APPLIED MICROBIOLOGY, July, 1966
Vol. 14, No. 4

Effects of Additives on the Survival of Lactic Streptococci in Frozen Storage[1]

C. A. GIBSON, G. B. LANDERKIN, AND PAMELA M. MORSE

Food Research Institute and Statistical Research Service, Research Branch, Canada Department of Agriculture, Ottawa, Ontario, Canada

Received for publication 28 March 1966

ABSTRACT

GIBSON, C. A. (Canada Department of Agriculture, Ottawa, Ontario, Canada), G. B. LANDERKIN, AND PAMELA M. MORSE. Effects of additives on the survival of lactic streptococci in frozen storage. Appl. Microbiol. **14**:665–669. 1966.—Three single-strain cultures, *Streptococcus lactis* C_2, *S. cremoris* R_1, and *S. diacetilactis* DRC_2, were frozen and stored in skim milk, in skim milk containing apple juice, and in skim milk containing one of the following additives: glycerol (10%, v/v), dimethyl sulfoxide (10%, v/v), L-malic acid (0.5 and 2.0%, w/v), acetamide (0.5 and 2.0%, w/v), or succinimide (0.5 and 2.0%, w/v). Cultures were frozen and stored at −23.3 C, frozen and stored at −196 C in liquid nitrogen, or frozen at −196 C and stored at −23.3 C. Cultures frozen and stored at −196 C in liquid nitrogen gave the greatest recovery of viable cells. The number of cells surviving after storage at −23.3 C was greater when the cells had been frozen in liquid N_2 than when they had been frozen at −23.3 C. All strains stored at −23.3 C showed a decrease in numbers of surviving cells; additives, particularly L-malic acid and apple juice, were advantageous in preserving the viability of the *S. lactis* C_2 and *S. cremoris* R_1 strains, but had little or no effect on the survival of *S. diacetilactis* DRC_2. L-Malic acid and apple juice stimulated acid production for all cultures in activity tests following incubation after thawing, whereas glycerol and dimethyl sulfoxide retarded its development.

Several investigators have studied the use of protective additives in the suspending medium for reducing the death rate of lactic streptococci during storage in the frozen state. Baumann and Reinbold (1) reported that the addition of glycerol, casein hydrolysates, yeast extract, or egg white to ripened milk cultures of lactic streptococci helped to preserve their activity during storage at −20 C. Kawashima and Maeno (6) observed that the addition of 1% L-glutamic acid to skim milk reduced the death rate of lactic streptococci frozen and stored at −15 to −20 C and that the rate of acid production was increased during incubation after thawing. Heinemann (4) found that the addition of glycerine improved subsequent activity of ripened lactic cultures after storage at 35, 5, and −20 F. Rudnick, Bucy, and Glenn (12) reported that ripened cottage cheese and buttermilk cultures, propagated in 16% skim milk with calcium carbonate added, had on the average the best activity after frozen storage at −20 ± 2 F for 190 days, whereas those in 10% skim milk were least active. Lamprech and Foster (8) observed that lactic acid organisms survived better in neutral than in acidic media, and that glycerol was helpful in overcoming the harmful effects of low *p*H levels but offered no benefit at *p*H 7.0.

Glycerol, dimethyl sulfoxide, acetamide, succinimide and malic acid have been reported to protect various types of cells, tissue cultures, and microorganisms against damage during freezing and freeze-drying (7, 10, 11, 13). This paper describes the effects of these substances on the viability of representative strains of lactic streptococci during frozen storage.

MATERIALS AND METHODS

Three single-strain cultures, *Streptococcus lactis* C_2, *S. cremoris* R_1, and *S. diacetilactis* DRC_2, were used in these studies. Routinely, all strains were subcultured twice weekly in sterile skim milk containing calcium carbonate; the inoculum was 1% and the incubation period was 16 hr at 21 C. Propagations were made on 3 successive days immediately prior to freezing.

Suspending media for the cells during freezing were

[1] Contribution No. 38 from the Food Research Institute.

reconstituted skim milks (10% total solids, w/v) with or without each of the following additives: glycerol (10%, v/v), dimethyl sulfoxide (10%, v/v), acetamide (0.5 and 2.0%, w/v), succinimide (0.5 and 2.0%, w/v), and L-malic acid (0.5 and 2.0%, w/v). Also, skim milk powder was added to commercial vitaminized (35 mg of vitamin C in 100 ml) apple juice [0.4% (w/v) malic acid] at the rate of 10% (w/v). Each medium was adjusted to a final *p*H of 6.8 with 1 N KOH or HCl; 99-ml volumes were dispensed into Pyrex dilution bottles for autoclaving at 116 C for 15 min.

