# CLOSTRIDIUM MULTIFERMENTANS IN CHOCOLATE CREAM CANDIES

GRACE A. HILL[1]

*Camp Kearny, California*

Received for publication September 15, 1924

The object of the investigations upon which this paper is based, was to try to solve a problem of confectioners—the cracking of chocolate creams.

We are here presenting a description of a saccharolytic anaerobe which we found to be uniformly present in the cracked chocolate creams; with an account of our experiments to determine its rôle in the spoilage.

## ISOLATIONS AND IDENTIFICATION

Of 24 samples of spoiled chocolate creams examined, 18 showed the presence of gas-producing organisms. Four of these showed good gas production in the initial tubes, but the gas-producers were not isolated. From 1 sample *C. bifermentans* was isolated; from two, *C. tertium;* and from the remaining 11, a saccharolytic anaerobe which we think to be the *C. multifermentans* of Stoddard (1915).

Where the gas-former was demonstrated but was afterwards lost, and where other gas-formers than *C. multifermentans* were isolated, there was every reason for believing, from morphology, stains and cultural characteristics, that it was present in the initial fermentation tubes, but was lost in the isolation process.

This organism was also isolated three times from "mazetta cream," a commercial preparation used by confectioners as a

[1] The author is indebted to Dr. Ivan C. Hall, with whose valuable council the work was executed, at the University of California, Berkeley, California, August to December, 1922.

foundation for cream fillings; and three times from egg albumin, which is in turn the foundation for mazetta cream. *C. sporogenes* was not met with at any time in our investigations.

All of the isolations, of the supposed species of *C. multifermentans*, from the candies, the egg albumin and the commercial cream, were cultured as a series. No differences were found and they were decided to be of the same species and type.

We are classing this organism as *C. multifermentans*[2] only tentatively. Perhaps it should be considered as a special type of this species, since it does not ferment inulin. This difference however, may be due to technique. Stoddard (1919) does not give his method for making the fermentation tests. Our method was that of Hall (1922). Meat-infusion broth was rendered sugar-free by the action of the Welch bacillus, and the carbohydrate, in solution in distilled water, was sterilized separately, and added just before inoculation.

## MORPHOLOGY

The organisms isolated, which were evidently, as will be shown later, those responsible for the splitting of the chocolate coat of cream-filled candies, and which we are considering to be a strain of *C. multifermentans*, are large, though slender, bacilli, occurring singly or in pairs. Filaments are formed in some media—most notably in those lacking sugar.

*Gram stain.* The organisms retain the Gram stain in young cultures, but quickly lose this property. In glucose broth after eighteen hours incubation there may be as many Gram-positive as Gram-negative individuals, but in twenty-four hours the culture is largely Gram negative. This is also true of glucose-agar cultures. (It is possible, however, that the Gram-negative rods are those that have been inhibited or killed by acid produced in the sugar media. This theory is supported by the fact that within a week glucose cultures are usually found to be

[2] The organism is so named to comply with Bergey's Manual of Determinative Bacteriology. We believe that it is more like the *Clostridium butyricum* of Prazmowski than the organism chosen by the committee as the type species, which is stated not to form the typical clostridia.

dead.) Infusion agar and Petroff's egg-medium cultures, on the other hand, retain the Gram's stain after three days of incubation.

*Shadow forms.* There is a tendency, even in young cultures, to the production of "shadow forms," which do not retain any stain well. These, especially in sugar media, frequently show deeper staining granules, sometimes one in each end of a pale swollen rod. Forms resembling the well-known *Vibrion septique* "citrons" are also formed.

*Starch granules.* If dilute iodine is added to a hanging drop made from a sugar medium culture, the rods are found to contain many granules which take a deep purple stain, showing the storing of a starch-like substance.

*Spores.* Spores are formed in twenty-four hours in brain media, blood agar, Petroff's egg and infusion agar. Spores are produced sparingly in glucose infusion agar shakes, and abundantly on glucose infusion agar plates. Spores have not been observed in any meat-extract, sugar media, although there is abundant growth with gas production.

The spores are rather straight-sided ovals, produced subterminally or, less frequently, in the center of the rod. The bacilli swell in their production to form clubs or spindles. Occasionally a thickened spore-bearing rod has straight sides. The spores appear as refractive bodies with the Gram stain, and are acid-fast. A great variety of forms are found in spore production on some media, most notably Petroff's egg medium.

