus is first in the depth, later till near the surface quite discoloured, to turn red again by shaking with air. The thickness of the red layer in the curdled milk admits an accurate measure of the intensity of the growth and of the reduction process. The thinner the red layer the more intensive both functions must be.

2. *Factors of variability.*

Many, perhaps all lactic acid ferments display a high degree of variability as well in physiological as in morphological properties. Nevertheless this variability in different stocks, coming from different isolations of the same species, is not always equal by far, which may give rise to trouble in the study of the specific properties. The circumstances causing the variability are but partly known; decidedly belongs to them an oxygen pressure, too far above or too far beneath the optimum for the vital functions, which may, especially for the bacterium of the long whey (*Lactococcus hollandiae*), be demonstrated with exceeding clearness.

This remarkable species is characterised by a vigorous slime formation when cultivated in milk or whey, but loses this power at temperatures above 20° C., as well at the ordinary pressure of the atmospheric oxygen, as at complete exclusion of it, if the changed influence is allowed to act during some time on the growing microbes. This is shown by cultivating the whey in a closed bottle; the upper layer, just beneath the stopper, where a little air can find access, becomes quite liquid and contains a hereditarily constant, common *Lactococcus*, forming little acid and no slime. Also by cultivating the long whey microbe in tubes of boiled milk with access of air, after one or two reinoculations, a *Lactococcus* is produced, which forms no slime at all. If the material for the reinoculation is secured from the depth of the cultures grown in closed flasks, at places where the access of air is impossible, and the inoculation is repeated once or more in the same way, a *Lactococcus* is likewise obtained which displays no trace of slime production.

At some depth beneath the surface, however, is a zone in which unchanged, slime forming, hereditarily constant material is found.

What in this case can be very easily ascertained, proves, at accurate investigation, also to be true for the other species of lactic acid ferments, namely, that they only then continue to display constant specific characters, when they are continuously cultivated at a certain pressure of the oxygen, else, these characters are seen to disappear, whilst in fact, or apparently, new ones originate. Hence, in some cases it may be proved, in others the probability is shown, that each species must occur in three varieties, joined by intermediate forms, i. e. the normal form, a "high pressure variant", and a "low pressure variant".

As in wholly different groups of bacteria corresponding facts may be observed, there is cause to assign a fundamental signification to them.

A decisive factor which may cause the production of variants is furthermore the temperature, for experience proves that a prolonged cultivation above the optimum temperature of growth, gives rise to the appearance of forms distinctly different from the original stock.

In other cases the cause of the variability is unknown; not seldom for example, we find at the very first culture of a species taken from nature, strongly varying colonies, which prove to belong to the same species only because many colonies by sector-variation display the genetic alliance of the variants to the wild stock.

But then, too, there is reason to admit that the new vital conditions, to which the microbes are subjected just by the change of oxygen pressure and temperature, are the chief factors of the variation process which is, as it were, seen in action. This observation is of so general a nature and is so closely related to the essence of life, that it must be considered as probable, that also in higher plants and animals, local changes in the access or exclusion of oxygen, in connexion with temperature, play an important part in the morphogenesis.

As the examination of other species of microbes shows that the absence of certain nutrient substances in the culture medium, at free aëration and during growth, may cause hereditary variation, for example in *Schizosaccharomyces octosporus*, which in old cultures changes into the spore-free variant, totally differing from the chief form, there is reason also to believe, that also the said factor must be considered to explain the great variability of the lactic acid ferments; but the observations there about are not yet fit for definite conclusions.

3. *Elective culture of the microbes of the slimy lactic acid fermentation.*

There is reason to assume that the slime producing lactic acid ferments are the normal forms and the non-slime formers, species or variants derived from them. Hence, the former deserve to be considered in the first place.

To the typical slime producing species belongs the microbe of the long whey (*Lactococcus hollandiae*), which particularly before the introduction of pure cultures in the dairy industries, played an important part in the fight against cheese defects in North-Holland, and is still here and there practically used to that end.