Each bacterial strain was prepared for freezing by inoculating 99 ml of substrate with 1 ml of culture. The dilution was shaken 25 times, and 4-ml samples were dispensed into 11 sterile polyethylene culture tubes (12 by 75 mm) with caps. One tube was sampled immediately for bacterial count and then incubated for the activity test. The remaining tubes were treated as follows: four tubes were frozen and stored at −23 C; four tubes were placed in liquid N_2 (−196 C) for 5 min, then removed and stored at −23.3 C; and two tubes were frozen and stored in liquid N_2.

After 3, 6, 12, and 24 weeks of storage at −23.3 C, single tubes frozen at this temperature and in liquid N_2 were thawed by immersion for 3 min in water at 37.5 C, and then sampled for bacterial count. After 12 and 24 weeks, tubes frozen and stored in liquid N_2 were also thawed and sampled for bacterial count. At the end of 12 and 24 weeks of storage, after samples had been taken for bacterial count, all tubes from all treatments were incubated for 16 hr at 21 C and then activity tests were performed.

The experiment was repeated once, starting with

TABLE 1. *Mean log counts of lactic streptococci frozen in skim milk with and without additives*[a]

Organism	Additive	Before freezing	Frozen and stored at −23.3 C		Frozen in liquid N_2, stored at −23.3 C		Frozen and stored in liquid N_2	
			12 weeks	24 weeks	12 weeks	24 weeks	12 weeks	24 weeks
Streptococcus lactis C_2	Skim milk (10% total solids)	7.37	6.32	5.82	6.94	6.82	7.33	7.32
	10% glycerol	7.36	7.02	7.02	7.13	7.04	7.07	7.08
	10% dimethyl sulfoxide	7.36	6.93	6.69	7.08	6.92	7.27	7.28
	0.5% L-malic acid	7.36	6.96	6.78	7.12	7.06	7.35	7.33
	2.0% L-malic acid	7.36	7.14	7.03	7.24	7.21	7.38	7.33
	0.5% acetamide	7.36	7.06	6.90	7.16	7.11	7.35	7.35
	2.0% acetamide	7.36	6.93	6.81	7.12	7.07	7.35	7.32
	0.5% succinimide	7.36	6.76	6.58	7.12	7.03	7.31	7.30
	2.0% succinimide	7.36	6.42	6.24	7.04	6.95	7.20	7.15
	Apple juice	7.36	7.22	7.20	7.27	7.25	7.02	7.03
S. cremoris R_1	Skim milk (10% total solids)	7.31	6.65	6.24	6.86	6.74	7.17	7.19
	10% glycerol	7.30	6.90	6.82	7.01	6.93	6.90	6.85
	10% dimethyl sulfoxide	7.30	7.09	7.02	7.10	7.05	7.13	7.10
	0.5% L-malic acid	7.30	6.98	6.89	7.05	6.95	7.20	7.16
	2.0% L-malic acid	7.31	7.14	7.08	7.17	7.09	7.26	7.16
	0.5% acetamide	7.30	6.93	6.63	6.98	6.89	7.14	7.12
	2.0% acetamide	7.31	6.94	6.68	7.09	7.02	7.10	7.07
	0.5% succinimide	7.30	6.77	6.60	7.04	6.92	7.10	7.11
	2.0% succinimide	7.30	6.38	6.09	6.82	6.54	7.07	7.00
	Apple juice	7.30	7.08	6.99	7.17	7.09	7.02	6.94
S. diacetilactis DRC_2	Skim milk (10% total solids)	7.13	6.78	6.65	6.83	6.77	7.10	7.09
	10% glycerol	7.13	6.79	6.72	6.78	6.68	7.07	6.98
	10% dimethyl sulfoxide	7.13	6.82	6.66	6.80	6.71	6.87	6.82
	0.5% L-malic acid	7.13	6.89	6.73	6.85	6.79	7.10	7.08
	2.0% L-malic acid	7.13	6.86	6.73	6.89	6.80	7.02	6.93
	0.5% acetamide	7.13	6.83	6.69	6.84	6.72	6.89	6.85
	2.0% acetamide	7.13	6.87	6.74	6.82	6.71	6.97	6.92
	0.5% succinimide	7.13	6.81	6.66	6.85	6.80	6.94	6.94
	2.0% succinimide	7.13	6.50	6.20	6.78	6.70	6.86	6.80
	Apple juice	7.13	6.85	6.69	6.88	6.75	6.70	6.65

