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Study of the microbial flora during the natural commercial fermentation of Italian-dry salami and identification of species suitable as starters.

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Italian-style dry salami is a low moisture, fermented sausage. In the U.S.A., it is mostly produced in the San Francisco area. It is neither cooked nor smoked. It is typically made from pork, beef, nonfat dried milk, dextrose, salt, spices, $NaNO_2$, and $NaNO_3$. Lactic acid bacteria (LAB) are the bacteria primarily responsible for fermenting salami and other fermented sausages (4,7,21). LAB help develop desirable appearance, texture, and flavor in fermented sausages. LAB are also important in insuring the safety of fermented sausages by forming acid and other compounds and suppressing the growth of bacteria harmful to human health (8,14). The most common microbial hazard of fermented sausages is Staphylococcus aureus, which is often present in sausage ingredients. A knowledge of S. aureus prevalence, growth, and enterotoxin formation in dry salami shows the need for a quick onset of fermentation (14,22,26).

The traditional source of LAB for fermented sausages has been a small amount of previously fermented sausage (natural starter) added to the new batch. Regarding the quality and safety of the sausage, inoculation with a pure starter bacteria of selected types, numbers and physiological conditions is superior to inoculation with the natural starter. The staphylococcal food poisoning outbreaks of the 70's due to the consumption of fermented sausages in the U.S.A. amplified the need for a change in fermented meat production and a blending of the art with science. One of the early targets of this change was the expansion in the use of pure starters to accelerate the fermentation process. Guidelines for "good manufacturing practices" (GMP) were developed by the American Meat Institute (1) for the industry. One of the early experiences of Italian dry salami manufacturers was the fact that commercial starters developed for rapid fermentation at high temperatures were not satisfactory for the production of this salami either because of both temperature and/or formulation effects on the rate of growth of the starter. It was for this reason that we decided to develop customized starters by isolating appropriate LAB during commercial processing based on natural starter. This practice has been utilized recently with success even by producers of commercially available starters.

In this paper we report on the progression of kinds and numbers of microorganisms growing in Italian dry salami during its natural commercial production by two companies, the isolation and identification of important LAB and their eventual use as pure starters to assure rapid fermentation, good product characteristics, and control of S. aureus.

MATERIALS AND METHODS:

1. General: For the two largest manufacturers of Italian-style dry salami in the San Francisco area, kinds and numbers of bacteria in fermenting salami were determined for 22 months. Each month, usually 6 batches, (2 companies, 3 salami sizes)(9,10) were microbiologically analyzed at ages 0,1,2,3,7,14,21 and 28 days. Salami samples were sent from the companies to the laboratory, refrigerated the same day, and analyzed for viable bacteria the next morning. Samples (25 g) were blended with 225 ml sterile distilled water. This mixture was further serially diluted with sterile 0.1% peptone, and appropriate dilutions were plated onto APT and Rogosa agar (Difco). The APT and Rogosa plates were then incubated three days at approximately 25°C, aerobically and in a candle jar, respectively.

2. Bacterial identification: Different types of bacterial colonies on the two media were distinguished and counted under a stereoscopic dissecting microscope and characterized into broad categories by checking representative colonies for cellular appearance (phase contrast microscopy), Gram reaction, and catalase test. Aerobic mesophiles capable of growth on APT agar were categorized as cocci, Gram + rods, or Gram - rods. Types of LAB were categorized and enumerated by the use of the two media as types A, B, C+D, E, F, and others. Counts for types B and E were usually taken from APT agar. Type F, only moderately aerotolerant, was usually counted on Rogosa agar; and type A, indistinguishable from types C and D on APT, was counted on Rogosa agar. Types C and D, each associated with a particular company but indistinguishable from each other without further testing, were lumped together as C+D and usually enumerated by a count on APT agar minus the type A count of Rogosa agar. All 6 of the major types, even type F if the APT agar was candled, had the ability to be recovered slightly better on APT agar than on the more acidic Rogosa agar. Individual cultures were lyophilized in porcelain beads and stored at -20°C in well-closed small tubes containing dry silica gel. Further identification of LAB included: a) Glucose, ribose, lactose, sucrose, cellobiose, xylose, sorbitol, and mannitol fermentation (13). b) Testing for lactic acid and short chain fatty acids in the extracts of APT broth culture supernatants by gas chromatography (12). c) Testing for the presence of D and L lactic acid dehydrogenases using 6 point standard curves. d) Litmus milk reaction, deamination of arginine, growth in various concentrations of ethanol, growth at 45 and 47°C (27). e) Determination of growth and acid production rates in APT broth. f) Effect of NaCl on growth rate.

