FREEZING AND FREEZE-DRYING OF LACTOBACILLI FOR SUCCESSFUL ITALIAN STYLE DRY SALAMI MANUFACTURING.

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SUMMARY

The viability and activity of frozen (F) freeze-dried (FD) lactobacilli preserved with cryoprotectants and used in making commercial Italian dry salami. Better survival of older than younger cells, at neutral than lower pH, at 0.5 M than 0.02-0.1 M K-phosphate buffer as suspending medium and with increasing cell concentrations was observed. Viability of FD cells improved with decreasing storage temperature (30 to -13°C). FD and F cultures stored at -13°C for 12 months did not differ in % of surviving cells. Of 10 cryoprotectants added to cell suspending buffer at 1 and 5% before FD, monosodium glutamate (MSG), sucrose, and i-inositol were the most protective (10-100% survival of initial population) at 30 and 5°C. When FD and F cells were stored for 12 months at -13°C all cryoprotectants (including buffer 0.1M, ph7), but malt extract and peptone contributed to the survival of 10-100% of the original population. Table salt, sugar and nonfat dried milk were satisfactory carrier powders for FD cells. *L. curvatus* at 10^6 cells/g salami kept frozen or FD in buffer with 2% MSG gave excellent fermentation under commercial conditions.

INTRODUCTION

Several *Lactobacillus* sp are the main organisms in the natural fermentation of Italian style dry salami made in the USA (Cahalan and Genigeorgis 1986). Commercial starters were unable at times to ferment and dominate the natural microbial flora of meat (Genigeorgis et al. 1986). This was due probably to product formulation or process temperature or both or loss of starter viability during storage. Most starters are marketed in the frozen state. This paper reports the development of suitable freezing and freeze-drying preservation methods for lactobacilli and their use in dry salami manufacturing.

MATERIALS AND METHODS

Preparation of frozen and freeze-dried (FD) cultures for survival studies.

L. curvatus strains A,C,D, *L. casei* strains E,F and *L. fermentum* strain B were isolated from naturally fermented Italian dry salami, biochemically identified (Cahalan and Genigeorgis, 1986) and used in this study. Stock cultures lyophilized on porcelain beads were kept at -20°C. A bead was transferred to 10 ml APT broth (Difco) incubated at 30°C for 24 h, subcultured to 80 ml APT broth and further incubated at 30°C for 36 to 60 h. Stationary phase cells were centrifuged and resuspended to original broth volume in cold, sterile 0.1 M potassium phosphate buffer, pH 7.2. The suspension was recentrifuged and resuspended in fresh 0.2 M buffer. Double-strength solutions of cryoprotectants were made with distilled water and then, except for filter sterilized sodium ascorbate, were sterilized at 121°C for 15 minutes. Cold solutions were mixed 1:1 with the cell suspensions to $1X10^9$ cells/ml. One ml of each suspension was placed in screw-capped vials, frozen quickly in a dry ice acetone bath and stored at -13°C. For freeze-drying, the frozen cultures were placed in a Virtis freeze-dryer for 8 h. Some freeze-dried (FD) cultures were mixed with carrier powders (lg/vial) after freeze-drying but before storage. All vials were sealed with plastic tape and kept in Mason jars along with ~100 g $CaSO_4$. Frozen and FD cultures to be tested without storage (0 month) were kept in a freezer or refrigerator, respectively, for < 18 h before testing for viable bacteria. For viable counts in survival studies FD cells were rehydrated with 10 ml sterile, distilled water at 25°C. After one min. vortexing and 10 min. rest, they were serially diluted with 0.1% peptone, plated onto APT agar in duplicate and incubated at 30°C for 3 days. Fresh and frozen cultures were plated similarly.

Preparation and use of fresh, frozen and freeze-dried starters for salami inoculation.

A porcelain bead was transferred to 10 ml APT broth and incubated at 30°C for 1 to 2 days (until OD_{600nm} was > 1). The 10 ml were added into 30 ml fresh broth and incubated for 14 h at 30°C. The cells were harvested,

Table 1. Survival of L. curvatus strain C cells[a] after freezing or freeze-drying in buffer with cryoprotectants and storage for 12 months at 30 to -15°C.

