Microbiology. — *"Fat-splitting by bacteria."* By Dr. N. L. SÖHNGEN. (Communicated by Prof. Dr. M. W. BEIJERINCK).

(Communicated in the meeting of November 26, 1910).

Our knowledge about fat-splitting by bacteria, a process interesting as well for its practical value for several industries, as for scientific reasons, must hitherto be considered as very deficient when compared with what we know of the breaking off of carbohydrates and proteids.

This relatively little knowledge should be ascribed to various causes.

In the first place there is no urgent motive to study fat-splitting by organisms for technical purposes, in as much as technical fat-splitting is without much trouble effected chemically or enzymatically. Moreover, in the splitting of fat do not result such striking conversion products as in the breaking off of carbohydrates and proteids by bacteria, so that this process may easily be overlooked by the experimenter.

To this we may add that the decomposition of fat proceeds in general slowly, so that a prolonged cultivation is required before a sufficient quantity of material is formed for making the analyses, which, besides, are by no means simple.

Fat-splitting by microbes is notwithstanding of great signification for the industry of fats by the highly prejudicial consequences accompanying this process.

So we should not be astonished that the greater number of investigations in this line have been made especially in behalf of the dairy industry; so that rancidity of butter and fat-splitting in cheese have best been studied.

1. HISTORICAL.

We shall only give a short survey of those investigations on fat-splitting which have been made in recent times.

REINMANN [1]) stated in 1900 that the rancidity of fats must not be ascribed to the influence of air as was then the common opinion. The action of microbes he did not mention. The growing tallowy of fats was after REINMANN caused by the action of light in presence of air.

JENSEN [2]) attributes rancidity wholly to microbic action. Of the

[1]) Centralblatt f. Bakt. 2 Abt. 1202. Bd. 6 S. 166.

[2]) Landw. Jahrb. d. Schweiz 1901. Bd. 15 S. 329.

organisms isolated from rancid butter *Oidium lactis*, *Cladosporium butyri*, *Penicillium glaucum* and *Streptotrix alba* proved vigorously to split fats, whilst *B. fluorescens liquefaciens*, *B. prodigiosum* and *B. mesentericus vulgatus* belong to the feebler splitters. According to JENSEN part of the fat in cheese is also splitted, especially at the outside.

The researches of LAXA [1]) with pure cultures of fat-splitting organisms inoculated into sterilised casein, made of unskimmed milk, showed that *B. fluorescens liquefaciens*, *Oidium lactis*, *Penicillium glaucum* and a *Mucor* species split butter.

KÖNIG, SPIECKERMANN and BREMER [2]) published researches on the decrease of fat percentage in cattle-cake by microbic action. According to them this percentage may be reduced from 10%—12% to a few per cents within the time of a year.

HUSS [3]) isolated from milk a *Bacterium lipolyticum* which in milk and butter causes a rancid, bitter taste. The description of this bacterium we briefly give as follows.

Bacterium lipolyticum is a coccus-shaped, 0.3—0.5 μ wide, and 0.7—1.4 μ long motile rod; liquefies gelatin slowly without film formation, coagulates milk at 20° C. in three days, then dissolves the casein; the culture is then dirty yellow, has a putrid smell and reacts alkaline. Indol is produced in slight quantity; methylene blue is reduced, nitrate is reduced to nitrite, lipase is secreted. Acid production occurs from glycerin, mannite, dextrose, saccharose, raffinose and xylose, not from lactose.

From a sample of quickly creaming milk WOLFF [4]) isolated a bacterium, the cause of this phenomenon. The shape of this microbe is like that of *B. lactis acidi*: size of the cells 0.6—0.8 $\mu \times 1$—1.5 μ. Slightly motile. On gelatin this bacterium grows out to small colonies having the form of a flower-head of *Bellis perennis*. Gelatin and casein are not liquefied. In milk this microbe grows well and forms a film; the reaction of the liquid is alkaline and it smells like soap.

Besides in the dairy industry, researches on fat-splitting in the soil have been made. In 1900 RUBNER [5]) published a treatise on this subject in which the splitting and assimilation of fat by microbes in the soil is stated. This experimenter found that in a year $\pm$ 50% of the fat added to the soil was split and $\pm$ 15% assimilated. A vigorous splitting of the additional fat also occurs in culture liquids, especially

1) Archiv. f. Hyg. Bd. 41 1902.
2) Zeitschr. f. Unters. der Nahrungs- und Genüssmittel 1901 Heft 16 S. 720.
3) Centralblatt f. Bakt. 2 Abt. 1908 Bd. 20 S. 474.
4) Milchwirtsch. Zentralbl. 1909 p. 500.
5) Arch. f. Hyg. 1900 Bd. 38 S. 67.

in those containing an easily assimilable source of nitrogen, such as peptone or proteids, after inoculation with garden soil. Addition of calcium nitrate to this culture medium proved very favourable for the process.

In his experiments with the pure culture of a fat-splitting bacterium in broth, calcium nitrate and 4.424 grs. of fat, after 35 days' cultivation at room temperature, more than the half was split and ± 0.7 gr. assimilated; after a cultivation of more than a year, however, nearly all the added fat was split, more than the half being assimilated.

The fatty acids and the glycerin formed from the fats are oxydised without intermediate products to carbonic acid and water.

BECHHOLD [1]) demonstrated that fats and soaps disappear in the sewage mud of the installations for the purification of water at Stettin, by bacterial action.

EYKMAN [2]) gives in a treatise on fat-splitting bacteria, beside other facts, a simple and nice method to demonstrate lipase secreted by micro-organisms. This method is based on the splitting action of diffusing lipase, produced by bacterial streaks on agar or gelatin, on a thin fat layer at the underside of this substratum. The fat, decomposed under the bacterial mass has the appearance of an opaque white stripe, very distinct from the non-decomposed fat.