3. Procedure for use of starter: To produce experimental salamis, pure starters were prepared from lyophilized master cultures. Using aseptic techniques, the starter was grown by steps in sterile APT broth (Difco) at 30°C. A typical preparation of starter began with the inoculation, from a master culture, of 10 ml APT broth, which was incubated until it was turbid (1-2 days). The 10 ml was inoculated into 80 ml APT broth, and this 90 ml was incubated approximately 24 hrs for type F and approximately 14 hrs for the other types. The appropriate amounts of each type of culture (to yield approximately 100,000 to 500,000 starter bacteria/g salami ingredients) were collected by centrifugation and were resuspended in 450 ml of cold (below 10°C), fresh brain heart infusion (BHI) (Difco) for a 300 lb batch. The resuspensions were kept chilled until use in salami production (appoximately 4.5 hrs). Each starter was checked microscopically for purity and plated on APT agar for purity and viable count check. In a typical experiment, 8 oz. salamis were stuffed with the startercontaining salami mix and incubated as follows: At company A at 59-65°F and 88-90% RH (relative humidity) for 24 hours and then at 73-75°F and 75-80% RH for 2 days and aged at 59-60°F and 75-80% RH for at least 17

additional days. At company B, the salamis were placed in the "green room" at 73-77°F and 70-75% RH for 3 days and aged at 64-66°F and 70-72% RH for at least 17 additional days. Commercial starters were used as recommended by the suppliers. The natural starter used routinely by the companies at the time of this study was made of chopped 18-19 day old 7.5 mm diameter ripening salami, analyzed before for total LAB and S. aureus and kept under vacuum for a maximum of 20 days. This starter was added to the salami mix to give 3-5 x 10^5 LAB/g. Experimental salami samples were taken at 0,1,2,3,7,14,21, and 28 days after preparation and shipped by car (about a 2 hr drive) to the laboratory for microbiological, chemical, and panel analysis as described before (10,14).

RESULTS:

During the study of 22 batches of salami for each salami size and company, 27 LAB were isolated and studied. Of these, 6 designated as A-F were the most frequently found during the fermentation-ripening period. All 6 grew well in APT broth at 30°C and had a doubling time of 1 hr for types A, C, and D, 0.8 hr for E, 1.5 hr for B, and 2 hr for F at this temperature. Using an initial inoculum of $1x10^6$/ml, they all decrease the pH of the broth to <5.0 within 24 hrs. All 6 produced D and L-lactic acids in various proportions. Types A, C, and D were classified as strains of Lactobacillus curvatus, types E and F as strains of L. casei, and type B as L. fermentum, a heterofermetative species. Table 1 presents the microbiological changes occurring during the salami fermentation-ripening under commercial conditions.

Numbers of bacteria other than LAB tended to peak at about two days after the salami ingredients had been chopped, mixed, and stuffed into casings. These numbers tended to rise to nearly 10^7/g for company A and about 10^6/g for company B. For non-LAB, growth of Gram - rods stopped first, then growth of Gram + rods, and finally growth of cocci. Numbers of LAB/g salami tended to peak at nearly 10^9/g after about 3 days for company A and after about 2 days for company B. Type B grew well in company B's salami during the early part of fermentation. Types C and D grew well during the early fermentation. Type F, although a slow grower, became the major part of the flora in older salami.

Once we identified the various species of LAB responsible for the fermentation, we proceeded in testing the ability of simple or combined pure cultures to induce a rapid fermentation leading to acceptable final product. We evaluated the use of fresh, frozen, and freeze-dried cultures. The use of the last 2 and the development of preservation methods for long term storage will be reported elsewhere. The use of fresh cultures was evaluated first. The counts of type C LAB in the original APT (a), in fresh APT (b), in BHI broth (c), and K-phosphate buffer (0.1M, pH 7.00) (d) kept at 2-3°C for 0,1,4,7 and 14 days of storage were found to be in millions: a) 167, 219, 178, 174, 70; b) 166, 176, 246, 228, 219; c) 171, 172, 163, 205, 188; d) 161, 166, 141, 87, and 7 respectively. Thus the viability of a culture grown in APT broth remained stable for up to 7 days and up to at least 14 days when fresh APT or BHI broths were used to resuspend the cells.