Further I have found that the popular food known in Norway as "tjaette molken", a sample of which I owe to the kindness of Mr. PENNINK of Rotterdam, consists of milk, in which the long whey microbe, or at least a nearly allied form, secretes acid and slime.

Other materials in which these and allied microbes occur, were till now unknown, evidently because of the uncertainty about culture conditions and the lack of a good accumulation method. Taking the idea "species" in the broad sense, I think there is no objection as to bringing the group of forms, found in the manner described below, to the species just mentioned.

Starting from the following properties, the most characteristic for the microbes of the slimy lactic acid fermentation:

1st. The optimum temperature for their growth is at 20° or lower,

2nd. they can only compete in anaërobic cultures with the other microbes, and

3rd. the medium must consist of substances containing peptones as nitrogen and carbonhydrates as carbon source, I succeeded in finding a method giving rise to their accumulation.

It is true that I only examined a single material in this way, the common baker's yeast, but the investigation of the soil of fermenting or fermented substances, in short of materials of most varying description may be done in a corresponding way.

The experiment is arranged as follows.

Into a 30 c.c. closed bottle, filled with maltextract, to which is added $^1/_2$% of peptone siccum and which contains c.a. 10% extract, a little pressed yeast is introduced, for instance $^1/_2$ gram. Placed at a temperature of 18° to 20° C. a quiet fermentation sets in, which is allowed to continue 24 to 72 hours, whereby, because of the absence of air the yeast hardly grows, but the various lactic acid ferments reproduce quickly. Other microbes do not develop. Not seldom in this first culture have the contents of the flask already become somewhat slimy.

Whether this be the case or not, a not too small quantity from it is transplanted into a bottle quite filled with boiled, air-free milk, for instance $^1/_2$ c.c. into 30 c.c. of milk. At the same low or a somewhat higher temperature only a flora of lactic acid ferments can develop, and if the slime-forming species is present, it is the most vigorous. We then see that after 2 or 3 days the milk become slimy and by inoculation into milk whey, a culture will start which sometimes differs so little from the ordinary long whey, that we may conclude to an identity of species.

Of course, I cannot foretell that such microbes occur in any yeast sample taken at random, hence I must add that for my experiments I used pressed yeast from the Yeast and Alcohol manufactory at Delft.

Such a culture in milk differs it is true in some respects from what is obtained by growing long whey from North Holland in milk, as in the former case short rods or oblong cocci are observed, and in the latter, shorter forms more reminding of the common micrococci.

I expect that by repeating this experiment various deviating varieties will be found, and by application of the method to other infection material perhaps new species of slime lactic acid ferments may be discovered.

4. *Elective culture of the lactococci of cream souring.*

As the lactococci and lactobacilli, which both occur in spontaneously or otherwise soured milk, in cheese, and various other dairy products, seem to grow nowhere better than in milk,[1]) the culture experiments here considered, should be taken with milk.

In order out of the innumerable microbes of the crude milk practically to come to a pure culture of *Lactococcus*, the management is as follows.

The optimum of growth is at 30° C. or lower, and as all species of *Lactococcus* (like those of *Lactobacillus*) are strongly microaërophilous, sometimes even anaërobic (i. e. cannot grow at all at full atmospheric pressure on plates), it is best to cultivate in absence of air.

A stoppered bottle is quite filled with commercial milk and placed at 30 C. After 24 hours or somewhat later a *Lactococcus*-flora begins to replace the other microbes, while not seldom a feeble fermentation of *B. coli* or *B. aërogenes* has preceded.