According to EYKMAN the following bacteria split fat: *B. pyocyaneum*, *Staphylococcus pyogenes aureus*, *B. prodigiosum*, *B. indicum*, *B. ruber*, *B. fluorescens liquefaciens*.

RAHN [3]) has studied fat-splitting by microbes on a culture medium of the following composition. In an inclined ERLENMEYER flask some fat is melted. After cooling the flask is put upright and a thin layer of an anorganic culture liquid is filled into it.

After inoculation with garden soil a good growth of moulds and bacteria results, which, after transference to a similar flask soon recommences.

Four fat-splitting moulds and two fat-splitting bacteria were isolated on culture plates of an anorganic nutrient liquid solidified with agar and containing tributyrin in finely divided state. On these plates a clear field appears around the colonies of fat-splitting microbes, in consequence of the splitting action of the diffusing lipase on the tributyrin, and of the solution of the products formed in the medium.

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[1]) Zeitschr. f. Angew. Chem. 1898 S. 849 cit. [1]).

[2]) Eighth Dutch Phys. and Med. congress 1901 p. 171.

[3]) Centralblatt f. Bakt. 2 Abt 1906 Bd. 15 S. 422.

DE KRUYFF [1]) has made a research at Buitenzorg on fat-splitting bacteria in the tropics. For the isolation of the microbes he first accumulated them in ERLENMEYER flasks provided with a thin layer of an anorganic culture liquid and finely divided fat. The inoculation was made with soil, water, or excrements; cultivation at 37° C. Transplantation to a same culture liquid produces an exclusive growth of fat-splitting organisms.

Nine fat-splitting species were isolated, all *Lipobacter*-species. Of these four are fluorescents, two correspond in many respects to the *Bacterium pneumoniae*, whilst *Lipobacter* N°. 4 is a yellow non-liquefying micrococcus.

Quantitative determinations show that by some species in 12 days all the fat is split and one half oxydised. In a paper on thermophilous bacteria DE KRUYFF [2]) mentions that also among these sporulating species some are found which secrete lipase,

II. PROOF OF THE FAT-SPLITTING POWER OF MICRO-ORGANISMS. LIPASE.

In two ways the fat-splitting power of micro-organisms may be stated: 1. by means of titration of the fatty acids split off by an organism from the fat; 2. by properly rendering visible the produced fatty acids and soaps.

The first method may be successfully applied in the investigation of preparations in which concentrated lipase occurs. It will always be necessary to apply it for quantitative determinations of fat-splitting. The second way, including the methods followed by RAHN, DE KRUYFF, and EYKMAN, should be preferred for qualitative determinations on account of its great sensitiveness.

On the plates some of EYKMAN's culture plate reactions are represented. The white fields of Plate *A* are formed on the left by a *B. lipolyticum a*, described on page 674, and on the right by *B. Stutzeri*, both cultivated on broth agar. Plates *B* and *C*, on which *B. lipolyticum β* and *B. denitro fluorescens non-liquefaciens*, contain broth agar with additon of respectively 1 % glucose and 4 % glycerin. The peculiar fields of *D* are obtained by allowing the culture plate to lie for some weeks after the agar layer has been removed.

These figures show that glucose and glycerin exert little influence on the degree of decomposition by lipase; this becomes, however,

[1]) Bull. du Départ. de l'Agric. aux Indes Néerland. 1907 cit. Centralblatt. f. Bakt. 2e Abt. Bd. XX S. 610.

[2]) Bull. du Départ. de l'Agric. aux Indes Néerland. 1909. N. XXX Microbiologie IV.

the case as soon as from those compounds acids are formed, as wil be shown below.

When considering attentively the decomposed portions of the culture plates we observe that around each white central part of a field, consisting of fatty acid, soap, and compounds of lipase with fatty acid, a less white band extends formed from fatty acids. Around this less white part another band is found characterised by still more clearness, it is even more transparent than the non-decomposed adjoining fat. This clear portion is formed in consequence of the disappearance of the fat-crystals, caused by the lipase action.

These bands are very well seen in the figure; on the culture plates themselves they can be distinguished still better.

The same is observed respecting little staves of fat placed in a lipase solution. Fig. 1, 2 and 3 Pl. 4 are drawings (24 times magnified) of pieces of dry fat kept for 30 days at 20° C. in test tubes of broth inoculated with fat-splitting microbes. On the outside of the fat (in the figure at the top) is a bacterial film under which a white saponaceous part (on the plate cross-hatched) then a layer consisting of fatty acids (hatched), and finally the non-decomposed fat.

The diffusion velocity of lipase in dry fat is, as the experiment shows, very small. In one month the lipase has penetrated into the fat not more than half a millimeter. In moist fat that velocity is much greater. The great difficulty however, evenly to emulsionate water and fat, makes comparative experiments as to the diffusion velocity of lipase in fat containing different quantities of water almost impossible.

As said above, the rate of acidity of the medium influences the degree of decomposition of fat by fat-splitting microbes. When using an acid culture agar for the lipase reaction after EYKMAN we see under and around the bacterial inoculation streaks in the fat layer a wide field appear, but we do not perceive a white central part as is the case when the plate is alkaline.

From a series of experiments which will be discussed in a separate communication, followed that for some micro-organisms two lipases are formed which, besides by their different diffusion velocity, are also characterised by their different behaviour towards acids.

Thus, *B. Stutzeri* and *B lipotyticum* secrete two lipases, *α-lipase* and *β-lipase;* the former diffuses more rapidly than the latter and splits fat as well in an acid as in an alkaline medium.

β-Lipase is formed in an acid medium, but does not decompose fat in it, it may however, become active again after neutralisation of the medium.

Fat-splitting moulds and yeasts can be accumulated in a feebly acid culture liquid, which, besides fat or fatty acids, contains only anorganic salts, with garden soil for inoculation material. They produce, together with *endo-lipase*, often also *α-* and *β-lipase*.

