VIABILITY OF THE COLON-TYPHOID GROUP IN CARBONATED WATER AND CARBONATED BEVERAGES

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The destructive effect of carbon dioxide on various microörganisms and the value of carbonation for the preservation of foods and beverages have claimed the interest of a number of workers since the first days of bacteriology. As early as 1885, Leone reported the examination of several commercial mineral waters which were under a slight pressure of CO_2. The number of microörganisms found to be present was always low. He also observed that after passing CO_2 gas through a drinking water the total count rapidly diminished.

Somewhat later than this a number of investigations were made of the destructive effect of CO_2 under relatively high pressures. Schaffer and Freudenreich (1891–1892), after studying the effect of pressures of 40 to 50 atmospheres of CO_2 combined with an increase of temperature, conclude that CO_2 has only a feeble bactericidal action. Sabrazès and Bazin (1893) found that cultures of *Bact. typhosum*, *Bact. coli*, *Staphylococcus aureus*, and the anthrax bacillus were able to develop after exposure to 60 to 70 atmospheres of CO_2 for several hours. These results are contradicted by D'Arsonval and Charrin (1893) who report that CO_2 under a pressure of 50 atmospheres sterilized cultures of *Ps. pyocyanea* in from six to twenty-four hours. Recently, Larson, Hartzell, and Diehl (1918), in a study of the effect of pressures upon bacteria, found that CO_2 under a pressure of 50 atmospheres would destroy *Bact. typhosum*, *Bact. coli*, *Mycobact. tuberculosis*, *Ps. pyocyanea*, staphylococci, strep-

tococci, and pneumococci, in a period of time ranging from one and one-half to two and one-half hours. Yeast cells were unaffected after an exposure of forty-eight hours.

Since these pressures are many times greater than those to which the ordinary carbonated beverages are subjected, there is the possibility that certain organisms may retain their vitality for a longer period. Several reports of the examination of carbonated beverages purchased in the open market have shown that occasionally there are encountered considerable numbers of microörganisms, including those indicative of pollution. Allen, LaBach, Pinnell, and Brown (1915) report a sanitary survey of the "soft drink" industry of Kentucky. Although carbonation was found to cause a distinct reduction in the numbers present, occasional high counts and the presence of *Bact. coli* were reported. Stokes (1920) recently examined a great variety of "soft drinks" and noted the frequent presence of *Bact. coli* in 10 cc. and 1 cc. quantities, with an occasional occurrence in 0.1 cc. The plate counts exhibited great variation, and while the majority of samples yielded counts of less than 100 per cubic centimeter, a few showed surprisingly high numbers. Gershenfeld (1920) reports similar results. Young and Sherwood (1911) have reported an experiment in which they determined the viability of *Bact. typhosum Bact. coli,* and *Erythrob. prodigiosus* in carbonated water to which lemon syrup had been added. Although the typhoid bacillus showed a considerable reduction in numbers after four hours exposure, a few viable cells were found after ten days. *Bact. coli* and *Erythrob. prodigiosus* were found to be somewhat more resistant than *Bact. typhosum.*

In the present investigation chief emphasis has been placed upon the colon-typhoid group for the purpose of determining the length of time one may expect the various members of this group to withstand the environment of the different types of commercial carbonated beverages.

The following organisms have been employed: *Bact. coli* (fecal origin), *Bact. paratyphosum* B, and *Bact. typhosum.* Also, as a matter of interest, two common spore forms were included; *B. mesentericus,* and a putrefactive anaerobe of the *Clostridium sporogenes* group.

The beverages were prepared and carbonated in the 7-ounce bottles commonly used in the industry. Since they were prepared as nearly as possible under commercial conditions and no effort was made to sterilize the various ingredients, control examinations of the product were made previous to experimental inoculation to determine the absence of the particular type of organism used in the investigation. In no instance was any difficulty of this kind encountered. Throughout the work commercial CO_2 was used for carbonation. As a test for any impurities in the carbon dioxide which might affect the death-rate of the organisms, tap water was carbonated as usual, then heated in the Arnold sterilizer for a short period to expel the CO_2 and finally the death-rate of *Bact. coli* in this water was compared to that in parallel samples of the original tap water. No discrepancies other than those which might be attributed to experimental variation were observed.

Small amounts of a suspension of the various test organisms in sterile tap water were used for inoculation. This was accomplished in one of the two following ways. The first method consisted of adding equal amounts of bacterial suspension to each bottle just before carbonation. In the second method the samples were prepared, bottled, carbonated, and capped as usual. They were then stored at 1°C. for several days until used, when the bottles were re-opened and inoculated. If opened while still cool, there was little loss of CO_2 gas. The first method was used for most of the experiments with *Bact. coli*. The second method was necessary when working with *Bact. typhosum* and *Bact. paratyphosum* B since by the first method there is more or less spattering of the material during the process of carbonation.

