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Growth, Sporulation, and Germination of *Clostridium perfringens* in Media of Controlled Water Activity[1]

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Requirements in terms of water activity (a_w) for the growth, sporulation, and germination of *Clostridium perfringens* were determined. Strain A48 was used in all phases, and in addition either NCTC 8239 or NCTC 8797 was used for growth, sporulation, and germination studies. The desired a_w of the test media was obtained by the addition of one of three solutes: glycerol, sucrose, or sodium chloride. The freezing point depression method was used to determine the a_w. The basal medium for growth and germination was Fluid Thioglycollate Medium. It had an a_w of 0.995 and produced maximum growth and fastest growth rate among the six levels of a_w tested. The lowest a_w supporting growth and germination of *C. perfringens* was between 0.97 and 0.95 in the test media made with sucrose or sodium chloride and 0.93 or below in the test media adjusted with glycerol. Spore production by *C. perfringens* in Ellner's or modified medium required a higher a_w than growth.

An understanding of the influence of the available water in foods on the growth, sporulation, and germination of *Clostridium perfringens* may aid in control of this microorganism as a cause of foodborne illness. The available water activity (a_w) in a food is affected by all of the constituents which have an affinity for water. These will include those which can be metabolized by the organism as well as those which cannot. In addition, food processing may alter the available water. Gough and Alford (5) tested the effects of NaCl, $NaNO_3$, and $NaNO_2$ on growth, survival, and heat resistance of several strains of *C. perfringens*. Growth occurred in concentrations of these salts which were higher than those used in the normal curing of meat. A NaCl concentration of 6% (w/v) in Fluid Thioglycollate Medium was required to inhibit growth significantly. The a_w of the growth medium may affect the rate of growth and the synthesis of cellular components (3). Scott (9) has reviewed the relation of a_w to the growth of food-spoilage microorganisms, including molds, yeasts, and bacteria. One factor which limits the interpretation of data is that the moisture requirements of bacteria may be affected by nutritional and environmental factors (9).

In this paper, the ranges of a_w for growth, sporulation, and germination of *C. perfringens* in laboratory media are reported.

MATERIALS AND METHODS

Preparation of inoculum. Three strains of *C. perfringens* were used. NCTC 8797 and NCTC 8239 were heat-resistant strains obtained from Betty Hobbs, Central Public Health Laboratory, London, England; A48, a strain that is not heat-resistant, was received from H. E. Hall, Robert A. Taft Sanitary Engineering Center, Cincinnati, Ohio. Stock cultures were carried in Noyes veal broth without glucose. Cultures of NCTC 8797, NCTC 8239, and A48 in the logarithmic-growth phase in Fluid Thioglycollate Medium were used after centrifugation and resuspension in diluent as the inoculum for growth and sporulation series. About 100 vegetative cells per ml were inoculated into growth media, and about 800,000 vegetative cells into sporulation media. Spore crops of NCTC 8239 and A48 were produced in a modified medium (7). The general method of Zoha and Sadoff (12) was followed. Spore suspensions after centrifugation were stored at 5 C, and numbers viable were checked periodically. The average number of spores was 160,000 per ml for NCTC 8239 and 120,000 per ml for A48.

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Media. Fluid Thioglycollate Medium (Difco) was the basal medium used for growth and was used as the heating medium for germination. Agar was added when it was used as the plating medium for the germination series. Media used for sporulation were Ellner's (4) and a modified medium (7). The test media were prepared by adding predetermined amounts of solutes to the basal media. The selected a_w levels of the test media for growth and sporulation were 0.98, 0.97, 0.95, 0.93, and 0.91. For germination and growth, a_w levels of 0.98 to 0.93 at 0.01 intervals were used. The a_w of the Fluid Thioglycollate Medium was 0.995; of Ellner's medium, 0.993; and of the modified medium, 0.992.

The test media with sodium chloride and glycerol were made by adding solutes directly to the basal medium before autoclaving. The test media containing sucrose were prepared by combining separately sterilized basal medium and sucrose solutions. The latter solution was sterilized by discontinuous steaming. To test the effect of added ground veal near the limiting levels of a_w, 2 g of freshly ground veal was added before autoclaving, and the a_w was adjusted to 0.95 with glycerol or sodium chloride in test media for growth and to 0.97 in test media for sporulation. Any evaporation losses during preparation of media were carefully made up with distilled water, and an allowance was made for the amount of inoculum that would be added.