In my experiments the fat-splitting power of bacteria was usually demonstrated by means of a method with fatcoated testtubes, which is carried out as follows.

The inner side of a sterile test tube is coated with a thin layer of fat; now a nutrient liquid is introduced in which the bacterium to be examined on lipase grows well and this bacterium is inoculated into it. If now lipase is produced in the culture we see after two or three days that portion of the fat which touches the liquid grows white; this appears first and most obviously at the place where the bacterial growth is strongest. Aërobes decompose the fat first near the surface of the liquid, anaërobes first at the bottom of the tube.

On the accompanying photographs Pl. 3 we see a series of fatted tubes in which various fat-splitting microbes have decomposed the fat. In 1 no fat-splitting bacterium is inoculated, hence the fat has remained unchanged; 2 and 3 contain cultures with ammonium chloratum as source of carbon resp. of *B. Stutzeri* and *B. denitrofluorescens non-liquefaciens*; 4 and 5 contain cultures of the same microbes but with kaliumnitrate as source of nitrogen; 6 contains a rough culture of a proteid putrefaction by inoculation with soil; 7 contains a rough culture of a pasteurised proteid putrefaction; 8, 9, 10, 11, and 12 contain cultures of various fat-splitting microbes isolated from soil and milk.

II. Fat-splitting microbes in the soil.

The microflora of the soil abounds in organisms which secrete lipolytic enzymes; a sowing of soil on a fatted plate after Eykman, or dilutions in fatted tubes show this most clearly. In one gram of humus we not seldom count some ten thousand fat-splitting ferments.

A. Accumulations in culture media with ammonium chloratum as source of nitrogen.

For the growth of bacteria, requiring beside fat as source of carbon only anorganic salts and ammonium chloratum as source of nitrogen, the following culture liquid was used.

100 tapwater
0,5 fat [1]) (finely divided)
0,5 calciumcarbonate
0,05 kaliumsulphate
0,1 magnesiumammonium-phosphate.

In this feebly alkaline medium the fat-splitting bacteria grow very well; the chalk and the magnesiumammonium-phosphate serve to neutralise the fatty acid and the acids resulting from glycerin.

Aërobic culture at 18°—25° *C.* If we inoculate a layer of the above medium, ± one centimeter thick, in an ERLENMEYER flask, with garden soil, sewage mud, canalwater or dung, and cultivate at 18°—25° C., a rapid increase of the bacteria introduced with the inoculation material ensues. After one or two transferences to a same medium there is an abundance of these microbes. The changes observed in the medium are the following: After one or two days the liquid becomes turbid by the bacterial growth and usually assumes a yellowish green colour; the floating pieces of fat sink down on saponification and subsequently change into slimy flakes.

Most of the bacteria present in the medium belong to the fat-splitting species; among them are melting and non-melting micrococci and fluorescents and species corresponding to *B. punctatum.* As well among the fluorescents as among the last named, stocks are found that split fat very vigorously, whereas others do this feebly or not at all.

If we take fatty acid instead of fat for source of carbon, the same flora occurs, whilst with glycerin very fine accumulations of *fluorescents* are obtained.

Aërobic culture at 30°—37° C. At these temperatures the culture presents quite another aspect as at those between 18°—25°. Thus we often find on the liquid a film of *Spirillum* especially when inoculating with sewage or canal water; it gets, however, lost after one or two transplantations. Evidently the spirilla have no lipolytic enzymes and grow at the expense of the products formed by other microbes. Sometimes a not inconsiderable growth of hay bacteria and butyric

[1]) The fat used for these experiments is the so-called "suif pressé", a product remaining behind after pressing the oleo margarine from tallow. The melting-point is ± 55° C., the saponificationnumber 193—195. In consequence of the high melting point it is easy finely to distribute this fat in the culture liquid by shaking it in melted state with the latter and quickly cooling it. Also for culture at high temperatures (± 52°), for anaërobic culture and for experiments on denitrification with fats, it shows advantages over easily melting fats.

acid ferments takes rise in the medium inoculated with rough material; this neither reoccurs after transplantation.

After the first or second transplantation the flora chiefly consists of a group of aërobic bacteria of which four species have been isolated; *B. lipolyticum* α, β, γ, and δ. These bacteria have the shape of short double rodlets, 0.15 μ—1 μ wide and 0.25—2.5 μ long, slightly motile and wrapped in a slimy envelope. The colonies show resemblance to those of *B. aërogenes*; often the middle part is somewhat elevated. On broth gelatin after 5 days' culture at 20° C. they grow out to white or greyish white, sometimes slimy colonies, which after 5 days get a diameter of 1.5—2 mm. On broth agar they are more transparent and flatter.

The growth optimum is ± 35° C. They cannot resist heating for 10 minutes at 60° They thrive better on broth gelatin or broth agar than on media with salts of organic acids (malic acid and lactic acid) as carbon source and ammonium chloratum as source of nitrogen. On slices of potato these microbes grow out to white or greyish white moist colonies. Broth becomes very turbid after inoculation, at the bottom of the test tube a sediment forms, no film at the surface.

They thrive very well in milk which becomes viscous, alkali being formed. *B. lipolyticum* γ and δ form chymosine, the two others not or very little. Trypsine is not produced, neither diastase nor ureasa. Indican or aesculine are not split. On whey gelatine the growth is good and alkali is formed whereby an iridescent film appears. Indol is not produced, nitrate not reduced to nitrite, glucose is not fermented. The optimum of the lipase action lies near ±65°. In culture liquids containing ammonium chloratum as source of nitrogen a good growth is obtained with the following carbon sources: alcohol, glycerin, glucose, saccharose calciummalate, -lactate, -stearinate, aethylacetate, aethylbutyrate, tributyrine, trioleïne.

