Relation of Fatty Acids to the Inhibition of *Clostridium botulinum* in Aged Surface Ripened Cheese[1,2]

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Toxin production was studied in two types of surface ripened cheese inoculated at various intervals of storage at 2 to 4 C with toxin-free spores of *Clostridium botulinum*. Cheese samples aged beyond 8 weeks became increasingly inhibitory to growth of *C. botulinum*, whereas prior to 8 weeks of storage the cheese supported growth (Grecz *et al.*, 1959).

The amount of free fatty acids in cheese progressively increases during aging of this product (Van Slyke and Price, 1952). This fact suggested that the observed inhibition of *C. botulinum* may be due to an accumulation of free fatty acids in the cheese during prolonged storage. Fatty acids were reported to inhibit germination of spores of *C. botulinum* (Humfeld, 1947; Foster and Wynne, 1948); subsequently other authors stated that oxidative rancidity of the fatty acids was the cause of the observed inhibition (Roth and Halvorson, 1952). The amount of free fatty acids formed during aging of cheese varies (Van Slyke and Price, 1952), and factors affecting the rate of fatty acid formation are little known (Babel and Hammer, 1945). Apparently, oxidative rancidity of fatty acids of aged type I cheese[3] has not been studied previously.

The present work is concerned with following the gradual changes in the amount of fatty acids in type I surface ripened cheese during aging at 2 to 4 C and correlating these changes with the inhibition of *C. botulinum*.

Materials and Methods

Cultural methods. A culture of type A *C. botulinum* (strain T) was used for experimental inoculation of cheese preparations. The methods of preparing spores and maintaining stock cultures are described elsewhere (Grecz *et al.*, 1959).

Cheese preparations. Surface ripened cheese, type I, was obtained as composite lots in polyethylene bags. The age of the cheese on the day of arrival at this laboratory was between 23 and 30 days. The bulk lots of type I cheese were stored at 2 to 4 C to study the effect of length of storage on the ability of the cheese to support growth of *C. botulinum*. Appropriate portions were removed from the composite lot at bi-weekly intervals and made up into an experimental medium as described by Wagenaar and Dack (1958). The normal salt content of type I cheese was in excess of 3 per cent. Reagent grade NaCl was added to some of the samples to yield approximately 3.0, 3.5, 4.0, 4.5, and 5.0 per cent salt. The cheese was pasteurized at 90 C for 10 min in a water bath, cooled, and inoculated with approximately 100 spores of *C. botulinum* per g of cheese. A sample of the cheese preparation was removed to determine fatty acids, rancidity, and fat prior to inoculation with *C. botulinum*.

The inoculated cheese was weighed in 30-g portions into sterile 25 by 150-mm culture tubes and incubated anaerobically at 30 C. Samples were removed for toxin and pH analyses after 3, 7, 14, 30, and 60 days. Toxin was assayed by intraperitoneal injection of white mice with 0.3-ml portions of the supernatant fluid from centrifuged 1:5 aqueous dilutions of the cheese. If the mice died within 4 days, the sample was similarly inoculated into additional mice plus a control animal which was given type A botulinum antitoxin.[4] It was assumed that the presence of detectable amounts of toxin reflected growth of *C. botulinum* and that growth which did not produce detectable amounts of toxin was insignificant.

To study the role of fat and fat soluble substances in cheese, the butterfat fraction of aged cheese was separated by heating (90 C) and centrifugation. The supernatant fat fraction was combined with fresh type I cheese in such proportions as to give a final combined weight equal to the weight of cheese from which the fat was obtained. Four fractions of type I cheese were prepared: (a) normal aged, (b) defatted aged, (c) normal fresh, and (d) fresh with added butterfat from aged cheese. The fractions were tested for their ability to support growth and toxin production of *C. botulinum* as described above.

Fatty acid analyses. Acetic, propionic, and butyric

[1] This study was supported by a grant from the National Cheese Institute.

[2] The data in this report were taken in part from material presented by N. Grecz in partial fulfillment of the requirements for the M.S. degree at the Illinois Institute of Technology, Chicago, Illinois.

[3] To avoid use of brand names, this surface ripened cheese has been classified type I. The flora of type I is made up of bacteria and yeasts.

