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Characterization and Behavior of *Salmonella javiana* During Manufacture of Mozzarella-type Cheese[1]

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ABSTRACT

A patient isolate of *Salmonella javiana* implicated in an outbreak of salmonellosis in Minnesota was characterized and used to examine its response to Mozzarella manufacturing conditions. The strain possessed biochemical-metabolic activities typical of *Salmonella* species. Growth was observed in 6.5% NaCl Trypticase Soy Broth (TSB) but not in 12% NaCl TSB. This *S. javiana* strain was resistant to two antibiotics, penicillin G and erythromycin. Pasteurization trials indicated the strain did not survive pasteurization and that pasteurization affected a log reduction of greater than 9 cycles. Mozzarella-type cheese was manufactured from milk inoculated with *S. javiana* at levels of 1×10^4 and 1×10^6 per ml milk. Manufacturing process was monitored by following pH, titratable acidity, and temperature. Survival of *S. javiana* was monitored using traditional enrichment procedures and direct plating procedures. *S. javiana* survived and grew through the acid-ripening phase, but temperatures attained in cheese mass during stretching and molding (60°C, 140°F) killed all *Salmonella* present. No subsequent process steps were found positive for *Salmonella*.

Mozzarella cheese was identified by epidemiological evidence as the vector in a recent outbreak of salmonellosis in Minnesota and Wisconsin (Osterholm, personal communication, 1989). The *Salmonella* serotype involved was identified as *S. javiana*. This particular serotype has rarely been found to be associated with dairy products.

Mozzarella cheese has been infrequently implicated as the vector in outbreaks of foodborne illness. Hard cheeses in general are seldom involved in salmonellosis outbreaks in North America. Three outbreaks within the past decade have been attributed to Cheddar cheese consumption. One occurred in 1976 in Colorado (*3*), and the cheese was found to be contaminated with *Salmonella heidelberg*. The other two occurred in Canada. One occurred in 1982 and involved Cheddar cheese made from unpasteurized milk. The cheese was found to be contaminated with *Salmonella muenster* (*12*). The second occurred in 1984, and the cheese was contaminated with *Salmonella typhimurium* (*2*). Pasteurized milk contaminated with raw milk was determined to be the source of *Salmonella* in this outbreak.

Species of *Salmonella* are not very heat resistant. Generally, heat processes in the range of 60 to 62°C (140-144°F) for 3.5 min are sufficient to destroy the most heat resistant *Salmonella* (*4*). Milk pasteurization temperatures exceed this heat treatment and should be sufficient to destroy any *Salmonella* that might be present in raw milk used for cheesemaking.

Little if any research has been done on growth and survival of *Salmonella* during manufacture and processing of Mozzarella cheese. White and Custer (*11*) and Park et al. (*6*) studied survival and growth of *Salmonella* during manufacture and ripening of Cheddar and Colby cheese. They determined that if milk was contaminated with *Salmonella* after pasteurization, the organism would survive and grow during the make procedure and remain viable in the cheese for some time during ripening and aging.

The objective of this study was to determine the behavior of *S. javiana* during manufacture, brining, and storage of Mozzarella-type cheese.

MATERIALS AND METHODS

Cultures

The *S. javiana* culture used in these experiments was a patient isolate from an outbreak of salmonellosis associated with consumption of cheese in Minnesota and Wisconsin. This culture was provided by Dr. M. Osterholm of the Minnesota Department of Health. *S. typhimurium* from our culture collection was used in salt tolerance studies. Inoculum was prepared by transferring cultures at least twice in fresh sterile RSM or Tryptic Soy Broth (TSB; Difco, Detroit, MI) and incubating at 37°C (98.6°F) for 16-18 h. Starter cultures *Lactobacillus bulgaricus* 880 and *Streptococcus thermophilus* C3 were propagated in sterile RSM and incubated at 37°C (98.6°F) for 16 h prior to cheese manufacture.