In a culture medium of the composition: 100 tapwater, 1 fat, 0,05 NH_4CL, 0,05 K_2HPO_4, 1 $CaCO_3$, after ten days' cultivation at 25° *B. lipolyticum* α and β had split respectively 130 and 105 mGs. of fat, and assimilated 20 and 21 mGs. In brothwater, 2 % peptone, 1 % $CaCO_3$, and 1 % fat, in ten days respectively 630 and 480 mGs. of fat were split, and resp. 40 and 80 mGs. were assimilated.

Aërobic cultivation at 45°—55°. At these temperatures fat-splitting is seldom observed even with addition of large quantities of inoculation material (5 Gr.) to the medium. The cultures in which fat-splitting occurs contain a species closely allied to *B. mesentericus*

nearly in pure culture; this species stands no transplantation to the same medium; after sowing out on broth agar with 2 % glucose, white or greyish white colonies result of 2—6 μ long rodlets, 1 μ wide. The spores resist boiling heat, gelatin is liquefied, diastase and lipase are secreted.

In a medium with mineral salts to which glucose, saccharose, glycerin, calciumlactate or amylum have been added, a good growth results after inoculation. Addition of peptone as source of nitrogen gives much stronger growth than ammonium chloratum. Stearinacid salts are not assimilated. The culture on slices of potato reminds of that of *B. mesentericus* on this medium, but the colour of the colony is whiter.

This microbe belongs to the group of hay bacteria and is distinguished by its lipase production; *B. mesentericus*, *B. subtilis* and *B. megaterium* isolated from potatoes do not secrete lipase.

Anaërobic culture. Under anaërobic conditions no growth of fat-splitting microbes occurs in a medium containing only fat as source of carbon and ammonium chloratum as source of nitrogen.

B. Denitrification with fats.

In our researches on denitrification with fats no other source of carbon was present in the medium composed of 100 tapwater, 1 kaliumnitrate, 0,05 bikaliumphosphate.

The culture was arranged as follows: about a gram of fat was melted in a carefully dried stoppered bottle with narrow mounth of ± 250 c.c. capacity; by turning the fat is evenly distributed over the inner surface. After cooling the bottle is filled with the said nutrient liquid which is subsequently inoculated with garden soil or some other infection material.

A series of experiments at temperatures between 20° and 45° C. proved that at 27°—30° C. the strongest denitrification was brought about; at this temperature the subsequent researches have been made.

If we inoculate with 3 grams of garden soil, sewage- or canal mud and cultivate at ± 28°, we see after one or two days the topmost edge of the fat layer near the stopper grow white. From here the decoloration proceeds to the bottom of the bottle. Soon gas bubbles arise from the inner wall of the fat layer, then they also form between the fat and the glass wall, by which the fat is separated from the glass. After 5 or 6 days we usually see the pieces of fat partly lying at the bottom. The fat, at first rather transparent, grows white, then dirty yellow and quite opaque. The culture liquid, partly pressed out of the bottle, is turbid and dirty yellow. Transplantations

of such a culture to a fresh medium, arranged in the same way, yield again a good growth, whereby the described changes of fat and culture liquid return.

The bacteria which cause the denitrification with fat may be studied better by sowing the culture on broth gelatine or tapwater gelatine with addition of 0,5 % tributyrine, 0,05 % K_2HPO_4, and 0,1 % KNO_3 or NH_4Cl.

On the two plates the same species of bacteria develop, but they thrive better on broth gelatine than on the other medium.

The most vigorous bacteria denitrifying with fat proved to be *B. Stutzeri* L and N, *B. pyocyaneum*, *B. punctatum*, and a bacterium producing lipase and diastase, a representative of *B. denitro fluorescens non-liquefaciens*, described by VAN ITERSON [1]).

By cultivation at ± 20° the denitrifying species are more obvious, whereas at ± 34° the same flora appears as at 28°, but the growth is less vigorous; *B. sphaerosporus* and *B. nitroxos* described by BEIJERINCK [2]) denitrify neither with fat nor with glycerin or fatty acid.

The varieties of *B. Stutzeri* and *B. denitrofluor. non-liq.* are characterised as well by the different structure of their colonies as by their different power of denitrification and fat-splitting.

The above mentioned microbes denitrify with glucose, fatty acid, calcium lactate, humate of natron, asparagine and peptone. The various stocks of *B. Stutzeri* denitrify besides with maltose, glycerin, glycol and mannite, aethylacetate, maltose and butylalcohol; no acid is formed from saccharose, lactose and raffinose.

B. denitro fluor. non-liq. produces a little acid from glucose, but none from any of the said compounds; nor does it denitrify with mannite, glycerin, maltose, or glycol.

The fat-splitting of both bacteria under aërobic conditions was ascertained by cultivation in an ERLENMEIJER flask of 300 cc. with the culture liquid: 200 cc. tapwater, 1 gr. finely divided fat, 0.1 gr. ammonium chloratum, 0.1 gr. bikaliumphosphate and 0.5 gr. chalk.

After inoculation with 5 cc. of a 48 hours old brothpeptone culture of these bacteria, after 30 days at 28° C.

B. Stutzeri . . . . . had split 630 mG. and assimilated 65 mG.
B. den. fluor. n. liq. had split 920 mG. and assimilated 80 mG.

These experiments show that by *B. Stutzeri* and *B. denitrofluor. non liq.* respectively 70 % and 100 % of the fat are split and

[1]) Accumulation experiments with denitrifying bacteria. Royal Ac. 1902.

[2]) Bildung und Verbrauch von Stickoxydul durch Bakterien. Centralbl. f. Bakt. Abt., 2, Bd. 25, S. 30.

that nearly $^1/_{10}$ is assimilated for the building of the bacterial bodies.

Splitting and assimilation of fat at the denitrification were determined as follows.