[4] Jensen-Salsbery Laboratories, Inc., Kansas City, Missouri.

acids were separated from each other and from closely related acids on a silica gel column using the method of Harper (1953). The C_5 and higher fatty acids were eluted from the chromatographic column as a composite group. A chloroform-butanol solvent system was employed. The quantity of eluted fatty acids was determined by titration with 0.01 N KOH in absolute ethanol, using phenol red as the indicator. The accuracy of the method and the location of the peaks for individual acids in the titration curves were determined by addition of known acids in trial runs. The identity of the fatty acids was also verified by the crystallographic microanalysis method of Klein and Wenzl (1932).

Rancidity determinations were made by the von Fellenberg aldehyde test, by the Kreis test for epihydrin aldehyde, and by the Lea test for peroxides (Lea, 1939). Samples for these tests were prepared by heating 100 g of the cheese to 90 C, and centrifuging the hot cheese in 50-ml Pyrex tubes. The fat layer was poured off and used for the analysis. If necessary, appropriate dilutions were made in liquid petrolatum, USP. A rancid sample of lard was used as a positive control. The fat content of cheese was determined by the Babcock method (Van Slyke and Price, 1952).

Analytical methods. The methods employed for pH determinations, NaCl analyses, and assay of *C. botulinum* toxin were described in an earlier paper (Grecz *et al.*, 1959).

NaCl brine concentration. The effective NaCl concentration in the experimental cheese preparations was expressed as NaCl brine concentration defined as:

$$\text{per cent brine} = \frac{\text{per cent salt} \times 100}{\text{per cent moisture} + \text{per cent salt}}$$

In the following discussion, reference will be made to brine concentration; this should not be confused with the initial salt concentration of the cheese (for example, 3.0 per cent NaCl in a cheese containing 57 per cent moisture corresponds to a brine concentration of 5.00 per cent).

Results

Rate of fatty acid formation. Three lots of type I surface ripened cheese were aged at 2 to 4 C for periods up to 8 weeks. Chromatographic column analyses conducted at bi-weekly intervals showed that the quantity of free fatty acids in individual lots of type I cheese did not increase at the same rate during aging. Also, there was not a proportional relationship between individual fatty acids which were recovered by the method of Harper (1953). However, all fatty acids (except acetic) showed a consistent increase at each 2-week interval during the entire 8-week observation period. The rates of formation of individual free fatty acids are shown in figure 1, and that of total free fatty acids in figure 2.

Each lot of type I cheese became inhibitory to growth of *C. botulinum* after 8 weeks of storage at 2 to 4 C. After this period the total free fatty acid content of 3 lots of type I cheese was between 1.10 and 2.16 per cent (figure 2).

At brine concentrations above 6.0 per cent the growth of *C. botulinum* was generally retarded (figure 3). The inhibitory action due to the age of the cheese seemed to be additive with the inhibitory effect of increasing concentrations of NaCl. Thus, in moderately ripened cheese, which was a good medium for the growth