The fermentative ability of liquid starters resuspended in fresh broth or kept in the original broth for 7 days at 2-3°C was tested next in salami inoculation experiments (processed according to company A procedures) and compared to the addition of no starter or to natural starter. Table 2 presents the pH changes of salamis inoculated with the various starters. All pure liquid starters decreased the pH of the salami to less than 5.30 within the first 2 days of fermentation in accordance with the GMP guidelines (1). No fermentation took place without any starter. Of the two batches of salamis made with natural starter, only one met the GMP guidelines. The data also showed that a liquid culture can be kept refrigerated for up to 7 days without any change of its ability to ferment.

Table 3 compares our starters with some commercially available ones with respect to their ability to dominate the microbial flora of the salami during the fermentation and aging periods. As the data show, the developed starters which have been adapted to the salami formulations used by both companies dominated completely the microbial flora during the fermentation and aging periods. One of the commercial starters was completely outnumbered by the natural salami flora and 2 were found satisfactory. In previous studies we found that additional commercial starters could not function well under the formulation and processing conditions used by the 2 companies and remained <1% of the total LAB counts by the 3rd day of fermentation. Salamis made with separate starters A, C, D, E and F and combinations of D+F, C+F, D+A, and C+A at levels of 1-2x10^5cells/g and divided into two groups and processed in both companies showed 28th day residual nitrite of 4-23 ppm. In salamis inoculated with single starters and processed in company A, naturally occurring S. aureus grew to a maximum of $4x10^3$/g, and to a maximum of $5.9x10^4$/g in uninoculated controls. In salamis processed in company B, S. aureus grew to a maximum of $7x10^2$/g. The rate of pH decrease in duplicate salamis was faster under the conditions of company B than A for all the single and combined starter experiments.

DISCUSSION:

Past studies have evaluated the microbiology of selected natural fermentations of sausages in the U.S.A.(7,21,28). LAB contributing to such fermentations included L. plantarum, pediococci, and micrococci. The microbiology of Italian dry salami fermentation has not been studied. Various studies have covered the natural fermentations of sausages in Europe. The most numerous kinds of bacteria observed in Hungarian salami were lactobacilli, micrococci, enterococci and aerobic spore-formers (30). Isterband, a Swedish type of fermented sausage contained bacteria mostly belonging to the genera Lactobacillus and Leuconostoc (20). Reuter (24) found that the most common group of lactobacilli in German processed and unprocessed meat belonged to the group to which Lactobacillus casei, L. plantarum, and related species belong. These kinds of lactobacilli were also the predominant kinds found in salami in Italy (2,5). In some fermented sausages, micrococci and LAB work together. Micrococci predominate during the early stages of the ripening of Fruskogorska sausage, and lactobacilli during the later stages (19). Micrococcal stimulation of lactic acid bacteria growth has been observed (11,16), but micrococcal inhibition of lactic acid bacteria has also been observed (19).

In this study the microbiology of the Italian dry salami fermentation was followed for 2 years in salami made commercially by 2 companies utilizing natural starter. Six types of

lactobacilli were frequently found in the salami. Type B was identified as Lactobacillus fermentum, a heterofermenter. The other 5 types were facultative homofermenters. Types A, C, and D were identified as L. curvatus. Types E and F were both strains of L. casei. Although salami size did not make much difference in the types of LAB present, the day of fermentation and the company producing the salami did make noticeable differences. At both companies, L. curvatus grew well in the salami, with type C growing to high numbers at company A and type D to high numbers at company B. However, at company B, type B usually reached even higher numbers than type D. Type E usually did not make much of a showing, except at company A during warm months. Type F was the most numerous type at the end of the fermentation at both companies. Catalase + rods present in the ingredients tended to not grow well during the fermentation, but catalase + cocci grew somewhat. Catalase + rods were usually more numerous in samples from company A than in samples from company B.

To speed up the process of fermentation and improve product uniformity, color, and flavor, the commercial use of pure LAB starters was introduced in the 50's. They included micrococci and combinations of micrococci and L. plantarum in Europe (17,19), and pediococci in the U.S.A. (18). Combination cultures which included Pediococcus cerevisiae, P. acetilactici, L. plantarum, and Micrococcus varians were introduced later in the U.S.A. (3). The early cultures were marketed as freeze-dried preparations while the frozen culture introduced in 1968 dominated the market since.

