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Use of rRNA Gene Restriction Patterns To Evaluate Lactic Acid Bacterium Contamination of Vacuum-Packaged Sliced Cooked Whole-Meat Product in a Meat Processing Plant

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Molecular typing was applied to an in-plant lactic acid bacterium (LAB) contamination analysis of a vacuum-packaged sliced cooked whole-meat product. A total of 982 LAB isolates from the raw mass, product, and the environment at different production stages were screened by restriction endonuclease (*Eco*RI and *Hin*dIII) analysis. rRNA gene restriction patterns were further determined for different strains obtained from each source. These patterns were used for recognizing the spoilage-causing LAB strains from the product on the sell-by day and tracing the sources and sites of spoilage LAB contamination during the manufacture. LAB typing resulted in 71 different ribotypes, of which 27 were associated with contamination routes. Raw material was distinguished as the source of the major spoilage strains. Contamination of the product surfaces after cooking was shown to be airborne. The removal of the product from the cooking forms was localized as a major site of airborne LAB contamination. Food handlers and some surfaces in contact with the product during the manufacture were also contaminated with the spoilage strains. Some LAB strains were also able to resist cooking in the core of the product bar. These strains may have an effect on the product shelf life by contaminating the slicing machine. The air in the slicing department and adjacent cold room contained very few LAB. Surface-mediated contamination was detected during the slicing and packaging stages. Food handlers also carried strains later found in the packaged product. Molecular typing provided useful information revealing the LAB contamination sources and sites of this product. The production line will be reorganized in accordance with these results to reduce spoilage LAB contamination.

Vacuum packaging is commonly used for increasing the shelf life and hygienic handling of cooked meat products. Due to the atmosphere created by the vacuum-packaging of cooked meat products, lactic acid bacteria (LAB) are the major spoilage-causing bacteria in these products (2, 3, 7, 12, 17, 20, 30, 37, 42). The activity of LAB produces the typical sour flavor and odor changes, making products eventually unfit for human consumption. These sensory changes are delayed until the stationary growth phase of spoilage LAB (21, 37), and a product is usually expected to retain good sensorial quality for at least 21 days. Production failures leading to spoilage LAB contamination and to spoilage of the product prior to the sell-by day cause considerable economic loss to manufacturers. Product circulation is limited and producer reputation is damaged due to recalls.

Detection of LAB contamination sites during the manufacture of cooked meat products is difficult. LAB populations are very low in an adequately cleaned production environment (25). Shortly after packaging, LAB populations in the product are usually below the routine detection limit (<10 CFU/g), even if the product later spoils quickly (4, 32). The cooking process (internal temperature of 68 to 73°C) inactivates LAB, and surfaces of the cooked products can be considered sterile (2, 25, 27). Products become recontaminated with LAB mainly during handling after the cooking process. LAB growth has been detected in the air, physical facilities, working surfaces, and workers' hands, and these sites have been assumed to be the sources of the spoilage LAB (9, 17, 18, 25, 26, 30, 32). There are, however, differences in the ability of different LAB species and strains to spoil meat products (8, 11, 13, 14, 16, 20, 23, 29, 31, 36–40, 42). On the basis of LAB growth alone, it cannot be determined if a sample contains species or strains later associated with the spoilage of a product. Very few attempts have been made to study recontamination by the spoilage-causing LAB species and strains in particular (4, 24, 26, 28). The sources of the spoilage LAB and the routes and sites of the recontamination in meat-producing plants are still poorly known.

DNA typing techniques produce characteristic "fingerprints" for bacterial strains, optimally allowing repeatable strain identification among isolates of different origins. These molecular typing methods have been used widely in epidemiological studies of pathogenic bacteria (41). In this study, molecular typing was applied to an in-plant LAB contamination analysis of a vacuum-packaged, sliced, cooked whole-meat product. This product is expected to retain good sensorial quality for 21 days, but sour off-odors and off-flavors were sometimes detected after 14 days (4). This study investigated spoilage strain contamination by distinguishing these strains among LAB sampled from different sites of product manufacture. Raw mass, the product at different manufacturing stages, and surface and air samples were studied. Restriction endonuclease analysis (REA) of chromosomal DNA, in combination with rRNA gene restriction patterns (ribotypes) (15), was chosen for this characterization on the basis of the knowledge about the genotyping of meat-associated spoilage LAB (4–6).