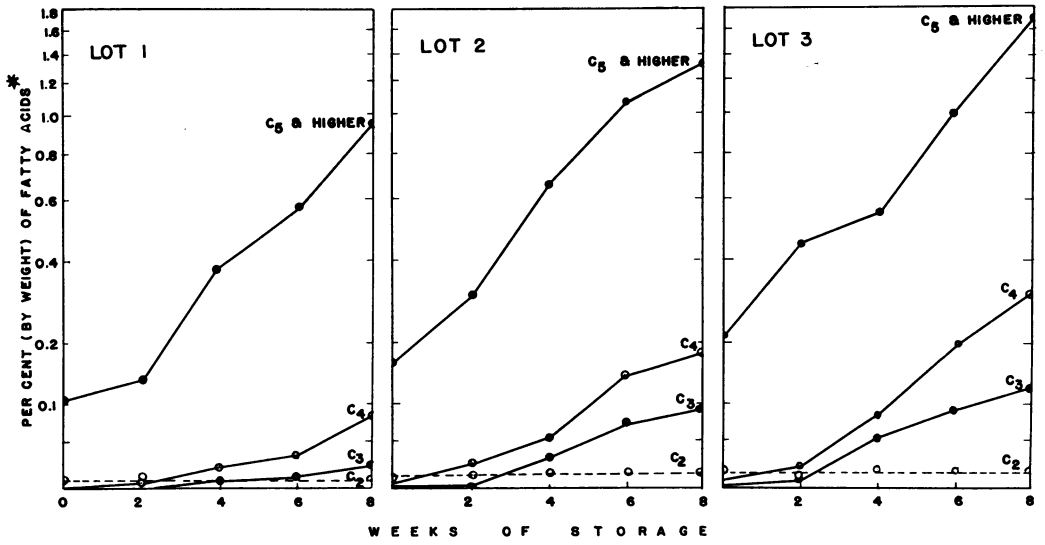

Figure 1. Formation of fatty acids in three lots of type I cheese during storage at 2 to 4 C. (Initial age of cheese before storage was approximately 4 weeks.)

* Molecular weights used in calculating percentage of fatty acid from titration values: acetic, 60.52; propionic, 74.08; butyric, 88.10; C^5 and higher, 267.48 (based on a weighted average of molecular weights of oleic, 282.45; palmitic, 256.41; stearic, 284.46; and myristic, 228.35, since these acids account for approximately 75 per cent of fatty acids present in butterfat) (Hilditch, 1947).

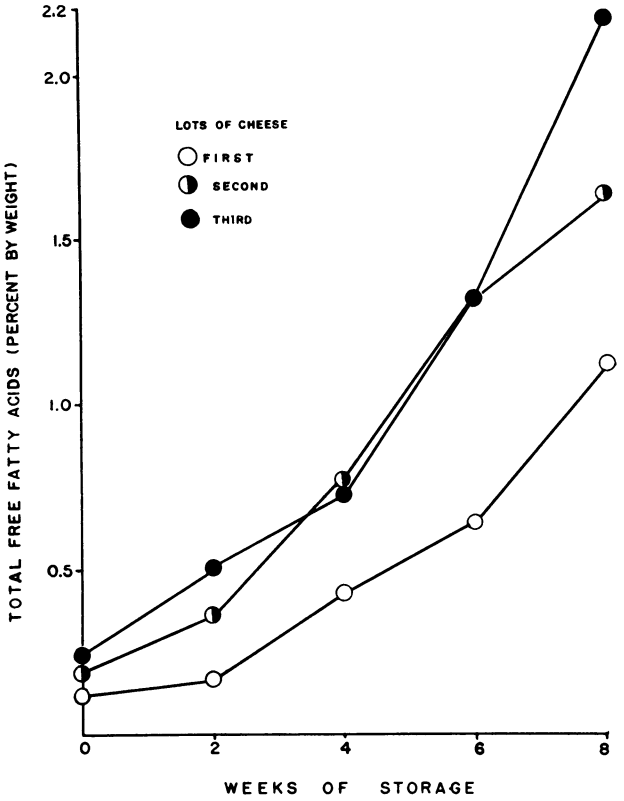

Figure 2. Total fatty acid formation in three lots of type I cheese during storage at 2 to 4 C.