A split plot design with three replications was planned for each phase of the experiment. An analysis of variance was done on the data for growth.

Determination and control of a_w. The a_w levels of the basal media and test media were determined from the freezing points of the media. The freezing point depression was measured by use of a Beckman thermometer graduated to 0.01 C and an H.B. Instrument engraved-stem thermometer, registering from −35 to 35 C and graduated to 0.1 C. The freezing point depression was measured in a DeWar flask with an alcohol, ice, and dry ice mixture as the cooling medium. The equivalent a_w was calculated from the freezing point depression by use of the equation given by Harned and Owen (6). The relationships of freezing point depression, a_w, and concentration of solutes in Fluid Thioglycollate Medium are shown in Table 1. The a_w values of the basal media as well as the test media combined with the molar concentrations of solutes theoretically giving the desired freezing points were first determined by the freezing point depression. Since theoretical values may deviate from ideal values in concentrated solutions, corrections were made by plotting the measured freezing point depressions against the molar concentrations of solutes added to the basal media. The values of the solute concentrations determined to give the desired levels of a_w were used throughout. The a_w of the test medium was checked by freezing point depression after each preparation. The maximal error in the a_w of a test medium was −0.007, and average experimental error was ±0.002. The three solutes selected were sodium chloride, glycerol, and sucrose. The latter two could be metabolized by *C. perfringens*.

Determination of growth and sporulation. The growth of *C. perfringens* after incubation at 37 C for 4, 12, 24, and 72 hr was determined by making appropriate dilutions in 0.1% peptone diluent. Plating was in iron sulfite-agar [SPS agar with no antibiotics added, Angelotti et al. (1)] with an overlay of agar. Anaerobiosis was obtained as described, by use of Case anaerobic jars (1). The colonies were counted after incubation at 37 C for 24 to 48 hr.

Determination of spore numbers was made after 24, 48, and 72 hr and 7 and 14 days of incubation at 37 C. For spores formed in Ellner's medium, 3 ml of sporulation medium was placed in a 5-ml ampoule. For tests with modified sporulation media, spores were first centrifuged and resuspended in 0.1% peptone diluent. After sealing, ampoules were heated for 15 min in a constant-temperature water bath adjusted to 80 ± 0.1 C. Heat-treated ampoules were immediately placed into cool water. After 2 to 5 min, ampoules were opened, samples were plated in iron sulfite-agar, and plates were incubated as for the previous series. No spores were recovered from 0-hr counts. In addition, direct phase microscopic observations were made on cultures from the modified medium.

Determination of germination and growth. The spore suspension was introduced into duplicate tubes containing 5 ml of Fluid Thioglycollate Medium adjusted with solutes to the desired a_w levels. This same medium was used for heating, diluting, and plating the spore inoculum. One tube was heat-activated immediately after inoculation, and the other, after 90 min of incubation at 37 C. Germination after heating spores for 15 min at 80 C and growth following germination were determined by plating each suspension in correspondingly adjusted Fluid Thioglycollate Medium with agar added. Plates were then incubated as above.

TABLE 1. *Relationships among freezing point depressions (θ), water activities (a_w), and molar concentrations of solutes in Fluid Thioglycollate Medium*

θ[a]	a_w[a]	Concn of solutes[b]		
		Glycerol	Sucrose	Sodium chloride
C		M	M	M
−0.51	0.995	0	0	0
−2.08	0.98	0.84	0.63	0.44
−3.14	0.97	1.23	1.05	0.72
−5.28	0.95	2.18	1.88	1.31
−7.46	0.93	3.13	2.58	1.86
−9.69	0.91	4.08	3.21	2.41

[a] Values were calculated from the equation given by Harned and Owen (6).

[b] Molar concentrations of solutes added to basal medium to give desired freezing point depressions.

RESULTS AND DISCUSSION

Growth. The growth of two strains of *C. perfringens* was determined in Fluid Thioglycollate Medium (basal medium) and in test media with

the a_w controlled by the addition of one of three solutes: glycerol, sucrose, or sodium chloride. The growth patterns at different a_w levels for strain NCTC 8797 are shown in Fig. 1, 2, and 3. Differences among the three replications were not significant at the 0.05 level.

