UNDERSTANDING THE BEHAVIOR OF PATHOGENIC BACTERIA ON CHEESES

BY

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ABSTRACT

Cheese is one of the ready-to-eat food categories that have been implicated in foodborne illness outbreaks. The use of unpasteurized milk for cheese manufacturing is one of the leading causes for outbreaks linked to cheeses. For cheeses made with pasteurized milk, the major causes of outbreaks are cross-contamination of cheeses after processing and the storage of contaminated cheeses under abusive temperature conditions. Preventive measures that can be taken to ensure cheese safety include the use of pasteurized milk for cheese production, the practices of good hygience during manufacturing and handling, proper implementation of time/temperature control, and the application of antimicrobial compounds or treatments for certain types of cheeses. Specifically, this work aims to provide information that can assist with the decision-making for implementation of time/temperature control on cheeses, and also to facilitate the development of antimicrobial strategies for high risk cheese. To achieve these aims, we investigated: (I) the influence of cheese composition upon the survival and growth of pathogenic bacteria, (II) the effect of storage environment upon bacterial survival/growth on cheeses, and (iii) the specific molecular mechanisms and responses that are important for survival/growth of *L. monocytogenes* on high risk cheese.

According to guidelines established in the U.S. Food and Drug Administration Food Code, food with pH <4.2 or water activity (a_w) < 0.88 does not require time/temperature control for safety (TCS). Foods with compositional values that fall outside these boundaries are labelled as TCS-food, and must be stored at <5°C to inhibit pathogenic bacteria growth. However, many cheeses that do not support pathogenic bacterial growth are labelled as TCS food according to the Food Code guidelines. This suggests a need of a more practical and specific guidelines to

evaluate the ability of cheeses to support pathogenic bacteria growth under extended storage without refrigeration.

Initially, we tested the ability of 67 natural cheeses to support the growth of *Listeria monoctogenes* (LM), *Salmonella* spp. (SALM), *Escherichia coli* O157:H7 (EC) and *Staphyloccocus aureus* (SA) at 25°C for up to 15 days. The types of cheeses that were tested include varieties of hard (Asiago, Cheddar, Parmesan), semi-hard (Colby, Havarti, Monterey Jack), and soft cheeses (fresh mozzarella, Queso Fresco/Blanco). Reduced fat and reduced sodium types of certain cheese varieties were also included in this study. Cheese slices, each weighed approximately 20-25 g, were inoculated with ~10^5 CFU/g of a single-pathogen cocktail (10 strains of LM, 6 of SALM, 5 of EC, or 5 of SA). The inoculated cheese slices were held at 25°C, and inoculum bacteria were enumerated by plating on selective agar every 3 days starting from day 0 up to day 15. To invesgiate the influence of compositional factors upon bacterial growth, the pH, % salt-in-the-moisture phase (%SMP), a_w, % titratable acidity and concentrations of background microflora were measured. Results showed that 53 of the 67 cheeses did not support pathogen growth. Among the 14 cheeses that supported growth, SA grew on all cheeses (0.57 to 3.08 log CFU/g growth), SALM on 6 (1.01 to 3.02 log CFU/g), LM on 4 (0.60 to 2.68 log CFU/g), and EC on 3 (1.01 to 3.02 log CFU/g). Combining our data with results from other publications, we were able to establish growth/no-growth boundaries using pH and %SMP as predictor variables, noting that more data were needed to confirm the significance of a_w and other compositional factors in predicting bacterial growth.

In next steps, we studied 35 additional cheeses, to increase the number of cheeses of a given type from different manufacturers, in order to enable the statistical analysis of cheese type as a predictor variable, and also to strengthen the statistical analyses of all compositional factors

as predictor variables. Additionally, pathogen survival/growth on cheeses was compared under (i) isothermal holding (25°C) vs. temperature cycling (alternating 4°C and 25°C in 12 h intervals), and (ii) aerobic vs anaerobic holding. Results showed that the pathogen growth outcomes under these holding environments (isothermal vs. temp. cycling; aerobic vs. anaerobic) were not significantly different ($p > 0.05$). Combination of results from this study, along with data from previous experiments/publications yielded a total of 437 trials involving 116 cheeses. Statistical analyses using this combined dataset showed that cheese type is not a definitive predictor of pathogenic bacteria growth, as compositional variation within a cheese type from different lots resulted in differences in growth outcomes. Analyses on cheese compositional factors as predictor variables indicated that cheeses would be safe for storage at ≤25°C up to 15 days, if the composition match the following criteria: pH is ≤5.1 and any % moisture, or pH is >5.1 and moisture ≤39%.

Compared to other cheese types, Queso Fresco, a fresh Hispanic-style cheese is linked to foodborne illness outbreaks at a greater frequency, due to its ability to support growth of LM to high concentrations if contaminated. LM as a psychrotolerant pathogenic bacterium is able to grow at low temperature, rendering refrigeration an ineffective hurdle. Queso Fresco typically has close-to-neutral pH (6.2-6.5) and low salt content (<1.5%), and application of antimicrobial treatments is recommended to control pathogen growth. In this project, transcriptomics analysis was conducted to characterize transcriptome in LM-Scott A grown on Queso Fresco at 7°C, compared to the same strain grown in a complex laboratory medium. Results showed that 494 and 209 genes were up- and down-regulated (false discovery rate [FDR] ≤0.01; $\log_2$ fold change ≥1.5) respectively, for LM-Scott A that was grown on Queso Fresco. As the RNA samples were extracted when cultures were at late-log phase under both growing conditions, it is assumed that

the transcriptomics results reflect only the influence of the growing environments, but not of the growth phases. Gene ontology enrichment analyses involving the differentially expressed genes suggest that LM-Scott A grown on Queso Fresco may be diversifying its intake of carbohydrate, activating anabolism of cobalamin and histidine, utilizing both ethanolamine and 1,2-propanediol as carbon and nitrogen sources in environment with competitive microflora, increasing iron intake, and upregulating the expression of virulence genes. On the other hand, genes related to flagellar formation, putrescine/spermidine transport, peptidoglycan synthesis, ribosomal protein and pyrimidine synthesis were downregulated in LM-Scott A that was grown on Queso Fresco, when compared to culture grown in TSB at 7°C. The results from this study could potentially facilitate the development of effective microbial control strategies for Queso Fresco.