After one or two re-inoculations under the same conditions, but

[1]) It is not impossible that there are "peptones" which, together with glucose or lactose, are still better food for the lactic acid ferments than milk itself. How very differently peptones of dissimilar origin act on microbes is easily observed in yeast species which in general grow better on "plant peptones" than on "animal peptones". The introduction of the word "bios" to denote those nitrogen compounds which are best fit as yeast food, is an attempt to circumscribe the peptoneproblem has been given. The relation between "peptones" and the lactic acid ferments is still closer than between these substances and the different yeasts; but it is here not the place to insist on this point.

into well boiled milk, which is done by transferring a trace of the first culture to the second bottle, quite filled with boiled, air-free milk, and so on, the lactococci free themselves completely from all foreign microbes and a material is obtained, which displays a high degree of purity and of practical usefulness. If the acidifying power of the microbes obtained by the experiment is lower than wished for, for example 5, whilst 8 to 10 c.c. of normal acid on 100 c.c. milk is desired, this must be attributed to the accidentally present stock. It is necessary then to begin a new experiment, following the same way as described, or it can be advisable to perform the first inoculation with some good butter-milk.

As buttermilk, however, very often contains lactose yeast, in the latter case a vigorous alcohol fermentation may at first be expected in the bottles. But it soon disappears by inoculation into milk rendered free from oxygen by boiling.

If in this way, thus in absence of air, the culture has been prolonged, a fairly constant acid amount is obtained at each renewed inoculation, which does not, however, rise above 10—12 c.c. normal in 100 c.c. of milk. On whey agar or whey gelatin plates the growth at the air of the thus obtained lactococci is different, as sometimes a great many aërobic colonies arise, which cause the same acidification as the cultures in the bottles, while in other cases nothing is seen to grow.

The first group corresponds with the usual commercial forms destined for the souring of cream, which commonly consist of cultures of the microbes dried on milk sugar or starch; moreover there are commercial aërobic pure cultures in milk or whey, which are sold in bottles.

The second group, that is the cultures non-growing at the air, may still better be used for the cream souring than the aërobic stocks, as the anaërobic forms of *Lactococcus* show more aptness to secrete the flavour desired in butter, than the more aërophilous bacteria. [1])

As well for this reason as for the great purity of the cultures made after this "bottle method", there is reason to prefer them in dairy work to the commercial so-called pure cultures, which for the greater part are by no means pure, but mostly contain, besides lactococci, numerous contamination germs of the milk. In consequence of frequent investigations I can therefore advise interested persons to use the here described method. Best would be if these cultures were

[1]) Of late I have also met with such like anaërobic lactic acid bacteria in commercial preparations.

prepared in the creameries themselves, but also the sellers of pure cultures, by following the above prescriptions, will obtain a better product than by the more usual way of selection of aërobic colonies. Besides, the management is simpler and more scientific.

To my opinion there is no satisfying ground to class the aërobic and anaërobic forms of *Lactococcus*, which can be produced after the said method, in separate species. They are but variants of one and the same species, whose oxygen requirements are different, which also appears from the fact that in the course of time one and the same stock shows considerable differences with regard to the said relation. Moreover, by several isolations all transitions between the more or less aërobic stocks may be obtained.

Finally it should be borne in mind, that by applying the "bottle method" at low temperature, in rare cases instead of a culture of real *Lactococcus* a *Lactobacillus* is obtained, which may likewise be had by colony selection from cheese. Using this *Lactobacillus* I did not observe at all the pleasant flavour of the anaërobic lactococci, so that I do not recommend these bacilli for cream souring.

5. *Elective culture of the lactic acid bacilli.*

If milk, soured spontaneously by *Lactococcus lactis*, or still better, buttermilk, is placed at exclusion of air in a thermostat of ca. 40° C., the original acid amount of 8 to 12 c.c. will in most cases rise after some days to about 18 or 20 c.c. per 100 c.c. of milk. For this experiment it is best to use a stoppered bottle of 250 to 300 c.c. capacity quite filled with milk. If for the first experiment a smaller quantity is used the result becomes uncertain, either by the disturbing influence of the air, or by the scarcity of the inferred bacteria.