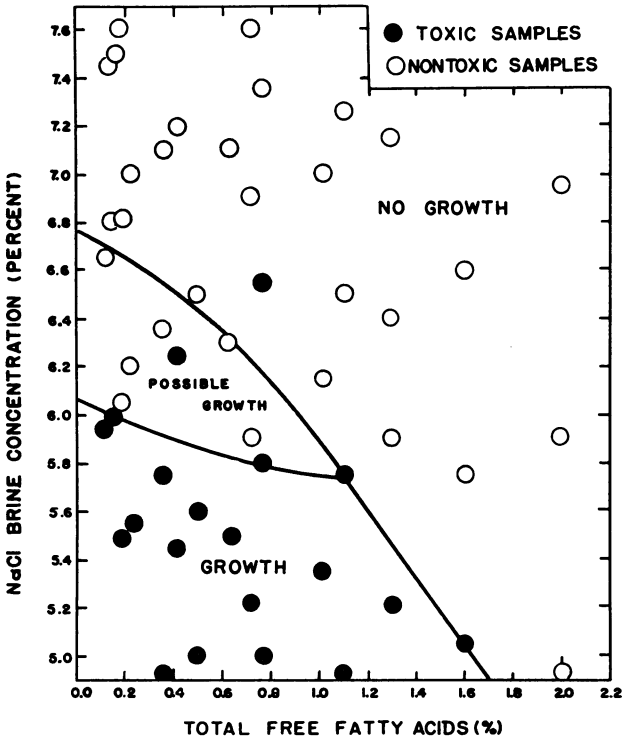

Figure 3. Relation of toxin production of *Clostridium botulinum* to fatty acid level and NaCl brine concentration in type I cheese (7 days of incubation at 30 C).

of *C. botulinum*, this organism grew at brine concentrations of 6.25 to 7.35 per cent. In aged cheese (8 weeks at 2 to 4 C), which was a poor medium for growth of *C. botulinum*, restricted activity of this organism took place only at 4.90 to 5.90 per cent brine concentration, and no growth occurred above this limit. Therefore, varying quantities of NaCl were added to obtain a gradation of the growth response in different samples in the same experiment. As illustrated by the above example, the bacteriostatic activity in cheese was reciprocally related to the highest level of brine concentration at which growth of *C. botulinum* took place. Therefore, the reciprocal of the highest level of brine concentration at which toxin production occurred was taken as an index of the inhibitory activity of a cheese sample. The relation of age of the cheese to the inhibitory activity is shown in figure 4. An inhibitory activity of 0.15, for example, means that production of toxin by *C. botulinum* occurred at brine concentrations below 6.6 to 6.7 per cent (that is, $x = 1/0.15$), and no toxin could be demonstrated in samples having a higher level of brine concentration than 6.7 per cent.

The total fatty acid concentration of 3 inhibitory lots of cheese after 8 weeks of storage at 2 to 4 C cor-

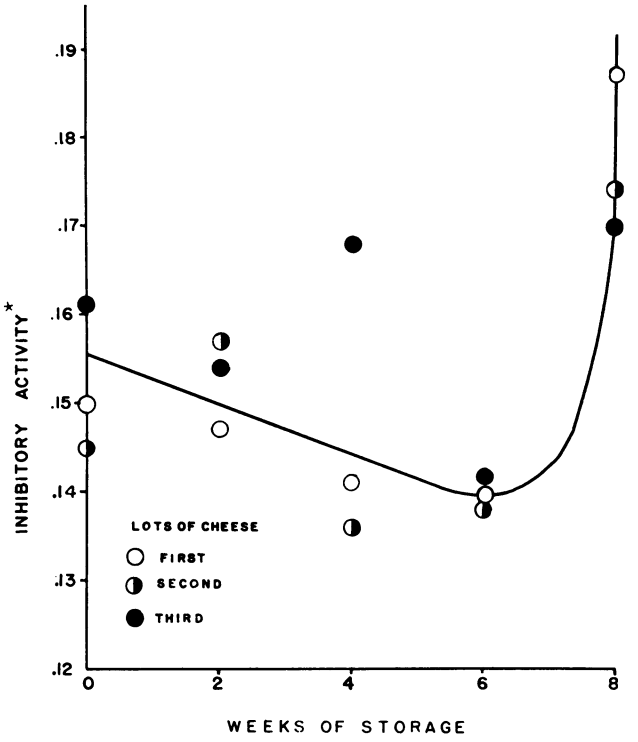

Figure 4. Inhibitory activity of type I cheese on growth and toxin production of *Clostridium botulinum* during storage of the cheese at 2 to 4 C.

* Inhibitory activity on growth of *C. botulinum* was defined as the reciprocal of the highest brine concentration at which type I cheese became toxic to white mice when inoculated with approximately 100 spores per g and incubated at 30 C. (Also see first footnote to table 1.)

related with the highest brine concentration at which toxin production occurred after 7 days of incubation at 30 C (table 1).

