

(4) *B. pyocyaneus* is less proteolytic in cream than it is in whole milk or skimmed milk.

(5) The presence of certain pathological bacteria, *B. typhosus*, and *B. paratyphosus alpha* and *beta*, cannot be detected in milk by the chemical changes which they induce in it.

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### STUDIES IN BACTERIAL METABOLISM.

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#### XXXVIII. Observations on Fat-Splitting in Milk by Bacterial Lipase.

A study of the metabolism of certain bacteria in sterile whole milk disclosed certain reactions, chiefly relating to the production of titratable acid in the presence of the progressive formation of basic products of protein breakdown, which could not be explained by any available information. *B. typhosus* and *B. paratyphosus alpha*, for example, produced a progressively acid reaction in milk, while *B. paratyphosus beta*, an organism very closely related, both qualitatively and quantitatively, produced a terminal alkalinity in the same medium under the same conditions. Similarly, *B. proteus* and *B. mesentericus* produced a progressive acid reaction, while *B. subtilis* produced a progressively alkaline reaction, notwithstanding the fact that all three organisms are strongly proteolytic. Several possibilities present themselves to explain these results:

(1) It might be assumed that there is a liberation of phosphoric acid, probably as acid phosphates, during the course of the bacterial digestion.

(2) The fermentation of fats in the Rubner sense,<sup>1</sup> with the liberation of fatty acids from the cream, might be a possibility.

(3) These organisms may secrete lipases, which break down the fatty constituents of the milk, perhaps independently of the protein metabolism.

(4) Other causes, as, for example, the possible formation of acid-reacting products, the result of protein decomposition.

There is no direct evidence for any of these assumptions, so far as these studies have shown: there is a certain amount of *à priori* objection to possibilities 1, 2, and 4, chiefly theoretical, however, and due to imperfect knowledge of the nature of intermediary metabolism of bacteria. To assume the presence of a fat-splitting ferment would appear to be a most logical hypothesis to consider first, and with this possibility in view the following experiments were made:

Broth cultures of *B. typhosus*, *B. coli*, *B. proteus*, *B. subtilis*, *B. mesen-*

<sup>1</sup> Rubner, *Arch. Hyg.*, 38, 67 (1900).

*tericus*, and *B. pyocyaneus*, respectively, in plain and dextrose broth were grown for ten days at 37° then filtered through Berkefeld filters to separate out the bacteria. Plain and dextrose broths were chosen because the products formed by all the organisms, except *B. subtilis*, and probably *B. pyocyaneus*, are widely different in the two media.<sup>1</sup> There is, furthermore, apparently about 0.1% dextrose in good grades of milk, an amount which the dextrose-fermenting organisms can soon use up, however, forcing them then to utilize other substances in their dietary.<sup>2</sup>

TABLE I.  
Filtrate.  
Cc. 0.02 N NaOH.

	Cream.				Ethyl butyrate.		Triacetin.	
	Neutralize start.	24 hours.	Neutralize start.	24 hours.	Neutralize start.	24 hours.	Neutralize start.	24 hours.
Plain broth.								
<i>B. typhosus</i> .....	+0.90	0.00	+0.80	0.00	+0.10	0.00	+0.50	+1.25
<i>B. coli</i> .....	+0.80	+0.05	+0.70	0.00	+0.20	0.00	+0.45	+1.15
<i>B. proteus</i> .....	+0.20	0.00	+0.20	0.00	-0.75	0.00	-0.10	+0.85
<i>B. subtilis</i> .....	+0.55	0.00	+0.65	0.00	+0.20	0.00	+0.40	+1.10
<i>B. mesentericus</i> .....	+1.15	+0.25	+1.25	+0.25	+0.50	+0.05	+0.60	+1.30
<i>B. pyocyaneus</i> .....	+0.15	0.00	+0.20	0.00	-0.50	+0.10	+0.15	+0.70
Cc. 0.02 N NaOH.								
Dextrose broth.								
<i>B. typhosus</i> .....	+2.70	+0.10	+2.90	+0.15	+1.65	+0.30	+2.10	+1.30
<i>B. coli</i> .....	+2.80	+0.05	+3.00	+0.05	+2.35	+0.15	+2.50	+1.10
<i>B. proteus</i> .....	+2.85	+0.20	+3.00	+0.20	+2.25	+0.35	+2.40	+1.30
<i>B. subtilis</i> .....	+0.30	0.00	+0.35	0.00	+0.45	0.00	+0.60	+1.35
<i>B. mesentericus</i> .....	+2.10	+0.20	+2.20	+0.20	+1.65	+0.25	+1.70	+1.15
<i>B. pyocyaneus</i> .....	+0.40	+0.20	+0.35	+0.25	-0.45	+0.10	+0.20	+0.95
Bacteria.								
Cc. 0.02 N NaOH.								
<i>B. typhosus</i> .....	+0.55	+0.25	+0.60	+0.20	0.00	+0.15	+0.30	+0.60
<i>B. coli</i> .....	+0.45	+0.15	+0.50	+0.15	-0.15	+0.15	+0.35	+0.80
<i>B. proteus</i> .....	+0.60	+0.40	+0.45	+0.45	-0.15	+0.30	+0.40	+1.15
<i>B. subtilis</i> .....	+0.60	+0.25	+0.65	+0.30	+0.10	+0.15	+0.50	+1.00
<i>B. mesentericus</i> .....	+0.50	+0.40	+0.40	+0.30	+0.10	+0.25	+0.40	+1.10
<i>B. pyocyaneus</i> .....	+0.60	+0.35	+0.55	+0.30	+0.05	+0.15	+0.40	+1.00
Controls.								
Cc. 0.02 N NaOH.								
<i>B. typhosus</i> .....	+0.50	0.00	0.00	0.00	+0.10	+0.05		