Characterization of the isolate

Subcultures of the isolate were used to inoculate API 20E Strips (Analytab Products, Plainview, NY) and were sent to the University of Minnesota, Veterinary Pathobiology Laboratory for antibiotic susceptibility testing. Minimum inhibitory concentrations (MIC) of 18 antibiotics were assessed.

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Salt tolerance

S. javiana or *S. typhimurium* were used at a level of 1% to inoculate TSB which contained 0, 0.5, 1.0, 1.5, 3.0, 6.0, and 12.0% added sodium chloride (w/v). Growth was recorded as turbidity after 24 and 48 h incubation at 37°C (98.6°F).

Laboratory pasteurization

Nine milliliters of 11% RSM (steamed 60 min at atmospheric pressure) was placed in each of five 16 x 150 mm test tubes and preheated to 62.8°C (145°F) in a Lab-Line 3086 water bath (Lab-Line Instruments, Inc., Melrose Park, IL). One milliliter of *S. javiana* inoculum was added to each preheated tube of milk, mixed, and immersed in the water for 30 min. Following heating, samples were cooled on ice. The entire sample was added to 90 ml lactose broth for *Salmonella* enrichment as described below. Number of *S. javiana* present in the inoculum was determined by spread plating appropriate 0.1% peptone water dilutions onto Tryptic Soy Agar (TSA, Difco) plates which were incubated at 37°C for 24 h.

Manufacture of Mozzarella-type cheese

Mozzarella-type cheese was manufactured following the procedure outlined in Cheese and Fermented Milk Foods (*5*). Each vat contained 6 L of pasteurized, homogenized 2% fat milk. Temperature was adjusted to 32.2°C (90°F). Starter cultures employed were *Lactobacillus bulgaricus* 880 and *Streptococcus thermophilus* C3. Cheese milk was inoculated with 0.75% of each starter culture and experimental vats were inoculated with 0.1 ml, 1.0 ml, and/or 10 ml of *S. javiana* grown in sterile RSM for 16 to 18 h. *Salmonella* inoculum levels were chosen to approximate levels of 1 x 10^4 CFU/ml milk, 1 x 10^5 CFU/ml milk, or 1 x 10^6 CFU/ml milk in the vat. Milk was allowed to ripen for 30 min in vats, after which single-strength rennet (Hi-C chymosin, Pfizer, Milwaukee, WI), diluted 1:40 with water was added at a level proportional to 85 ml/1000 lb (0.085 L/453.6 kg) milk. Curd was cut 30 to 60 min after rennet addition when a smooth, thick curd of proper strength was obtained. After cutting the coagulum with 0.95 cm (3/8") wire knives, curd was allowed to heal with periodic, gentle agitation for 15 min. After healing, curds were cooked with gentle agitation for 35 min during which temperature was increased to 41.1°C (105°F). Once 41.1°C (105°F) was attained, curds were allowed to settle and mat over a period of 10 min. Whey was drained off after matting and matted curd was cut into two small blocks. Curd blocks were ripened at 42.2°C (108°F) until a titratable acidity (TA) of 0.60-0.70% (expressed as % lactic acid) was attained and pH dropped to 5.1-5.3. During ripening curd blocks were turned every 30 min. Acid-ripened curd blocks were sliced with sterile knives into strips about 12 cm by 3 cm by 1 cm and placed into 73.9°C (170°F) water for stretching and molding. Cheese strips were manually stretched into a smooth, plastic, glossy mass. When the cheese mass reached an internal temperature of 60°C (140°F), the mass was formed into two small balls approximately 10 cm in diameter and cooled in cold water until firm. Mozzarella balls were placed separately into 3 L of saturated salt brines (23% NaCl [w/v]) for 4 h at 4°C. Manufacturing procedure was monitored for titratable acidity, pH, temperature, and for the presence and population of *Salmonella* at intervals specified in Table 2. Compositional analysis of the cheese was not performed.