Early starter cultures had optimum temperatures of growth (35-40°C) higher than was traditionally employed in the fermentation phase of the process, especially with dry sausages (15.6-23.9°C). The use of higher temperatures created human health problems mostly due to the growth of S. aureus (8,9). As a result, the first starter cultures were utilized in some semi-dry products where higher temperatures and natural smoking were employed (18). The heavy smoke application during fermentation tended to minimize S. aureus growth. Further culture developments resulted in strains that were effective at lower temperatures (3,23). Currently, a variety of starters are utilized with temperatures from 10-46°C, depending upon culture, product, and establishment (3,6). Early efforts to use commercial starters for manufacturing Italian dry salami were not successful due to the inability of these starters to grow and dominate the flora under the processing conditions. It was for this reason that we developed the custom cultures based on isolates from salami fermentations, which replaced the natural starters as soon as sufficient data on their performance became available. The data of Tables 2 and 3 demonstrate the practical usefullness of the isolated lactobacilli. Types C and D were found to be the best with respect to rapidity of fermentation and salami smell and flavor. Type B was excluded as a heterofermenter. Type F, which grew slowly, was used only in combination with starters C and D. Production of Italian dry salami by most of the industry is now based on pure starters. A recent study by Food Safety and Inspection Service, U.S.D.A. of 40 fermented sausage plants have shown that all finished products (120 samples) had S. aureus levels of <100/g (25).

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Table I. Typical bacterial changes during natural salami fermentation in two companies

	Size inch.	DAY	Lactic Acid Bacteria Types							Catalase-Positive Bacteria			
			A	B	C-D	E	F	Other	Total	Cocci	Gram Pos. Rods	Gram Neg. Rods	Total
COMPANY A	2.09	0	4.15*	4.30*	5.20	4.30	5.08	4.85	5.84	5.00	5.57	5.18	5.70
		1	4.95	5.78	6.00	5.48	5.86	5.00	6.61	6.00	5.60	5.30	6.30
		2	7.15	7.00	8.26	7.26	7.37	7.00	8.60	6.48	< 6	< 6	6.48
		3	7.38	7.00	8.28	7.00	7.70	7.00	8.72	6.85	< 6	< 6	6.85
		7	6.69	7.00	7.79	7.00	7.81	< 6	8.50	6.30	< 6	< 6	6.30
		14	6.60	6.88	7.36	7.00	7.77	< 6	8.17	6.00	< 6	< 6	6.00
		21	7.12	6.93	7.43	6.83	7.96	< 6	8.29	< 6	< 6	< 6	< 6
	2.50	0	3.78	4.13	5.13	4.00	4.77	4.30	5.53	4.78	5.40	5.31	5.78
		1	4.85	5.00	5.58	5.00	5.85	5.23	6.31	6.00	5.78	5.30	6.38
		2	7.04	7.00	8.00	6.78	7.43	7.00	8.48	6.95	< 6	< 6	6.90
		3	7.21	7.00	8.28	7.00	7.70	< 7	8.65	6.45	< 6	< 6	6.45
		7	6.43	7.00	7.49	6.30	7.74	6.00	8.08	6.00	< 6	< 6	6.00
		14	6.15	6.70	7.20	6.00	7.79	< 6	8.04	5.60	< 5	< 5	5.60
		21	6.13	6.48	7.36	6.00	7.81	< 6	8.04	5.70	< 5	< 5	5.70
	3.07	0	3.70	4.00	4.78	4.00	4.78	4.00	5.58	5.11	5.04	5.04	5.59
		1	4.48	5.00	5.43	4.30	5.61	5.00	6.18	6.00	5.00	5.00	6.30
		2	7.49	7.30	8.53	< 7	7.30	< 7	8.76	6.70	< 6	< 6	6.78
		3	7.46	7.30	8.56	< 7	7.58	< 7	8.72	6.15	< 6	< 6	6.15
		7	6.58	6.90	7.37	6.00	7.86	< 6	8.08	6.00	< 6	< 6	6.00
		14	6.40	6.30	7.15	6.00	7.90	< 6	8.00	5.48	< 5	< 5	5.48
		21	6.00	6.08	6.90	< 6	7.96	< 6	8.04	5.15	< 5	< 5	5.15
		28	5.90	6.00	7.00	6.00	7.91	< 6	8.11	5.00	< 5	< 5	5.00
COMPANY B	2.07	0	3.45	4.50	4.88	< 4	4.98	4.00	5.48	4.00	4.88	4.18	5.13
		1	5.00	6.78	6.04	5.30	5.74	<5	6.88	5.00	5.00	5.00	5.30
		2	7.25	8.50	8.05	<7	7.56	<7	8.76	6.30	< 6	< 6	6.30
		3	6.65	8.08	7.67	<7	8.01	<7	8.51	6.00	< 6	< 6	6.00
		7	6.00	7.82	7.00	<6	8.17	<6	8.40	< 6	< 6	< 6	< 6
		14	6.30	7.89	7.04	6.23	8.11	<6	8.36	< 6	< 6	< 6	< 6
	2.32	0	3.30	4.59	4.36	< 4	4.66	4.00	5.07	4.00	5.12	5.22	5.50
		1	4.00	6.30	5.70	< 5	5.48	<5	6.85	5.00	5.30	4.70	5.48
		2	7.19	8.37	8.06	7.00	7.55	<7	8.65	6.00	< 6	< 6	6.00
		3	6.24	8.11	7.56	6.50	7.92	<6	8.49	< 6	< 6	< 6	< 6
		7	6.00	7.63	6.80	6.58	8.17	<6	8.36	< 6	< 6	< 6	< 6
		14	5.78	7.15	6.60	6.00	8.15	<6	8.23	< 6	< 6	< 6	< 6
	3.02	0	3.00	4.00	4.00	<3	4.57	3.48	4.92	4.00	5.11	5.04	5.34
		1	4.78	6.39	5.30	5.00	5.39	< 5	6.65	5.39	5.15	5.00	5.75
		2	6.95	8.28	7.90	7.00	7.30	< 7	8.71	< 6	< 6	< 6	< 6
		3	6.67	8.23	7.49	7.00	7.95	< 7	8.52	< 6	< 6	< 6	< 6
		7	6.04	6.96	6.88	6.00	7.90	< 6	8.06	< 5	< 5	< 5	< 5
		14	5.70	6.68	6.06	6.00	7.98	< 6	8.11	< 5	< 5	< 5	< 5
		21	5.00	6.34	6.00	5.70	8.04	< 6	8.04	< 5	< 5	< 5	< 5
		28	5.30	6.35	6.00	<6	8.09	< 6	8.09	< 5	< 5	< 5	< 5