<sup>1</sup> Kendall, Day and Walker, THIS JOURNAL, 35, 1243 (1913).

<sup>2</sup> Kendall, Day and Walker, "Studies in Bacterial Metabolism, XXXI," THIS JOURNAL, 36, 1920 (1914).

The details of the technique used in testing lipolytic activity of bacterial growth have been described in detail elsewhere<sup>1</sup> and will not be referred to here other than to mention the general procedure.

One cc. of the sterile filtrate was introduced into large, clean test tubes containing toluol water (10 cc.). It will be seen that, theoretically at least, but  $\frac{1}{100}$  of the total reactive substance of the original culture is thus brought under observation, for the initial volume of each culture was uniformly 100 cc. To these diluted cultures 0.25 cc. neutral ethyl butyrate and triacetin, respectively, were added, and the whole thoroughly mixed by vigorous shaking, neutralized to phenolphthalein with 0.02 *N* NaOH or HCl, as the reaction warranted, and incubated at 37° for 24 hours. The increase in acidity is taken as a measure of the esterase activity of the solution in which the determination was made. Appropriate controls, using sterile (uninoculated) media of like kind and amount with ester, and ester controls in water, were made at the same time, and inoculated under the same conditions. These controls were uniformly negative, that is, they did not break down the esters, indicating clearly that the reactive substance is present only in the filtrates of the cultures.

To determine the action of these filtrates in cream, 1 cc. of 40% certified cream was sterilized in the autoclave, after the addition of 10 cc. of distilled water. One cc. of the respective filtrates were added to this sterile, diluted cream, together with toluol, and examined according to the same procedure with suitable controls.

The results follow: Table VIII shows the amount of 0.02 *N* NaOH required to neutralize the acid liberated from the ester, the glyceride and the cream (duplicate), respectively, as these substances were acted upon by the sterile filtrates of the organisms mentioned above. For the sake of completeness, the initial neutralizing values are given, which indicate the amounts of acid or alkali necessary to bring the mixtures of filtrate and ester to neutrality prior to incubation.

#### Discussion.

*B. mesentericus* appears to be the only organism which elaborates a reactive substance (lipase) in plain broth, which forms acid by breaking down the glycerides in the cream. None of the plain broth filtrates of the various organisms appear to liberate acid from ethyl butyrate, while all act to a considerable extent on triacetin. Grown in dextrose broth, the filtrates of all the organisms, except *B. coli* and *B. subtilis*, produce a certain amount of acid in cream after 24 hours, *B. typhosus* somewhat less than the remaining bacteria. *B. typhosus*, *B. proteus* and *B. mesentericus* filtrates (dextrose) act more energetically than the other organisms on ethyl butyrate, while all the types studied liberate consider-

<sup>1</sup> Kendall, Walker and Day, to appear in *J. of Infectious Diseases*, November, 1914.

able amounts of acid from triacetin, as was the case with the plain broth filtrates. It will be seen from Table VIII that the filtrates of those bacteria which ferment dextrose are more active lipolytically than the corresponding plain broth filtrates. This may be attributable, in part at least, to the greater luxuriance of growth in dextrose broth. These results do not fully meet the requirements of the hypothesis, however, for it is conceivable that those bacteria whose filtrates do not exhibit any appreciable action on the cream might still contain within their bodies an endo-ferment, which was not present in the culture media separated from them by filtration through porcelain.