[a] Standard errors of means for comparisons within strains and temperatures: (i) for vertical comparisons, 0.045; (ii) for other comparisons, 0.036 (180 degrees of freedom). Standard error of means for comparisons between temperatures within strains: 0.051 (180 degrees of freedom).

TABLE 2. *Effect of two concentrations of additives on survival of lactic streptococci after 24 weeks of frozen storage*

Additive	Culture	Differences in mean log counts (2.0% concentration minus 0.5% concentration)		
		Frozen and stored at −23.3 C	Frozen in liquid N_2, stored at −23.3 C	Frozen and stored in liquid N_2
L-Malic acid	*Streptococcus lactis* C_2	0.25***	0.15*	0.00
	S. cremoris R_1	0.19**	0.14*	0.00
	S. diacetilactis DRC_2	0.00	0.01	−0.15*
Acetamide	*S. lactis* C_2	−0.09	−0.04	−0.03
	S. cremoris R_1	0.05	0.13	−0.05
	S. diacetilactis DRC_2	0.05	−0.01	0.07
Succinimide	*S. lactis* C_2	−0.34***	−0.08	−0.15*
	S. cremoris R_1	−0.51***	−0.38***	−0.11
	S. diacetilactis DRC_2	−0.46***	−0.10	−0.14*

* $P \leqq 0.05$.
** $P \leqq 0.01$.
*** $P \leqq 0.001$.

different subcultures of each strain and different preparations of each suspending medium.

Numbers of viable organisms were estimated, by use of the lactic agar of Elliker, Anderson, and Hannesson (2). The medium contained (w/v): 2% tryptone; 0.5% yeast extract; 0.25% gelatin; 0.5% of each of dextrose, lactose, and sucrose; 0.4% sodium chloride; 0.15% sodium acetate; 0.05% ascorbic acid; and 1.5% (Noble) agar; the *p*H was 6.8. Duplicate plates were poured at three dilutions, and the plates were incubated at 21 C for 96 hr.

Activity tests were performed in duplicate on incubated samples by use of the method of Horrall and Elliker (5). Starter was added at the rate of 0.3 ml per 10 ml of sterile skim milk, and titratable acidities were determined after 3.5 hr at 37.8 C.

Results for different concentrations of additives were compared by use of the *t* test. Overall comparisons among the additives were made with Duncan's multiple range test. There are, in general, difficulties in applying and interpreting the results of the latter test when, as here, the treatments differ both qualitatively and quantitatively, but the test provides a convenient way of summarizing the results and ranking the treatments. The significance levels should not be interpreted too rigidly, but the results of the tests were clear and consistent enough that they should not be misleading.

RESULTS

The bacterial counts were converted to logarithms, and subjected to analysis of variance. There were significant interactions between all treatment factors; the results, therefore, are summarized and assessed from tables of means, averaging only over duplicate plates and the two replicates of the experiment. Different components of error variance enter into different types of comparison, and are incorporated in the standard errors given in the tables.

The mean log bacterial counts after 12 and 24 weeks of storage are shown in Table 1 for the different strains, additives, freezing treatments, and storage temperatures. The results at 3 and 6 weeks followed the same pattern as those shown, although the differences between strains and treatments were not as marked.

For all treatments, the numbers of viable cells decreased with increased time in frozen storage, in a manner which varied with the additive and method of freezing and storage. Cultures frozen and held in liquid N_2 and then rapidly thawed, with or without additives, gave the greatest recovery of viable cells. When cells were initially frozen in liquid N_2 and subsequently held at −23.3 C, the number of survivors was increased over that of cells frozen and stored at −23.3 C.