Cryoprotectant	Log_{10} counts at 0 day		Log_{10} (%) survivors after 12 months storage at 30°C		5°C		-13°C	
			Freeze-dried cultures					
	1	5[b]	1	5	1	5	1	5
Gelatin	1.38	1.65	-4.74	-3.34	-1.64	-0.14	1.11	1.60
Peptone	0.59	0.23	-1.52	-1.06	-0.08	-0.54	0.52	0.20
Yeast extract	1.97	2.05	-0.96	-1.06	1.68	1.91	2.02	2.07
MSG	1.92	1.95	1.18	1.74	1.94	2.02	1.97	1.98
Na Ascorbate	1.67	1.74	-2.68	-0.72	1.36	1.32	1.82	1.46
Skim Milk	1.97	1.96	-1.82	-3.33	1.49	0.20	1.81	1.48
Malt extract	1.74	1.49	-1.82	-2.85	1.53	0.92	1.69	1.56
Sucrose	1.85	1.79	1.26	0.82	1.75	1.53	1.75	1.68
glycerol	1.85	1.93	-6.15	-6.15	0.59	-3.39	1.85	1.49
i-inositol	1.97	1.89	1.28	1.04	1.85	1.62	1.94	1.84
Buffer only	1.64		-1.85		1.23		1.59	
			Frozen cultures					
Gelatin	1.81	1.94					1.84	1.85
Peptone	0.41	-0.05					0.42	-1.09
Yeast extract	1.97	2.01					1.87	1.94
MSG	1.92	2.02					2.02	1.94
Na ascorbate	1.87	1.72					1.59	1.46
Skim milk	2.02	2.02					1.87	1.93
Malt extract	1.83	1.84					-0.05	0.36
Sucrose	1.88	1.88					1.70	1.76
glycerol	1.83	1.96					1.84	1.04
i-inositol	1.87	1.86					2.03	1.81
Buffer only	1.97						1.88	

a = 40 hr. APT culture, cells suspended to original concentration in K-phosphate buffer, 0.1M, pH 7.2 with or without 1 and 5% cryoprotectant before freeze-drying or freezing.

b = Cryoprotectant concentration in %.

desirable levels/g of salami were resuspended in 450 ml of cold (C) brain heart infusion (Difco) broth for a 300 lb salami batch and used within 4.5 h. Starter purity and counts were checked on APT agar. Frozen cultures were stored at -13°C until use. Freeze-dried starters with cryoprotectants were stored with or without carrier powders at 5 or -13°C. The carriers included non-iodized table salt, table sugar, nonfat dried milk and gum arabic with or without ascorbic acid (5%) or BHT (0.1%). One gram of powder was added to each 1 ml of FD culture vial. Sausage formulations and commercial processing were the same as reported (Metaxopoulos et al., 1981). Frozen cultures were thawed rapidly and FD cultures were rehydrated 30 min. before use. All starters were diluted in 450 ml of cold potable water and added slowly to each 300 lb sausage batch at the chopper level for maximum uniformity in cell distribution. Individual refrigerated sausage samples were brought to the University and analyzed the next morning.

RESULTS AND DISCUSSION

Effect of age, harvesting method, suspending buffer and process.

Plating a day after freezing or freeze-drying, *L. curvatus* and *L. casei* cells demonstrated better survival for frozen than FD cells ($P < 0.001$) and for late stationary than younger cells ($P < 0.001$). Broth culture cells resuspended in buffer (0.1 M pH 6.5) survived better than those harvested from agar plates or left in the growth medium ($P < 0.01$). The buffer pH (5-8) affected ($P < 0.001$) survival after freezing or FD with pH 7 supporting better viability. At pH 6.5, 0.5 M buffer was more protective than 0.02-0.1M. Slow freezing to -13°C as compared to rapid freezing at -60°C of *L. curvatus* type C cells in the presence of sucrose, YE, or only buffer did not affect significantly the viability of cells during storage at -13°C for 6 months. Extending lyophilization from 6 to 24 h. decreased the level of surviving.

