Journal of Food Protection, Vol. 46, No. 5, Pages 420-425 (May 1983)

Acetoin and Diacetyl Production by Homo- and Heterofermentative Lactic Acid Bacteria

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(Received for publication August 23, 1982)

ABSTRACT

Eleven strains of homofermentative and heterofermentative lactic acid bacteria were screened for acetoin (A) and diacetyl (D) production from pyruvate and citrate in a peptone-yeast extract-glucose broth. The homofermenters, except *Streptococcus faecalis* subsp. *liquefaciens*, produced much more AD from pyruvate than from citrate; the opposite was true for the heterofermenters. Acetoin and diacetyl were produced from pyruvate as soon as growth was initiated. The production was exponential up to 24 h. Destruction of the accumulated AD coincided with entry into the stationary phase. Production of AD from citrate did not begin until 6 h of the logarithmic phase of growth. Formation of gas from citrate by *Lactobacillus plantarum* did not implicate greater ability to form AD from citrate than from pyruvate. Fifty μmoles ml^{-1} citrate caused about 50% inhibition of growth of *Streptococcus lactis* subsp. *diacetylactis*. All strains examined for ability to use pyruvate as a sole source of carbon were able to do so. Acetate (50 μmoles ml^{-1} generally stimulated AD formation from pyruvate. With the exception of a *Pediococcus* sp. and *S. faecalis* subsp. *liquefaciens*, acetaldehyde (100 μg ml^{-1}) enhanced AD production but not growth. Concentrations higher than 100 μg ml^{-1} had different effects.

Production of acetoin and diacetyl from citrate and pyruvate by homo- and heterofermentative lactic acid bacteria is undoubtedly strain-dependent, depending on its genetic capacity. Strains of *Lactobacillus casei* and *Lactobacillus plantarum* have been found by Hegazi and Abo-Elnaga (*16*) to show great difference in their capability of producing acetoin and diacetyl (AD) from citrate and pyruvate in milk. Strains which showed no evidence of gas production from citrate gave larger amounts of AD from citrate than from pyruvate; still some of these behaved otherwise. Of 9 strains of *Leuconostoc lactis* and *L. mesenteroides*, Cogan et al. (*10*) also found four cultures producing no diacetyl from citrate and only traces of acetoin. The remaining five strains produced high levels of acetoin and diacetyl from citrate, although one produced no diacetyl.

Moreover, production of AD from pyruvate is strongly influenced by the composition of the culturing medium. Homofermentative lactobacilli have been found to give consistently much more AD from pyruvate in peptone-yeast extract-glucose broth than in skim milk (*16*).

Reports on the ability of free-living organisms to produce both types of compounds are limited, although diacetyl has been repeatedly mentioned as an important constituent of Cheddar cheese flavor (*6,7,20*), and probably the flavor of other cheese varieties.

Salt-tolerant lactic acid bacteria play an important role in the ripening and flavor development of white pickled cheese of the Domiati type. Active cultures freshly isolated from salted raw milk were therefore tested for their ability to produce acetoin and diacetyl from citrate and pyruvate. *Streptococcus lactis* subsp. *diacetylactis* DRC3 was included in this study for comparison. Pyruvate breakdown products, e.g. acetaldehyde and acetate, were also examined for a possible stimulatory effect on acetoin and diacetyl synthesis.

MATERIALS AND METHODS

Microorganisms

Homo- and heterofermentative lactic acid bacteria were isolated and characterized by Hegazi (*15*). They came from different samples of raw milk containing 3 to 12% salt. After incubation at 30°C for 4 to 21 d the cultures were used. *S. lactis* subsp. *diacetylactis* DRC3 was obtained from the West of Scotland Agricultural College, Auchincruive, Ayr, UK. *Streptococcus faecalis* subsp. *liquefaciens* was from N. I. Z. O., Ede, The Netherlands.