In an ERLENMEYER flask of 300 cc. capacity a certain quantity of fat was weighed and the flask closed with a rubber stopper, fitted with a bent glass tube for gas outflow. After the apparatus had been sterilised and heated the fat was spread over the inner surface by rotation. After cooling 300 cc. of the following nutrient liquid was introduced: 100 tapwater, 1 kaliumnitrate, 0,05 bikaliumphosphate; inoculation took place with one of the denitrifying bacteria. The cultivation took place at 28° the evolving gases were caught over paraffin oil or caustic alkali.

Before the evolving of gas begins the oxygen above the culture is assimilated, the fat first undergoing hydrolytic splitting.

From June 28, 1909 to July 8 *B. denitrofluor. non liq.* formed 57 cc. nitrogen and 1.5 cc. carbonic acid with 0.4385 gr. of fat. The rapidity of the process had then decreased so much that it was resolved cautiously to renew the culture liquid without loss of fat. The decanted liquid reacted feebly alkaline, and contained nitrate, nitrite and glycerin, whilst per 25 cc. liquid 5.1 cc. carbonic acid was present. After the refreshing the fermentation velocity increased again, thereupon it decreased slowly. The total production until August 18 with the 0.4385 gr. of fat that had nearly disappeared, was 230 cc. nitrogen and 142.6 carbonic acid. If we neglect the very slight quantity of the still present saponaceous substances, about $^1/_6$ of the carbon of the fat is transformed by the denitrification into carbonic acid and $\pm^5/_6$ into bacterial matter and insoluble organic compounds. In the culture liquid no intermediate products could be pointed out; evidently, also in this process the oxydation of the fatty acid only produces carbonic acid and water.

In Pl. 4 fig. 4 the rate of rapidity of the process is graphically represented. The evolved gas volumina per 6 hour's, cultivation are noted on the abscis; one centimeter representing a cm.^{3} of gas. On the ordinate the time is noted, one day being represented by one cm. We can very well perceive the increase in rapidity of the gas production after the refreshing of the culture liquid on July 8; yet the angle of inclination of the line is much smaller than that at the beginning of the process.

The cause of this falling must not be attributed to the formation of soluble secretion products of the culture, as is often the case in bacterial cultures, but to the presence of a thick layer of bacterial

slime which has set off on the fat. This disturbs the movement of the substances and retards the rapidity of the process.

A denitrification by *B. Stutzeri* was set up in a similar way with 0.547 gr. of fat; the evolved gases were collected above caustic alkali, the medium was not renewed. From 9 to 28 October 1909 102 cc. of nitrogen were caught; on the 28th of October the fermentation produced less than a half cc. of gas per 24 hours, hence the experiment was stopped although part of the fat was still present.

In fig. 5 the rate of velocity of the gas evolution is represented in a way similar to that of the previous experiment.

Denitrifications with fatty acids proceed correspondingly to those with fats, on the other hand, denitrification with glycerin by *B. Stutzeri* occurs rapidly and completely; it is quite finished within a few days. About $^2/_3$ of the carbon of the glycerin is then converted into carbonic acid and $^1/_3$ is assimilated for the building up of organic material.

IV. Fat-splitting bacteria in milk.

Milk belongs to the most favourable culture media for fat-splitting microbes; beside a finely divided and very easily saponifying fat, it contains excellent sources of nitrogen and carbon for these bacteria.

The number of fat-splitting organisms varies exceedingly in spontaneously infected milk, some hours after the milking we can already point out from 180—20000 per cc., among which may occur the species mentioned above.

Two factors chiefly influence the growth of fat-splitting bacteria in milk, namely temperature and admission of oxygen.

If we wish to accumulate fat-splitting microbes in milk the surest way is by aërobic culture at 10°—15°; at which grow chiefly *fluorescents*, *B. punctatum*, *micrococci* and so-called *aromatic bacteria* [1]). Also at aërobic cultivation at 27°—30° a considerable growth of fat-splitting microbes may occur, but this depends on the primitive composition of the bacterial mixture. At anaërobic cultivation, however, the lactic acid ferments in a short time overgrow all other species.

In connection with the lactic acid ferments we can admit as a general rule that the conditions of growth for fat-splitting organisms in milk are wholly opposed to those wanted for the thriving of lactic acid ferments.

[1]) Beijerinck. Fermentation lactique dans le lait. Archives Néerl. des Sciences exactes et naturelles Serie II, t. XIII.