Effect of cryoprotectant.

Table 1 presents the effect of type and amount of cryoprotectant on the viability of frozen or FD *L. curvatus* cells stored for 12 months at 30, 5 and -13°C. Cell viability was affected by type and amount of cryoprotectant, temperature and time of storage ($P < 0.001$). Freeze-dried cultures survived better at -13 than 5 or 30°C. The viability of frozen and FD cultures at -13°C did not differ significantly. MSG, sucrose, i-inositol and YE were the most protective compounds for FD or frozen cells. Protection was most noticeable at 30°C. Thirty-nine to 100% of initial frozen cells survived 12 months at -13°C in the presence of all cryoprotectants but peptone and malt extract. In 20/30 experimental settings of FD cultures (Table 1), increasing the cryoprotectants from 1 to 5% decreased the level of surviving cells. This was true for only 50% of the frozen cultures. Plain buffer was more protective to frozen cells after 12 months at -13°C than 15/20 other cryoprotectants. The effect of 0.1 to 5% YE, MSG, skim milk, sucrose and i-inositol in buffer on the survival of $1X10^9$ and $1X10^{10}$/cc *L. curvatus* cell concentrations, freeze-dried and stored at 30°C for 2 months were evaluated. Best protection was given when at least 0.5% of a cryoprotectant was added to the buffer, by MSG than sucrose and when concentrated cells were used. We also evaluated the effect of 1% YE, MSG, sucrose skim milk and i-inositol in buffer (0.1M pH 7.2) in all possible combinations (up to three) on the survival of frozen and FD *L. curvatus*. After 6 months storage of

Table 2. Percent survival of six strains of lactobacilli after freezing or freeze-drying in the presence of absence of 2% mono-sodium glutamate (MSG) and stored frozen at -13°C or freeze-dried (FD) at 5°C for 4 months.

Process	Additive	% survival after 0 or 4 months storage											
		0 months						4 months					
		Lactobacillus strains[a]											
		A[b]	B	C	D	E	F	A	B	C	D	E	F
Frozen	None	96	81	101	89	80	49	70	82	94	86	21	27
"	MSG	93	75	103	100	113	47	123	87	112	113	47	35
FD	None	44	29	36	72	50	3	9	8	5	4	10	0.4
"	MSG	77	81	84	96	81	26	115	70	84	94	45	12

a = 40 to 46 cultures ($1x10^9$/cc) centrifuged and resuspended in K-phosphate buffer 0.1M pH 7.0 with or without 2% MSG.

b = L. curvatus strains A, C, D, L. casei strains E, F and L. fermentum strain B.

Table 3. Comparison of the fermentation activity (growth and pH change) of starter cultures preserved by freeze-drying, freezing or used as liquid fresh cultures in commercially processed salami.

Starter type	Fermentation Day								
	0	1		2		3		7	
	pH	LAB[a]	pH	LAB	pH	LAB	pH	LAB	pH
1. Fresh C cells (5.91)	6.13	6.1	6.08	8.5	4.86	7.7	4.75	7.6	4.50
2. Frozen C cells (6.05)	6.14	6.0	6.11	9.0	5.52	9.1	4.97	7.9	4.65
3. FD C cells (6.03)	6.13	5.8	6.13	9.0	5.23	8.9	4.81	8.0	4.70
4. Fresh C cells (6.03)	6.22	6.3	6.16	9	5.05	8.4	4.74	7.6	4.63
5. Frozen C cells (5.92)	6.21	6.1	6.16	8.6	5.61	9.0	5.21	8.5	4.84
6. FD C cells (6.09)	6.21	6.2	6.16	8.4	5.61	9.1	5.00	8.0	4.73
7. Frozen C+F cells (6.04 C + 5.21 F)	6.23	6.3	6.17	8.8	5.60	8.9	5.06	7.9	4.72
8. FD C+F cells (6.03 C + 5.24 F)	6.23	6.1	6.17	8.9	5.35	8.3	4.92	7.9	4.64
9. Fresh C+F cells (5.92 C + 4.48 F)	6.23	6.29	6.16	9.1	5.06	8	4.74	7.7	4.66
10. No starter added	5.95		5.85		5.80		5.70		5.8