Immediately after inoculation and at definite intervals thereafter plate counts were made. To prevent the considerable loss of CO_2 upon repeated opening of the same bottles, especially those held at room temperature, a number of bottles were inoculated with equal amounts of bacterial suspension and, at each time interval, different sets of two were opened and samples withdrawn for plating. When the numbers had become so

reduced as to give negative results upon plating 1 cc. quantities, larger amounts, 5 cc. and 10 cc., were introduced into broth to determine, insofar as possible, the final disappearance of the organisms in question. This was done by streaking Endo plates from the broth cultures, fishing any typical colonies, and finally applying the usual methods used for the identification of the various members of this group of organisms.

EXPERIMENTAL

Since temperature may be expected to exert a marked influence upon the death-rate, experimental samples were held at two different temperatures, namely, in cold storage at 1°C. and at room temperature, 19° to 23°C. Tables 1 and 2 present data showing the viability of *Bact. coli* in carbonated water at several different pressures and also, for purposes of comparison, in plain tap water. It is evident that carbonation causes a speedy destruction of the colon bacillus and that this effect is dependent upon the temperature at which the samples are held, being much more pronounced at room temperature than at 1°C. Furthermore, the different degrees of pressure of CO_2 mentioned in these tables apparently exerted little or no influence upon viability, for the organisms were killed as speedily in water saturated with CO_2 (at both 20°C. and 1°C.), but under no excess pressure, as they were in the carbonated samples under pressures of 28 and 41 pounds per square inch. In fact, where the pressure was released the plate counts frequently were less than those of the samples held under pressure (table 2), a phenomenon which was regularly observed upon several repetitions of the experiment.

To gain an idea of the hydrogen-ion concentration of carbonated water the indicators brom-phenol-blue and methyl-red were added to different bottles which were then filled with carbonated water at these several pressures. In this way the value was roughly determined as pH 4.0–4.4. Release of the pressure, as indicated in table 2, was followed by very little, if any, immediate change in the hydrogen-ion concentration when meas-

ured in this way. When, however, such samples are held for a period of one or two weeks at 19° to 20°C. there is a gradual escape of CO_2 gas as evidenced by a decrease in the hydrogen-ion concentration. It is believed that under the conditions of our experiments the acidity of the dissociated carbonic acid is

TABLE 1

Showing the comparative viability of Bact. coli in carbonated tap water under pressure and in plain (non-carbonated) tap water

	TIME INTERVAL	CARBONATED TAP WATER PRESSURE 41 POUNDS PER SQUARE INCH (2.78 ATMOSPHERES) AT 18°C.		CONTROLS, PLAIN TAP WATER	
Held at room temperature (20–21°C.)	At once	181,000*	156,000	204,000	190,000
	4 hours	79,000	80,000		
	24 hours	950	6,600	203,000	194,000
	4 days	1 cc. plate negative	26	34,200	18,000
	7 days	10 cc. 0†	10 cc. +	14,800	10,000
		5 cc. +	5 cc. 0		
		1 cc. 0			
	14 days	10 cc. 0	10 cc. 0	2,200	1,000
		5 cc. 0			
Held at 1°C.	At once	163,000	181,000	200,000	260,000
	24 hours	51,000			
	4 days	25,500	23,000	166,000	180,000
	7 days	2,700	5,400	190,000	Lost
	14 days	1 cc. plate negative	30	100,000	Lost
	26 days	5 cc. +	1 cc. +	17,000	Lost
		1 cc. 0	0.1 cc. +		

* Figures represent numbers of *Bact. coli* per centimeter.

† 0 indicates the absence, and + the presence, of *Bact. coli* as determined by transferring the specified amount of water (10, 5 or 1 cc.) to broth. This was done when the numbers had become so reduced as to give negative results upon plating 1 cc. quantities.

the main factor responsible for the death of the bacteria. Other factors, such as differences in osmotic pressure, may also play a part.