The results of the effect of additive concentration on cell survival after 24 weeks of storage are given in Table 2. Three additives, L-malic acid, acetamide, and succinimide, were each tested at two concentrations (0.5 and 2.0%, w/v). In general, the higher concentration gave better results for L-malic acid, poorer results for succinimide, and no consistent difference for the acetamide.

Table 3 presents the multiple-range tests on log counts after storage for 24 weeks. The additives and amounts used are listed in apparent order of effectiveness; additives which are not linked by the same line gave significant differences at the 5% level. The percentages of bacteria surviving frozen storage were derived from the means of the transformed data, and are also given in the table.

With cultures frozen and stored in liquid N_2,

TABLE 3. *Percentage survival of lactic streptococci after 24 weeks of frozen storage in skim milk with and without additives*[a]

Organism	Frozen and stored at −23.3 C		Frozen in liquid N_2, stored at −23.3 C		Frozen and stored in liquid N_2	
	Additive[b]	Per cent	Additive	Per cent	Additive	Per cent
Streptococcus lactis C_2	AJ	69.0	AJ	78.3	ACE-0.5	98.0
	LMA-2.0	46.7	LMA-2.0	70.3	LMA-2.0	93.3
	GLY-10	45.9	ACE-0.5	56.2	LMA-0.5	92.3
	ACE-0.5	34.8	ACE-2.0	51.8	ACE-2.0	91.6
	ACE-2.0	28.1	LMA-0.5	50.4	SKM	91.0
	LMA-0.5	26.0	GLY-10	47.9	SUE-0.5	87.5
	DMSO-10	21.8	SUE-0.5	47.5	DMSO-10	83.0
	SUE-0.5	16.6	SUE-2.0	39.1	SUE-2.0	62.2
	SUE-2.0	7.6	DMSO-10	36.9	GLY-10	53.3
	SKM	2.8	SKM	28.7	AJ	47.3
S. cremoris R_1	LMA-2.0	58.9	AJ	61.0	SKM	76.3
	DMSO-10	52.6	LMA-2.0	60.8	LMA-0.5	73.3
	AJ	48.5	DMSO-10	55.2	LMA-2.0	71.1
	LMA-0.5	38.9	ACE-2.0	51.4	ACE-0.5	65.5
	GLY-10	33.0	LMA-0.5	44.8	SUE-0.5	64.2
	ACE-2.0	23.4	GLY-10	42.6	DMSO-10	62.4
	ACE-0.5	21.6	SUE-0.5	40.8	ACE-2.0	58.2
	SUE-0.5	19.7	ACE-0.5	38.9	SUE-2.0	50.0
	SKM	8.6	SKM	27.2	AJ	43.9
	SUE-2.0	6.2	SUE-2.0	17.5	GLY-10	35.4
S. diacetilactis DRC_2	ACE-2.0	41.2	SUE-0.5	46.5	SKM	90.0
	LMA-2.0	39.8	LMA-2.0	46.1	LMA-0.5	89.3
	LMA-0.5	39.5	LMA-0.5	45.6	GLY-10	69.3
	GLY-10	38.4	SKM	43.2	SUE-0.5	63.4
	AJ	36.4	AJ	41.8	LMA-2.0	62.8
	ACE-0.5	35.8	ACE-0.5	39.1	ACE-2.0	62.5
	DMSO-10	34.2	ACE-2.0	38.6	ACE-0.5	52.0
	SUE-0.5	34.0	DMSO-10	38.0	DMSO-10	49.3
	SKM	33.3	SUE-2.0	37.0	SUE-2.0	46.3
	SUE-2.0	11.6	GLY-10	35.3	AJ	32.7

[a] Differences between log values corresponding to those linked by the same vertical line did not reach the 5% level of significance by Duncan's multiple-range test.

[b] SKM = skim milk (10% total solids); GLY-10 = 10% glycerol; DMSO-10 = 10% dimethyl sulfoxide; LMA-0.5 = 0.5% L-malic acid; LMA-2.0 = 2.0% L-malic acid; ACE-0.5 = 0.5% acetamide; ACE-2.0 = 2.0% acetamide; SUE-0.5 = 0.5% succinimide; SUE-2.0 = 2.0% succinimide; AJ = apple juice.

only *S. cremoris* R_1 showed any significant loss in viability for the control, and none of the additives gave any improvement. Some additives, in fact, gave poorer results: glycerol, 2.0% succinimide, and apple juice for *S. lactis* C_2 and *S. cremoris* R_1, and all additives except 0.5% L-malic acid and possibly glycerol for *S. diacetilactis* DRC_2.