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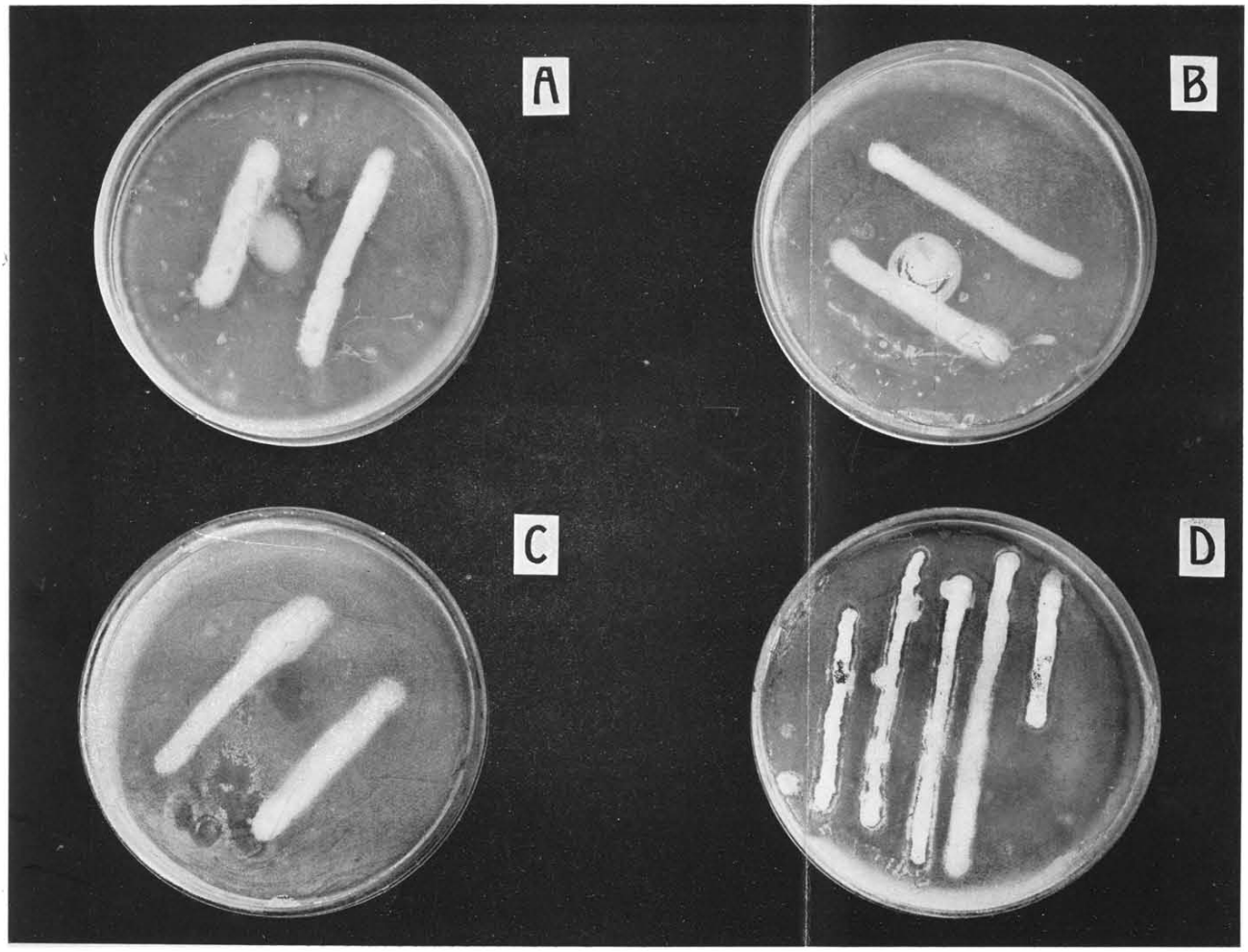

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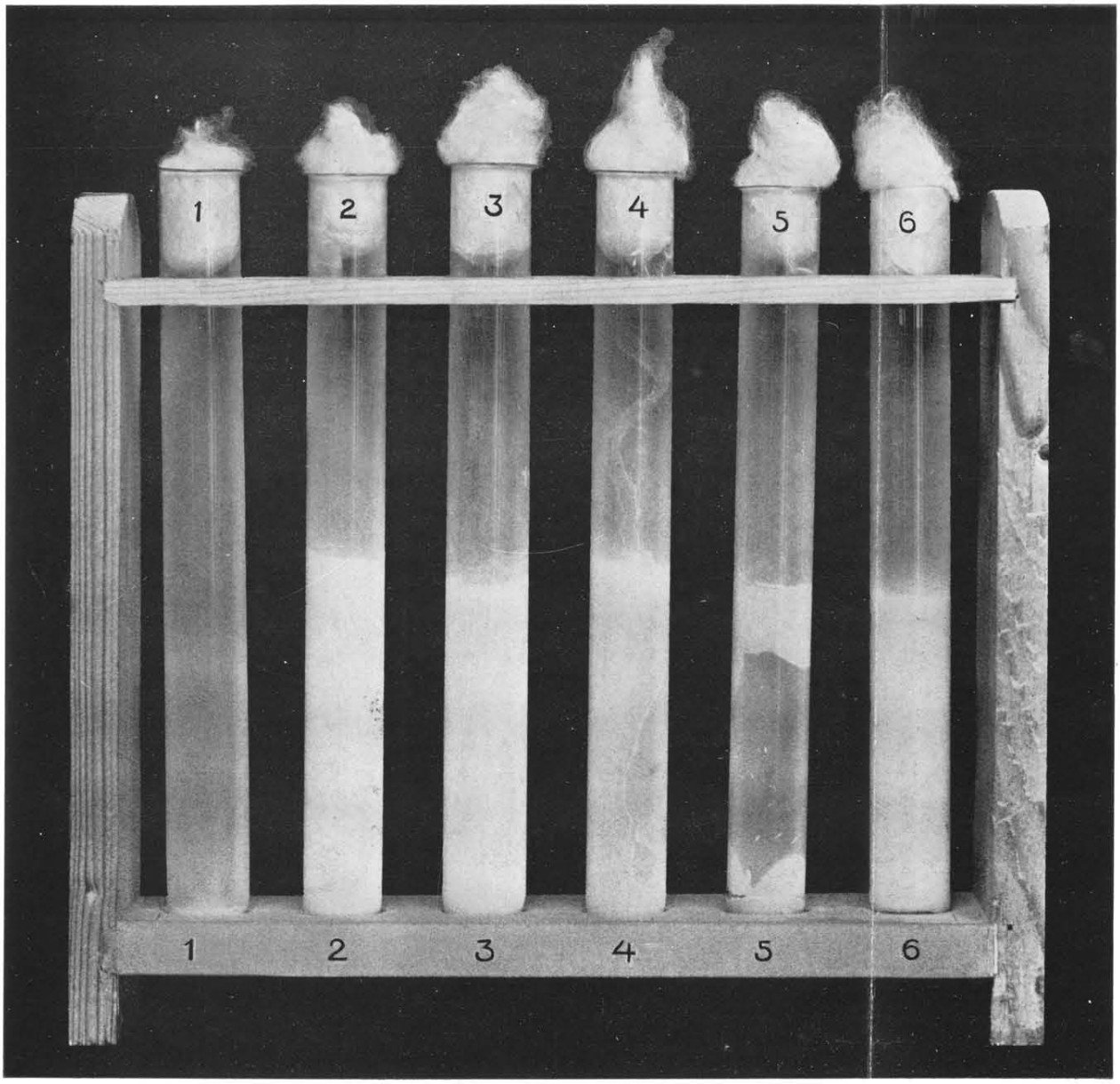