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MATERIALS AND METHODS

Manufacture of the product and the sampling sites. The product studied was a sliced, cooked whole-meat product. Raw meat was macerated overnight in

TABLE 1. Number of strains detected among LAB isolates from each source by REA with *Eco*RI and *Hin*dIII

Source	No. of strains/no. of isolates from:					
	Lot I				Lot II	
	Day 1	Day 2	Day 3	Day 4	Day 3	Day 4
Macerating room						
1. Air near the macerating pool	3/10				4/4	
2. Macerated mass, pool 1	5/15				9/15	
3. Macerated mass, pool 2	4/15				5/15	
4. Macerated mass, pool 3	6/15				7/15	
Cooking room surfaces						
5. Shelf trolleys 1 and 2, shelf surfaces and side pillars	4/30					5/30
6. Products cooked day before, three bars	3/20					NG[a]
7. Worker 1, glove, sleeve, apron	2/20					2/10
8. Worker 2, glove, apron	2/20					5/20
9. Working table	2/10					2/10
10. Pillar adjacent to oven	1/10					1/10
11. Door of oven-refrigerator	NG					NG
Air samples						
12. Area near the casing machine	NS[b]				1/1	
13. Form removal area	NG	1/1			NG	7/10
14. Form removal area						5/26
15. Inside cold room 1	NG				NG	
Core of the cooked products, three sites per bar						
16. Bar 1		NG				1/10
17. Bar 2		2/20				4/20
18. Bar 3		3/20				2/20
Transport to the packaging department						
19. Air, inside the vehicle		2/2				2/4
Cold-room 2 adjacent to slicing-packaging						
20. Air, inside the cold-room		NG	2/2			1/1
21. Cooked product, three bars			2/20			NG
Air in slicing-packaging department						
22. Air, near the packaging line		NG		NG		NG
Surfaces in slicing-packaging department						
23. Cooked product, three bars				2/20		4/30
24. Pillar adjacent to the packaging line				1/10		NS
25. Peeler 1, glove, apron				1/10		NS
26. Peeler 2, glove				NS		1/10
27. Peeler 3, apron, sleeve				NS		3/20
28. Peeling table				1/10		2/10
29. Slicing-packaging machine				7/30		5/50

[a] NG, no growth.
[b] NS, not sampled.

nitrite- and phosphate-containing brine. The next day, the meat mass was stuffed into an artificial nonproliferative casing (a three-layer laminate having a nylon layer as an oxygen barrier), pressed into forms, and cooked until the core temperature reached 72°C. The average weight of one bar was 3 to 4 kg. Chilling was started in an oven-attached system, and the cooked lot was later transferred to an adjacent cold storage room (cold room 1). The next day, the cooking forms were removed, and the lot was transferred to the cold room of the slicing-packaging department (cold room 2); slicing and vacuum-packaging were done during the next few days. Casings were removed manually on a table adjacent to the slicing-packaging line. The expected shelf life of the product stored at 6°C is 21 days.

This study was done in a normal production situation in a large-scale meat plant. LAB contamination of two production lots (lots I and II) was monitored by sampling the LAB in the raw mass, product, and environment at different production stages. A detailed description of the sources is presented in Tables 1 and 2. The production was monitored from raw-mass macerating until slicing-packaging, covering all stages of the manufacture. Packages sampled from these two lots were stored at 6°C as recommended by the manufacturer and studied on the sell-by dates. Environmental samples from the different areas were taken when the lots were handled in that area or when the lots were entering the area. The two lots had some differences in the total handling time. Lot I (cooked on day 1) was sliced on days 3 and 4, while lot II (cooked on day 3) was already sliced on day 4. Sampling for both the lots in the slicing-packaging department was performed on day 4. Both lots were handled on the same slicing-packaging line. Lot I entered the line first, and before lot II was packaged, a lot cooked on day 2 was handled. The packaging line was not completely washed and disinfected between different lots, although it was occasionally sprayed with alcohol during the work shift breaks.