*Spore germination.* On infusion-glucose-agar plates spores may apparently be formed, become distended, and germinate within forty-eight hours. Forms have been observed fulfilling all of the stages pictured in Prazmowski's classical illustration of the polar germination of *Clostridium butyricum.* These cultures are Gram negative except for what appear to be spores in the early stages of development within the rods, and also for the small new rods as they leave the old distended spore cases.

*Motility.* Motility is variable. It is uniformly absent in glucose broth. A few motile individuals may usually be observed in preparations from young brain cultures, especially if some of the solid portions of the medium are carried over to

the slide. The majority of individuals in any field are non-motile.

CULTURAL CHARACTERISTICS

*Infusion agar.* No growth occurs in extract agar, nor indeed in any medium that is sugar-free. When colonies are produced in infusion agar shakes, they are irregularly lenticular masses. Surface plate colonies on infusion agar, at forty-eight hours, appear as small round transparent dew-drops. The hand-lens reveals irregular edges.

*Glucose agar.* In glucose-agar stab-cultures, in twenty-four hours, gas is produced that fragments the agar so violently as, not infrequently, to push out the cotton plug. Colonies are knobby and irregularly lenticular. They are commonly broken up by subsequent gas production. In from two to three days gas production fragments the agar.

On a glucose-infusion-agar slant or plate the surface colonies are small, white, opaque and raised. These cultures, however, are never very satisfactory because the agar becomes torn with gas bubbles, and some liquefaction of the media takes place, which overflows the surface.

Fermentation in all sugar media is accompanied by a distinct odor of butyric acid.

*Petroff's egg medium.* In forty-eight hours colonies are formed abundantly. They are small, averaging 1 mm. in diameter, opaque, yellowish in color, ameboid in shape, and viscid in consistency.

*Blood agar.* Dew-drop colonies are formed which have irregular edges and are non-haemolytic.

*Gelatin.* Gelatin is not liquefied, even when sugar is present.

*Brain.* Gas is produced in brain media, but no blackening takes place.

*Broth.* No growth takes place in sugar-free broth. In glucose broth 80 per cent or more of gas is produced in twenty-four hours. Even before rapid gas production ceases the organism has a tendency to clump and settle to the bottom of the tube, so that in forty-eight hours the broth is clear. A hanging drop

of a twenty-four-hour, glucose-broth culture usually shows this clumping, so that the preparation has the appearance of an agglutinating Widal. Because of this property immunization-agglutination tests for differentiation could not be used.

*Milk.* In twenty-four hours there is acid and gas, with a fairly firm clot below the marble seal. At this time a clot may be forming above the seal, or it may not appear for several days. In forty-eight hours the clot below the seal is firmer than at twenty-four hours, and is somewhat riddled with gas bubbles; although it is not so firm nor so shredded as in the typical *C. Welchii* reaction.

*Starch agar.* These organisms produce a strong diastase. Large halos appear about the colonies on starch agar plates. These are accentuated by the addition of dilute iodine. In sugar-free, 1 per cent starch agar-shake-cultures gas is produced with a clearing of the medium, and after forty-eight hours of vigorous growth the starch is found, by the iodine test, to have disappeared completely.

*Fermentation.* The organism ferments, with the formation of acid and gas, glucose, lactose, sucrose, raffinose, salicin, glycerol and starch; but not mannitol or inulin.

*Pathogenicity.* The organism was found to be non-pathogenic for guinea pigs. Eleven of the isolations were inoculated as twenty-four-hour glucose-broth-cultures, intraperitoneally, 1 cc. each, into as many (11) guinea pigs. The pigs were apparently unaffected.

## THE SPLITTING OF THE CHOCOLATE COAT

In an attempt to help solve the problem of a local confectioner who was having trouble with the cracking of chocolate creams, we made a cream filling in the laboratory, following his receipt. His "mazetta" was used as a foundation.

*Clostridium multifermentans* was isolated, without difficulty, from the mazetta used. Also a sample of the egg albumin that had been used in making the mazetta, was found to contain this organism to the number of more than 10,000 per gram.

About 10 cc. of the cream filling made in the laboratory was

put into each of twenty sterile test tubes, and a layer of paraffin was added to simulate the chocolate coat. Ten of these were inoculated with *C. multifermentans*, and ten were left for controls. Within a week gas was produced in eighteen tubes, inoculated and uninoculated alike, breaking or tipping the paraffin coat, with subsequent oozing.

Two tubes, one inoculated, and one control, failed to respond. It was noticed that these had been given paraffin coats of unintentional thickness. After three weeks incubation, on the way from the incubator to the discard, they met with a jarring accident. A few days later they were noticed to have undergone a stormy fermentation, with a lifting of the paraffin coat to the cotton plug. This was undoubtedly made possible by the jar and loosening of the paraffin. This is recorded as support for the theory that a strong chocolate covering may prevent splitting even when other conditions are favorable for gas production.