The first change commonly observed in the sour milk is a moderately vigorous alcoholic fermentation, caused by the hardly ever lacking lactose yeast, and at the same time a complete separation of the caseine, which is driven to the surface of the liquid by the carbonic acid.

Microscopically we find that the lactococci present at first, are succeeded by more lengthened forms, truncated at the ends and united in chains, whereby the acid titer may considerably diminish, for instance in 12 hours from 8 c.c. to 6 c.c., which should be ascribed to the lactose yeast, for which the free lactic acid can serve as carbon food. By transference, at exlusion of air, the lactose yeast, as in the elective culture of lactococci, is rapidly dispelled by the then stronger lactic acid ferments.

Real lactobacilli mostly appear after 2 or 3 days and then the acid rises rapidly parallel to their multiplication to 20, even to 25 c.c. normal per 100 c.c. of milk. When this degree of souring is reached, there is usually no further increase observed, not even after several days, and whenever this does take place, there should be thought of aëration, by which the growth of vinegar bacteria and acetic acid formation from alcohol, have become possible.

The pure culture of lactobacilli is sometimes easy, in other cases, with more anaërobic stocks, it is more difficult. Always, however, it is troublesome with these pure cultures to obtain a considerable souring in milk and there is most chance of success (but even then the success is not quite certain) by souring lactobacilli together with *Lactococcus* which serves for the first souring to 8 c.c. If this amount of acid is reached, and the pressure of the oxygen sufficiently diminished, which in a stoppered bottle is likewise brought about by the presence of the lactococci, the lactobacilli can develop and cause further souring.

From the observation that by the described experiment more or less perfectly anaërobic lactobacilli are obtained, follows that here as in the case of *Lactococcus* different varieties may be expected. At a continued research the differences prove to extend over other characteristics also and may become so great, as well from a morphologic as from a physiologic point of view, that it seems necessary to create new species.

Especially the dimensions of the rods, the more or less branched state of the colonies on agar plates, the slime formation, the either or not originating of carbonic acid as fermentation gas beside the lactic acid, and the action or non action on different sugars give rise to this consideration. The deeper however we enter into these distinctions, the more troublesome it becomes to devise such descriptions as are wanted to present to other investigators an image of the results of our own researches; so numerous become the forms which nature, or better perhaps, which culture produces, and so slight are the differences by which these forms are distinguished, if we do not confine ourselves to the extremes of the groups. [1])

If the latter is done, two distinct forms call attention, which on a former occasion I named [2]) *Lactobacillus caucasicus* and *L. longus.*

[1]) For further information see W. Henneberg, Zur Kenntniss der Milchsäurebakterien. Sonderabdruck aus Zeitschrift für Spiritusindustrie. No. 22--31, 1903. Parey, Berlin.

[2]) Sur les ferments lactiques de l'industrie. Archives Néerlandaises. Sér. 2, T. 6, p. 212, 1901.

Without attributing a special value to this classification I yet wish to keep to it as I think that the facts to be mentioned are fairly well comprised thereby.

The longusgroup is characterised by its not acting on maltose, so that in maltextract no, or very little acid it formed, but it does decompose milksugar. In milk the forms of this group, if grown after a previous culture of *Lactococcus* which has produced 5 to 8 c.c. of lactic acid per 100 c.c. of milk, will once more produce a certain, even a like quantity of acid so that ca. 16 c.c. may be titrated, the latter amount being however an exception. Generally no evolution of carbonic acid is observed but sometimes it is, and then so much gas can arise that a milk beverage is acquired foaming like champagne.

By a series of transitions, the longus forms obtained at 40° C., are joined with lactobacilli which at a lower temperature find their optimal vital conditions, but which are rarer in milk.