Medium

MRS medium (*13*) was used for culturing of lactobacilli, leuconostocs and pediococci. *S. lactis subsp. diacetylactis* and *S. faecalis* subsp. *liquefaciens* were routinely grown in litmus milk at 30°C.

Screening of homo- and heterofermentative lactic acid bacteria for acetoin and diacetyl production.

The medium of Cato and Moore (*8*) consisting of 2% peptone, 1% yeast extract and 1% glucose (PYG) supplemented with 0.0575% $MgSO_4 \cdot 7H_2O$, 0.012% $MnSO_4 \cdot 2H_2O$ and 0.0034% $FeSO_4 \cdot 7H_2O$ was used. Either one or more of the following substrates was added to the medium: tripotassium citrate (BDH, AR), sodium pyruvate (Merck) and sodium acetate (Mallinckrodt) at a concentration of 50 μmoles ml^{-1}; acetaldehyde (Hopkin & Williams) 11.4 μmoles ml^{-1} (100 μg ml^{-1}). After the addition was made the medium was distributed in 16 × 160 mm test tubes (screw-capped for acetaldehyde) and autoclaved at 121°C for 10 min. The tubes were then inoculated with 2% of the culture under study, grown in MRS broth or PYG for 24 h, and incubated at 30°C for 24 h.

TABLE 1. *Acetoin and diacetyl production (μmoles ml^{-1}) from pyruvate and citrate (50 μmoles ml^{-1}) by homo- and heterofermentative lactic acid bacteria in PYG broth after 24 h at 30°C.*

Species	Strain	Pyruvate			Citrate		
		AD[a]	D	Recovery[b](%)	AD	D	Recovery[b](%)
L. casei	333C	5.2	0.6	21.0	2.1	0.2	4.0
	354C	5.2	0.1	21.0	1.1	0.2	2.0
	341C	2.0	0.4	8.0	0.7	0.1	1.0
	351C	5.2	0.1	21.0	2.0	0.3	4.0
L. plantarum	2310	5.2	0.3	21.0	0.5	0.2	1.0
	238	4.7	0.1	19.0	2.6	0.2	5.0
Motile strepto-bacterium	I17	6.0	0.3	24.0	2.6	0.3	5.0
L. brevis	421	0.2	0.1	0.8	8.8	0.1	18.0
Leuconostoc para-mesenteroides	II47	0.1	0.0	0.4	2.9	0.3	6.0
	III29	0.2	0.2	0.8	0.7	0.3	1.0
S. faecalis ssp. *liquefaciens*	Dutch	1.9	0.3	8.0	2.1	0.2	4.0

[a]PYG = peptone - yeast extract - glucose broth; A = acetoin; D = diacetyl; [b]=assuming 2 moles of citrate ⟩ 1 mole of acetoin + 1 mole of diacetyl, and 4 moles of pyruvate ⟩ 1 mole of acetoin + 1 mole of diacetyl.

Production of acetoin and diacetyl during growth

PYG broth containing 50 μmoles ml^{-1} of either sodium pyruvate or tripotassium citrate was placed in 16 × 160 mm screw-capped test tubes, 10-ml quantities, inoculated (after sterilization) with 1% of the culture under study and incubated at 30°C. A sample was withdrawn periodically during growth and subjected to the various analyses.

Determination of acetoin and diacetyl

The total acetoin and diacetyl and the amount of diacetyl were separately determined on acid deproteinated samples. To 1 ml of culture 2 ml of distilled water, 1 ml of 2/3 *N* H_2SO_4 and 1 ml of a 10% soultion of $Na_2SO_4 \cdot 2H_2O$ were added and the sample was centrifuged at 3000× *g* for 20 min using a Janetzki centrifuge, piccolo. The supernatant fluid was used for colorimetric determination of the total acetoin and diacetyl by the method of Westerfeld (*21*) at 530 nm, and diacetyl by the method of Krampitz (*18*) at 470 nm, using a Bausch & Lomb spectronic 20 spectrophotometer.