Isolation of LAB, selection of the strains, and evaluation of the packages on the sell-by day. LAB in the air were sampled on MRS-S agar (Oxoid, Basingstoke, United Kingdom)-containing strips by using an RCS air sampler (Biotest AG, Dreieich, Germany). The sampling time was 8 min (320 liters of air), and all 17 sites were sampled in parallel with two pieces of equipment. The strips were incubated inside plastic packages at 25°C for 5 days. All isolates recovered (see Table 1) were cultured pure for DNA analysis, with the exception of two sample strips containing 33 and 45 colonies. From both of these strips, 13 randomly selected colonies were further characterized.

Surface sampling (56 sites) was performed by enriching LAB from the surfaces

in MRS broth (Difco, Detroit, Mich.) as described by Björkroth and Korkeala (4). Samples from the core of the cooked product were taken aseptically (33) from the middle and both ends of three cooked product bars from both lots and enriched in MRS broth at 25°C for 5 days, and growth was observed daily. Enriched MRS broths were plated to produce single colonies, of which 10 colonies from each sample were randomly selected and cultured pure for DNA analysis.

Lot I and II raw mass samples for LAB analysis were taken from each of the three pools used in the manufacture. The pH, sensory quality, and LAB were determined on the sell-by dates for 22 lot I and II packages randomly collected earlier. The packages were stored at 6°C until culturing. Sensory evaluation by three trained judges and pH measurements were performed as described by Korkeala et al. (22). LAB were determined as described by Korkeala and Lindroth (19). Fifteen colonies recovered from each raw mass and package sample were selected randomly for DNA analysis.

Isolation of DNA, REA, and determination of rRNA gene restriction patterns (ribotyping). Before the isolates were genotyped, they were confirmed to be LAB by the Gram stain and catalase reaction (34). DNA was isolated by the guanidium thiocyanate method of Pitcher et al. (35) as modified by Björkroth and Korkeala (4) with combined mutanolysin (Sigma Chemical Co., St. Louis, Mo.) and lysozyme (Sigma) treatment. Restriction endonuclease digestion of 5 μg of DNA for both the REA and ribotyping was done with *Eco*RI and *Hin*dIII as specified by the manufacturer (New England BioLabs, Beverly, Mass.); these restriction endonucleases are known to characterize meat-associated LAB well (4, 5). REA, genomic blots, and the rDNA probe for rRNA gene restriction patterns (ribotypes) were prepared as described by Björkroth and Korkeala (4).

A total of 982 LAB isolates were characterized by *Eco*RI and *Hin*dIII REA. Isolates from the same source were screened by electrophoresis in parallel in the same gel. Different strains recovered from each source were further characterized by ribotyping. Tables 1 and 2 show the number of strains selected from each source for ribotyping.

RESULTS

LAB counts in raw mass varied from 2.7×10^3 to 3.8×10^3 CFU/g for lot I and from 8.5×10^4 to 1.5×10^5 CFU/g for lot II. The 1-log-unit difference between these raw mass LAB populations was not reflected in air counts obtained from the macerating room. The average of the two parallel samples from the macerating room contained 16 CFU/m^3 when lot I was produced and 6 CFU/m^3 when lot II was handled. Generally, the LAB air counts were very low, or, at six sites, growth was not detected at all. LAB were also not detected in the air during the transfer of lots I and II from the oven to cold room 1 or in the air of cold room 1 20 to 40 min after the lots had entered the cold room. The highest air counts (141 and 103 CFU/m^3) were obtained 1 day after transfer from the cooking room, when the cooking-form removal from lot II was nearly finished. Air sampled from the slicing department did not show LAB growth, and only three isolates were obtained from the air of cold room 2, which was adjacent to the slicing-packaging room.

Of the 56 total surface samples, 15 did not show growth after enrichment. These sample sites were located randomly in the production area (Table 1). Growth was detected in five of the six product bars after the enrichment procedure; 9 of the total 18 core samples showed growth.

The number of CFU of LAB per gram of product on the sell-by date, together with sensorial results, pH values, and isolates showing different ribotypes, are presented in Table 2. A slight decrease in the pH values of the spoiled products was noticed. On the sell-by day, the packages of lot I showed better sensory quality than those of lot II. None of the eight lot I packages were deemed to be unfit for human consumption, and only two were found to have sensory changes, whereas only 4 out of 14 lot II packages were evaluated as good.