The caucasicus group comprises those lactobacilli, which are able, independently of lactococci to produce in milk a very high acid formation. At 37 to 40° C. it is possible after three days of their action to titrate 20 to 25 c.c. of normal acid per 100 c.c. of milk. When that amount is reached further acid formation stops. In this case, too, there is a parallel form which, beside much lactic acid, also evolves carbonic acid. What by-product is then formed from the lactose molecule beside the carbonic acid is not yet clear; probably it is aethylalkohol. G. BERTRAND has proved that these ferments can produce succinic acid. They greatly owe their notoriety to their presence in kephir, which subject I have touched before[1]). Later however I have come to the conclusion [2]) that their distribution is by no means restricted to kephir only, but that they also occur in our climate, sometimes in buttermilk, in cheese and even in common baker's yeast.

6. *Yoghurt and maya.*

The use of soured milk as drink and food is so familiar to many Eastern countries, and dates from so remote an antiquity that there can be no doubt as to its favourable effect on health, and the establishment of various societies which try to popularise new preparations of that nature, seems to prove that the attention of the Western nations begins to be drawn towards it.

Both in the preparations of the Eastern nations and in those of

[1]) Sur le Kefyr. Archives Néerlandaises. T. 23, p. 428, 1891.

[2]) Ferments lactiques de l'industrie l. c.

industry are always found lactic acid ferments of the genus *Lactobacillus,* mostly of *Lactococcus* too. These lactic acid ferments alone determine the character of the "leben raïb" of Egypt, [1]) of the "yoghurt" of Bulgary,[2]) and probably also that of the "prostokwacha" and the "véranetz" of Russia, which METCHNIKOFF mentions. In the "kephir" of the Caucasus, the "koumys" of Central Asia, [3]) and the "mazun" of Armenia, [4]) occurs moreover, lactose yeast, which may, however, under certain circumstances be wanting, without the character of these beverages being lost. All other microbes, which are mentioned in literature as occurring in the said beverages or their ferments, such as *Oidium, Mucor,* other moulds, torula, red yeast, vinegar bacteria, butyric acid ferment, proteolytic bacteria, are only present by deficient preparation, so that it may be said that in all examined cases a pure lactic acid fermentation proves to be the wanted process, whilst eventually also an alcoholic fermentation is wished for or suffered [5]).

Hence, in the commercial preparations which start from yoghurt, only lactic acid ferments are cultivated. I have in particular investigated the products of "Le Ferment", mentioned beneath, as also a substance, sold as "maya" or Bulgarian ferment, [6]) to which my attention was drawn by Dr. DE LINT at Scheveningen. Here I will shortly describe the latter preparation.

It consists in a yellowish strongly acid reacting powder, composed, after chemical, microscopical and bacteriological examination, of caseine lactic acid, lactose, fat and lactic acid bacteria; it is evidently nothing

---

1) Annales de l'Institut Pasteur. T. 16. p. 65, 1902.

2) MASSOL et GRIGOROFF, Revue médicale de la Suisse romande 1905 p. 716. BERTRAND et WEISWEILLER, Action du ferment Bulgare sur le lait. Ann. de l'Institut Pasteur, T. 20, p. 977, 1906.

3) For Kephir and Koumys see WEIGMANN in LAFAR Technische Mykologie. Bd.2. p. 128. 1905.

4) Centralblatt für Bacteriologie, 2te Abt. Bd. 15, p. 577, 1906.

5) The study of literature leads at first view to a quite other result, as many microbiological descriptions are made by beginners, not sufficiently acquainted with the properties of lactic acid ferments, and who have attributed an exaggerated weight to the different kinds of infections named above.

6) On the bottle stands: Maya bulgare, Société de la maya bulgare, GARNIER & Co., Paris, 16 Rue Popincourt. The Société de Pury, Montreux, brings into commerce a ferment of the same nature under the name of "maya bacilline", and the Société HENNEBERG, Geneva, a liquid preparation as "lacticose". Besides there are to be had in Paris Lactobacilline de METCHNIKOFF in "Le Ferment", Fournisseur de l'Assistance publique, 77 Rue Denfert-Rochereau, who sells also, the "Biolactyle" of FOURNIER and the "Bacilline paralactique" of TISSIER (the preparations of this firm make a very good impression).

else but yoghurt evaporated at low temperature, perhaps in the vacuum. As to the preparation of the "yoghurt" itself by means of this ferment, it is done as follows and gives good results.