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CHAPTER 1:

Research Background and Introduction

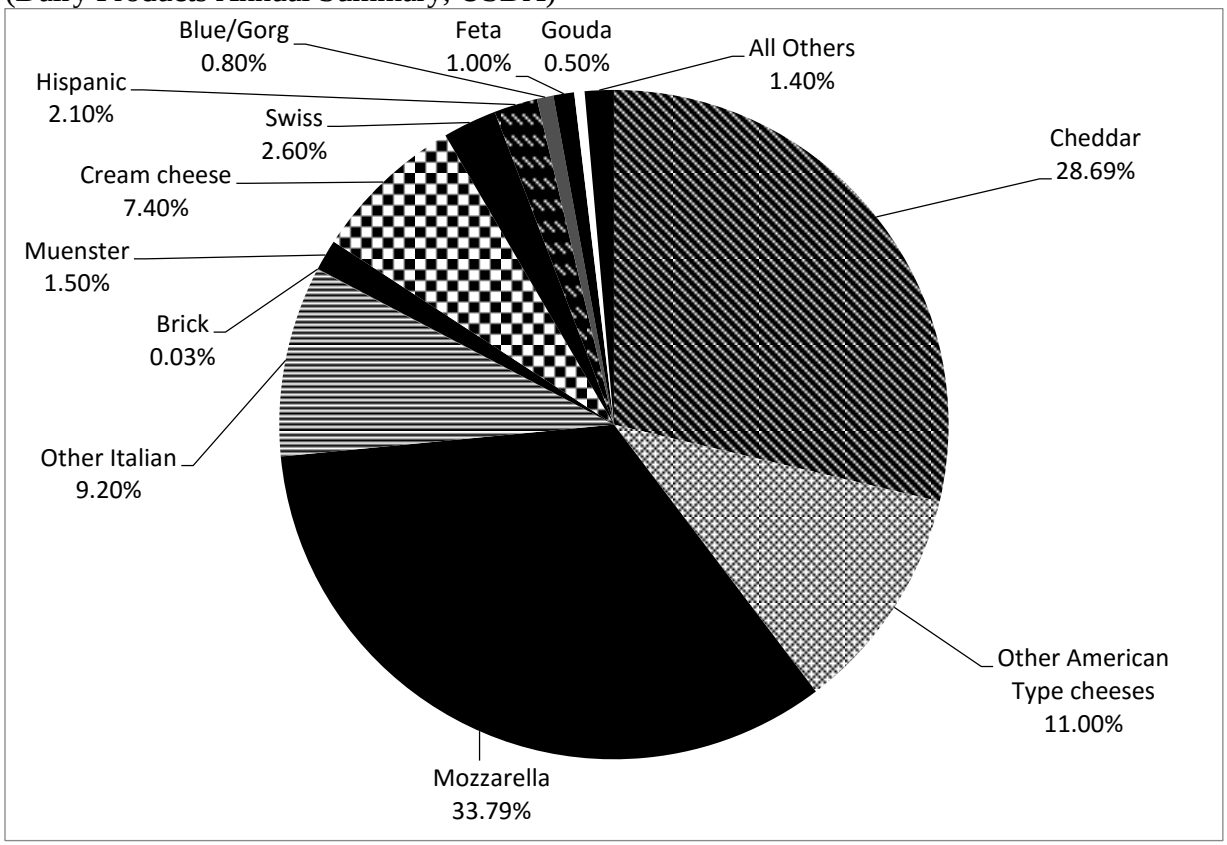
1.1 CHEESE

The earliest evidence of cheese-making was found in northern Europe and dated back in the 6th millennium B.C.E, with the discovery of milk fat in vessels that resembled modern cheese strainers (80). Cheese-making was practiced on local farms in the United States beginning in the 1600's after the English Puritans carried with them cheeses and the knowledge of cheese-making to the New World. The first cheese factory in the United States (U.S.) was established in 1851 in Oneida County, New York. Since then, the cheese industry has grown rapidly, so that now it is an important segment of the U.S. economy especially in New York, Vermont, Ohio and Wisconsin (45).

Annual cheese production in the U.S. has increased tremendously, from 216 million pounds back in 1880 to 11,838 million pounds in 2015, almost doubling in the last two decades, from 6917 million pounds in 1995 to 11,838 million pounds in 2015. The major cheese producing states are: Wisconsin (25.9% of U.S. annual production), California (20.6%), Idaho (8%), and New York (6.5%) (96). In the U.S., more than one-third of all milk produced is allocated for cheese production (45). The demand for cheese by consumers in the U.S. has grown steadily over the years, with consumption increasing from 24.6lb cheeses per capita in 1990 to 35.3 lb per capita in 2015. Cheese consumption is expected to further increase to 37.1lb per capita by 2025(95).

The International Dairy Federation recognizes 510 cheese varieties, with differences based on dairy animal, moisture content and ripening style (20). Currently the most popular cheese types in the U.S. are Cheddar, Mozzarella, Other American-type cheeses, Italian-style and Swiss (96; Fig 1-1).

Figure 1-1: Percentages of cheese varieties that were produced in the United States in 2015. (Dairy Products Annual Summary, USDA)



1.1.1 STANDARDS OF IDENTITY

Part 133 of CFR (Code of Federal Regulations) Title 21 elaborates the definitions and Standard of Identity (S.O.I.) for cheeses and related cheese products. The criteria for meeting a standard of identity include: percent moisture and milk fat limits, minimum ripening time, standard ingredients, standard manufacturing and processing procedures, and labeling requirements. In this research project that forms the basis for this dissertation, a variety of hard, semi-soft, and soft cheeses were tested for their ability to support the growth of pathogenic bacterial growth under non-refrigerated conditions. The two major cheese groups tested in the study: hard and semi-soft cheeses were defined in the CFR section 133.150 (99), as shown below:

21 CFR Part 133.150

I. Hard cheeses contain ≤ 39% moisture; with solids comprised of ≥ 50% milkfat.

II. Milk (cows, goat's and/or sheep's) for cheese manufacturing can be pasteurized or used raw, in which case the cheeses must be cured at temperature ≥ 35 °F for ≥ 60 days.

III. Milk for cheese-making is subjected to the action of safe lactic-acid-producing or flavor-producing bacteria.

IV. Salt may be added to curds at some point of manufacturing after drainage of whey.

V. Mold-inhibiting ingredients can be added to the surface of cheese, and subject to limits under the current good manufacturing practices.

21 CFR Part 133.187

I. Semi-soft cheeses contain 39% < moisture ≤ 50%; solids comprised of ≥ 50% milkfat.

II. Milk (cows, goat's and/or sheep's) for cheese manufacturing can be pasteurized or used raw, in which case the cheeses must be be cured at temperature ≥ 35 °F for ≥ 60 days.

III. Milk for cheese-making is subjected to the action of safe lactic-acid-producing or flavor-producing bacteria.

IV. Salt may be added to curds at some point of manufacturing after drainage of whey.

V. Mold-inhibiting ingredients such as sorbic acid, potassium sorbate, and sodium sorbate can be added up to 0.3 % by weight (21 CFR133.187).

Specifications listed are relevant to the hard and semi-soft cheeses tested in this study. In addition, a few soft cheeses such as Feta, Queso Fresco and Queso Blanco were also included in our research study. CFR lists the standard requirements for soft ripened cheeses such as Feta, but not for fresh soft cheeses such as Queso Fresco and Queso Blanco. In this study, 27 types of cheese from different manufacturers, and different batches of a given variety from

similar manufacturer were inoculated with pathogenic bacteria in challenge studies to evaluate pathogen behavior. Of the 27 cheese types tested in this study, ten cheese types have S.O.I. specified in the CFR, as shown in Table 1-1. The remaining 17 cheeses that do not yet have an established S.O.I. are listed in Table 1-2.