The two different strains were very much alike in their growth patterns in response to the solutes and to the a_w except that cell counts for strain A48 decreased less sharply from the maximal levels. The *F* value for strains was not significant at the 0.05 level. At the highest a_w, 0.995, the greatest total numbers of cells as well as the most rapid growth rate were produced. At a lower a_w, the growth pattern of *C. perfringens* was similar to that in the basal medium but with a depression in numbers. The major characteristics observed in the media of lower a_w were extended

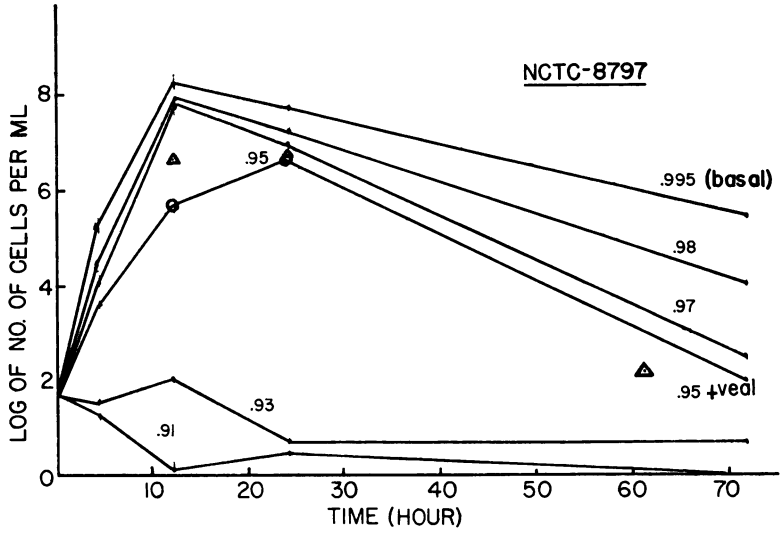

FIG. 1. *Growth of C. perfringens in Fluid Thioglycollate Medium, a_w adjusted with glycerol (average of three replications). At the a_w level of 0.95, ⊙ represents cell counts in the medium; △ represents cell counts in the medium plus veal after incubation at 37 C.*

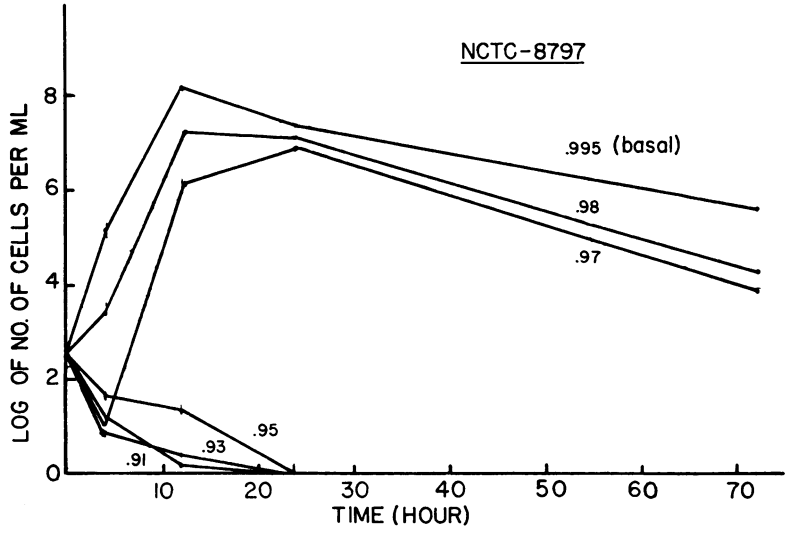

FIG. 2. *Growth of C. perfringens in Fluid Thioglycollate Medium, a_w adjusted with sucrose (average of three replications).*