Bacteriological analysis

Enrichment procedure. Twenty-five grams of curd or 25 ml of milk, whey, cooling water, or brine were used to inoculate 225 ml of sterile lactose broth (Difco) and were incubated for 24 h at 37°C (98.6°F). One-milliliter portions were transferred to tubes containing 10 ml of selenite cysteine (Difco) and to 10 ml tubes of tetrathionate broth containing brilliant green dye and iodine solutions (*9*). After 24 at 37°C (98.6°F), selective enrichments were streaked onto Xylose Lysine Desoxycholate (XLD) plates. Representative black colonies present on XLD plates after 24 h at 37°C (98.6°F) were used to inoculate Lysine Iron agar (LIA, Difco) and Triple Sugar Iron agar (TSI, Difco) to confirm the presence of *S. javiana*.

Direct plating procedure. Populations of *S. javiana* were determined using the Strantz-Zottola method (*10*). Additional samples were serially diluted in 0.1% peptone water and spread plated onto pre-poured TSA (Difco) plates with sterile glass hockey sticks. Plates were incubated at room temperature (22°C, 72°F) for 2 h and then overlaid with 10 to 15 ml of XLD agar tempered to 45°C (113°F). After solidification of overlay, plates were incubated at 37°C (98.6°F) for 24 h. Populations of *S. javiana* present in samples were determined by counting black colonies on overlay plates. Representative black colonies from overlay plates were used to inoculate LIA and TSI slants for confirmation of the presence of *S. javiana*.

RESULTS AND DISCUSSION

The *S. javiana* strain obtained for use in this study was subjected to a variety of tests to characterize it and to evaluate whether it possessed any distinguishing or unusual traits. Reactions in API 20E strips indicated this *S. javiana* strain possessed no unusual biochemical activity. Sodium chloride tolerance for this *S. javiana* strain was tested and compared to resistance for the strain of *S. typhimurium* responsible for the Hillfarm outbreak in 1985 (*1*). There was strong growth through 3.5% NaCl TSB, weak growth in 6.5% NaCl TSB, while 12.5% NaCl TSB completely inhibited growth of both *S. javiana* and *S. typhimurium*. Response in sodium chloride demonstrated growth of *S. javiana* in the saturated brine solution used in Mozzarella manufacture will not occur, making subsequent contamination of the cheese at this point unlikely.

As frequency of multiple antibiotic resistant *Salmonella* isolates has been increasing over the past years (*7*), the *S. javiana* isolate was subjected to an antibiotic resistance assay to determine Minimum Inhibitory Concentration (MIC) levels for 18 different antimicrobial agents (Table 1). Resistance was noted only for penicillin G and erythromycin. *S. javiana* was sensitive to the other 16 antibiotics tested. The resistance pattern was not unusual (*7,8*). The number of antibiotic resistances observed is low when compared to the *S. typhimurium* isolated during the Hillfarm outbreak of salmonellosis (*1*).

With the characterization phase of the study completed, attention was focused on behavior of *S. javiana* during Mozzarella-type cheese manufacture. To eliminate the possibility that pasteurized cheese milk was the source of contamination, bench top vat pasteurization at 62.8°C (145°F) for 30 min was employed to evaluate thermal destruction characteristics of the strain. None of the five trials using *S. javiana* showed survival. The original inoculum was 2.6 x 10^9 CFU/ml milk. Consequently, laboratory pasteurization treatment affected a population reduction in excess of 9 log cycles. Thus, properly pasteurized milk is an unlikely source

TABLE 1. *Results of antibiotic susceptibility tests of* S. javiana.

Antimicrobic Agent	MIC (μg/ml)	Susceptibility
Amikacin	<2	S
Ampicillin/Amoxicillin	2	MS
Ceftiofur (Cefotaxime)	4	S
Cephalothin	4	S
Erythromycin	>8	R
Gentamicin	<0.5	S
Lincomycin	>8	S
Nitrofurantoin	<16	S
Penicillin G	>16	R
Tetracycline	4	MS
Trimethoprim-sulpha	2/38	MS
Tobramycin	<0.5	S
Chlorotetracycline	16	MS
Cefamandole	8	S
Cefoxitin	4	S
Cefaperazone	4	S
Piperacillin	16	S
Carbenacillin	32	S

S = Susceptible.
MS = Moderately Susceptible.
R = Resistant.

of contamination of finished cheese.