Milk is evaporated to half its volume, cooled to a (not nearer indicated) temperature, for which I took 40°, as 45° proved too high and 37° too low, and on a quantity of 250 c.c., so much ferment is strewn as can be put in a little spoon distributed with the *flacon containing the maya. After* 6 hours already the curdling of the milk becomes perceptible, after 24 hours I titrated 12 c.c. and after 3 $\times$ 24 hours 20 to 23 c.c. of normal lactic acid per 100 c.c. of the evaporated milk, which by that time is changed into yoghurt.

As a titer of 10 c.c. corresponds to 0.9 % of lactic acid, the titer 20 corresponds to somewhat less than 2 % of the vanished milk sugar. Supposing that the evaporated milk contains about 9.6 % of milksugar it follows that 7 % of milksugar has remained undecomposed. The caseine is of course curdled and the whole has changed into a solid but soft, sweet tasting mass.

The evaporation of the milk is not necessary, but when prepared from ordinary milk, the yoghurt remains more liquid, and as the acid formation is equally strong as in evaporated material, there remains about 2.5 % of the original 4.8 % milksugar, so that in this case the taste is much less sweet.

If in the said way yoghurt has been prepared in the presence of air and is re-inoculated into a new quantity of milk, then the result is yoghurt of the same acidity as the first time. But after 3 or 4 transferrings difficulties arise and only with great quantities of infection material further souring can be obtained. The experiment succeeded much better when the yoghurt was prepared in a quite filled stoppered bottle; the transferring can then be longer continued, but I do not know whether this will do in the long run. Evidently the difficulty here, too, is the right choice of oxygen pressure, whereby the inferred lactic acid bacteria preserve their properties unchanged; and this difficulty is still increased by the presence of two different forms, with unequal optima as to temperature, and probably as to oxygen pressure also.

One of these forms is again a *Lactococcus*, the other a *Lactobacillus*.

The former deviates somewhat from the common *Lactococcus*, in as much as it is more extended, reminding of short rods, and furthermore by possessing a higher optimum as to the temperature whereby the growth is quickest, which optimum proves nearer to 37° than to 30° C. Hence, this form is as it were a transition to a *Lactobacillus*. Isolation on milk agarplates was very easy, even at 30° C.

As to the second species, the *Lactobacillus* proper of yoghurt, it was troublesome to grow its colonies on milk agar plates, but on malt extract agar it was more easily obtained. In literature it has been named *Bacillus Massol* by GRIGOROFF, but I think that name superfluous as the characters correspond fairly well with those of the kephir bacilli which also occur in our country; for instance, as has been observed before, in yeast and buttermilk. Sown in slightly soured milk this *Lactobacillus* can produce the strong acid mentioned above, without the help of other bacteria. Evolution of carbonic acid does not take place and the product has a very pure taste, although a beginning of fat cleavage seems inevitable at such a high amount of acid.

METCHNIKOFF ascribes a very favourable influence to the use of yoghurt, as it diminishes the phenomena of autointoxication starting from the intestinal canal, and he explains this effect by accepting that the *Lactobacillus*, after passing the stomach, continues active in the intestine, and checks [1]) the formation of the obnoxious products which derive from other bacteria species. I do not doubt but this may be brought about by the lactic acid, but I think it highly improbable that the presence of the lactic acid bacteria from the yoghurt themselves should be required in the intestine. I think this conclusion is necessary, first because, without the use of yoghurt or other soured milk preparations, there occur in the intestine lactic acid ferments of different species, and second, because the conditions for lactic acid formation by the active - ferments are wanting or must at least be very unfavourable there.

As to the first point I refer to the following experiments.