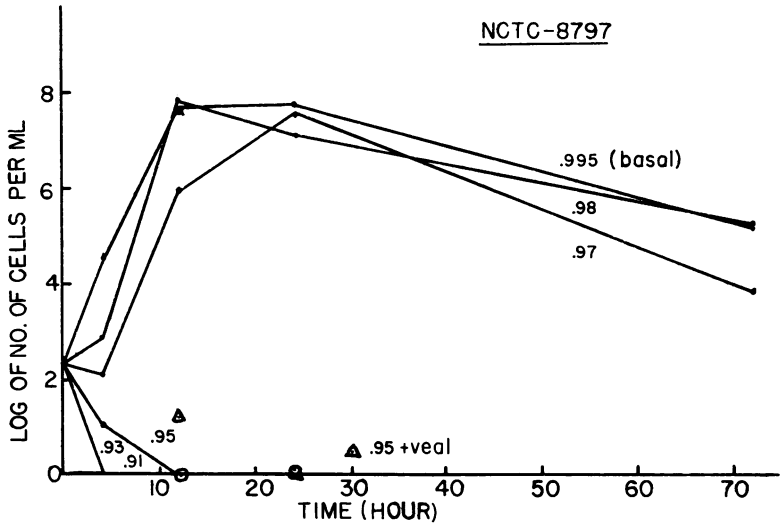

FIG. 3. *Growth of C. perfringens in Fluid Thioglycollate Medium, a_w adjusted with sodium chloride (average of three replications). At the a_w level of 0.95, ⊙ represents cell counts in the medium; △ represents cell counts in the medium plus veal after incubation at 37 C.*

lag phases, smaller populations at the maximal growth stage, and faster death rates as compared with the basal medium. The lowest a_w permitting growth of *C. perfringens* was between 0.97 and 0.95 in the test media prepared with sucrose or sodium chloride as the controlling solute. For strain A48, the final cell count was as high in the medium with an a_w of 0.97 controlled by the addition of NaCl as in the control. The addition of glycerol for an a_w of 0.93 permitted a small increase in numbers at 12 hr only. The range of a_w permitting growth of *C. perfringens* is in general agreement with the range of a_w given for bacteria by Scott (9).

The solute used to control a_w produced differences in the growth rate and in the lowest a_w permitting growth of *C. perfringens*. Although sucrose and glycerol could be metabolized, the initial role of these solutes appeared to be their effect on the available water in addition to an effect related to the specific solute. The analysis of variance indicated that the mean numbers of cells were different at the 1% level of significance in test media made with different solutes and among the six levels of a_w tested. Baird-Parker and Freame (2) observed a similar difference between the effect of glycerol and sodium chloride in supporting the growth of *C. botulinum* at lower a_w values.

The Fluid Thioglycollate Medium in itself may be inhibitory under certain conditions. When a small amount of ground veal was added before autoclaving to the test medium of 0.95 a_w that had been made with glycerol, an increased growth rate was observed, although the final number of viable cells was lower. However, the growth rate was not comparable to that at an a_w of 0.97. In a test medium of the same a_w prepared with sodium chloride, the death rate of *C. perfringens* was slower when veal had been added to the medium. Others have noted increased growth rates and an extended range of a_w with an increased concentration of nutrients or a further addition of growth elements.

Sporulation. Sporulation at different a_w levels at time intervals of 24, 48 and 72 hr and 7 and 14 days is shown in Tables 2, 3, and 4. Ellner's medium, used as the basal medium for sporulation, supported approximately 100% sporulation for strain A48 and less than 1% for strain NCTC 8797. The modified medium supported sporulation of both A48 and NCTC 8239. There was little or no further increase in spore numbers beyond a period of 24 hr of incubation in Ellner's medium as the basal medium or with additives, except for an a_w of 0.98 with sodium chloride for A48 (Table 4).

In the modified medium, few spores that germinated and grew after 15 min at 80 C were found at the lowered a_w values, although isolated spores were found as low as 0.93 a_w. In Ellner's medium, the lowest a_w permitting sporulation of *C. perfringens* was 0.98 for strains A48 and NCTC 8797. There was little difference among the test media made with different solutes, although for Ellner's medium sucrose supported better sporu-

TABLE 2. *Sporulation of three strains of C. perfringens in Ellner's medium or modified medium with the a_w adjusted with glycerol*[a]

Strain	Incubation time	Replications	a_w of Ellner's medium			
			0.993	0.98	0.97	0.97 + veal
	hr					
A48	24	1	6.3×10^5	34	0	14
		2	1.7×10^5	7	3	4
		3	2.6×10^5	120	0	0
	72	1	6.8×10^5	0	0	24
		2	2.6×10^5	1	4	2
		3	7.8×10^5	1	64	0
	336	1	4.7×10^5	0	0	—
		2	1.7×10^5	0	0	—
		3	6.3×10^5	1	2	—
NCTC 8797	24	1	5	0	0	1
		2	92	0	0	0
		3	0	0	0	0
	72	1	31	0	0	0
		2	2	0	0	0
		3	0	0	0	0
	336	1	2	0	0	—
		2	150	0	0	—
		3	0	0	0	—
			a_w of modified medium			
			0.992	0.98	0.97	0.96
A48	24	1	8.8×10^5	2	0	190
		2	3.0×10^4	3	6	3
		3	3.1×10^5	0	0	0
NCTC 8239	24	1	2.1×10^6	1	1	140
		2	7.0×10^5	28	0	23
		3	7.4×10^5	130	52	7