Table 1-1: Standard of Identity (S.O.I.) and typical compositions of 10 cheese types that were included in this research study (Adapted from Bishop and Smukowski 2006).

Cheese	S.O.I.[1]	CFR Moisture limit (%)[2]	Typical Moisture (%)[3]	Typical pH[4]
Asiago (Fresh)	133.102	45	40	5.2-5.5
Asiago (Medium)	133.103	35	32-34	5.2-5.5
Brick	133.108	44	43	5.3
Cheddar	133.113	39	38	5.2
Colby	133.118	40	39	5.2
Gouda	133.142	45	41	5.3
Monterey Jack	133.153	44	38-42	5.25
Muenster	133.160	46	43	5.2
Parmesan	133.165	32	31	5.4
Provolone	133.181	45	42.5	5.2

[1]S.O.I. : Standard of identity for cheeses listed in FDA CFR 133.
[2]CFR Moisture Limit (%): the maximum moisture content allowed for specific cheeses as listed in FDA CFR 133.
[3]Typical moisture percentage in cheeses
[4]Typical pH in cheeses.

Table 1-2: Cheeses (17 types) that were included in this study that do not yet have a S.O.I. specified in FDA CFR Title 21, Part 133.

1. Cheddar-Mozzarella	7. Reduced-fat Cheddar	13. Queso Blanco
2. Colby Jack	8. Reduced-fat Colby Jack	14. Queso Chihuahua
3. Farmer's	9. Reduced-sodium Colby Jack	15. Queso Fresco
4. Feta	10. Reduced-Fat Provolone	16. Queso Quesadilla
5. Havarti	11. Reduced-Sodium Provolone	17. String
6. Pepper Jack	12. Provolone-Mozzarella	

The S.O.I. specify the limits for moisture and milkfat content in cheeses, whereas other compositional factors that could influence bacterial behavior such as pH and salt content are not specified, suggesting that cheese type may not be a reliable factor in assessing cheese safety in all cases. Compositional variation among different batches and different manufacturers of a

similar cheese type has yet to be explored, and a reliable predictor for pathogen growth hasn't yet been established.

1.2 REGULATORY FRAMEWORK OF CHEESE SAFETY ASSESSMENT

1.2.1 Time/Temperature Control for Safety

Based on scientific findings, the FDA (U.S. Food and Drug Adminstration) has developed a framework using pH and water activity as criteria to facilitate the safety assessment of food (Table 1-3). The tolerable ranges shown in Table 1-3 were established by taking into account the possible contamination of spores and pathogenic bacteria on food products that are not heat-treated, or are heat-treated but not packaged (100). According to the FDA Food Code, food products that are classified as Time/Temperature Control for Safety (TCS) foods must be kept at ≤ 5°C. If a TCS food is removed from refrigeration, it can be held up to 6h if kept under 21°C, after which the food must be discarded (100).

Table 1-3: Interaction of pH and a_w for determining shelf stability of ready-to-eat food products that have not been heat-treated or heat-treated but without packaging to protect against microbial contamination (Adapted from FDA Food Code)

Critical a_w values	Critical pH values			
	< 4.2	4.2 to 4.6	>4.6 to 5.0	>5.0
<0.88	Non-TCS	Non-TCS	Non-TCS	Non-TCS
0.88 to 0.90	Non-TCS	Non-TCS	Non-TCS	Product Assessment Required
>0.90 to 0.92	Non-TCS	Non-TCS	Product Assessment Required	Product Assessment Required
>0.92	Non-TCS	Product Assessment Required	Product Assessment Required	Product Assessment Required

Most natural cheeses, once manufactured, do not undergo further processing steps that would eliminate pathogenic bacteria. When TCS status of cheese is assessed based on Table 1-3,

most cheese varieties are classified as TCS food and thus require time/temperature control. The evaluators have the option of reformulating TCS food products to create a less favorable condition for pathogen growth, and achieving the non-TCS status (100). However, cheese manufacturers are unlikely to attempt product reformulation given the S.O.I. of certain cheeses and also the desire of manufacturers to preserve the sensory quality of cheese. Hence, the common action that has been taken is to conduct a further product assessment to confirm the safety status of cheeses.

FDA listed out the factors to be considered during a product assessment:

I. Potential pathogens
II. Intrinsic inhibitory factors in the food product, including preservatives, antimicrobials, humectant, acidulants and nutrients;
III. Extrinsic factors, including packaging, atmosphere, use/shelf life, and temperature range of storage and use
IV. Effectiveness of the processing for control of pathogens; and
V. Possible post-process recontamination opportunities that may be present.

According to the FDA, TCS status of food products such as cheeses could not be determined solely based on historical information, e.g., implication in foodborne illness cases etc., especially in the case where the intrinsic and extrinsic factors of food are not constant. Microbial growth models such as the USDA Pathogen Modeling Program and ComBase Predictor (63) could be useful in evaluating the potential behavior of pathogenic bacteria in food, but the use of these models could be restrictive due to a lack of data on specific pathogenic bacteria. To further confirm the TCS status of a food product such as cheese, the FDA recommends gathering data from challenge studies (100). There are many factors to be

considered when conducting a challenge study. The National Advisory Committee on Microbiological Criteria for Foods (NACMCF) has published guidelines on conducting product assessments or challenge studies to understand pathogen behavior in a variety of foods (68).

1.2.2 FDA RISK ASSESSMENT OF LISTERIA MONOCYTOGENES IN READY-TO-EAT FOOD

In a risk assessment of *Listeria monocytogenes* on ready-to-eat (RTE) foods conducted by FDA, cheeses were categorized into six groups, see below (97). The categorization was based on cheese moisture content, and some of these categories were defined in Title 21 of CFR as well.

I. Fresh soft cheese: >50% moisture (i.e., Queso Fresco, Queso de Puna, Queso de Creama)
II. Soft unripened cheese: >50% moisture (i.e., Cottage cheese, Cream cheese, Ricotta)
III. Soft Ripened cheeses: >50% moisture (21 CFR 133.182) (i.e., Brie, Camembert, Feta)
IV. Semi-Soft cheeses: 39-50% moisture (21 CFR 133.187) (i.e. Brick, Monterey, Muenster)
V. Hard cheeses: ≤ 39% moisture (21 CFR 133.150) (i.e., Cheddar, Colby, Parmesan)
VI. Processed cheeses: ≤43% moisture (21 CFR 133.169) (i.e., cheese foods, spreads and slices)

Factors that were considered by the FDA in the risk assessment included the amount of consumption, frequency and level of contamination at retail, potential of food to support growth during storage, and the typical storage length prior to consumption. These factors were characterized for the six cheese categories (Table 1-4) and were used by the FDA panels/experts to predict the relative listeriosis risk associated with each cheese category (Figure 1-2).