Determination of growth

Growth was measured by determining the increase in absorbance at 660 nm.

RESULTS AND DISCUSSION

Eleven strains of certain homo- and heterofermentative lactic acid bacteria were screened for diacetyl and acetoin production from pyruvate and citrate. As shown in Table 1, with the exception of *Leuconostoc paramesenteroides* II 47, all homo- and heterofermentative strains tested, were able to produce both acetoin and diacetyl (AD) from pyruvate and citrate. The homofermenters, except *S. faecalis* subsp. *liquefaciens*, produced much more AD from pyruvate than from citrate, the opposite was true for the heterofermenters of which *Lactobacillus brevis* was the most active. However, only a slight detectable amount of diacetyl was produced from citrate by this organism on incubation for 24 h, resembling in this respect the two strains examined by Keenan and Lindsay (*17*). The highest recovery of A obtained here was from pyruvate by the homofermentative lactobacilli, presumably the remainder was reduced to 2,3-butanediol by 2,3-butanediol dehydrogenase which may be proportionately less active in strains showing high recoveries. Recovery of acetoin from citrate found by Cogan et al. (*10*) ranged from 14 to 56%. The two strains of *Lactobacillus plantarum* were capable of producing gas from citrate within 3 d at 30°C, as evidenced by Durham tube results, but, contrary to expectation, much greater amounts of AD were formed from pyruvate than from citrate. Production of gas from citrate does not implicate greater ability to form AD from citrate than from pyruvate.

Lactobacillus casei strain 333C and *L. plantarum* 2310 produced relatively high amounts of AD, and were selected for further study. Production of acetoin and diacetyl by these two strains and by *S. lactis* subsp. *diacetylactis* from pyruvate and citrate (50 μmoles ml^{-1}), during growth is shown in Fig. 1 to 3. Similar results were obtained for *L. casei* and *L. plantarum*. Growth of both in pyruvate or citrate broth was identical, whereas Branen and Keenan (*3*) found ≥40 μmoles ml^{-1} citrate to be completely inhibitory to *L. casei*. Much greater amounts of acetoin and diacetyl were produced in the presence of pyruvate than in the presence of citrate. Pyruvate use with simultaneous production of AD and D began as soon as growth was initiated with acetoin being quantitatively the more important compound. Little of the pyruvate used during growth was recovered as diacetyl. In agreement with the results of Branen and Keenan (*5*), production of AD was exponential up to 24 h, after which it slowed down. These results conflict with the findings of Bassette et al. (*1*), who found that the

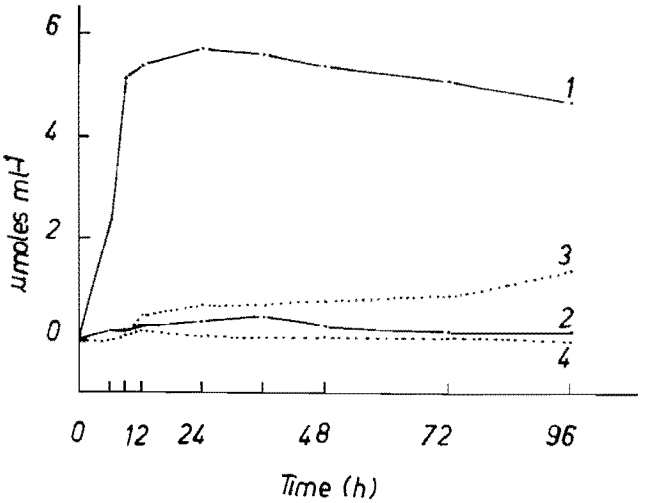

Figure 1. *Acetoin and diacetyl production from pyruvate and citrate (50 µmoles ml^{-1}) in PYG broth by* L. casei *subsp.* pseudoplantarum *333C during growth. 1-Total acetoin and diacetyl from pyruvate; 2-Diacetyl from pyruvate; 3-Total acetoin and diacetyl from citrate; 4-Diacetyl from citrate.*

