Evidence of "Flat Sour" Spoilage by Obligate Anaerobes in Marketed Canned drinks*1

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According to the latest statistics by the Canners Association of Japan, the production of canned drinks in Japan increased by 3.7 times during the last five years, the gross production in 1978 being 140×10^4 tons. This rapid increase is due to the rapid propagation on vending machines in the soft drinks market, especially of hot vendors which accelerated the consumption of canned drinks in winter. Among canned goods developed for hot vending are coffee, *shiruko* (sweet bean drink), cocoa, tea, etc. In 1978 the production of these products, of which canned coffee was about 95 percent, was 44×10^4 tons and 31.4 percent in all canned drinks[1].

It has been suggested that prolonged storage of canned foods in the vending machines at the serving temperature (approximately 60°C) caused bacteriological problems[2], which have only recently become a source of concern to the canning industry in Japan[3]. Thermophilic spoilage microorganisms, both gas-forming and flat sour causing, grow vigorously at this temperature. As a matter of fact, in some of the cans kept hot in the vending machines flat sour spoilage was strongly evidenced.

Flat sour spoilage has been well known for about 50 years, and described as caused by *Bacillus stearothermophilus* in low-acid foods and by *Bacillus coagulans* in acid foods[4]. However, it was found in our laboratory that no colonies of microorganisms were detected when the aliquots of the spoiled canned drinks are inoculated on the dextrose tryptone agar (DTA), which is known to be a suitable medium for the growth of these bacilli[5,6].

Some results on the flat sour spoilage of canned coffee have been already reported in a short paper[7]. In this paper the detailed results obtained from the investigation of the marketed canned drinks for hot vending are reported.

MATERIALS AND METHODS

Marketed canned drinks used for incubation test at 55°C

All the samples were marketed canned drinks. As shown in Table 1, the samples were the products of 9 manufacturers and contained 17 kings of canned coffee, 3 kings of canned *shiruko*, 2 kinds of canned cocoa, and each one kind of tea, milk shake and *amazake* (fermented rice-gruel). Twelve cases of each were subjected to the test. The numbers of cans per case were either 30 or 40. So, each sample contained 360 cans or 480 cans and the number of cans used for the test

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amounted to 10,800 in all.

Incubation test

All samples were incubated at 55°C and 4 cases of each kind were tested every ten days. PH values and vacuums of 10 percent of cans were measured and others were observed visually.

Isolation of bacteria from spoiled cans

Contents of spoiled cans were withdrawn under aseptic conditions. For the detection of aerobes 1-ml aliquots of the content were mixed with 15–20 ml of melted DTA in sterile Petri-dishes. For the detection of anaerobes 1-ml aliquots of the content were inoculated into test tubes containing 10ml of the modified fluid thioglycollate medium (mTGC). The formula of mTGC is shown in Table 2. Two plates and two test tubes were used for a spoiled can. The plates were incubated for 5 days and the test tubes for 10 days at 55°C. For purification and isolation, mTGC involving grown bacteria was inoculated on the plate of mTGC. The plate was incubated for 10 days at 55°C anaerobically in a Tomy's anaerobic jar containing room temperature catalyst[9], and the colonies were transferred into mTGC. The isolated bacteria were maintained in mTGC.

Table 1. All Samples Used for Incubation Test at 55°C

Manufacturer	Sample No.	Contents			Cans
A	1	Coffee	190 g	5227*	40 × 12
	2	Coffee	190 g	5224, 5322	40 × 12
	3	Coffee	250 g	5220	30 × 12
	4	Coffee	190 g	5326	40 × 12
	5	Milk shake	190 g	5301	40 × 12
	6	*Amazake*	200 g	4Z19	40 × 12
	7	Cocoa	190 g	4Z15, 4Z16	40 × 12
	8	*Shiruko*	200 g	4Z20	40 × 12
B	11	Coffee	250 g	5414	30 × 12
	12	Coffee	190 g	5410	40 × 12
	24	Coffee	250 g	5Y18	30 × 12
C	15	Coffee	250 g	5404	30 × 12
	17	Coffee	190 g	5401	40 × 12
	18	*Shiruko*	200 g	5325	40 × 12
D	21	Coffee	190 g	5020	40 × 12
	22	Coffee	250 g	5015	30 × 12
	23	*Shiruko*	200 g	5028	40 × 12
E	9	Coffee	250 g	5127, 5131	30 × 12
	10	Cocoa	250 g	5305	30 × 12
F	13	Coffee	250 g	5407	30 × 12
	25	Tea	250 g	5Z12	30 × 12
G	19	Coffee	190 g	5605	40 × 12
	20	Coffee	190 g	5204	40 × 12
H	14	Coffee	200 g	5118	40 × 12
I	16	Coffee	250 g	5412, 5415	30 × 12

* Japanese code for the date of production: First, second, third and forth numbers show the year, month, and day, respectively. Y is November and Z is December.