A definite pattern of growth of *C. botulinum* at different levels of fatty acids could be established. Higher brine concentrations were tolerated by *C. botulinum* in one series which contained the lowest level of fatty acids than in the other 2 series. In series 3, which contained the highest level of fatty acids, the tolerance of brine concentrations by *C. botulinum* was lowest. This indicated that the quantities of fatty acids in the experimental range had an inhibitory effect on growth of *C. botulinum*. The degree of inhibition was related to the level of fatty acids in type I cheese.

As a rule, *C. botulinum* toxin was demonstrated in cheese preparations inoculated with *C. botulinum* spores after 7 days of incubation at 30 C. The results obtained after 7 days of incubation were more closely related to the initial fatty acid concentration of the cheese than the results obtained after longer incubation.

An analysis of the relation of individual fatty acids to the number of positive samples indicated that the rate of development of propionic and butyric acids was correlated with the inhibitory effect on growth and toxin production of *C. botulinum*. A good example supporting this view was furnished by the third series of experiments. Here the level of propionic and butyric acids remained low for the first 2 weeks while the C_5 and higher fatty acids developed rapidly (figure 1, lot 3). At the same time, the number of toxic cheese samples in the corresponding experiments rose from 8 to 10 (table 2). When the same cheese was stored at 2 to 4 C for 2 additional weeks, the quantity of the lower fatty acids increased considerably, but the amount of C_5 and higher fatty acids remained relatively low. As a result of this combination, the number of positive samples decreased from 10 to 7. After 4 weeks of storage at 2 to 4 C, the rate of development of the lower fatty acids remained constant but the level of C_5 and higher fatty acids rose rapidly. During this period the number of positive samples rose from 7 to 10. A similar pattern was observed in the other 2 series of type I cheese experiments. When the cheese was stored for longer than 6 weeks, the increase in total free fatty acids enhanced the inhibitory effect. The small quantities of lower fatty acids, together with the small number of positive samples, gave consistent results indicative of the same general trend.

As indicated in table 1, the relation of the total free fatty acid concentration to the inhibition of *C. botulinum* in aged type I cheese after 7 days of incubation at 30 C appeared to be a straight line function. The results obtained after incubation for longer than 7 days showed that the cheese of lot 3, which had the highest initial level of fatty acids, was considerably more inhibitory to growth of *C. botulinum* throughout the entire 60-day observation period than lots 1 and 2. No such relationship could be established with lots 1 and 2. However, if the results obtained from experiments with 8-week old cheese in series 1, 2, and 3 were compared with each other, the rate of development of butyric acid was inversely proportional to the number of positive samples obtained in these experiments. The rate of development of butyric acid was highest in the third series, lowest in the second series, and intermediate in the first series. The number of toxic samples produced was lowest in the third series (3 toxic samples), highest in the second series (7 toxic samples), and

TABLE 1

Relation of total fatty acids to toxin production by Clostridium botulinum in aged type I cheese (7 days of incubation at 30 C)

Lot	Total Fatty Acids	NaCl Brine Conc		Inhibitory Activity of Cheese Samples*
		Toxic sample	Nontoxic sample	
	%	%	%	$\times 10^{-1}$
1	1.10	5.35	6.15	1.87
2	1.63	5.05	5.75	1.98
3	2.16	—†	4.95	2.02

* Inhibitory activity (x) was defined as the reciprocal of the highest brine concentration at which type I cheese became toxic to white mice when inoculated with approximately 100 spores per g and incubated at 30 C. Thus, when toxin was produced at 5.35 per cent brine concentration, but not at the next higher level, the inhibitory activity was calculated as $x = 1/5.35 = 0.187$ arbitrary units.

† The lowest NaCl brine concentration tested was 4.95 per cent. No toxin was produced at this NaCl brine concentration after 4 days of incubation but toxin was produced after 14 days of incubation at 30 C.

TABLE 2

*Number of toxic samples in type I cheese inoculated with spores of Clostridium botulinum**

Weeks of Storage at 2 to 4 C	No. of Toxic Samples†		
	Lot 1	Lot 2	Lot 3
None	7	10	8
2	7	9	10
4	9	15	7
6	10	11	10
8	4	7	3

* Inoculum = approximately 100 spores per g; *C. botulinum*, type A, strain T.