Table 1-4. Summary of data used to model *L. monocytogenes* exposure for each cheese category relative to one another. (Adapted from FDA 2003 risk assessment of ready-to-eat potential hazardous foods)

Cheese Categories[1]	Annual Servings[2]	Median Amount Consumed[3]	Contamination Frequency[4]	Contamination level at retail[5]	Growth rate during home storage[6]	Storage time[7]
Fresh soft	Low	Low	Low	Low	Low	Moderate
Soft Unripened	Moderate	Low	Moderate	Moderate	Low/NA	Long
Soft ripened	Moderate	Low	Moderate	Low	Low/NA	Long
Semi-soft	Moderate	Low	Moderate	Low	Low/NA	Long
Hard	Moderate	Low	Low	Low	Low/NA	Long
Processed	High	Low	Low	Low	Low/NA	Long

[1]Cheese categories: Cheeses were categorized largely based on moisture content. "Fresh soft", "Soft Unripened" and "Soft ripened" contain > 50% moisture; "semi-soft" 39-50%; "Hard" ≤39%.
[2]Number of Annual servings: "Low" = ≤1 x 10^9; "Moderate" = >1 x 10^9 to < 1 x $10^{10;}$ "High" ≥ 1 x 10^{10}
[3]Median amount consumed per serving (g): "Low" = ≤40g
[4]Contamination frequency: "Low" ≤ 2%, "Moderate" > 2% to <5%.
[5]Exponential growth rate at 5°C (log CFU/day): "Low" = ≤ 0.1. "NA"= Decline of *L. monocytogenes* during storage.
[6]Storage time: "Moderate: = >2-5 days; "Long" = ≥6-10 days.

Among all cheese categories, the "Soft Unripened Cheese" category comprised of cottage cheese, cream cheese, and ricotta, etc., was the only group designated as high risk (Figure 1-2). These cheeses have moderate contamination frequency and level at retail, and the long storage time may allow sufficient time for *L. monocytogenes* to grow to a high concentration. The overall consumption of these cheeses by the larger population is high, and this increases the predicted risk per annum for this cheese category. However, the low serving size for this cheese group helps decrease the risk per serving. A wide pH variation among different types of soft unripened cheese could result in significant differences in terms of the cheese ability to support growth. Designation of risk based on cheese category as a whole may overestimate the risk for soft unripened cheese with low pH, such as cream cheese (pH 4.8) which was shown not to support growth of *Listeria monocytogenes*. Hence, the FDA recommended subdivision of soft unripened cheese for better risk assessment (97).

Figure 1-2. Two-dimensional matrix of food categories based on cluster analysis predicted per serving and per annum relative rankings (Adapted from FDA 2003 risk assessment of ready-to-eat potential hazardous foods)

Decreased Risk per Annum →

Decreased Risk per Serving ↓

Cluster A and B	Cluster C and D	Cluster E	
Very High Risk (Cluster 1-A, 1-B) Deli Meats Frankfurters (not reheated)	**High Risk (Clusters 1-C, 1-D)** Pate and Meat Spreads Unpasteurized Fluid Milk Smoked Seafood	**Moderate Risk (Cluster 1-E)** No food categories	**Cluster 1**
High Risk (Clusters 2-A, 2-B) High Fat and Other Dairy Products Pasteurized Fluid Milk Soft Unripened Cheese	**Moderate Risk (Clusters 2-C, 2-D)** Cooked RTE Crustaceans	**Moderate Risk (Cluster 2-E)** No food categories	**Cluster 2**
Moderate Risk (Clusters 3-A, 3-B) No food categories	**Moderate Risk (Clusters 3-C, 3-D)** Deli-type Salads Dry/Semi-dry Fermented Sausages Frankfurters (reheated) Fresh Soft Cheese Fruits Semi-Soft Cheese Soft Ripened Cheese Vegetables	**Low Risk (Cluster 3-E)** Preserved Fish Raw Seafood	**Cluster 3**
Moderate Risk (Clusters 4-A, 4-B) No food categories	**Low Risk (Clusters 4-C, 4-D)** No food categories	**Very Low Risk (Cluster 4-E)** Cultured Milk Products Hard Cheese Ice Cream and Other Frozen Dairy Products Processed Cheese	**Cluster 4**

Three cheese groups: "Soft Unripened", "Soft-Ripened" and "Semi-Soft" cheeses were categorized as moderate-risk RTE foods due to their low contamination frequency and level at retail, and low growth rate of *L. monocytogenes* in these cheeses. "Soft Unripened" cheese such as Queso Fresco has been implicated in foodborne illness outbreaks in the past, notably the 1985

outbreak in Los Angeles that resulted in 142 listeriosis cases and 28 deaths. Despite the frequent association of Queso Fresco with outbreaks of listeriosis, the risk for "Soft Unripened" cheeses was characterized as moderate instead of high because the data used in this risk assessment do not represent the outbreak-linked soft cheeses, which were made mostly with unpasteurized milk. "Soft Unripened" cheeses include mold- and surface-ripened cheeses such as Brie, Camembert, and white brined cheese such as Feta, which contain moisture > 50%. "Semi-soft" is a large cheese category, comprised of cheese varieties with moisture ranging between 39-50%, including Brick, Edam, Gouda, Havarti, and Monterey Jack (97). "Hard" cheeses typically have high salt and low moisture content (<39%), and this presents an unfavorable condition for pathogen growth. In addition, given the low contamination level and frequency at the retail, this cheese group has been consistently labeled as low risk. Examples of hard cheeses include Cheddar, Emmentaler, Parmesan, Romano, and Swiss (97).

The FDA assessment of the risk of growth of *L. monocytogenes* has provided a good foundation to understanding the relative risk associated with the consumption and handling of different cheese categories. However, the broad categories could be too general for reliable use in assessment of cheese safety. Also, the FDA assessment was conducted based on bacterial growth outcomes under refrigerated conditions. To better understand the safety status of cheeses under nonfrigerated condition and to avoid overgeneralization, more scientific data are needed to analyze the influence of cheese compositional factors upon bacterial survival.