Table 2. Formula of mTGC, TF, TSiF and TSaF

Ingredients / Media	mTGC	TF	TSiF	TSaF
Trypticase (BBL)	17.0 g/l	17.0 g/l	17.0 g/l	17.0 g/l
Phytone (BBL)	3.0	3.0	3.0	3.0
Glucose	6.0	6.0	6.0	6.0
NaCl	2.5	2.5	2.5	2.5
Na-Thioglycollate	0.5	0.5	0.5	0.5
Agar	0.7	0.7	0.7	0.7
L-Cystine	0.25			
Na_2SO_3	0.1		1.0	
Na_2SO_4				1.0
Ferric Citrate*		0.5	0.5	0.5

* Sterilized separately.

Biochemical examination

Gram staining, production of acids from sugars, production of indole and hydrogen sulfide, coagulation of milk, reduction of nitrate and hydrolysis of gelatin were examined by the ordinary methods[10]. Digestion of coagulated egg albumin was tested by the methods described by Burnett and others[11]. However, for the test of indole production, mTGC in which phytone and glucose were omitted was used; for hydrogen sulfide production, SIM medium[12]; for coagulation of milk, 10 percent skim milk with iron; and for nitrate reduction, the fluid nitrate medium[13] in which proteose peptone was replaced with phytone.

Appearance of flat sour spoilage caused by isolated bacteria

Ten ml aliquots of the content of a normal can were introduced into sterile test tubes with tubes with plastic caps under aseptic conditions. The test tubes were heated in boiling water for 10 min to remove dissolved oxygen and sterile liquid paraffin was added to each tube to seal the content. The isolated bacteria were inoculated respectively into the test tubes containing the normal canned coffee samples corresponding to those from which the bacteria were isolated. Ten test tubes were used for one isolate, five were for the control and the other five were for the examination. The test tubes were incubated for 15 days at 55°C and observed visually.

Preparation of spore suspensions of the isolated bacteria

mTGC in which agar was omitted was used as the medium for sporulation. As soon as the medium was autoclaved, sterile melted paraffin (M.P. 42–44°C) was added to seal the mdium from air. Isolates were cultured at 55°C in the mdium.

The spores were harvested after 10 days by centrifugation, washed three times with sterile deionized water of the same volume as the mdium and suspended in sterile deionized water of the same volume. The suspensions were filtered through sterile Toyo No. 2 filter paper to remove clots of spores and paraffin. These procedures were carried out at 0–4°C. The filtrates were heated in a boiling water bath for 10 min to destroy vegetative cells. The spore suspensions obtained were stored in a refrigerator.

Determination of D values of the spores at 120°C

One ml aliquots of the spore suspensions were introduced into small test tubes (7×105mm), which were sealed by heat immediately. The sealed test tubes were immersed in an oil bath at

120±0.1°C and were removed after each of the heating periods. These tubes were cooled in cold water, broken aseptically and the number of survivors were determined by the MPN method with mTGC.

Reduction of sulfite and sulfate

For the test of reduction of sulfite and sulfate by isolates, the iron sulfite agar (ISA)[14], the Starkey's medium[15], the Postgate's medium[16], TF medium (TF), TSiF medium (TSiF), and TSaF medium (TSaF) were used. TF is mTGC in which L-cystine and sodium sulfite were replaced with ferric citrate. TSiF is TF with 0.1% sodium sulfite added and TSaF is TF with 0.1% sodium sulfate added. Table 2 shows the formula of these media. When an isolate does not blacken TF but does TSiF, it is sulfite-reducing. When an isolate does not blacken TF but does TSaF, it is sulfate-reducing.