[a] Results show the number of spores per milliliter after heating for 15 min at 80 C, plating in iron sulfite-agar, and incubating at 37 C for 24 to 48 hr. The average initial numbers of vegetative cells inoculated were 9×10^5 per ml.

lation than did the test media made with glycerol or sodium chloride at a_w 0.98. The average numbers of spores of strain A48 formed at a_w 0.98 was 54 in test media made with glycerol, 2.3×10^4 with sucrose, and 1 with sodium chloride after 24 hr incubation. Sporulation did not appear to occur at an a_w of 0.95 or 0.93 in Ellner's medium for either strain. The addition of veal to Ellner's medium at an a_w of 0.97 had little effect on spore numbers. Spore production in Ellner's medium did not increase with a prolonged incubation of 2 weeks except for strain A48 at an a_w of 0.98 in the sodium chloride test medium in two of three replications (Tables 3 and 4).

However, the small and variable numbers of spores limit the generalizations which can be drawn. Direct observation with phase microscopy indicated that larger numbers of spores were produced in the modified medium than were counted as viable by the method used. Spores produced in the modified medium appeared normal, with larger proportions of spores to vegetative cells at a_w levels of 0.98 and 0.97 than in the basal medium; spores were present in the modified medium at an a_w of 0.96 for both

TABLE 3. *Sporulation of two strains of C. perfringens in Ellner's medium with the a_w adjusted with sucrose*[a]

Strain	Incubation time	Replications	a_w		
			0.993	0.98	0.97
	hr				
A48	24	1	6.7×10^6	5.3×10^2	0
		2	1.5×10^6	6.9×10^4	1
		3	4.0×10^6	1.1×10^2	0
	48	1	7.4×10^6	4.5×10	2
		2	1.5×10^6	2.0×10^4	0
		3	1.3×10^6	1	0
	72	1	6.0×10^6	1.3×10^2	0
		2	2.0×10^6	4.0×10^4	2
		3	2.0×10^6	8	0
	168	1	3.5×10^6	4.4×10	0
		2	1.6×10^6	2.3×10^4	0
		3	1.8×10^6	3	0
	336	1	9.1×10^6	7	0
		2	2.3×10^6	4.5×10^4	0
		3	1.7×10^6	0.7×10^6	0
NCTC 8797	24	1	1.0×10	0	0
		2	1.7×10^3	1.9×10^2	20
		3	2.0×10^2	7	0
	48	1	1.8×10	0	0
		2	2.5×10^3	8.2×10^2	1
		3	1.8×10^2	1	0
	72	1	2.2×10	0	0
		2	3.3×10^3	6.1×10^2	0
		3	1.7×10^2	0	0
	168	1	1.4×10	0	0
		2	4.5×10^3	7.5×10^2	2
		3	3.2×10^2	0	0
	336	1	5.1×10	0	0
		2	5.7×10^2	5.1×10^2	0
		3	5.8×10	0	0

[a] Results show the number of spores per milliliter after heating for 15 min at 80 C, plating in iron sulfite-agar, and incubating at 37 C for 24 to 48 hr. The average initial numbers of vegetative cells inoculated were 8.1×10^5 per ml for strain A48 and 1.0×10^6 per ml for strain NCTC 8797.

strains. A more protective heating medium perhaps would have yielded higher viable counts. Weiss and Strong (10) observed that spores heated in Ellner's medium had reduced heat resistance as compared with those in other substrates.