† Each series consisted of 25 inoculated samples (5 samples each at 5 NaCl brine concentrations spaced at approximately 1 per cent intervals) plus 5 uninoculated controls. A total of 450 samples was analyzed. The lowest brine concentration was approximately 5.00 per cent. A set of samples was removed for toxin analyses after 3, 7, 14, 30, and 60 days of incubation at 30 C.

intermediate in the first series (4 toxic samples). This may imply that the development of butyric acid was related to the inhibition of *C. botulinum*. Such processes may be independent of lipolysis, that is, they may originate from normal microbial metabolic activities in which the production of propionic and butyric acid is incidental to the inhibitory mechanism by which it affects *C. botulinum*. This would be in agreement with the hypothesis that the rate of formation of these fatty acids and not the absolute amount could be related to the inhibition of *C. botulinum*. As far as the effect of fatty acids per se is concerned, the results indicated that a certain minimal initial quantity (between 1.10 and 2.16 per cent) was necessary to noticeably contribute to the inhibition of growth of *C. botulinum* in type I cheese. Such a conclusion would be in agreement with data shown in figures 3 and 5. In these figures, the brine concentration at which toxin production by *C. botulinum* took place was plotted against the corresponding fatty acid concentration. The growth limiting fatty acid concentration obtained from figures 3 and 5 by graphic extrapolation was 1.72 per cent of total fatty acids for 7 days of incubation at 30 C and 2.16 per cent for 14 days of incubation at the lowest brine concentration employed in these experiments (that is, 4.95 per cent—the approximate normal NaCl brine concentration of type I cheese). After 14 days of incubation, no appreciable change in the toxicity of the samples took place.

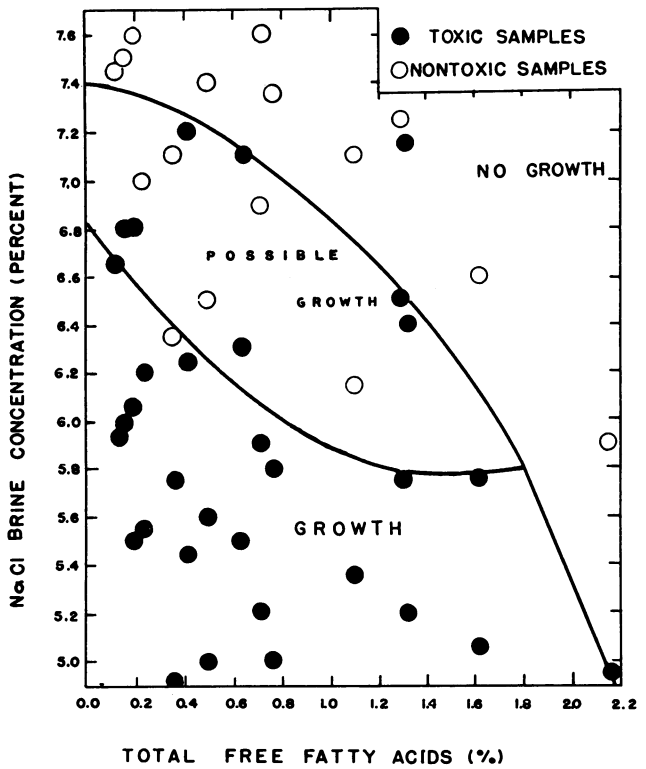

Figure 5. Relation or toxin production of *Clostridium botulinum* to fatty acid level and NaCl brine concentration in type I cheese (14 days of incubation at 30 C).

The variations of the effect on *C. botulinum* of fatty acid concentrations of less than the growth limiting amounts could be explained by the assumption of some inhibitory mechanism in aged cheese which develops independently of fatty acids. The results indicated that the inhibitory action of fatty acids and of the unknown principle(s) affected *C. botulinum* additively. The assumption of a second inhibitory mechanism in aged type I cheese explains the absence of inhibition of *C. botulinum* in cheese, aged for 6 weeks at 2 to 4 C, containing as much as 1.32 per cent total fatty acids. In this case, additional storage time was probably necessary for the development of the second inhibitory principle which in combination with the fatty acids exerted a total inhibitory effect on *C. botulinum* after the cheese was stored at 2 to 4 C for 8 weeks.[5]

pH. The existence of two types of processes occurring in type I cheese during aging at 2 to 4 C was also indicated by pH changes; for example, one process was associated with a trend toward an alkaline pH and a second process was associated with a trend toward an acid pH. Generally, the pH of type I cheese became consistently more alkaline during storage at 2 to 4 C. The rate of this change, however, was retarded by the accumulation of fatty acids. This resulted in an inverse

[5] Subsequent preliminary experiments verified this assumption.