Table 3. PH Value and Vacuum of Normal Cans

Manufacturer	Sample No.	pH			Vacuum		
		10 Days*	20 Days*	30 Days*	10 Days*	20 Days*	30 Days*
A	1 (Coffee)	6.2	6.1	6.0**	27	27	29**
	2 (Coffee)	6.1	6.0	6.0	27	18	20
	3 (Coffee)	6.3	6.1	6.1	40	26	27
	4 (Coffee)	6.0	6.0	5.9	28	16	20
	5 (Milk Shake)	6.3	6.2	6.2	23	25	24
	6 (*Amazake*)	4.5	4.7	4.9	31	30	30
	7 (Cocoa)	6.3	6.2	6.1	38	28	26
	8 (*Shiruko*)	5.5	5.1	5.2	31	27	27
B	11 (Coffee)	6.2	6.1	6.1	47	36	39
	12 (Coffee)	6.5	6.4	6.4	37	27	30
	24 (Coffee)	6.3	6.3	6.3	43	42	38
C	15 (Coffee)	6.3	6.2	6.2	31	23	27
	17 (Coffee)	6.4	6.4	6.3	39	32	33
	18 (*Shiruko*)	5.9	5.9	5.9	39	40	43
D	21 (Coffee)	5.9	6.2	6.0	36	38	36
	22 (Coffee)	6.0	6.1	6.0	27	27	25
	23 (*Shiruko*)	6.0	5.4	5.2	36	34	33
E	9 (Coffee)	6.2	6.1	6.0	47	38	40
	10 (Cocoa)	6.4	6.3	6.3	53	40	38
F	13 (Coffee)	6.1	6.0	6.0	21	16	17
	25 (Tea)	6.6	6.5	6.4	24	21	22
G	19 (Coffee)	6.1	6.0	6.0	31	29	30
	20 (Coffee)	6.1	6.0	6.1	20	23	25
H	14 (Coffee)	6.3	6.1	6.1	8	8	10
I	16 (Coffee)	6.4	6.3	6.2	30	23	23

* Days of incubation at 55°C.
** Average values.

RESULTS

Incubation test

PH values and vacuums of normal cans were shown in Table 3. PH values of normal canned coffee ranged from 5.9 to 6.5, those of canned *shiruko* from 5.1 to 6.0, canned cocoa from 6.1 to 6.4, and canned tea, milk shake and *amazake* 6.5, 6.2 and 4.7, respectively. The vacuums of normal cans varied remarkably as shown in the table from a minimum of 8 cmHg (Sampel No. 14) to a maximum of 53 cmHg (Sample No. 10).

Number of spoiled cans and spoilage ratio of each sample were shown in Table 4. Spoiled cans were found in six kinds of canned coffee samples, No. 12, 24, 21, 22, 13 and 14, from 4 manufacturers. The numbers of spoiled cans were 5, 9, 9, 13, 1 and 3, respectively. These amounted to 40 cans in all. Spoilage ratios ranged from 0.3% of sample No. 13 to 3.6% of sample No. 22.

Table 5 shows pH values and vacuums of spoiled cans. PH values of the spoiled cans were lower than those of normal ones. Maximum drop of pH values was about 1.0. Vacuums of 8 spoiled cans measured, except for can No. 22-11, were lower than those of normal ones and the decrease of vacuums was 10 to 20 cmHg.

Some contents of spoiled canned coffee samples were more whitish than the normal ones, and

Table 4. Number of Spoiled Cans and Spoilage Ratio

Manufacturer	Sample No.	Number of Spoiled Cans			Spoilage Ratio
		10 Days*	20 Days*	30 Days*	
A	1 (Coffee)	0	0	0	
	2 (Coffee)	0	0	0	
	3 (Coffee)	0	0	0	
	4 (Coffee)	0	0	0	
	5 (Milk Shake)	0	0	0	
	6 (*Amazake*)	0	0	0	
	7 (Cocoa)	0	0	0	
	8 (*Shiruko*)	0	0	0	
B	11 (Coffee)	0	0	0	
	12 (Coffee)	3	1	1	1.0%
	24 (Coffee)	4	1	4	2.5%
C	15 (Coffee)	0	0	0	
	17 (Coffee)	0	0	0	
	18 (*Shiruko*)	0	0	0	
D	21 (Coffee)	6	1	2	1.9%
	22 (Coffee)	2	3	8	3.6%
	23 (*Shiruko*)	0	0	0	
E	9 (Coffee)	0	0	0	
	10 (Cocoa)	0	0	0	
F	13 (Coffee)	1	0	0	0.3%
	25 (Tea)	0	0	0	
G	19 (Coffee)	0	0	0	
	20 (Coffee)	0	0	0	
H	14 (Coffee)	0	1	2	0.6%
I	16 (Coffee)	0	0	0	

* Days of incubation at 55°C.