Bacterial spores have long been known for their capacity to withstand drier conditions than vegetative cells. In contrast to the survival of bacterial spores in dry atmosphere, the moisture requirement for the formation of spores has received little attention. In the present experiment, a higher level of a_w appeared to be required for the formation of spores than for vegetative growth. Leifson (8), in his study of the effect of inorganic salts on sporulation, found that almost invariably growth of *C. botulinum* occurred at a higher concentration of inorganic salts than did sporulation. Williams and Purnell (11) observed that growth and spore formation by sporulating bacteria did not necessarily parallel each other. At a moisture concentration

TABLE 4. *Sporulation of three strains of C. perfringens in Ellner's medium or modified medium with the a_w adjusted with sodium chloride*[a]

Strain	Incubation time	Replications	a_w of Ellner's medium			
			0.993	0.98	0.97	0.97 + veal
	hr					
A48	24	1	1.8×10^6	0	0	0
		2	5.6×10^5	0	0	0
		3	5.9×10^5	3	0	0
	72	1	1.4×10^6	38	1	1
		2	6.6×10^5	0	0	0
		3	1.1×10^6	2	3	1
	336	1	8.7×10^5	440	0	—
		2	7.9×10^5	0	0	—
		3	6.5×10^5	190	0	—
NCTC 8797	24	1	7.3×10^2	0	0	0
		2	1.6×10^2	0	0	0
		3	5.9×10	0	0	0
	72	1	3.2×10^2	0	0	0
		2	5.4×10^2	0	0	0
		3	2.4×10^2	0	0	0
	336	1	2.4×10	0	0	—
		2	9.7×10^2	0	0	—
		3	2.3×10	0	0	—
			a_w of modified medium			
			0.992	0.98	0.97	0.96
A48	24	1	4.0×10^5	6	5	7
		2	1.2×10^5	0	0	0
		3	7.8×10^6	150	0	0
NCTC 8239	24	1	3.7×10^5	9	4	0
		2	3.4×10^6	1	1	1
		3	3.3×10^5	650	16	0

[a] The results show the number of spores per milliliter after heating for 15 min at 80 C, plating in iron sulfite-agar, and incubating at 37 C for 24 to 48 hr. The average initial numbers of vegetative cells inoculated were 8.0×10^5 per ml.

of 45%, the growth of *C. botulinum* took place but no spore formation was detected.

Germination and growth. The numbers of germinated spores of both strains in media of all a_w values tested were very similar, whether heating was done immediately after inoculation or after 90 min of incubation at 37 C; therefore, data for strain NCTC 8239 from the former only are presented in Table 5. Strain A48 followed a similar pattern except that counts averaged only 73 per ml in media adjusted with NaCl to an a_w of 0.96 and, in contrast, were higher with glycerol added to an a_w of 0.96 (4.7×10^3). As the germination of spores was determined by following growth in an agar medium adjusted to the same a_w level, germination that might occur at a lower a_w than that required for growth could not be observed in this experiment. Williams and Purnell (11) concluded on the basis of a decrease in spore count at 35% total moisture that spores of *C. botulinum* could germinate at moisture concentrations which did not permit growth. Using a different criterion, Baird-Parker and Freame (2) reached a similar conclusion.

It would appear that germination occurs at a_w levels as low as allow growth of vegetative cells. The addition of glycerol as the means of lower-

TABLE 5. *Germination of spores of C. perfringens NCTC 8239 in Fluid Thioglycollate Medium of adjusted a_w levels*[a]

Solute	Replication	a_w						
		0.99	0.98	0.97	0.96	0.95	0.94	0.93
Glycerol	1	1.9×10^5	2.0×10^5	1.7×10^5	1.2×10^5	1.1×10^5	1.9×10^2	0
	2	2.2×10^5	1.1×10^5	6.5×10^4	1.8×10^5	1.5×10^5	3.3×10^3	0
	3	2.2×10^5	1.8×10^5	1.7×10^5	1.4×10^5	1.3×10^5	2.0×10^3	1
Sucrose	1	1.8×10^5	6.8×10^4	2.3×10^3	1.7×10^3	0	—	—
	2	1.9×10^5	1.3×10^4	3.3×10^4	1.9×10^3	0	—	—
Sodium chloride	1	2.1×10^4	1.0×10^4	1.2×10^4	1.2×10^3	0	—	—
	2	1.6×10^5	1.2×10^5	8.7×10^4	3.3×10^4	0	—	—
	3	1.2×10^5	1.5×10^5	7.2×10^4	3.5×10^3	0	—	—

[a] Results show the number of germinated spores per milliliter after heating for 15 min at 80 C, plating in Fluid Thioglycollate Medium adjusted to a corresponding a_w with agar then added, and incubating at 37/C for 24 to 48 hr.

ing the a_w offered much less inhibition to germination and growth than did either sucrose or sodium chloride for both strains. Colonies at the lowest a_w, 0.93, were very small and atypical in form, although they were confirmed to be *C. perfringens*.

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