1.3 CHEESE SAFETY WITHOUT TIME/TEMPERATURE CONTROL

From manufacturing to retailing, there are scenarios where cheeses might not be held under refrigerated temperature for practical reasons. For example, cheeses are often held under non-refrigerated condition in the warehouse or during distribution in order to support the

ripening process or for energy conservation (69). In some food service situations, cheese slices are tempered under room temperature for up to 8 hours to shorten the melting time during the preparation of hot sandwiches or burgers (102). In retail markets, there is a huge incentive for retailers to have the flexibility to display cheeses in eye-catching areas in order to attract the attention of customers. A marketing study conducted by the Wisconsin Milk Marketing Board (WMMB) indicated a significant increase in sales (1300%) for a type of hard cheese, during the month when the participating grocers introduced the "Barrel Program" (61). In this program, the grocers displayed vacuum packaged non-refrigerated pre-cut hard cheese wedges on top of a wheeled barrel during the day; the grocer would wheel the barrel back into the cooler at night. The sales of the particular cheese remained strong (800% increase) during the following 2 months after the 1-month barrel program ended and the cheeses were placed back in the refrigerated display case in the grocery store. Due to the impressive outcome, the "Barrel Program" has been launched in liquor stores to enable the display of cheese and wine pairings outside refrigerated areas. With this successful example, it is not surprising that other cheese retailers would be very interested to follow similar strategies. All these situations however led to questions about the safety of cheeses under extended non-refrigerated conditions.

The safety and regulatory concern about non-refrigerated holding of cheese has been longstanding, and was discussed in a publication by Nelson back in 1983 (69). Nelson argued that the decision for holding cheese out of refrigeration can be made based on cheese moisture content. It was suggested that hard cheeses with less than 42% moisture can be kept $\leq 25^{o}C$ for no more than 1 week, whereas cheeses with > 42% should be kept refrigerated for quality preservation. The author also suggested that cheeses in opened packages should be protected from contamination and held refrigerated, regardless of the moisture content. In addition to low

moisture content, Nelson also stated that a proper fermentation process using active and rigorous starter culture contributed to the safety of natural cheeses, and one way to confirm the fermentation status is by measuring the pH of cheeses 24h post-production (69).

In a more recent publication titled “Storage Temperatures Necessary to Maintain Cheese Safety”, J.R. Bishop and M. Smukoswki from the Wisconsin Center for Dairy Research (CDR), approached the topic of non-refrigerated cheese safety based on literature findings and historical outbreak data (17). Consistent with Nelson’s claim, the authors suggested that fermented dairy products such as cheese present unfavorable conditions for the growth of pathogenic bacteria. Factors that could inhibit pathogenic bacteria growth include the presence of active starter culture that serves as competition, the production of an lactic acid that decreases the pH of cheese, and the production of antimicrobial metabolites (17).

Recognizing the compositional variation among cheese varieties, Bishop and Smukowski approached the safety analysis by assessing cheese type individually. The authors discussed each of the following cheeses: Asiago (medium and old), Cheddar, Colby, Feta, Monterey Jack, Mozzarella, Muenster, Parmesan, Provolone, Romano, Swiss, Brick, Blue and Soft/Hispanic cheeses (Table 1-5). There were very few or no scientific data available to indicate if pathogenic bacteria would grow on the following cheeses: Asiago (medium/old), Monterey Jack, Muenster, and Provolone. For cheeses that were tested previously by other studies, the authors suggested that cheeses that contain <50% moisture, made with active starter cultures and with a typical level of pH, salt, fat, etc., would not support pathogenic bacterial growth when held between 4 and 30°C (17). With these findings, the authors recommended time/temperature control for safety exemptions for the following cheeses: Asiago (medium/old), Cheddar, Colby, Feta, Monterey Jack, Muenster, Pasteurized process, Parmesan, Provolone, Romano, and

Swiss/Emmentaler. The recommendation was made with the assumption that these cheeses fulfilled the following criteria:

I. Using pasteurized or heat treated (≥63°C for ≥16s) milk,
II. Following Good Hygienic Practices (GHP) and Good Manufacturing Practices(GMP),
III. Under applied Hazard Analysis and Critical Control Points (HACCP) systems,
IV. With active starter cultures, and
V. Meeting any applicable Standard of Identity specified in 21 CFR Part 133 (101).

Table 1-5: List of cheeses reviewed by Bishop and Smukoswki in determining the safety of holding cheese under non-refrigerated condition (<30°C), with the assumption that these cheeses were made with pasteurized or heat-treated milk (≥63°C for ≥ 16s), active starter cultures and following GMP, GHP, HACCP, and CFR specifications.

Categories	Cheeses	Typical Moisture content (%)	Recommendation
Hard: ≤39% moisture	Asiago (aged)*	32-34	Potentially Safe; more research needed
	Cheddar*	38	To be Exempted from TCS
	Parmesan*	31	To be Exempted from TCS
	Romano	33.5	Potentially Safe; more research needed
Semi-soft: >39% to ≤ 50% moisture	Asiago (fresh/soft)*	40	Potentially Safe; more research needed
	Blue	43	Potentially Safe; more research needed
	Brick*	43	Potentially Safe; more research needed
	Colby*	39	To be Exempted from TCS
	Monterey Jack*	38-42	Potentially Safe; more research needed
	Muenster*	43	Potentially Safe; more research needed
	Provolone*	42.5	Potentially Safe; more research needed
	Swiss*/ Emmentaler	38	To be Exempted from TCS
Soft Ripened: >50% moisture	Feta*	53	To be Exempted from TCS
	Mozzarella*	45-52	Potentially high risk
Soft Unripened: ≤55% moisture	Queso Fresco*, Queso Blanco*	50-55	Potentially high risk
Process: ≤43% moisture	Pasteurized process	40	To be Exempted from TCS

*Cheeses were tested for their ability to support pathogenic bacteria growth in this study.

The review paper (17) provided a comprehensive summary of the ability of cheeses to support the growth of pathogenic bacteria under non-refrigerated conditions. However, the analysis in this article mostly involved cheeses that have a S.O.I.. According to the International Dairy Federation, there are more than 500 varieties of cheeses being commercialized in the United States (20). Moreover, cheeses of the same type could potentially have significant variation in pH and moisture content from batch to batch, and even greater variation from manufacturer to manufacturer. Furthermore the variation of composition is likely to be greater for cheeses that have no S.O.I.. The reliability of cheese safety assessment based on types of cheese remained to be confirmed.

1.4 PATHOGENS OF INTEREST

1.4.1 FOODBORNE ILLNESS OUTBREAKS RELATED TO CHEESES

Cheeses have been implicated in foodborne illness outbreaks in the past. In 2014, a review paper titled “Outbreaks attributed to cheese: differences between outbreaks caused by unpasteurized and pasteurized dairy products, United States, 1998-2011” was published by researchers from the Centers for Disease Control and Prevention (38). During one 14-year period from 1998 to 2011, there were 90 outbreaks linked to cheeses, with 38 outbreaks (42%) involving cheeses manufactured with unpasteurized milk, and 44 outbreaks (49%) implicating cheeses made with pasteurized milk. The pasteurization status for cheeses in 8 outbreaks (9%) remained unidentified. Effective pasteurization should eliminate pathogenic bacteria from milk, hence contamination during manufacturing and handling procedures after pasteurization are likely the causes of outbreaks involving cheeses made from pasteurized milk. The following discussion will focus on cheeses made from pasteurized milk, which is the subject of this dissertation.