Table 5. PH Value and Vacuum of Spoiled Cans

Sample No.	Can No.	pH	Vacuum	Day*
	12-1	5.3	10	10
	12-2	5.1		10
12	12-3	5.2		10
	12-4	5.4	20	20
	12-5	5.5		30
	24-1	5.2		10
	24-2	5.1		10
	24-3	5.2		10
	24-4	4.9		10
24	24-5	5.3	29	20
	24-6	5.3	28	30
	24-7	5.3		30
	24-8	5.6		30
	24-9	5.2		30
	21-1	5.6		10
	21-2	5.2		10
	21-3	5.2		10
	21-4	5.9		10
21	21-5	5.1		10
	21-6	5.1		10
	21-7	5.2		10
	21-8	5.3		20
	21-9	5.1	27	30
	22-1	5.4		10
	22-2	5.4		10
	22-3	5.3		20
	22-4	5.4		20
	22-5	5.6		20
	22-6	6.0		30
22	22-7	5.5		30
	22-8	5.6		30
	22-9	5.5		30
	22-10	5.5		30
	22-11	5.5	26	30
	22-12	5.5		30
	22-13	5.1		30
13	13-1	5.1	8	10
	14-1	5.4	0	20
14	14-2	5.3		30
	14-3	5.3		30

* Days of incubation at 55°C.

Table 6. PH Values after 15 Days of Incubation

Strain No.	Control					Inoculated				
12-5	5.9	5.8	5.8	5.8	5.8*	5.3	5.3	5.3	5.3	5.3*
24-1	6.0	6.0	6.0	6.0	6.0	4.9	4.7	4.7	4.8	4.7
24-5	6.0	6.0	6.0	6.0	6.0	5.9	4.8	4.8	5.8	5.8
24-6	6.1	6.1	6.1	6.1	6.1	4.9	4.9	4.9	5.0	5.1
24-7	6.1	6.1	6.1	6.1	6.1	5.9	5.9	5.9	5.9	5.8
24-9	6.0	6.0	6.0	6.0	6.0	5.8	5.4	5.2	5.3	5.5
21-2	6.1	6.1	6.1	6.1	6.1	5.3	5.1	5.1	5.2	5.3
21-5	6.1	6.1	6.1	6.1	6.1	5.1	5.2	5.2	5.2	5.2
22-2	6.0	6.0	6.1	6.0	6.0	5.3	5.2	5.4	5.6	6.0
22-3	6.0	6.0	6.1	6.0	6.0	6.0	6.0	6.0	6.0	6.0
22-13	6.0	6.0	6.1	6.0	6.0	6.0	6.0	6.0	6.0	6.0
13-1	5.9	5.9	5.9	5.9	5.9	4.8	4.8	4.8	4.8	4.8
14-1	6.1	6.1	6.0	6.0	6.0	6.0	5.1	5.2	5.2	5.2
14-2	6.1	6.1	6.0	6.0	6.0	5.9	5.0	5.0	5.3	5.5
14-3	6.1	6.1	6.0	6.0	6.0	5.0	5.1	5.1	5.1	5.1

* Incubated for 5 days.

the others were separated into clear supernatants and precipitates, but the change of flavor was very slight.

Isolation of bacteria from spoiled cans

From the spoiled cans no colonies were detected on DTA but some bacteria were detected in mTGC. From 15 out of 40 spoiled cans some spore-forming obligate anaerobes were isolated. These 15 cans were as follow.

12-5	24-6	21-2	22-3	14-1
24-1	24-7	21-5	22-13	14-2
24-5	24-9	22-2	13-1	14-3

Each single strain was selected at random from some isolates from each can and the strains were numbered the same as the cans from which the strains were isolated. Thus, 15 strains were obtained.

Appearance of flat sour spoilage caused by the isolates

Fifteen strains isolated were respectively inoculated into the normal canned coffee samples, corresponding to those from which the strains were isolated, and incubated at 55°C. After incubation for 3 to 5 days, the test tubes containing the strains became whitish and after 10 days precipitates were formed at the bottoms and the supernatants were clear in some of the test tubes, whereas even after 15 days the controls remained unchanged. The change of the appearance is similar to that of the contents of the spoiled canned coffee samples.