a = Lactic acid bacteria $\log_{10}$ colony counts/g salami; FD = freeze-dried cells in buffer (0.1M pH 7.2) with 2% MSG stored for 1 month for starter No. 3, 3.5 months for starters No. 6 and 7 (type C) and 2 months for starter No. 7 (type F) at 5°C; b = frozen cells in buffer kept for 1 month for starter No. 2, 3.5 for starters No. 5 and No. 8 (type C) and 2 months for starter No. 8 (type F) at -13°C. Type C cells = L. curvatus, F cells = L. casei.

frozen cultures at -13°C and FD cultures at 30°C, none of the combinations of cryoprotectants gave any better protection than when tested alone. Table 2 presents survival rates of lactobacilli suspended in buffer (0.1M pH 7.2) with or without 2% MSG and stored frozen (at -13°C), or FD (at 30°C) for up to 4 months. Analysis of data indicated the significant ($P < 0.001$) positive effect of MSG and use of freezing as a preservation method.

Effect of carrier powders.

Carrier powders were used to increase the bulk of FD cultures. *L. curvatus* type C cells (1 cc with $1X10^9$) were suspended in buffer (0.1M pH 7.2) with or without 1% sucrose. After freeze-drying the surviving cells (73% in the presence of sucrose and 15% without), were mixed with 1g of salt, sugar, dry milk, gum arabic (G), G + 5% ascorbic acid, G + 0.1% BHT, or without anything and stored at 30°C for up to 6 months. In the presence of sucrose the % of surviving cells after 6 months as affected by the above powders was 12, 16, 23; 8.5, 6.1, 1.3, and 23, respectively. In the absence of sucrose the corresponding percentages of duplicate experiments were 0.071, 0.38, 0.24, 0.012, 0.003, 0.002 and 0.16, respectively.

Effect of cryoprotectant and preservation method on growth of lactobacilli in broth and salami.

The growth rates of fresh, 2-month-old frozen (in buffer), and 2-month-old FD (in 1% MSG or sucrose kept at 5°C) *L. curvatus* type C cells were evaluated. After 12 h at 30°C, initial APT inocula of $\log_{10}$ 4.79, 4.92, 4.84 and 5.15/cc reached levels of 7.16, 7.20, 6.75 and 8.36 for the FD-MSG, FD-sucrose, frozen and fresh cultures, respectively. Two-month-old frozen cultures in buffer, or 1% YE, MSG, skim milk, sucrose or i-inositol did not differ in cell yield after inoculation into APT broth and incubation at 30°C for 7 h. The cell yield in APT broth of 2-month-old (stored at 30°C) cultures was affected significantly by the nature of cryoprotectant with MSG giving the best yield after 7 h at 30°C. The growth rates and fermentation activity of frozen of FD starters were compared to fresh in commercially made dry salami. As Table 3 shows fresh starters outperformed FD and frozen ones in both rapidity of growth and pH decrease. The added starters dominated the bacterial flora of the salami throughout the fermentation and aging period. Since the starters were added to the salami formulation at approximately 10^6/g, their fermentative performance monitored by pH decrease was considered excellent. No fermentation was observed in salami made without starter. In the presence of starters, catalase + bacteria, mostly micrococci found in the periphery of salami, reached maximum levels of $\log_{10}$ 7/g by the 2nd day of fermentation and then decline to g10 6/g. All FD starters in buffered solution were stored in a refrigerator. For simplicity, frozen cultures had been made in plain buffer and stored at -13°C. Cultures frozen with MSG or YE and stored at -70°C would probably perform even better than the starters of Table 3.

Overall, this study demonstrated that customized frozen and FD lactobacillus starters with very good fermentation activity for salami making are feasible. Such starters can remain active for a long time and give the benefit of being adaptable to plant formulation and processing conditions. Yet, production of such cultures should be left to laboratories with appropriate facilities and expertise.

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