Among the 44 outbreaks linked to cheeses made with pasteurized milk, cheese type was reported in 36 outbreaks. Fresh soft cheeses such as Queso Fresco, other Mexican-style cheeses, cream cheese, and Mozzarella were the leading cheese types linked to the outbreaks, causing 13 outbreaks total. Despite having lower moisture content, the hard cheeses Cheddar and Swiss were implicated in 3 outbreaks each. The majority of the illness outbreaks were caused by *Listeria monocytogenes* (8 cases, 24% of total outbreaks) and *Salmonella* spp. (6 cases, 18%). Each of the following pathogenic bacteria was implicated in one outbreak during the 14-year period: Shiga toxin-producing *Escherichia coli* (STEC), *Staphylococcus aureus*, *Bacillus cereus*, *Campylobacter* and *Shigella* (38).

In other publications, pathogenic bacteria such as *L. monocytogenes*, *Salmonella* spp., pathogenic *E. coli* and *S. aureus* have been described as the major concerns for the dairy industry (29, 44). Based on surveillance data from multiple countries, including France, the United States, and several other European countries, De Buyser et al. summarized the 60 outbreaks linked to contaminated dairy products, including milk and cheeses, which happened in 1980-1997(29). The researchers focused their study solely on pathogenic bacteria that were considered as a major threat to dairy food safety, and the following pathogenic bacteria were reported for cheeses made with pasteurized milk: *Salmonella* spp. caused 2 outbreaks, *L. monocytogenes* 1 outbreak, pathogenic *E. coli* 1 outbreak, and *S. aureus* 2 outbreaks.

Clearly, outbreak data and summaries from these studies suggest that *L. monocytogenes, Salmonella* spp., pathogenic *E. coli,* and *S. aureus* are the relevant pathogenic bacteria to be considered when assessing cheese safety.

1.4.2 *Escherichia coli* O157:H7

E. coli is a Gram negative, non-spore forming facultative anaerobe. This rod shaped bacterium belonging to the family Enterobacteriaceae is naturally present in the intestine of warm-blooded animals as a harmless species, and can be released to the environment along with feces (90). Hence, *E. coli* has been taken as an indicator for fecal contamination in water and food (31). The majority of *E. coli* strains are non-pathogenic to humans. The first recognition of Shiga-toxin producing *E. coli* (STEC) O157:H7 as a foodborne pathogen was when this serotype was linked to a hemorrhagic colitis outbreak in 1982 associated with contaminated hamburgers sold at a fast-food chain (79). Other serogroups of pathogenic *E. coli* that have been recognized as leading causes of infection in the United States include *E. coli* O26, O103, O111, O121, O45, and O145 (62). Farrokh et al. stated that the dairy industry considered *E. coli O157:H7* as a major threat due to its high virulence, with infectious dose as low as 5-50 cells (16, 30).

Infection by pathogenic *E. coli* can lead to symptoms and illnesses including abdominal cramp, mild to bloody diarrhea, and hemolytic uremic syndrome (HUS) (7). In the past, *E. coli* O157:H7 outbreaks have been associated with consumption of undercooked meat, contaminated water, raw milk, cheeses made with raw or unpasteurized milk, and other reay-to-eat food that were contaminated post-processing (30). Table 1-7 shows that *E. coli* O157:H7 was implicated in 3 outbreaks that involved cheeses made with unpasteurized milk; while three non-O157 STEC serotypes were implicated in 1 outbreak related to processed cheese made with pasteurized milk (22).

In fact, the frequency of outbreaks involving *E. coli* O157:H7 in cheese is low in comparison to *Salmonella* spp. and *Listeria monocytogenes* (Table1-6 to 1-8), and this might be a result of a low prevalence of *E. coli* O157:H7 in the dairy processing environment. In a

surveillance study conducted by Ansay et al. (3), *E. coli* was found in 35% of the total samples taken from six varieties of soft and semi-soft cheeses: Brie, Camembert, Colby, Havarti, Muenster and Monterey Jack, but none of these samples tested positive for *E. coli* O157:H7. In addition, 42 raw milk samples and 1104 environmental samples also tested negative for *E. coli* O157:H7. Based on these results, the researchers concluded that *E. coli* O157:H7 is not prevalent in dairy ingredients, cheeses, and the dairy processing environment (3). Despite the low prevalence of *E. coli* O157:H7, good manufacturing and hygiene practices should be strictly followed in dairy manufacturing and processing plants, and preventive controls should be in place to prevent cross-contamination.

E. coli O157:H7 is capable of growing under acidic conditions, with minimum pH for growth ranging between 4.0-4.5, depending on the influence of other growth parameters (19). The optimum temperature for *E. coli* O157:H7 growth is 37°C, however it is also able to grow at temperature as low as 7-8°C under optimal conditions (72). Most studies (60, 78, 81) investigated the survival of *E. coli* O157:H7 on cheeses made with raw milk, because of the previous linkages between *E. coli* O157:H7, dairy cattle, raw milk (51, 54, 109). However, there are fewer studies on post-processing contamination of *E. coli* O157:H7 on cheeses made with pasteurized milk, probably due to the very low outbreak frequency linked to these cheeses (Table 1-6). Kasrazadeh et al. investigated the growth of *E. coli* O157:H7 on Queso Fresco, a soft Hispanic-style unripened cheese. Growth of *E. coli* O157:H7 was observed in this high pH cheese (pH 6.6) when it was vacuum packaged and stored at 10, 12, 16, 20 and 30°C, whereas no growth was observed on cheeses that were kept at 8°C for two months (50). More studies are needed given the high virulence of *E. coli* O157:H7 and also the lack of data for its survival on cheeses under post-processing contamination scenarios.

Table 1-6: Pathogenic *Escherichia coli* outbreaks linked to cheeses from 1998 to 2015. Data were sourced from CDC Foodbone outbreaks online database (FOOD).

Year	State	Serotype	Illnesses	Hospitalizations	Food Vehicle
1998	Wisconsin	O157:H7	63	24	Cheddar cheese, unpasteurized
2007	Colorado	O121; O26; O84	135	10	American cheese, pasteurized
2010	Multistate	O157:H7	38	15	Gouda cheese, unpasteurized milk
2010	Multistate	O157:H7	8	-	Multiple cheeses, unpasteurized

1.4.3 *Listeria monocytogenes*

Listeria monocytogenes, first reported in 1926 in Cambridge, UK, is a Gram positive, non-sporulating, facultative anaerobic pathogenic bacterium (66, 108). Among the multiple species under the genus *Listeria*, only *L. monocytogenes* and *L. ivanovii* are recognized as mammalian pathogens. *L. ivanovii* is less threatening for the human in comparison to *L. monocytogenes*; it is rarer and mostly causes illnesses in ruminants (71). *L. monocytogenes* has the capability to tolerate and grow under a wide ranges of temperatures (-0.1 to 45°C), pH (3.0-9.5), and salt concentrations (up to 10%) (108) The ability of *L. monocytogene*s to grow at standard refrigeration temperature (≤4°C) makes this pathogen a hazard for the ready-to-eat food industry must consider under a food safety system.