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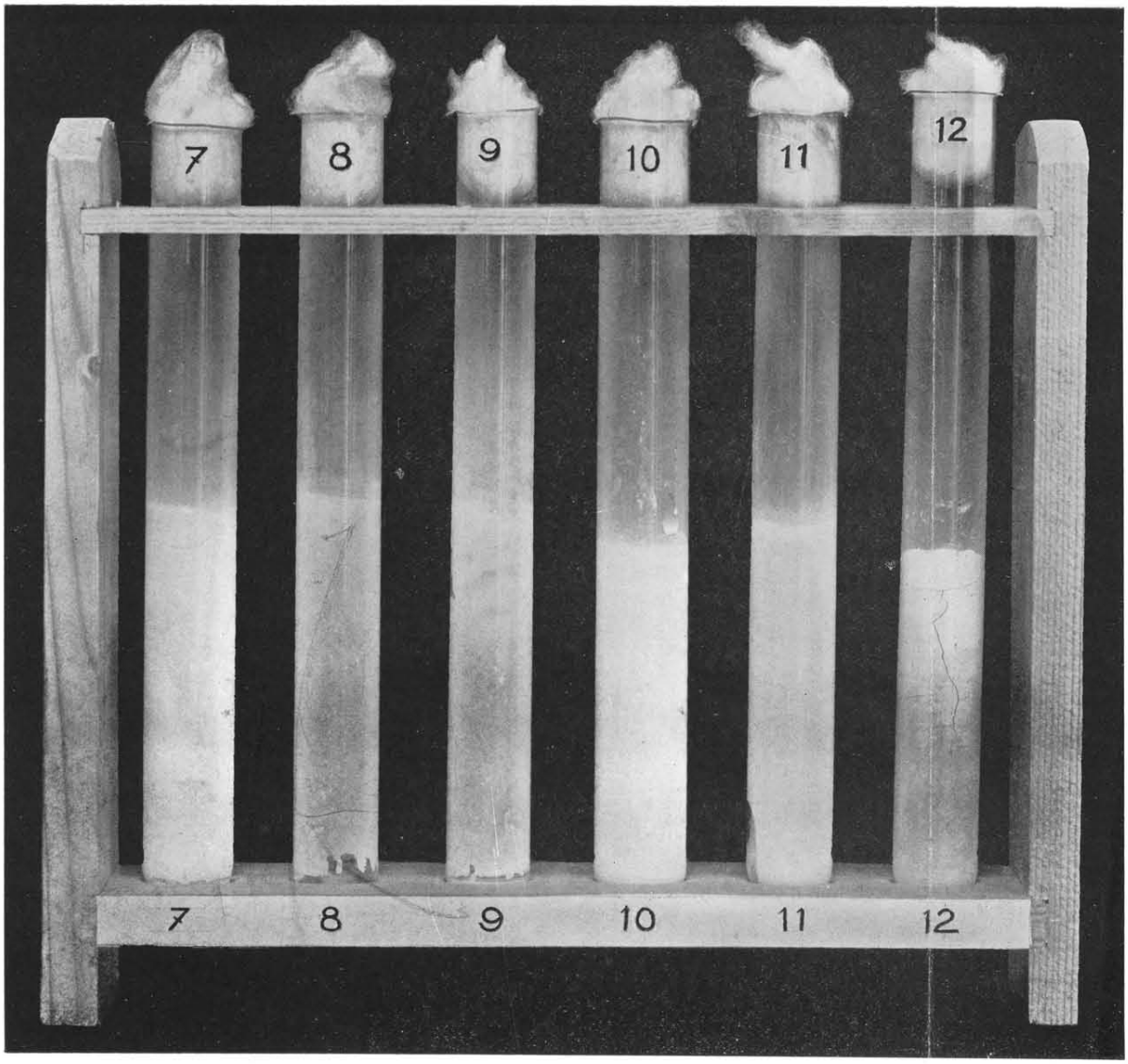

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Fig. 1.

Fig. 2.

Fig. 3.

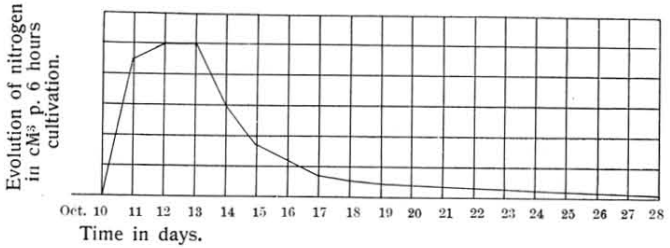

Fig. 5. Graphic representation of the denitrification with 0,547 gr. fat by B. Stutzeri (L. and N.).

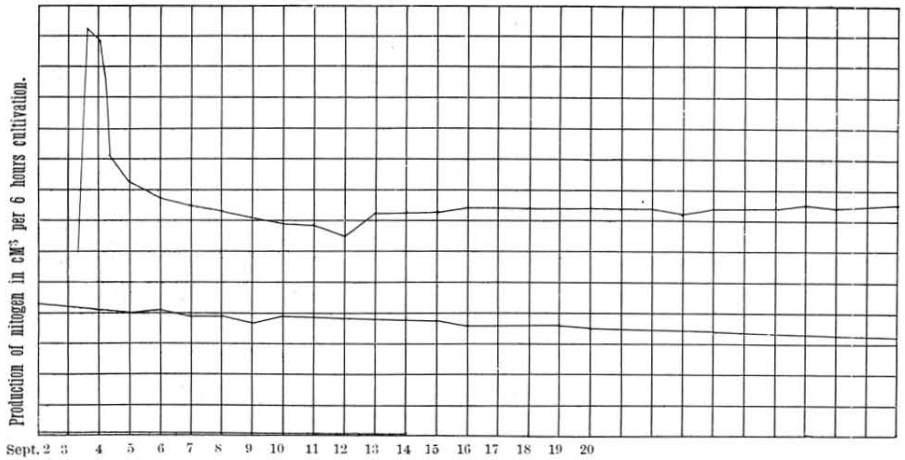

Fig. 4. Graphic representation of the dinitrification with 0,4385 gr. fat by B. dinitro-fluorescens.