TABLE 3

Relation of fatty acids to pH in type I cheese after storage for 8 weeks at 2 to 4 C

Lot	Total Fatty Acids	pH
	%	
1	1.10	6.96
2	1.63	6.31
3	2.16	6.20

TABLE 4

Effect of butterfat from aged cheese on growth and toxin production of Clostridium botulinum*

Expt No.	Samples†	No. of Toxic Samples after Indicated Days of Incubation				
		3	7	14	30	60
1	Normal fresh cheese	0	0	0	1	1
	Fresh cheese plus butterfat	0	0	0	0	0
2	Normal fresh cheese	0	0	0	2	2
	Fresh cheese plus butterfat	0	0	0	0	1
3	Normal fresh cheese	1	3	3	3	3
	Fresh cheese plus butterfat	0	1	3	3	3

* Type I cheese aged for 3 months at 2 to 4 C.

† Normal fresh type I cheese contained 22 to 25 per cent butterfat. Fresh type I cheese plus butterfat from aged cheese contained 34 to 36 per cent butterfat.

relation of the final pH values to the quantities of fatty acids in aged cheese (table 3). The pH values per se were not sufficiently lowered to inhibit the growth of *C. botulinum*; on the contrary, on the scale of the total pH variations in type I cheese, the inhibition of *C. botulinum* occurred at the most favorable pH range for the growth of this organism in each individual lot of cheese (Grecz *et al.*, 1959). The removal of the lipid fraction from aged type I cheese did not alter the pH of the defatted cheese nor did the addition of such butterfat to fresh type I cheese affect the pH of the fresh cheese.

Butterfat fraction. Since most fatty acids of cheese are generally associated with the lipid portion of this product, the relation of the butterfat of aged type I cheese to the inhibition of *C. botulinum* was also studied. The results are summarized in table 4. The butterfat portion of type I cheese stored at 2 to 4 C for 3 months was obtained as previously described, and added to relatively fresh type I cheese. Three experiments were conducted in which normal fresh type I cheese was compared with fresh type I cheese to which the butterfat portion from old cheese was added. Since the basic fresh type I cheese employed in these three experiments varied considerably in ability to support growth and toxin production of *C. botulinum*, the data were tabulated separately for each experiment. As was evident from table 4, the addition of butterfat from old type I cheese resulted consistently in definite inhibition of *C. botulinum* in fresh type I cheese to which the butterfat was added as compared with the normal control. The inhibitory property of the nonlipid cheese residue of aged type I cheese remained essentially the same as that of normal aged cheese.

Rancidity. The butterfat fraction of type I cheese was tested for rancidity by the von Fellenberg test, the Kreis test, and the Lea test (Lea, 1939). These tests demonstrated that type I cheese did not become rancid during storage at 2 to 4 C for as long as 3 months. Furthermore, no rancidity could be detected in type I cheese which was pasteurized after 3 months of storage at 2 to 4 C, inoculated with 100 spores of type A *C. botulinum* per g, and anaerobically incubated at 30 C for 60 days. Similar results were obtained with a second type of cheese (type III) which is surface ripened primarily by bacteria.

Discussion

The quantities of fatty acids present in type I cheese in which inhibition of growth of *C. botulinum* was observed were considerably larger than the amounts customarily employed in experiments with laboratory media (0.1 to 200 ppm; approximately 0.0001 to 0.02 per cent) (Nieman, 1954). Foster and Wynne (1948) found that 100 μg of oleic acid per ml of a brain heart infusion broth were inhibitory to the germination of spores of six strains of *C. botulinum*. This quantity corresponded to approximately 0.01 per cent. In general, linoleic and linolenic acids acted in the same manner as oleic acid. The effectiveness of fatty acids varied in suppressing germination of *C. botulinum* spores in two different lots of the same medium. In contrast to the above findings, Roth and Halvorson (1952) reported that as much as 1.0 per cent of nonrancid methyl esters of oleic, linoleic, and linolenic acids were noninhibitory to spores of *C. botulinum*, whereas oxidized samples of all 3 acids caused significant inhibition at 0.01 per cent concentrations.