Of the 982 isolates obtained, 90 were recovered from raw mass, 62 were recovered from air, 410 were recovered from surfaces, 90 were recovered from core samples of the five cooked product bars, and 330 were recovered from the packages analyzed on the sell-by date. Of these isolates, 169 were ribotyped, yielding 72 different patterns when ribotypes generated by *Eco*RI and *Hin*dIII digests were both used for strain identification. Surface isolates contained the largest number of different ribotypes (a total of 29). Twenty-seven different ribotypes were obtained from the LAB of raw mass, 22 were obtained from LAB from air, 13 were obtained from LAB from the product on the sell-by dates, and 7 were obtained from LAB from the cooked core samples.

Of the 72 ribotypes, 27 were detected in more than one source (Tables 1 and 2, sources 1 to 51). Strains associated with more than one sampling site (raw mass, air, surface, core, or packaged product) are marked with a capital letter in the ribotype (A to X). The occurrence of these strains in the different isolation sources is shown in Table 3, together with the product flow chart. Strain A (Fig. 1, lanes 5, 6, 8, and 13) was the only strain clearly dominating in the nonenriched raw mass and air samples 2. Of the total 60 raw-mass isolates characterized, 32 displayed this ribotype. A large number of strain A isolates (16 of 26 isolates) was also found in the air sample taken during the form removal of lot II.

The remaining 44 LAB types were detected only once and could not be used for detecting product contamination routes. Seventeen of these isolates originated from raw mass, 10 originated from air, 12 originated from surfaces, 3 originated from cooked core samples, and 2 originated from sliced products on the sell-by day. Sixteen of these strains were found in the cooking room, mainly during removal of forms, and seven detected in the slicing-packaging department.

TABLE 2. Characteristics of the vacuum-packaged, sliced, cooked whole-meat product on the sell-by dates

Source	LAB population (CFU/g)	Sensory quality	pH	Ribotype[a] (no. of isolates)
Products cooked on day 1				
30. Package 1	3.7×10^5	Good	6.2	R (15)
31. Package 2	3.9×10^4	Good	6.2	V (14), a (1)
32. Package 3	1.9×10^6	Good	6.1	b (15)
33. Package 4	1.5×10^5	Good	6.2	Q (15)
34. Package 5	1.2×10^6	Good	6.2	V (13), c (2)
35. Package 6	6.7×10^5	ES[b]	6.2	V (6), a (9)
36. Package 7	4.4×10^5	Good	6.5	V (5), d (10)
37. Package 8	5.4×10^6	ES	6.2	K (3), e (13)
Products cooked on day 3				
38. Package 1	4.1×10^6	ES	6.1	K (5), e (10)
39. Package 2	8.0×10^4	ES	6.2	T (5), a (10)
40. Package 3	1.6×10^6	Good	6.3	b (15)
41. Package 4	7.0×10^3	Good	6.4	T (13), f (2)
42. Package 5	1.0×10^5	ES	6.4	Q (10), T (5)
43. Package 6	8.0×10^4	ES	6.3	Q (5), T (10)
44. Package 7	5.0×10^5	ES	6.4	T (12), V (3)
45. Package 8	1.0×10^7	Spoiled[c]	6.0	A (15)
46. Package 9	2.7×10^6	ES	6.3	A (15)
47. Package 10	2.2×10^7	Spoiled	6.0	G (15)
48. Package 11	2.0×10^7	Spoiled	6.1	A (13), G (2)
49. Package 12	2.0×10^3	Good	6.2	T (3), V (1), f (10)
50. Package 13	2.3×10^7	Spoiled	6.1	A (15)
51. Package 14	9.0×10^3	Good	6.4	T (15)

[a] Types were named on the basis of the information obtained from both *Eco*RI- and *Hin*dIII-generated ribopatterns. Capital letters indicate association with a detected contamination route (Table 3).

[b] ES, early spoilage changes in aroma and/or taste, but the product was still scored fit for human consumption.

[c] The product was scored unfit for human consumption by all three judges.