Several of the simpler carbonated beverages were used in the next experiments. It should be realized that certain acids—

usually citric, tartaric, phosphoric, or lactic—are added to some types of beverages and that these acids may affect the longevity of the organisms in question. A comparison of the viability of *Bact. coli* in a non-acid and in an acid-containing beverage is shown in table 3. It will be noted that the hydrogen-ion concentration of the latter is considerably greater than that of the former. The colon bacillus is killed much more speedily in the acid-containing beverage, the effect being especially marked at the higher temperature. Apparently the rapid de-

TABLE 2

Viability of Bact. coli in carbonated tap water under pressure and with pressure released

	TIME INTERVAL	PRESSURE 28 POUNDS PER SQUARE INCH (1.9 ATMOSPHERES) AT 24°C.		EXCESS CO_2 ALLOWED TO ESCAPE AT THE RESPECTIVE TEMPERATURES BEFORE INOCULATION	
Held at room temperature (22–24°C.)	At once	440,000	360,000	440,000	500,000
	24 hours	1,500	1,360	870	130
	3 days	13	18		36
	7 days	10 cc. +	10 cc. 0	10 cc. +	10 cc. +
		5 cc. 0		5 cc. 0	5 cc. 0
	18 days	10 cc. 0	10 cc. 0	10 cc. 0	10 cc. +
					5 cc. 0
Held at 1°C.	At once	500,000	390,000	300,000	540,000
	24 hours	34,000	14,700	8,200	10,300
	3 days	3,600	900	83	71
	7 days	100	88	1 cc. +	1 cc. +
	18 days	1 cc. +	1 cc. +	1 cc. +	1 cc. +

struction of *Bact. coli* in the acid-containing lemon soda is due mainly to the dissociation of the citric acid present, for in additional experiments in which this acid was omitted the death rate was found to be comparable to that of the non-acid vanilla soda. Also in this case the hydrogen-ion concentration had decreased from pH 3.0 to pH 4.0–4.4 by the omission of the citric acid.

Experiments with other acid-containing beverages have demonstrated that as the amount of acid is increased above that indicated in table 3, the more readily is the colon bacillus killed.

In one instance in which 0.156 per cent lactic acid (5 grains per 7-ounce bottle) was employed the numbers of *Bact. coli* dropped from several hundred thousand to several hundred per cc. in

TABLE 3

Showing the viability of Bact. coli in a non-acid and in an acid type of carbonated beverage

	TIME INTERVAL	VANILLA SODA (NON-ACID), PRESSURE 24 POUNDS (1.6 ATMOSPHERES) AT 21°C., pH 4.0–4.4		LEMON SODA (ACID TYPE), PRESSURE 19 POUNDS (1.3 ATMOSPHERES) AT 23.5°C., pH 3.0	
Held at room temperature (21–24°C.)	At once	132,000	136,000	245,000	160,000
	24 hrs.	20,900	32,700	900	200
	3 days			1 cc. negative	1 cc. negative
	4 days	140	20	10 cc. negative	10 cc. negative
	7 days	1 cc. positive	1 cc. positive		
	14 days	10 cc. negative	10 cc. negative		
Held at 1°C.	At once	140,000	127,000	225,000	215,000
	24 hrs.	68,000	46,000	115,000	144,000
	3 days			87,000	25,000
	4 days	17,200	13,500		
	7 days	11,100	11,200	6,800	4,900
	14 days	390	600	600	
	1 mo.	1 cc. positive	1 cc. positive	10 cc. negative	10 cc. negative
	2 mos.	10 cc. positive; 1 cc. positive	10 cc. positive; 1 cc. negative		

Composition of the above beverages:

Vanilla soda: 10 mgm. c.p. vanillin (0.0048 per cent) and 10 grams sucrose (4.8 per cent) per 7-ounce bottle (207 cc.).

Lemon soda: 0.5 cc. commercial lemon flavor, 3 grains citric acid (0.094 per cent), and 20 grams sucrose (9.6 per cent) per 7-ounce bottle.

Carbonated water added to make the finished beverage.

4 hours at 20°C. When plain non-carbonated tap water was substituted for the carbonated water in the acid beverages, the death-rate of *Bact. coli* remained practically the same. That is, in these instances the added acids are the chief causative agents

in the destruction of the colon bacillus, irrespective of the effect of carbon dioxide.[1]

Since it was found that *Bact. coli* is able to withstand carbonation for an appreciable period, the next step was to investigate the viability under similar conditions of several of the pathogenic members of the colon-typhoid group. In these experiments *Bact. paratyphosum* B and *Bact. typhosum* were used. It was at once apparent that both of these types are considerably less resistant to the destructive effect of CO_2 than is the colon bacillus. Table 4 presents results which are characteristic of a number of similar experiments. One point of particular interest is the persistence, at 1°C., of the last few surviving organisms. These were too few in number to be estimated by plating and their presence could be detected only by the cultivation of 10 cc. amounts in glucose broth. Additional experiments with acid-containing beverages have shown that in these the typhoid bacillus is killed almost instantly. Thus in a carbonated lemon soda containing 0.156 per cent lactic acid, *Bact. typhosum* decreased in numbers from an initial inoculum of 27,400 per cubic centimeter to 10 per cubic centimeter within one hour and after two hours its presence could not be detected.