Table 6 shows pH values after 15 days of incubation. PH values of the contents with inoculated strains were lower than the controls except for strain No. 24-7, 22-3 and 22-13. Thus, all the strains except three caused the flat sour spoilage.

Heat resistance of spores of isolates

The strains of which D values at 120°C of spores could be measured by the method described above are shown in Table 7. These values ranged from 5 to 25.

Table 7. Heat Resistance of Spores of the Isolates

Strain No.	D value at 120°C
12-5	10 min.
24-1	25
24-5	5
24-6	17
24-7	14
24-9	6
21-5	16
22-2	10
13-1	6
14-1	21

Table 8. Characteristics of the Isolates

Strain No.	12-5	24-1	24-5	24-6	24-7	24-9	21-2	21-5	22-2	22-3	22-13	13-1	14-1	14-2	14-3
Gram stain	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Acids from															
Glucose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Lactose	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Salicin	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Sucrose	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Production of															
Indole	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
H_2S	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Coagulation of															
Milk	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Digestion of															
Albumin	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Meat	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
NO_2 from NO_3	−	−	+	+	+	+	−	−	+	+	+	−	−	−	−
Hydrolysis of															
Gelatin	−	−	−	−	+	−	−	−	−	−	−	+	−	−	−

Characteristics of isolates

Table 8 shows the characteristics of the strains. All of these were Gram-negative and produced acids from glucose but not from lactose, sucrose or salicin. These did not produce indole, but did hydrogen sulfide. The tests for coagulation of milk, and digestion of albumin and meat gave all negative results. However, some of the strains reduced nitrates to intrites but others did not. Some hydrolyzed gelatin but others did not.

All the strains showed the activities of formation of hydrogen sulfide. Moreover, when these were inoculated into ISA, all blackened it. As a preliminary experiment, some strains were inoculated into the Starkey's medium and the Postgate's medium, the media for the test of reduction of sulfate. But the present strains did not grow well in these media. When all the strains were inoculated into TF, TSiF and TSaF, these grew well but blackened only TSiF. So all the strains are sulfite-reducing but not sulfate-reducing.

DISCUSSION

After our short paper[7] which evidenced a new type of flat sour spoilage, was presented, Matsuda et al.* and Mizoguchi et al.* found similar spoilage of hot canned drinks individually. These findings indicated that the spoilage is widely taking place in Japan. Tables 3, 4 and 5 in this paper are for further confirmation of the problem.

As shown in Table 6, among 15 isolated strains which could be obtained by mTGC but not by DTA from spoiled cans, 12 strains caused flat sour spoilage in the normal canned coffee samples corresponding to those from which the strains were isolated. D values at 120°C of the spores of 10

* The annual meeting of the Canners Association of Japan on Oct. 25, 1977, Tokyo.

strains were obtained. These values ranged from 5 to 25 (Table 7). It was said that F_0 values of these cans at thermal process ranged from 15 to 20. Accordingly there is a chance that the spores survive the sterilization processes. From these results, it is concluded that at least 9 strains (12-5, 24-1, 24-5, 24-6, 24-9, 21-5, 22-2, 13-1, 14-1) are the bacteria that cause the flat sour spoilage. This flat sour spoilage caused by some obligate anaerobes is called "A New Type of Flat Sour Spoilage" (O.A. Flat sour spoilage) by the authors.

Some principal characteristics of these strains were thermophilic, obligate anaerobic, spore-forming, Gram-negative and producing hydrogen sulfide (Table 8). So one might conclude that *Desulfotomaculum nigrificans* plays a role. However, the genus *Clostridium* and genus *Desulfotomaculum* are classified by the ability of reduction of sulfate in the Bergey's Manual of Determinative Bacteriology (Eighth Edition). All the strains did not reduce sulfates but did sulfites in the test of using TF TSiF and TSaF. So it is difficult to determine these strains by the Bergey's Manual.

These results indicate that the new type of flat sour spoilage of canned coffee is not caused by known facultative anaerobes, but by some obligate anaerobes. So better care must be taken of canned foods sold in preheated conditions to prevent contamination by the causative micro-organisms in the ingredients.

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