* Log_{10} of median CFU/g salami

Table 2. Comparison of the fermentation activity of pure liquid starter preparations as determined by pH changes in the salami.

Starter Type	FERMENTATION DAY 0	1	2	3	7	14	21
No starter	5.95	5.85	5.80	5.70	5.80		
Fresh type C cells*							
4.8 x 10^5/g salami	5.92	5.39	4.64	4.82	4.85	4.69	4.90
7-day old culture C*							
4.8 x 10^5/g salami	5.91	5.36	4.65	4.80	4.72	4.80	4.80
Natural starter**							
8.1 x 10^5/g type C	5.90	NT	4.86	NT	4.71	4.80	4.81
7.2 X 10^5/g type C	6.13	6.12	5.43	4.96	4.60	4.63	
Fresh type C(8.2 x 10^5/g)	6.13	6.08	4.86	4.75	4.50		
Fresh type C(1 x 10^5/g)	6.00	5.75	4.60	4.45	4.30		
Fresh type C(1 x 10^6/g)	6.00	4.75	4.20	4.20	4.30		
Fresh type A(3.4 x 10^5/g)	6.10			4.85	4.65		
Fresh type D(1.1 x 10^5/g)	6.10			4.80	4.70		
Fresh type E(3.4 x 10^5/g)	6.15			5.00	4.80		
Fresh type C + F							
(8.3 x 10^5/g + 3x 10^5/g)	6.23	6.16	5.06	4.74	4.66		

* means of triplicate samples ** at least 50% of microbial flora made of type C cells

Table 3. Ability of new starter cultures to dominate the microbial flora during dry salami fermentation expressed as percentage of the total flora.

Fermentation day	Type of starter used: Fresh starter A 1x10^5/g*		Fresh starter C 2x10^5/g		Fresh starter D 7.5x10^5/g		Commercial freeze-dried L. plantarium 1.2x10^6/g		Commercial frozen L. plantarum 9.1x10^6/g P. cerevisiae 1.35x10^7/g			Commercial frozen P. cerev. 1.1x10^7/g	
	A	Others	C	Others	D	Others	L.plant	Others	L.plant	P.cere	Others	P.cere	Others
0	97	3	100	0	100	0	100	0	40	60	0	100	0
3	100	0	100	0	100	0	0	100	87	13	0	100	0
7	98	2	100	0	99	1	0	100	88	12	0	30	70
14	96	4	100	0	100	0	0	100	79	14	8	4	96
21	99	1	100	0	100	0	0	100	73	18	9	0	100
28	99	1	97	3	100	0	0	100	63	10	27	2	98

* Initial starter/g salami.