If sterile milk is infected with faeces of different origin (man, cattle) and treated as described for the elective culture of *Lactococcus*, without access of air and repeatedly reinoculated at a temperature between 23° to 26° C., the said genus of microbes is indeed obtained by which as good cream souring can be obtained as with the pure cultures prepared in the before described way.

If sterile milk is infected in a corresponding way and exposed to the conditions wanted for *Lactobacillus*, that is, if cultivated in absence of air at 40 to 45° C., a fermentation of coli will first arise and later or simultaneously a butyric acid and no lactic acid fermen-

---

[1]) Quelques remarques sur le lait aigri. Rémy, Paris, 1907. In this paper METCHNIKOFF gives many assertions but no decisive experiments. Besides, his bacteriological elucidation, p. 26, is not clear. The elaborate and interesting work of Dr. A. COMBE, L'autointoxication intestinale, Paris 1907, is neither quite convincing from a micro-biological point of view.

tation, which latter would inevitably arise if the lactic acid ferments were present in a rather considerable number. Only by repeated transferences *Lactobacillus* is produced, which after some inoculations forms 10 to 13 c.c. of normal acid.

Hence, there is no doubt as to the presence of *Lactobacillus* and *Lactococcus* in normal faeces. They are, however rare, and belong by no means to the intestinal flora proper, like *coli*, but to the accidental flora, which consists of all that is introduced and is able to pass the stomach and intestines alive; without multiplying. There seems to be no cause to attribute any important influence to this fact.

As to the second point, why in the intestinal canal the conditions for the growth of the active lactic acid ferments are wanting, it is that in the contents of the intestines an alcalic reaction exists, and that the sugars which are formed or introduced there, in as much as they are not absorbed by the intestinal wall, will surely be attacked by *coli*, which in these circumstances is the stronger and dispels all competitors.

Why *coli* (and *aërogenes*) so completely defeat the lactic acid ferments, should, to my opinion, be explained by the important fact, not sufficiently considered in literature, that the first mentioned species can quite well live on peptone only, and multiply at its expense, while the active lactic ferments completely lack this faculty and, beside peptone, require a carbonhydrate for food.

If, moreover, it is borne in mind that *coli* in the presence of a carbonhydrate can also feed on other sources of nitrogen than peptone, for example on amines and ammonium salts, whereas the active lactic acid ferments cannot, and decidedly want peptones for nitrogen food, it is clear that for the different forms of *coli* practically every where in the intestinal contents a good feeding material is present, and that in the few localities where it would also be sufficient for the lactic acid ferments, it will be seized upon by *coli*. Where only peptones occur, *coli* will moreover increase the already alcalic reaction of the contents and thus, not for itself but for the lactic acid ferments, render the conditions of life more unfavourable.

Hence it seems evident why in the intestinal canal a coliflora can exist but no lactic acid flora.

The yellow coloured faeces of babies during the lactation period may be alleged to support this view. They consist microscopically almost solely of bacteria, for far the greater part of common colibacteria [1]), among which there occur real lactic acid ferments, but

[1]) For different children not always the same varieties; sometimes, for instance non-fermenting forms reminding of *Lactobacillus*, for which I before indeed took such bacteria.

as in the case described before in quite an inferior number. This fact acquires a special significance when we consider that ESCHERICH, the discoverer of the colibacillus, has proved that this condition exists directly behind the baby's stomach, where *coli* and *aërogenes* are predominant which, in reference to the preceding, necessitates the conclusion that even at those portions of the intestines where a lactic acid flora should first be looked for, it is evidently unable to sustain itself.

There is no doubt but here too, the strongly disinfecting action of the stomachal hydrochloric acid plays a part, as this acid, at a much lower titer than the lactic acid checks the growth of the lactic acid ferments, but hence can be neutralised by much less alcali, which is not indifferent to *coli*, which produces alcali.