It should be emphasized that throughout all of the foregoing experiments the water used for carbonation and for preparation of the various beverages was an ordinary city supply of low mineral content. Under certain conditions, as for example in carbonated water of high mineral content, it is possible that non-spore-forming organisms may remain alive for longer periods than those herein reported. This possible influence of certain inorganic salts upon the viability of microörganisms in a carbonated environment has not been studied in the present investigation.

[1] The usual methods of bacteriological analysis could not be applied when larger quantities of the highly acid beverages were to be examined. It was found that sufficient amounts of acid were carried over to the culture medium to cause a distinct increase in the H-ion concentration, sufficient, indeed, to effect a retardation or even complete inhibition of growth. By the use of larger quantities of broth in flasks, instead of the usual amounts ordinarily contained in test tubes, this difficulty was largely overcome.

TABLE 4

Comparative viability of Bact. typhosum in carbonated and in plain (non-carbonated) tap water

TIME INTERVAL	CARBONATED TAP WATER, PRESSURE 25 POUNDS (1.7 ATMOSPHERES) AT 24°C.				CONTROL, PLAIN TAP WATER	
	Held at room temperature (22–24°C.)		Held at 1°C.		Held at room temperature (22–24°C.)	
At once	45,000	36,500	44,400	27,000	42,000	63,000
24 hours	1 cc. plate negative	1 cc. plate negative	30	1 cc. plate negative	41,000	22,000
48 hours	10 cc. negative	10 cc. negative	10 cc. positive	10 cc. positive	27,000	12,000
4 days			10 cc. positive	10 cc. positive		
7 days			10 cc. negative	10 cc. negative		

Comparative viability of Bact. typhosum and Bact. paratyphosum B. in vanilla soda

	PRESSURE 23 POUNDS (1.56 ATMOSPHERES) AT 24°C.							
	B. typhosum				B. paratyphosum B			
	Held at room temperature (21–24°C.)		Held at 1°C.		Held at room temperature (23–25°C.)		Held at 1°C.	
At once	175,000	166,000	237,000	144,000	170,000	116,000	194,000	200,000
4 hours					16,200	800		
24 hours	40	270	10,300	21,600	1 cc. plate negative	3	2,600	7,300
48 hours	1 cc. plate negative	1 cc. plate negative	310	170	10 cc. pos. 1 cc. neg.	10 cc. pos. 1 cc. neg.	430	860
3 days	10 cc. plate negative	10 cc. plate negative	10 cc. pos. 1 cc. neg.	10 cc. pos. 1 cc. neg.	10 cc. negative	10 cc. negative	10	15
4 days	10 cc. negative	10 cc. negative	10 cc. positive	10 cc. positive	10 cc. negative	10 cc. negative	10 cc. positive	10 cc. positive
6 days			10 cc. positive	10 cc. positive				
7 days							10 cc. positive	10 cc. positive
10 days			10 cc. negative	10 cc. negative			10 cc. negative	10 cc. positive
14 days							10 cc. negative	10 cc. negative

The resistance of spores to the conditions described in this paper is of some interest when compared to that of *Bact. coli* and *Bact. typhosum*. The spores of both *B. mesentericus* and *Clost. sporogenes* were found to be quite resistant, for after one month in carbonated water no reduction in numbers could be detected. In one experiment the spores of *B. mesentericus* survived in a citric acid beverage (pH 3.0) for one month with little, if any, diminution in numbers.

It must be stated emphatically that the results obtained in this investigation do not warrant the conclusion that water of a low sanitary quality can be used by the industry in the preparation of carbonated beverages, or that carbonation can be relied upon to destroy evidence of pollution. In many instances, particularly during the summer months, beverages are consumed within a few hours after their preparation and it is obvious that under these conditions pathogenic organisms, if originally present in the water, may survive carbonation and reach the consumer.

SUMMARY

Under the conditions of these experiments carbonation exerts a distinctly harmful effect upon the members of the colon-typhoid group and their period of viability in carbonated water is much shorter than that in plain tap water. The destructive effect of the CO_2 is especially marked at room temperature, 19° to 23°C., and less so at 1°C.

In a "non-acid" beverage, the organisms may persist for a slightly longer period than in carbonated water. In beverages containing 0.094 per cent or greater amounts of citric or lactic acids, the death-rate is very rapid and is apparently due to the effect of these acids, irrespective of the CO_2.

Bact. typhosum and *Bact. paratyphosum* B are more readily destroyed by CO_2 than is *Bact. coli*.

The spore forms of a common aerobe, *B. mesentericus*, and of a common anaerobe, *Clost. sporogenes*, were found to be quite resistant to carbonation, surviving one month at room temperature with no apparent diminution in numbers.

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