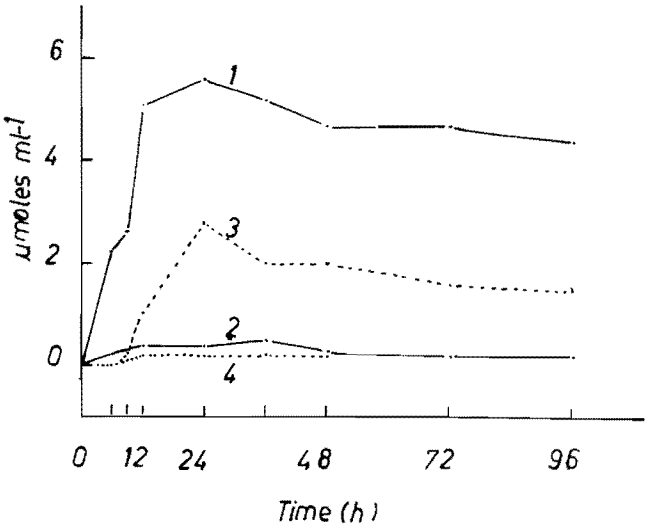

Figure 2. *Acetoin and diacetyl production from pyruvate and citrate (50 µmoles ml^{-1}) in PYG broth by* L. plantarum *2310 during growth. 1-Total acetoin and diacetyl from pyruvate; 2-Diacetyl from pyruvate; 3-Total acetoin and diacetyl from citrate; 4-Diacetyl from citrate.*

diacetyl level produced by *L. casei* in milk became very high after 100 h. Destruction of diacetyl may be due to diacetyl reductase, as was found for *L. casei* by Branen (*2*), who reported that acetoin was the end product of diacetyl reduction. Destruction of accumulated acetoin observed here is probably a consequence of the activity of NADH + H-linked acetoin reductase [D(-)-butanediol dehydrogenase, EC 1.1.1.4]. Acetoin reductase and diacetyl reductase have been reported by Cogan (*9*) to be constitutively present in cells of several strains of *S. lactis* subsp. *diacetylactis*.

Citrate utilization by *L. plantarum* did not begin until the middle of the exponential phase of growth. Drinan et al. (*14*) also found *L. plantarum* used no citrate until late in the exponential phase. They assumed that it may be due

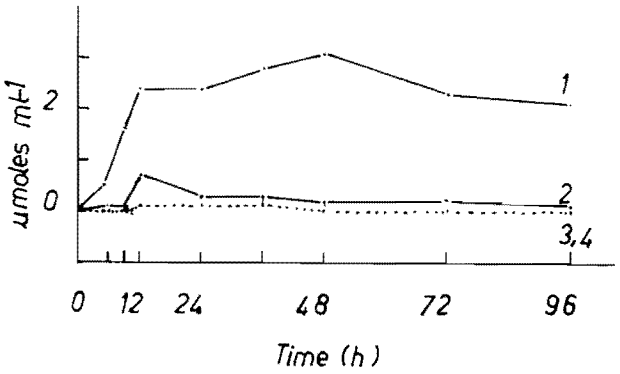

Figure 3. *Acetoin and diacetyl production from pyruvate and citrate (50 µmoles ml^{-1}) in PYG broth by* S. lactis *subsp.* diacetylactis *DRC3 during growth. 1-Total acetoin and diacetyl from pyruvate; 2-Diacetyl from pyruvate; 3,4-Total acetoin and diacetyl as well as diacetyl from citrate.*

to passive transport of citrate, as was found for *L. casei* (*4*). It is unlikely to be due to the need for inducible enzymes since the inocula were grown in the presence of citrate and induction is usually very rapid, requiring only 90 s for the β-galactosidase of *Escherichia coli* (*19*).