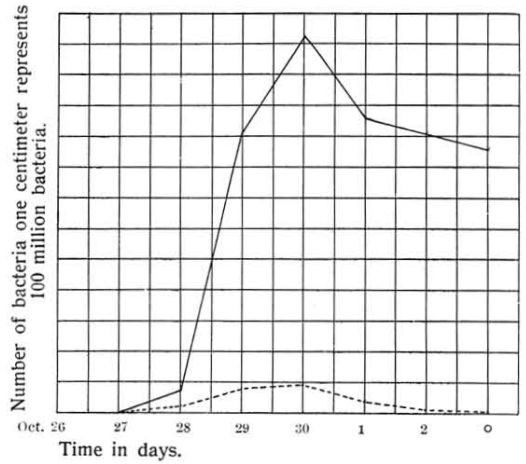

Fig. 6. Graphic representation of the development of lactic-acid ferments and fat-splitting bacteria in spontaneously acidifying milk.

In fig. 6 we see the rate of development of fat-splitting and that of the lactic acid ferments in milk kept under circumstances of temperature and oxygen pressure, such as is usually done in practice. (On the abscis 100 million bacteria are represented by one cm., on the ordinate 24 hours by 1 cm.). For this a 1 liter flask of 800 cc. of good clean milk was kept in a cellar at ± 10°; after every 24 hours the number of lactic acid ferments and that of the fat-splitting microbes was determined.

The bacterial ciphers used to construct the graphics, show that the proportion of the lactic acid ferments to the fat-splitting bacteria in the milk at the beginning was $^2/_3$; that same day after 8 hours $^9/_{10}$ and the 7 following days respectively $^3/_1$, $^5/_1$, $^{12}/_1$, $^{17}/_1$, $^{25}/_1$, $^{100}/_1$, $^{100}/_1$. During the first five days the number of fat-splitting microbes increased, but less rapidly than that of the lactic acid ferments; thereupon a relatively rapid destruction takes place at an acid degree of ± 85. The regularly increasing rate of acid appears to do little harm to the lipase producing bacteria, so sensitive to acidity, although these at a direct inoculation into acid milk, titrating 50 cc. $^1/_{10}$ N per 100 cc., no more grow; hence it is evident that they adapt themselves to the acid degree. To the fat-splitting species in this milk belonged: *fluorescents*, *micrococci* and the group of *B. lipolyticum*, described page 674.

The bacteria of putrefaction happening to get into the milk with the faeces of the cows or from the air of the stable, contain for the greater part lipolytic enzymes as for instance the aërobic species *B. vulgare*, *B. prodigiosum*, *B. fluorescens liquefaciens*, *B. pyocyaneum* and the anaërobic *Bacillus putrificus*, which last species is often met with in pasteurised milk.

The changes provoked in milk by the fat-splitting microbes are, excepting those caused by the lipolytic influence of the microbes, more particularly to be ascribed to their peptonising action. To milk they give a cheesy or soapy smell and often a bitter taste; they cause rapid creaming and render the suspended fat rancid as also the butter made from it.

Hence we must consider these bacteria as the most dreaded enemies of the dairy- and fat industry.

Summary.

1. The fat-splitting power of aërobic as well as of anaërobic micro-organisms and their power of denitrifying with fat, may in a simple way be determined by means of fatted test-tubes.

2. By a large number of bacteria, universally spread in nature, fats may be broken off anaërobically, oxydised aërobically, or if nitrates or nitrites are present be denitrified.

3. All these processes are caused by the secretion of "lipase" by microbes; glycerin and fatty acids separated by the action of this enzyme are then further converted by the organisms.

4. Several fat-splitting organisms produce two lipases, α- and β-lipase; the former diffuses more rapidly than the latter and splits fat as well in an acid as in an alkaline medium; β-lipase is formed in an acid medium but does not decompose fat in it; it may however, become active again after neutralisation of the medium.

5. Lipase diffuses through water-free fat; but the diffusion velocity is very small.

6. The aërobic fat-splitting bacteria thrive well in culture media containing exclusively fat as source of carbon and ammonium chloratum as source of nitrogen.

7. To the already known fat-splitting bacteria we can add: *Bacillus putrificus* (BIENSTOCK), a representative of the *mesentericus group*, *B. Stutzeri*, and *B. denitro fluorescens non-liquefaciens*.

8. Milk is a favourable medium for fat-splitting microbes.

9. In spontaneously infected milk, kept under circumstances usual in practice, growth and destruction of fat-splitting bacteria and lactic acid ferments occur about simultaneously; they are chiefly dependent on the acid production.

10. The injurious influence of fat-splitting microbes on the quality of dairy products is chiefly owing, besides to their lipolytic properties, to the formation of bitter tasting and badly smelling products from proteids and casein by these microbes.

This subject will be more elaborately treated in the "Centralblatt für Bakteriologie".

Botany. — "*On the cause of dimorphism in Oenothera nanella.*" By H. H. ZEIJLSTRA FZN. (Communicated by Professor HUGO DE VRIES).

In 1905 I occupied myself with an investigation of the dimorphism of *Oenothera nanella* which in consequence of many other duties before my departure to India, could not be completed. Although I intend to continue this inquiry next year, I think it desirable already to communicate the following preliminary results.

In "die Mutationstheorie" DE VRIES in a description of the species of *Oenothera* arisen by mutation has made us acquainted with a