The final phase of this study was to make Mozzarella-type cheese contaminated with *S. javiana*. The cheesemaking process was monitored according to the schedule in Table 2. Make times, pH, and titratable acidity followed guidelines from a traditional Mozzarella process (*6*). Microbial population data and pH profile during manufacture are summarized for control (Fig. 1), low inoculum (Fig. 2), and high inoculum vats (Fig. 3). Acid development during manufacture was appropriate (Fig. 1-3). Acid ripening was terminated when pH below 5.2 and titratable acidity above 0.60% were achieved. Levels of *S. javiana* found in experimental vats were a function of inoculum size. No *Salmonella* was isolated from control vats (Fig. 1) by either direct plating or traditional enrichment procedures. Low inoculum vats (Fig. 2) started with approximately log 4.5 CFU/ml milk. Levels remained stable through curd cutting, though there were levels about 1 log cycle lower in whey. Numbers of *S. javiana* increased during cooking to around log 5.5 in the

TABLE 2. *Sampling plan during Mozzarella manufacture.*

Process step	pH	TA	CFUs/ enrichment	Process step in Fig. 1-3
inoculation	+[1]	+	+	1
renneting	+	+	+	2
cutting, curds			+	3
cutting, whey	+	+	+	4
begin cooking	+	+	+	
end cooking, curds			+	5
end cooking, whey	+	+	+	6
end draining/ acid-ripening	+	+	+	7
after molding			+	8
molding water			+	
after brining			+	9
brine solution			+	

CFUs = samples taken for microbiological analysis.
[1] = Analysis made at this time.

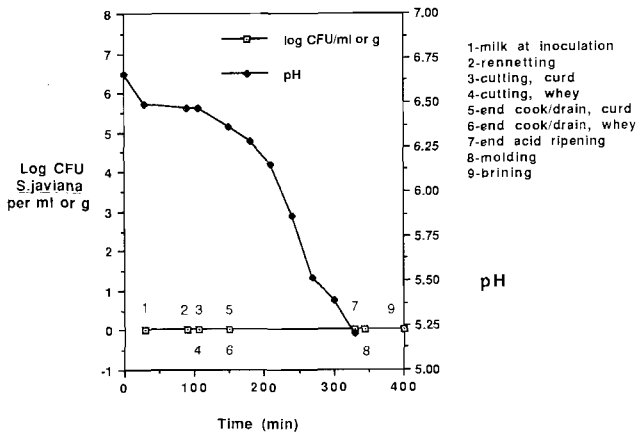

Figure 1. *Growth of* S. javiana *and pH profile during manufacture of control Mozzarella-type cheese (no* S. javiana *added to cheese milk).*

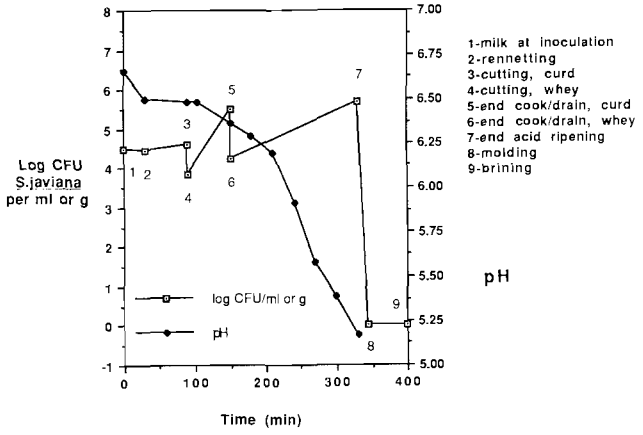

Figure 2. *Growth of* S. javiana *and pH profile during manufacture of Mozzarella-type cheese from milk inoculated with approximately* 10^4 S. javiana/*ml.*