The value of 50 µmoles ml^{-1} citrate used in these experiments was quite high for *S. lactis* subsp. *diacetylactis* and suggests that, if anything, citrate is a more effective inhibitor of growth of *S. lactis* subsp. *diacetylactis* than of *L. casei* and *L. plantarum*. Beside ca. 50% inhibition of growth of *S. lactis* subsp. *diacetylactis*, presence of 50 µmoles ml^{-1} tripotassium citrate in PYG broth before inoculation resulted in traces of acetoin, but no diacetyl production by *S. lactis* subsp. *diacetylactis*.

Production of total AD and D from pyruvate and their subsequent destruction by *S. faecalis* subsp. *liquefaciens* is also shown graphically in Fig. 4.

Several strains each of homo- and heterofermentative lactic acid bacteria were screened for their ability to produce AD and D from pyruvate. Table 2 presents data confirming the ability of both *L. casei* and *L. plantarum* strains to produce noticeable amounts of diacetyl from

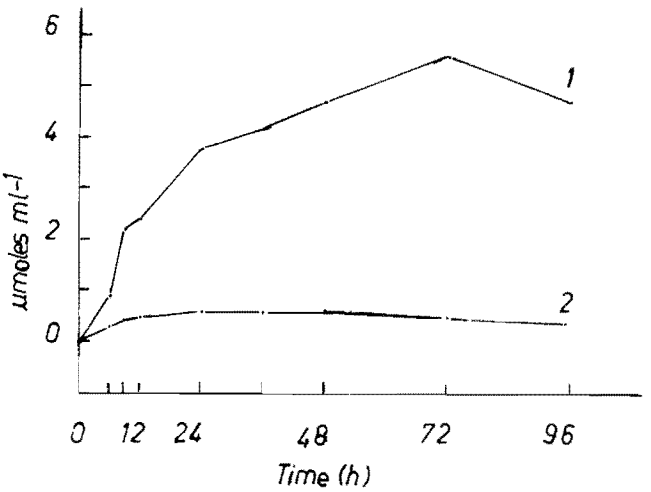

Figure 4. *Acetoin and diacetyl production from pyruvate (50 µmoles ml^{-1}) in PYG broth by* S. faecalis *subsp.* liquefaciens *during growth. 1-Total acetoin and diacetyl; 2-Diacetyl.*

TABLE 2a. *Acetoin and diacetyl production (μmoles ml^{-1}) from pyruvate (50 μmoles ml^{-1}) by various strains of* L. casei *and* L. plantarum *in PYG broth after 24 h at 30°C.*

Lactobacillus casei			*Lactobacillus plantarum*		
Strain	AD	D	Strain	AD	D
333C	5.2	0.6	2310	5.0	0.2
354C	5.2	0.4	I310	4.1	0.2
II23C	2.8	0.3	238	5.1	0.3
II15C	0.5	0.2	I 31	4.7	0.2
I 34C	2.9	0.4	32	4.4	0.2
I315	4.4	0.3	I 19	5.3	0.3
I 21	1.8	0.5	I 37	4.5	0.3
I 23	4.1	0.4	I 38	5.9	0.6
I 22	0.8	0.4	I312	4.6	0.5
245	4.6	0.4	I314	4.7	0.3
2415	4.8	0.1	234	6.2	0.2
227	4.5	0.4	235	5.2	0.2
I 33C	1.3	0.3	2315	2.4	0.6
III15C	0.4	0.2	2311	6.2	0.1
-	-	-	41P1	3.4	0.3
-	-	-	433P1	3.9	0.3

TABLE 2b. *Acetoin and diacetyl production by motile streptobacteria,* L. brevis *and* L. paramesenteroides. *Conditions the same as in Table 2a.*