Illness linked to *L. monocytogenes* is known to have a high mortality rate, approximately 20-30% of infection cases, and ingestion of contaminated food was thought to be responsible for 99% of the reported causes of listeriosis (8, 64). Symptoms of infection such as gastroenteritis are usually self-limiting for healthy individuals, whereas the infection can be life-threatening for immunocompromised individuals, including infants, pregnant women, and the elderly (74). *L. monocytogenes* is capable of surviving the contact with stomach acid and crossing the intestinal

barrier and spreading to lymph nodes, liver, and spleen. In healthy hosts with strong immune systems, macrophages will work to eliminate the bacterial pathogen from the liver. For immunocompromised individuals, the attack by macrophages may not be as effective, leading to survival of some *L. monocytogenes* cells, which subsequently can proliferate to high concentrations in the liver, and spread to attack the central nervous system. As a result, diseases such as sepsis, meningitis, cerebritis, and rhombencephalitis could arise and cause fatalities (39, 106). Stillbirths and miscarriages can happen when pregnant women contracted severe infection from *L. monocytogenes*, as the bacterium is able to infect the fetus through the placenta (10)

In the past, the types of food that have been implicated in *L. monocytogenes* outbreaks included dairy (soft ripened and unripened cheeses, ice cream, pasteurized chocolate milk, pasteurized and unpasteurized milk), meat (hot dogs, processed meat), fish and shellfish (smoked salmon, cooked shrimp), vegetables (potato salad, coleslaw) and fruits (cantaloupe, caramel apple) (34). The largest listeriosis outbreak in the U.S. involved contaminated cantaloupe, resulting in 143 hospitalizations and 33 deaths (21). Other major outbreaks include the 1983 outbreak in Boston, Massachusetts in which pasteurized milk was implicated, and the 1985 outbreak in Los Angeles, California that involved Queso Blanco. These outbreaks in 1980s prompted the FDA to a establish zero-tolerance policy for *L. monocytogenes* in cooked and ready-to-eat food (82). On the other hand, the Codex Alimentarius Commission (CAC) has adopted a limit of 100 CFU/g *L. monocytogenes* in food that will not support growth of the organism (24). This recommendation aligns with the conclusion of a 2004 FAO/WHO *Listeria* risk assessment, which suggested that 100 CFU/g of *L. monocytogenes* does not threaten public health (33). Discussions have been ongoing to revise the zero-tolerance policy, but the FDA has

remained firm on the policy up to this point due to uncertainties if the 100 CFU/g limit would effectively protect the immunocompromised population (73).

In the background document that supported the Center for Food Safety and Applied Nutrition (CFSAN) Food Advisory Committee (FAC) meeting in 2015, hard cheeses with <39% moisture were labelled as ready-to-eat food that do not support growth of *L. monocytogenes*, and soft unripened cheeses with >50% moisture content was stated to be able to support growth of *L. monocytogenes* (5). Queso Fresco/Mexican-style cheeses with typical high moisture content (>50%) reportedly are very likely to support growth of *L. monocytogenes* (50). And these are also the major cheeses types implicated in outbreaks linked to *L. monocytogenes* from 1998-2015, as shown in Table 1-7 (22). Previous research study has evaluated the survival of *L. monocytogenes* on a variety of cheeses, including Parmesan, Cheddar, Colby, Provolone, Monterey Jack, Colby, Muenster, String cheese, and Queso Fresco, under different storage conditions (37). While these data are helpful, more data are needed to create useful and reliable guidelines to assess the likelihood of growth of *L. monocytogenes* on cheeses under extended non-refrigerated holding.

Table 1-7: *Listeria monocytogenes* outbreaks linked to cheeses from 1998 to 2015. Data were sourced from CDC Foodbone outbreaks online database (FOOD).

Year	State	Illnesses	Hospitalizations	Deaths	Food Vehicle
2003	Texas	12	12	1	Queso fresco, unpasteurized
2006	Oregon	3	2	1	Other cheese, pasteurized
2008	Multistate	8	8	0	Mexican style cheese, pasteurized
2009	Multistate	8	3	0	Mexican style cheese, pasteurized
2009	Multistate	18	11	0	Mexican-style cheese
2010	Multistate	6	4	1	Mexican style cheese, pasteurized
2010	Multistate	34	32	4	Cheese, pasteurized
2011	Michigan	2	2	1	Ackawi cheese, pasteurized; chives cheese, pasteurized
2011	New Jersey	2	2	0	Mexican style cheese, pasteurized
2011	Multistate	15	1	1	Blue-veined cheese, unpasteurized
2012	Multistate	23	21	5	Ricotta salata cheese

2013	Multistate	6	6	1	Cheese-le frere
2013	Multistate	8	7	1	Latin style soft cheese
2013	Multistate	9	8	1	Mexican style cheese, pasteurized
2014	Washington	3	2	1	Mexican style cheese, pasteurized

1.4.4 *Salmonella* spp.

Bacteria in the genus *Salmonella* are Gram-negative, rod-shaped, and non-sporulating pathogens. These flagellated facultative anaerobes belong to the family Enterobacteriaceae. The genus *Salmonella* consists of two species: *S. enterica* and *S. bongori* (91). Six subspecies have been recognized under the species *S. enterica*, they are *S. enterica* subsp: *enterica*, *salamae*, *arizonae*, *diarizonae*, *indica* and *houtenae*, or I, II, IIIa, IIIb, IV, and VI, respectively (75). Almost all of the *Salmonella* infections in warm blooded animals are linked to *S. enterica* subsp. *Enterica* (Subspecies I). More than 2300 serovars have been identified and classified under the Subspecies I, while the three serovars that cause the majority of the infections are Typhimurium, Enteritidis and Newport (23, 76). Depending on an individual's immune system, the infectious dose can range from 10^3-$10^{5.5}$ CFU (32). Usually, the symptoms of salmonellosis including nausea, vomiting, diarrhea, and enteric fever, are self-limiting; however chronic diseases such as aseptic reactive arthritis and Reiter's syndrome can also be induced by *Salmonella* infection (2).

Salmonella spp. are able to survive and grow under temperature ranging from 5-45°C under ideal conditions, with a more limited temperature range, 25-35°C, at pH 3.94 and a_w at 0.942 (53, 59). *Salmonella* spp. are the leading foodborne pathogenic bacteria in the United States, causing the highest number of outbreaks, hospitalizations, and deaths (9). Chicken, pork, eggs, fruits and nuts are commonly associated with salmonellosis outbreaks (22). De Buyser et al reviewed outbreaks linked to milk and milk products that happened in France and 6 other countries in 1983-1997, and found that *Salmonella* spp. were also the leading cause of outbreaks linked to dairy products (29). Similar findings have been reported in a more recent study that

focused on outbreaks linked to cheeses in the United States, where study authors noted that *Salmonella* spp. and Queso Fresco or other Hispanic-style cheeses made with unpasteurized milk were the most common cheese-pathogen pairs, causing 10 outbreaks in total (38). The CDC database shows that cheeses were implicated in 23 *Salmonella* outbreaks from 1998-2015. Details of the cheeses were not specified for some cases, but it is shown that cheeses made with either pasteurized or unpasteurized milk were involved in outbreaks previously (Table 1-8).