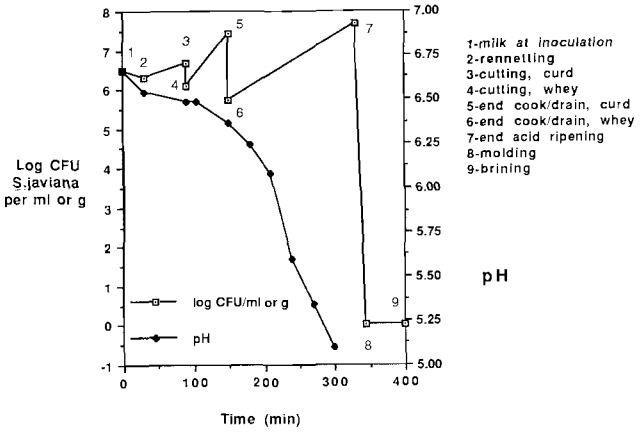

Figure 3. *Growth of* S. javiana *and pH profile during manufacture of Mozzarella-type cheese from milk inoculated with approximately* 10^6 S. javiana/*ml.*

curd with levels of log 3.5-4.5 found in the whey at draining. Curds exhibited peak population levels at the end of draining. However, the heat treatment given curd during stretching and molding (internal temperature 60°C, 140°F) completely eliminated any *Salmonella* from all further processing steps.

A similar response to processing was seen in cheeses made with higher levels of *S. javiana* (Fig. 3). Populations of *S. javiana* increased during manufacture, reaching maximal populations at the end of draining. Again, heat treatment during stretching and molding completely eradicated any *Salmonella* from further processing steps. As shown in Fig. 3, by the time an internal temperature of 60°C (140°F) was attained in the cheese mass, a reduction in *S. javiana* population of over 8 log cycles had been achieved.

In final analysis, it appears there is nothing distinctive from a biological or physical standpoint about this strain of *S. javiana* which would allow it to behave differently from any other *Salmonella* species. *S. javiana* is susceptible to heat inactivation as demonstrated by pasteurization trials, where a decrease in excess of 9 log cycles was observed. This eliminates properly pasteurized milk as a source of the *S. javiana*. *S. javiana* is capable of growth, in spite of increasing environmental acidity, during the manufacture of Mozzarella-type cheese. However, a stretch/molding temperature of 60°C (140°F) will effectively eliminate *S. javiana* contamination. Furthermore, this stretching/molding temperature is on the conservative side. Other procedures indicate stretching temperatures of up to 80°C (176°F) are employed. Consequently, fresh Mozzarella cheese should be *Salmonella*-free due simply to thermal inactivation, even if starter failed and there was low or no acid production. Therefore, internal temperature at molding is the major critical control point to be monitored in the process. It should be stressed that if proper HACCP and GMP guidelines are followed, the probability of *S. javiana* contamination in properly manufactured Mozzarella cheese is extremely remote.

CONCLUSION

The strain of *S. javiana* implicated in a salmonellosis outbreak in Minnesota possessed typical biochemical-metabolic activity of *Salmonella* species. Growth was observed in 6.5% TSB but not in 12% NaCl TSB. This *S. javiana* strain was resistant to two antibiotics, penicillin G and erythromycin. Pasteurization trials indicated *S. javiana* does not survive pasteurization and pasteurization reduces the population level more than 9 log cycles. *S. javiana* was inoculated into milk at levels varying from 1×10^4 to 1×10^6 CFU/ml milk. Mozzarella-type cheese was manufactured from this milk according to a traditional process. The process was monitored by following pH, titratable acidity, temperature, and *S. javiana* population. *S. javiana* survived and grew through the acid-ripening phase, but temperatures attained in cheese mass during stretching and molding killed all *Salmonella* present. No subsequent process steps were found positive for *Salmonella*. Temperature attained at stretch/mold is the major critical control point.

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