In this instance a fermentation tube inoculated from the control cream, one month after it had been put into the tube, produced *C. multifermentans* in pure culture.

Fifteen samples of chocolate creams, from as many batches (none of which showed spoilage), were obtained from six different confectioners. When inoculated with *C. multifermentans*, 9 of the 15 cracked characteristically. The organisms inoculated were recovered from the split candies, fulfilling Koch's postulates.

It had previously been observed that apparently good candies, from batches having typically cracked candies, usually were found to contain *C. multifermentans*, although they themselves showed no spoilage. Also the organism was at no time found to be present in samples from batches in which none of the candies showed spoilage. It seemed logical to expect, however, that occasionally *C. multifermentans* might be found in candies from batches which had shown no spoilage; so we took the precaution of inoculating fermentation tubes from the centers of the candies that had been opened, aseptically, to receive the inoculation. None of the samples inoculated were found to contain *C. multifermentans* previous to the inoculation.

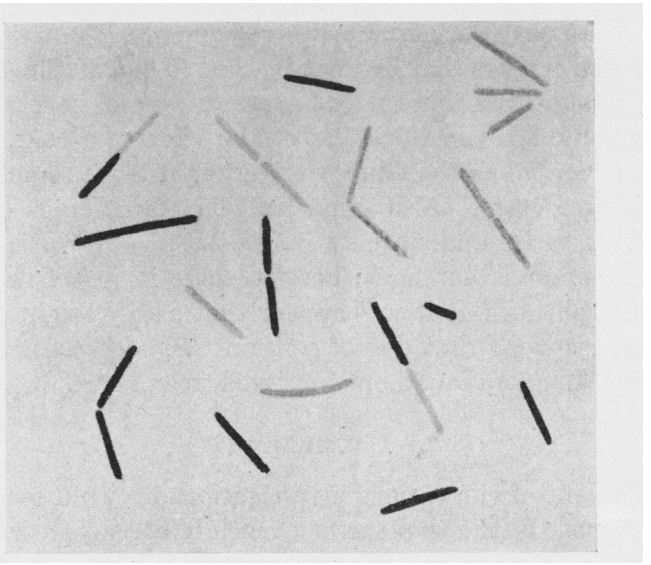

FIG. 1. C. MULTIFERMENTANS. GRAM STAIN. TWENTY-FOUR HOURS IN GLUCOSE BROTH

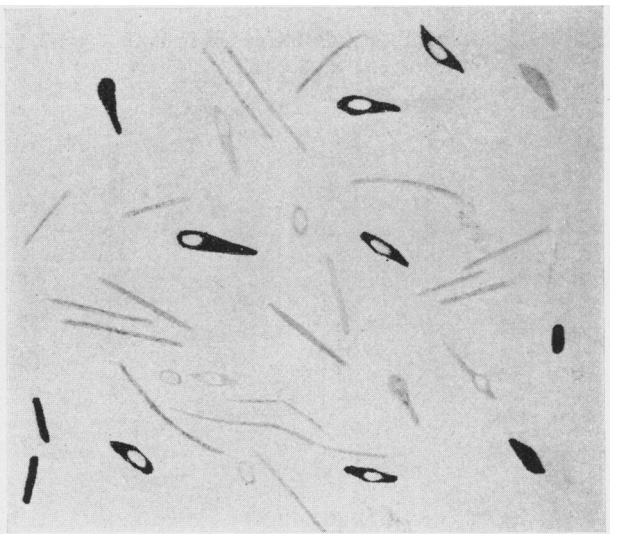

FIG. 2. C. MULTIFERMENTANS. GRAM STAIN. FORTY-EIGHT HOURS ON PETROFF'S EGG MEDIUM

As to the candies that had not reacted (6 out of the 15 inoculated), it appeared there may have been one of several reasons for the failure. The thickness of the chocolate coat, and the moisture content of the filling were thought to be sometimes the controlling factors. Also some fruit-flavored fillings are made so acid as to be undoubtedly inhibitive.

The attempt is not made here to solve in detail the confectioner's problem, but it will appear that safety might lie either in making creams that do not contain *C. multifermentans*, or in rendering them unfavorable for its development.

## SUMMARY

In the light of our various investigations it would seem probable that most of the spoilage of chocolate creams through splitting is due to a gas-forming microorganism whose morphology and cultural characteristics correspond to those of *Clostridium multifermentans* (Stoddard).

## REFERENCES

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