In so far as the theory of METCHNIKOFF and COMBE is right, after which yoghurt or other sour milk preparations counteract the auto-intoxication from the intestinal canal, it seems certain that here should more be thought of the influence of a milk diet and the free acid taken up with the milk, than of a specific intestinal flora. But in how far the apparently proved decrease of indol and phenol, whose quantity is considered as determining the degree of auto-intoxication, deviates, at a nutrition with soured milk preparations instead of meat, from this decrease when non-soured milk is used, — to my opinion the real core of the question, — has not been considered by the said authors.

Admitting that the soured preparations really deserve to be preferred, I think that especially in Holland, it must be possible with good buttermilk in as simple a way to reach the wished for end, as with the various exotic ferments, whose descriptions give the impression that the preparators are but imperfectly acquainted with the general phenomena of the lactic acid fermentation in milk.

Although I see no fundamental difference between the use of buttermilk and yoghurt, it is certain that the latter may be prepared in a very simple way under medical control, and hence, to my meaning, deserves to be recommended in certain cases.

Summarising the preceding I come to the following conclusion.

In milk three chief forms of lactic acid fermentation, determined by temperature, are to be distinguished, namely at very low temperature, the slimy lactic acid fermentation; at a middle temperature the common lactic acid fermentation caused by *Lactococcus*; and at higher temperature the lactic acid fermentation by *Lactobacillus*.

The elective culture of the microbes of the slimy fermentation, succeeds by cultivating baker's yeast in absence of air between 15°

and 18° C. in malt extract and transferring to boiled milk or whey at a somewhat higher temperature. The acidity obtained remains low and amounts to 3 to 5 c.c. of normal acid per 100 c.c. of milk.

The elective culture of *Lactococcus* takes place by allowing milk to sour in a stoppered bottle at 20° to 25° C. and transfer it repeatedly to boiled milk at that temperature. The thereby obtained stocks of *Lactococcus lactis* are mostly anaërobic but specifically not to be distinguished from the more aërobic forms which may be produced by the same experiment. The acid mostly remains at about 8 c.c. of normal acid per 100 c.c. of milk, but may become 10 to 12 c.c.

The elective culture of *Lactobacillus* succeeds best by cultivating buttermilk in absence of air at 37° to 40° C. and inoculating it into boiled milk, at 30° C. and higher, the acidity can rise from 18 to 25 c.c. of normal acid per 100 c.c. of milk.

The active lactic acid ferments are very variable; as factors of hereditary constant variation are recognised cultivation at too high or too low oxygen pressure, and cultivation at a temperature above the optimum of growth.

Lactic acid ferments do not lack in the intestinal flora, but play there an inferior part.

A considerable difference between Eastern and Western lactic acid ferments does not exist.

Yoghurt and other such like sour milk preparations deserve the attention of hygienists.

Chemistry. — *"On the course of the plaitpoint line and of the spinodal lines, also for the case, that the mutual attraction of the molecules of one of the components of a binary mixture of normal substances is slight"*, by Mr. J. J. VAN LAAR. (Communicated by Prof. H. A. LORENTZ).

(Communicated in the meeting of April 26, 1907).

1. In the latest volume of These Proceedings [1]) Dr. KEESOM (also in conjunction with Prof. KAMERLINGH ONNES) stated some important results, inter alia concerning his investigation on the special case that one, e.g. a_1, of the two quantities a_1 and a_2 is very small; which is

[1]) KAMERLINGH ONNES and KEESOM, These Proc., Dec. 29, 1906, p. 501—508 [On the gas phase sinking in the liquid phase etc. (Comm. 96*b*)]; KEESOM, Ibid. p. 508—511 [On the conditions for the sinking etc. (Comm. 96*c*)]; KEESOM, Ibid. March 28, 1907, p. 660—664 (Comm. 96*c* continued); KAMERLINGH ONNES and KEESOM, Ibid. of April 25, 1907, p. 786—798 [The case that one component is a gas without cohesion etc. (Suppl. N°. 15)].