Our observations generally agree with the views of Roth and Halvorson (1952) concerning the failure of 1.0 per cent of nonrancid fatty acids to noticeably affect germination of spores and subsequent growth of *C. botulinum*. However, aged type I cheese (8 or more weeks) contained considerably higher amounts of nonrancid fatty acids than were tested by Roth and Halvorson. Furthermore, examination of the original data of Roth and Halvorson indicated that, contrary to the conclusions of these authors, the addition to the plating medium of 1.0 per cent of nonrancid lard, corn oil, methyl oleate, methyl linolenate, and oleic acid consistently resulted in somewhat lower average counts than those obtained from control plates; namely, 2.5 to 8.5 per cent decrease. This appears to be in agreement with the possibility of a more pronounced inhibition of growth of *C. botulinum* by amounts of nonrancid fatty acids higher than one per cent, such as were present in aged type I cheese.

In view of the complexity of the cheese mixture it would be premature to draw more definite conclusions concerning the relation of fatty acids per se to the inhibition of *C. botulinum* in aged type I cheese. The data presented in this paper may be interpreted as an indication of the probability that high levels of free fatty acids developing in type I cheese during aging do contribute to the inhibition of *C. botulinum* in this product. The inhibitory dose seems to be 1.72 to 2.16 per cent of total free fatty acids.

During storage at 2 to 4 C, type I cheese was inhibitory to growth of *C. botulinum* at two separate stages: (a) present initially and lasting approximately 2 weeks, and (b) appearing after 8 weeks of storage. The inhibition of growth of *C. botulinum* present in type I cheese during the initial 2 weeks decreased on additional storage. The cheese was most favorable for growth of *C. botulinum* after 4 to 6 weeks (figure 4). Since the level of fatty acids in fresh cheese was low, it may be suspected that the inhibition of growth of *C. botulinum* at this stage was due to some other factor(s) than fatty acids.

As mentioned, in the chromatographic procedure (Harper, 1953) the fatty acids containing more than 5 carbon atoms were eluted as a composite group. An

approximation of the relative quantities of the individual components comprising this group could be made by the use of the data of Hilditch (1947) for the average fatty acid composition of butterfat. Such an approximation might be misleading and was not used in the interpretation of our results. However, the method of Harper (1953) for fatty acid analysis was followed since it was rapid and gave reproducible data.

ACKNOWLEDGMENT

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SUMMARY

Inhibition of the growth of *Clostridium botulinum* was correlated with the level of free fatty acids in type I aged surface ripened cheese. The level of free fatty acids in this cheese rose consistently during 8 weeks of storage at 2 to 4 C. The amount of increase of total and of individual fatty acids varied in different lots of cheese. By graphical interpolation, the growth limiting total fatty acid concentration in aged type I cheese was found to be 1.72 and 2.16 per cent with 7-day and 14- to 60-day incubation periods, respectively. Lower concentrations of fatty acids gave variable results. This may be explained by the existence of some concurrent inhibitory mechanism which develops in type I cheese independently of the accumulation of fatty acids.

The observed pH changes substantiated the existence of two types of processes occurring in type I cheese during aging, one contributing to the alkalinity and another contributing to the acidity of the product. The fatty acids were shown to contribute to the process associated with acidity of type I cheese. The final pH depended on the rates of the two processes.

Addition of the fat fraction taken from thoroughly aged cheese to relatively fresh cheese resulted in definite inhibition of *C. botulinum*. The inhibitory properties of the nonlipid residue of aged cheese were not reduced by the removal of butterfat.

No rancidity could be detected in type I cheese during storage at 2 to 4 C for as long as 3 months. Furthermore, this cheese remained nonrancid when inoculated with 100 spores of *C. botulinum* per g and incubated anaerobically at 30 C for an additional 2 months.

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