The percentage loss in viability of cultures stored in skim milk alone at −23.3 C ranged from 97% for *S. lactis* C_2 frozen at −23.3 C to 57% for *S. diacetilactis* DRC_2 frozen in liquid N_2. When *S. lactis* C_2 and *S. cremoris* R_1 were frozen and stored at −23.3 C or frozen in liquid N_2 with storage at −23.3 C, all additives except 2.0% succinimide improved the percentage survival. The best storage results were obtained most consistently with apple juice and 2.0% L-malic acid. Dimethyl sulfoxide improved the survival rate of *S. cremoris* R_1 but not *S. lactis* C_2. Intermediate results were obtained with glycerol, 0.5% L-malic acid, and acetamide. When *S. diacetilactis* DRC_2 was stored at −23.3 C, no additive improved the percentage survival; in fact, 2.0% succinimide resulted in greater loss in viability (88%) than the control.

In earlier studies (3), where the activity tests agreed with bacterial survival, there was a general decrease in apparent rate of acid production as the storage time increased. Although some additives markedly affected the activity test, the results for each additive, relative to skim milk

alone, were similar for all three strains and for all three freezing treatments and, on the whole, changed little with storage time. Glycerol and dimethyl sulfoxide apparently inhibited acid production; acidity values in the activity test with cultures frozen, stored, and incubated in media containing these additives were only 20 to 30% of those of the control. However, media containing either apple juice or both concentrations of the L-malic acid yielded acidity values in the activity test that were 15 to 30% higher than those of the control. Succinimide apparently stimulated acid production initially, but by the end of storage appeared to result in inhibition.

DISCUSSION

This study shows that the most significant factor influencing the survival of lactic streptococci was the feeezing and storage treatment, and thus agrees with the results of Baumann and Reinbold (1). Greatest recovery of viable cells was obtained when cultures were thawed rapidly after being frozen and stored in liquid N_2 in skim milk, with or without additives. The number of survivors was greater when cells were frozen in liquid N_2 and stored at −23.3 C than when cells were frozen and stored at −23.3 C.

The differences exhibited in cell survival clearly indicate that certain additives and freezing and storage treatments are more suitable than others for the frozen storage of lactic streptococci. The additives glycerol, dimethyl sulfoxide, L-malic acid, acetamide, succinimide, and apple juice were, in general, advantageous in preserving the viability of *S. lactis* C_2 and *S. cremoris* R_1 strains frozen and stored at −23.3 C, but had little or no effect on the survival of *S. diacetilactis* DRC_2. The additives glycerol, casein hydrolysate, yeast extract, and egg white were found by Baumann and Reinbold (1) to be beneficial in preserving the activity of *S. lactis* and *S. cremoris* strains stored at −20 C.

At the cell concentrations used, storage at −23.3 C resulted in significant decreases in the numbers of viable cells after 24 weeks of storage in skim milk with or without the addition of glycerol or other additives. This conflicts with the findings of Lamprech and Foster (8), who reported that concentrated cell suspensions of *S. lactis* or *S. diacetilactis* did not lose appreciable viability when stored at −20 C in skim milk with or without glycerol at *p*H 7.0, after 9 months. This disparity may be attributed in part to the lower concentration of cells used in this study. Major, McDougal, and Harrison (9) demonstrated that *Escherichia coli* is more resistant to death at temperatures of −20 and −22 C as the cell concentration is increased.

When *S. lactis* C_2 and *S. cremoris* R_1 cultures were frozen and stored at −23.3 C or frozen in liquid N_2 and held at −23.3 C, L-malic acid and apple juice stimulated acid production during incubation after thawing as well as exerting a protective effect against loss of viability during storage. Using L-glutamic acid, Kawashima and Maeno (6) reported similar results with *S. lactis* and *S. cremoris* strains. Although glycerol was found to have a protective effect on *S. lactis* C_2 and *S. cremoris* R_1 cultures stored at −23.3 C, it was observed that this compound as well as dimethyl sulfoxide retarded acid development during incubation after thawing.

ACKNOWLEDGMENTS

We acknowledge the skilled assistance received from M. D. Johnson, E. Montoya, and D. M. Stewart.

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