TABLE 3. Stages of the process and ribotypes named on the basis of both *Eco*RI and *Hin*dIII patterns detected at different production stages of lot I and II vacuum-packaged, sliced, cooked whole-meat product

Production area	Stage of process	Ribotype(s) (sources)[a]	
		Lot I	Lot II
Macerating room	Macerating		
	Air	A, B, C	A, F, G, H
	Raw material	A (2, 3, 4), B (4), C (2), F (2, 4), K (4), M (2), N (4)	A (2, 3, 4), G (2), O (4), P (2, 3, 4)
Cooking room	Casing	ND[b]	H (12)
	Cooking and chilling (oven)	ND	ND
	Unloading from oven	ND	ND
Cold room 1	Chilling overnight	ND	ND
Cooking room	Form removal	A (5), D (8), K (5, 6, 8, 10), L (6), R (5), S (9)	A (8, 13, 14), I (13), J (5, 13), K (5, 14), L (8), M (7), N (9), O (9), P (8), T (5, 7), X (5, 8)
	Product core	G (18), K (15), Q (18)	I (17)
Vehicle	Transport	D	ND
Cold room 2	Cold storage prior slicing and packaging	E (20), J (21), M (21)	L (20)
Slicing-packaging department	Transport to slicing; product surfaces prior casing removal	U	A, P
	Removal of casing	E (25), T (28)	R (27), U (26), X (28)
	Slicing-packaging machine	K, Q, R, S, U	Q, U, V
Terminal, delivery	Ready products		
	LAB determined on the sell-by day	K (37), Q (33), R (30), V (31, 34, 35, 36)	A (44, 45, 47, 49), G (47, 48), K (38), Q (42, 43), T (39, 41, 42, 43, 44, 49, 51), V (44, 49)

[a] Sources (numbered as in Tables 1 and 2) are presented in parentheses if more than one source was located in that area.
[b] ND, not detected.

DISCUSSION

All the packages deemed to be unfit for human consumption contained meat with LAB that exceeded 10^7 CFU/g, which is in agreement with previous studies dealing with LAB spoilage of vacuum-packaged cooked meat products (21). The small decrease in the pH of the spoiled products may result from the buffering capacity of the product and the early stage of the spoilage process. LAB types A (Fig. 1, lanes 5, 6, 8, and 13) and G (lanes 7 and 9) associated with lot II packages were the only strains detected in spoiled packages unfit for human consumption. In a previous study (4) assessing *Lactobacillus sake* starter strain contamination at the same slicing department, type A was already found to be associated with rapid spoilage of this cooked whole-meat product. The same study also found type G contaminating a sliced sausage product. These two strains can be considered to cause rapid spoilage of the product. Strains K, Q, R, T, V, a, and e were found in packages showing early spoilage changes. The LAB growth detected in these packages varied from 8.0×10^4 to 5.4×10^6 CFU. These strains cannot be stated to be associated with the rapid spoilage of the product as clearly as strains A and G, because they were found mainly in variable combinations with the other strains (Table 2). Strains K, Q, R, T, a, and e may have a lower growth rate than strains A and G, and the clear spoilage changes do not occur during the 21-day shelf life.

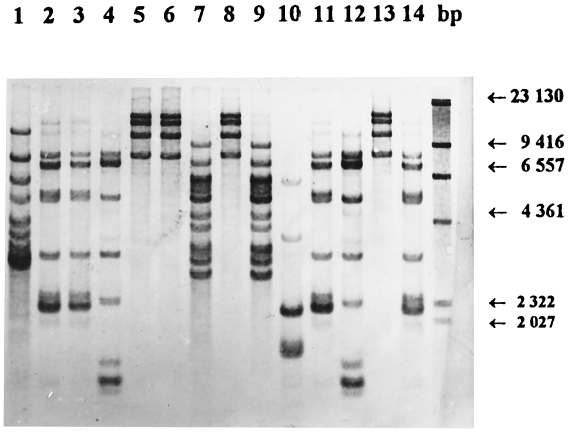

FIG. 1. Ribotypes obtained by *Eco*RI digests. Lanes: 1, strain Q; 2, 3, 11, and 14, strain T; 4 and 12, strain V; 5, 6, 8, and 13, strain A; 7 and 9, strain G; 10, strain f. Phage lambda DNA cleaved with *Hin*dIII was used as a fragment size marker.