To examine this possibility, the organisms mentioned above were inoculated upon slanted plain nutrient agar, the area of which was about 50 sq. cm. per organism. The bacteria were washed from the agar after three days' incubation at 37° with 5 cc. of sterile toluol water. This killed the organisms. The suspension was then thoroughly shaken to distribute the bacteria uniformly, and 1 cc. of this suspension of dead organisms was added to the cream (in duplicate), ethyl butyrate and triacetin, respectively, as outlined above. The reaction was adjusted to the neutral point of phenolphthalein, then incubated at 37° for 24 hours.

The results appear in Table VIII. All the organisms, except *B. mesentericus*, produced more acid in cream than did the filtrates of the broth cultures, either plain or dextrose. The killed culture of *B. mesentericus* liberated no more acid from cream than did the dextrose filtrate. The killed culture of *B. proteus* was about as active as the killed culture of *B. mesentericus* in cream, and both these organisms exhibited greater lipolytic activity than the remaining bacteria. Generally speaking, the less proteolytic bacteria of this series are less active lipolytically, particularly in cream.

It has been claimed that the butter fat of milk is an emulsion of extremely finely divided fat droplets, each droplet being encased in a protein-like envelope. The possibility presents itself, therefore, that the actively proteolytic bacteria may have produced, parallel with the lipase, an exo-proteolytic ferment, and that this ferment may have dissolved the protein-like envelope, exposing the fat to the direct action of the lipase. The sterile filtrates of the broth cultures (plain and dextrose) of the various organisms, consequently, were tested for an exo-proteolytic ferment, using carbol gelatin (0.50% carbolic acid, 8% gelatin) as a substrate, 1 cc. of the dextrose and plain broth filtrates, respectively, being added to a column of this (sterile) carbol gelatin whose dimensions were 60 × 15 mm., and incubated at 37° for two days. The results follow:

LIQUEFACTION.		
	Plain broth.	Dextrose broth.
<i>B. typhosus</i> .....	.....	.....
<i>B. coli</i> .....	.....	.....
<i>B. proteus</i> .....	Complete	.....
<i>B. mesentericus</i> .....	Complete	.....
<i>B. pyocyaneus</i> .....	.....	Complete

It will be observed that *B. proteus* and *B. mesentericus*, which ferment dextrose, produced no demonstrable gelatinase in dextrose broth, while in plain broth (of precisely the same composition and reaction, except for the dextrose) a very active gelatinase was demonstrable. This suggests strongly that the sparing action of dextrose for protein also influences the production of proteolytic ferment.<sup>1</sup> The production of an active gelatinase by *B. proteus*, *mesentericus*, and *pyocyaneus* in filtrates of broth cultures simultaneously with active lipases would appear to warrant the tentative assumption that these proteolytic ferments might dissolve the protein-like envelope surrounding the droplets of fat, thus facilitating the lipolytic splitting of this fat.

#### Conclusions.

(1) The sterile filtrates of broth cultures of certain bacteria split certain esters and glycerides with the liberation of acid.

(2) Generally speaking, the filtrates of dextrose broth cultures of these organisms are more active than the corresponding plain broth filtrates.

(3) This increased activity of the dextrose filtrates may be attributable, in part at least, to the greater luxuriance of growth of the bacteria in this medium.

(4) Autolyzed killed cultures of the same bacteria also split esters and fats; and absolute measure of the respective lipolytic activity of the filtrates and bacteria, is not available.

(5) Certain proteolytic bacteria, *B. proteus*, *B. mesentericus*, and *B. pyocyaneus* appear to split cream somewhat more actively than the less proteolytic organisms. They produce a soluble, active gelatinase in media containing no utilizable carbohydrate.

(6) The presence of this gelatinase in cultures containing active lipase appears to be associated with a more extensive liberation of acid from butter fat (cream), but not from ethyl butyrate and triacetin.

(7) The extent of the splitting of ethyl butyrate and triacetin by all the bacteria studied (both filtrates and killed organisms) appears to be independent of their relative proteolytic activity.

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<sup>1</sup> Kendall, *Boston Med. and Surg. J.*, 163, 322 (1910); *J. Med. Research*, 25, 117 (1911).