Motile streptobacteria			*Lactobacillus brevis*		
Strain	AD	D	Strain	AD	D
I 17	4.3	0.2	I 22	0.1	0.0
I 311	2.1	0.6	II 23	0.1	0.0
227	3.9	0.4	II 25	0.0	0.0
I 26	6.2	0.2	II 24	0.0	0.0
Leuconostoc paramesenteroides			II 27	0.0	0.0
I 28	0.0	0.0	III27	0.3	0.0
I 31	0.0	0.0	323	0.1	0.0
I 43	0.1	0.0	324	0.3	0.0
I 47	0.3	0.0	414	0.9	0.0
II 41	0.1	0.0	421	0.0	0.0
II 47	0.8	0.1	425	0.0	0.0
II 43	0.1	0.0	-	-	-
III29	0.2	0.0	-	-	-

pyruvate. These results suggest their probable contribution to the production of the diacetyl normally found in Cheddar cheese (*20*).

Some strains were studied further for their ability to use pyruvate as a sole source of carbon. The results given in Table 3 indicate that all of them grew better in PYG than in PY broth, even though the motile streptobacterium and *S. faecalis* subsp. *liquefaciens* were able to achieve considerable growth in the latter. Furthermore, with the exception of *L. casei* 354, addition of 0.5% sodium pyruvate to PY improved growth of microorganisms. Higher concentrations, up to 2%, generally had a stimulatory effect. De Cardenas et al. (*12*), however, found inhibition of growth of *L. acidophilus, L. casei, L. fermentum* and *L. brevis* by 2% pyruvate.

Production of AD from pyruvate, in the presence of acetate and acetaldehyde, by homo- and heterofermentative lactic acid bacteria is shown in Table 4. In general, acetate stimulated AD formation. It also enhanced diacetyl and acetoin formation by whole-cell suspensions of *L. casei* examined by Branen and Keenan (*5*), but not by us. With the exception of a *Pediococcus* sp. and *S. faecalis* subsp. *liquefaceins*, presence of acetaldehyde at concentrations as low as 100 μg ml^{-1} enhanced AD production but not growth, as reflected in the optical density of the culture. The findings of Collins and Speckman (*11*), that acetaldehyde (100 μg ml^{-1}) approximately doubled the production of acetoin plus deiacetyl by an active culture of *Leuconostoc citrovorum (cremoris)* probably by increasing the availability of hydroxythiamine pyrophosphate and acetyl-coenzyme A, have been confirmed. Concentrations of acetaldehyde higher than 100 μg ml^{-1} had different effects on AD production, from inhibition as seen with *Pediococcus* sp. and *S. faecalis* subsp. *liquefaciens* to the indifferent behavior seen with *Leuconostoc paramesenteroides*. Except for the latter, the highest concentration of acetaldehyde (2000 μg ml^{-1}) resulted in a marked inhibition of AD production by all strains compared with the control.

TABLE 3. *Use of pyruvate as a sole source of carbon by homo- and heterofermentative lactic acid bacteria after 6 h incubation at 30°C (growth expressed as O.D).*[a]

Species	Strain	PYG	PY	PY + 0.5%	PY + 1.0%	PY + 2.0%
L. casei	333C	0.37	0.05	0.08	0.09	0.14
	354	0.44	0.08	0.08	0.09	0.19
L. plantarum	2310	0.40	0.08	0.10	0.11	0.17
	238	0.37	0.09	0.11	0.12	0.13
motile Streptobacterium	I 17	0.29	0.14	0.15	0.18	0.18
Leuconostoc paramesenteroides	III29	0.21	0.09	0.14	0.16	0.17
	II47	0.19	0.05	0.12	0.12	0.19
Pediococcus sp.	452	0.38	0.08	0.09	0.11	0.17
S. faecalis ssp. *liquefaciens*	Dutch	0.13	0.10	0.17	0.21	0.29

[a]Peptone yeast extract gluclose broth (PYG) and peptone yeast extract (PY) with and without 0.5, 1.0 and 2.0% sodium pyruvate was inoculated with 2% of a 17 h culture.