Detection of contamination routes for LAB types A, G, and K were the most complete (Table 3). The contamination source of these spoilage strains seems to be the raw mass bringing the bacterial load to the production facilities. LAB type A was especially abundant (approximately 50% of the isolates characterized). With the exception of the cold rooms, types A, G, and K could also be detected in all the processing rooms (Table 3). Types A and G were found in the air in the macerating room (Table 3). During the overnight macerating, the raw mass is constantly stirred in open pools, allowing aerosol formation and bacterial transmission to the air. Even though lot II mass contained more LAB, the diversity of spoilage strains detected was not higher (Table 3). LAB strains B,

C, F, and H, also isolated from the raw material and/or the air sampled from the macerating room, could be detected only during day 1 of production (Table 3). The spoilage-causing A and G strains may survive better in the production environment. The high contamination level by strain A compared to the other isolates also affects the detection of this LAB strain.

Airborne contamination of the product with strains A and K and many other LAB types after cooking was shown to be possible. Until the slicing stages, these LAB will come in contact with the casing of the product transporting the spoilage LAB load to the packaging department. The variation of the strains and the level of airborne contamination in the form removal area during handling of lot II were remarkably high. Strain A was detected in both samples taken at approximately 3-h intervals, suggesting that this area is a major site of airborne strain A recontamination. The occurrence of strain A in the latter air sample was very high, clearly demonstrating the presence of an undesirable air flow from the macerating room to the cooking room. Only one LAB isolate was found in the air during the form removal of lot I, the lot which had better quality on the sell-by day.

Destro et al. (10) did not find that food handlers played a major role in disseminating *Listeria monocytogenes* throughout a shrimp processing plant. In the present study, we showed that workers handling both lots I and II after cooking were contaminated with strains A and K, which are associated with spoiled products (Table 3). Strains A and K were also detected on shelf trolley surfaces coming in contact with the casing of the cooked product.

Cold rooms were not major LAB air contamination sites, as stated in some works dealing with cooked ring sausage contamination (32). Only three isolates were recovered from 1.6 m^3 of air sampled from cold rooms. Prolongation of the storage of cooked products in cold-storage rooms has also been stated to be important for product shelf life (1). In our study, however, the 2-day-longer cold storage of lot I before slicing was not found to affect the lot quality; on the contrary, the lot possessed better sensory quality. This may be due to the nonproliferative casing protecting the product surface from direct contamination during cold storage. Products with natural casings are usually packaged with the casing, leaving all airborne surface contaminants inside the package.

Four strains (G, K, I, and Q) possess heat tolerance and survived the cooking process. When cooked ring sausages were studied, viable LAB were also found in the core of the products (19, 27). Korkeala and Lindroth (19) showed, however, that the LAB causing spoilage of cooked ring sausages grow only on the surfaces of the sausages. Strains surviving the cooking in the core of the sausages were not able to grow during cold storage. A lower a_w inside the product compared to the surface was suggested to be the reason for these growth differences (19). If the product is sliced, as in the present study, the viable bacteria inside the meat bar may have a different effect on the shelf life. Strains K and Q were also found to contaminate the slicing-packaging machine, and strains G, K, and Q were detected in the packaged product on the sell-by date. It is difficult, though, to evaluate the importance of core contamination only, because airborne surface contamination with these strains was detected as well.

When the contamination in the packaging department was studied, only surface samples yielded viable LAB and could provide information. Airborne LAB contamination in this room is very limited. Contamination of workers removing the casing with strains U and R, later isolated from the packaging machine and/or products, was detected. Removal of the casing on the peeling table was considered a possible site of contamination, where the worker may transfer contaminants. At this stage, when the protective casing is removed, surface contamination from the food handler (strains U and R) and the peeling table (strain T) is transmitted directly to the product entering the slicing-packaging machine.

Some of the strains detected on the slicing-packaging machine and in the products on the sell-by day could not be isolated during previous production stages. Lot II products, in particular, contained type Q, S, and V LAB that were not associated with the previous production stages of this lot. Type Q was found in the core of the lot I bar, and type S was found in the form removal of the same lot. Lot II was sliced after lot I on the same line, indicating the occurrence of cross-contamination at slicing stages between different lots.

Molecular typing revealed the probable routes of contamination by spoilage LAB. When these methods are employed in LAB contamination analysis at meat processing plants, sampling of LAB occurring at low levels must be carefully planned. The diversity of the LAB detected showed the importance of characterizing these isolates to obtain accurate information about contamination by spoilage LAB. The production line will be reorganized in accordance with these results to reduce contamination of this product by spoilage LAB.

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