TABLE 4. *Acetoin and diacetyl production (μmoles ml^{-1}) from pyruvate (50 μmoles ml^{-1}) in the presence of acetate and acetaldehyde in PYG broth, by homo- and heterofermentative lactic acid bacteria (after 24 h at 30°C).*

Species	Strain		Pyruvate (control)	Pyruvate + acetate (50 μmoles ml^{-1})	Pyruvate + acetaldehyde (μg ml^{-1})					
					100	300	500	700	1000	2000
L. casei	333C	AD[a]	5.4	6.4	6.90	6.70	6.50	6.40	6.40	4.10
		O.D	1.10	?	1.00	1.00	0.96	0.96	0.95	0.45
	354C	AD	5.40	6.70	5.60	5.60	5.20	5.10	5.10	3.40
		O.D	1.0	?	1.00	0.99	0.96	0.95	0.94	0.37
L. plantarum	2310	AD	5.90	7.00	7.20	7.00	6.40	6.40	5.70	4.90
		O.D	1.00	?	1.00	0.98	0.95	0.95	0.90	0.45
	238	AD	6.4	11.30	6.5	6.50	6.50	6.20	6.00	3.30
		O.D	1.0	?	1.0	0.95	0.90	0.85	0.68	0.27
Motile strepto-bacterium	I 17	AD	5.4	5.00	5.9	5.70	5.70	5.20	4.90	2.00
		O.D	1.1	?	1.1	1.05	1.00	0.80	0.66	0.19
Leuconostoc para-mesent eroides	II47	AD	0.79	0.85	0.86	0.86	1.05	1.19	2.24	1.78
		O.D	0.59	?	0.59	0.52	0.48	0.40	0.31	0.31
	III29	AD	0.20	?	0.39	0.39	0.33	0.66	1.98	1.65
		O.D	0.95	?	0.90	0.90	0.90	0.90	0.85	0.80
Pediococcus sp.	452	AD	6.70	8.50	2.80	2.60	2.60	2.30	1.80	1.50
		O.D	1.20	?	1.20	1.20	1.20	1.20	1.00	0.30
S. faecalis ssp. *liquefaci-ens*	Dutch	AD	2.80	3.00	2.20	1.80	1.50	0.50	0.40	0.30
		O.D	0.59	?	0.59	0.52	0.48	0.40	0.31	0.25

[a]AD = acetoin and diacetyl; O.D = optical density; ? = not determined.

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The storage trials illustrate the hardiness of human enteroviruses in environments outside their host. The reduction in poliovirus titer observed in prawns stored frozen or chilled was insufficient to substantially affect the public health risks associated with contamination of this nature. A proportion of added poliovirus remained infective at 4-6°C despite gross microbial spoilage of the prawns. The rate of inactivation of enteric bacteria, e.g. salmonellae, in frozen prawns is likely to be considerably faster than the rate of inactivation of poliovirus observed here (*4*).

The above conclusions are probably also applicable to human enteroviruses other than poliovirus 1. Although the various enteroviruses do differ in their behavior in foods, the differences are usually not large and the behavior of poliovirus 1 is usually representative of the behavior of a range of other enterovirus types.

Although several viral diseases are known to be transmitted by food, foods other than molluscan shellfish have rarely been surveyed for contamination with human enteric viruses. The evidence for involvement of cooked foods in transmission of viral disease is almost entirely epidemiological. The failure to detect viruses in cooked prawns during the present survey is in accord with the experience of Kostenbader and Cliver (*8*), who obtained negative results in the most thorough recorded attempt to isolate viruses from retail food samples. After examining 60 retail food samples and a variety of other related samples, they concluded that the incidence of viruses in foods marketed in the U.S.A. was probably so low that surveys of this kind are unlikely to yield positive results. Thus, although foods such as cooked prawns are quite capable of transmitting viral diseases, the available evidence suggests that such transmission is not a frequent occurrence.

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