The intestinal tract of farm animals is a major niche for *Salmonella* spp., and fecal shedding of *Salmonella* spp. in dairy farms has been reported (18, 34, 110). The results of these studies indicate that contaminated milk in the farm is likely to be the cause of outbreaks related to raw milk cheeses. In comparison to *L. monocytogenes*, there were fewer findings of *Salmonella* spp. within dairy processing plants; samples from dairy plants were tested negative for *Salmonella* spp. in the study by Cotton et al. (26). The low prevalence of *Salmonella* spp. in dairy processing plants plus the effectiveness of standard pasteurization in killing the pathogen could explain the lower number of outbreaks linked to *Salmonella* in cheeses made with pasteurized milk (6 outbreaks) in comparison to raw milk cheeses (13 outbreaks) (38).

A few research groups have investigated the survival of *Salmonella* in cheeses made with raw milk, and found that the pathogen is capable of surviving and persisting in this food matrix (1, 4, 65). For Cheddar cheeses made with raw milk and pasteurized milk inoculated with *Salmonella Enteritidis*, Modi et al. observed a 2-log decrease of the pathogen under 8°C storage, and the bacterial population remained at a relatively high concentration (10^3 CFU/g) after 99 days of storage (65). Several studies have investigated the survival of *Salmonella* spp. as a post-processing contaminant of cheeses that were made with pasteurized milk (49, 56, 84). On the following cheeses that were inoculated post-processing, Mozzarella, Cheddar and Swiss, with pH

5.3, 5.2, and 5.6 respectively, populations of both acid-adapted and non-adapted *S. typhimurium* decreased significantly or died off under 5°C storage up to 80 days (56). Another study reported growth of *Salmonella* spp. on Queso Fresco (pH 6.6) stored at 8-30°C with storage of up to 70 days (49). As there is a lack of understanding on behavior of *Salmonella* spp. on many other hard and semi-hard cheeses during non-refrigerated holding, this information would be helpful in establishing guidelines for cheese safety assessment.

Table 1-8: *Salmonella* spp. outbreaks linked to cheeses from 1998 to 2015. Data were sourced from CDC Foodbone outbreaks online database (FOOD).

Year	State	Serotype	Illnesses	Hospitalizations	Food Vehicle
1998	Oregon	Typhimurium	8	0	homemade cheese, unspecified
1998	Florida		2	1	other cheese, unspecified
1999	Maryland	Typhimurium	49	6	other cheese, unspecified
2000	Pennsylvania	Muenster	27	5	other cheese, unspecified; meat, unspecified
2001	Connecticut	Newport	4	1	cheese, pasteurized
2001	Multistate	Newport	27	12	multiple cheeses, unpasteurized
2002	Michigan	Bovismorbificans	7	2	homemade cheese, unspecified
2003	Wyoming	Heidelberg	65	14	swiss cheese, pasteurized; mushrooms, canned
2003	Florida		4		pizza, cheese
2003	California	Typhimurium	50	7	queso fresco, unpasteurized
2005	North Carolina	Newport	14	1	cake, cream cheese; cheese bread
2006	Illinois	Newport; Meleagridis	96	36	other cheese, unpasteurized
2006	California	Dublin	4	4	mexican cheese (queso fresco and/or other)
2007	Rhode Island	Enteritidis	2	1	blue cheese dressing
2007	Virginia	Typhimurium	33	4	cheese, unspecified; shami kabob
2007	Pennsylvania	Typhimurium	13	1	raw milk; cheese
2007	Multistate	Montevideo	20	9	shredded cheese, pasteurized
2007	Colorado	Montevideo	6	2	multiple cheeses, pasteurized
2008	Arizona	Montevideo	101	21	cheese, unspecified; cilantro, unspecified; chicken, raw
2008	New Jersey	Java	70	0	cheddar cheese
2014	California	Typhimurium	5	1	other cheese, unpasteurized
2015	Florida	Enteritidis	14	0	fettucine alfredo (cream, parmesan cheese)
2015	Multistate	Newport	25	3	latin style soft cheese

1.4.5 *Staphylococcus aureus*

Staphylococcus aureus is a Gram- and catalase-positive bacterium that usually appears in grapelike clusters under microscopic observation (13). This non-sporulating facultative anaerobe is commonly found in the environment, and has been recognized as commensal bacteria in nasal passages and on the skin of approximately 32.4% of the human population (25, 58). The genus *Staphylococcus* can be classified into coagulase positive and coagulase negative groups. *S. aureus*, the main causative agent of staphylococcal food poisoning outbreaks, belongs to the coagulase-positive group (58).

S. aureus is capable of growing under wide ranges of temperature (7-48°C) and pH (4-10) (43). This pathogen is also notable for its ability to survive and grow in salt concentration up to 20%, and under a wide range of water activities (0.83-0.99) (94). Previous studies suggested that the presence of microflora in cheeses could inhibit the growth of *S. aureus* due to the production of acid that leads to pH reduction, the release of inhibitory compounds, and the competition over limited nutrients (36, 40). The inhibitory effect is influenced by the ratio of the population of competitive microflora and the population of *S. aureus*, as well as the environmental temperature (36, 85).

The enterotoxigenic *S. aureus* can produce sufficient toxin to cause food poisoning when growing in a favorable food environment and reaching around 10^5-10^6 CFU/g (13). More than 20 types of staphylococcal enterotoxin have been identified so far, and these toxins have been found to be unaffected by conditions that are effective in killing the bacterial cells, including heat treatment and acidic conditions (42, 89). Typically, in cases of staphylococcal food poisoning, the following five conditions are met: (I) raw ingredients or carrier contain enterotoxin-producing *S. aureus*, (II) the bacteria were cross-contaminated from sources to food, (III)

intrinsic factors within the food promote growth and toxin production, (IV) sufficient time and temperature to allow growth and toxin production, (V) consumption of food that contains sufficient toxin to cause sickness (42). Staphyloccocal food poisoning is characterized by rapid onset (30 min - 8 h) of abdominal pain, diarrhea, vomiting, dizziness, general weakness and occasionally fever, and typically these symptoms are self-limiting within 24-48 h among healthy individuals, whereas hospitalization might be warranted for infants and the elderly (67).

S. aureus has been recognized as the major cause of mastitis among dairy cattle (107), and this pathogen has been isolated from bulk milk samples from dairy farms, dairy processing plants and cheeses (27, 41, 47, 93). The first recorded Staphyloccocal poisoning outbreak was linked to Cheddar cheese consumption in Michigan in 1884 (12). In a surveillance study by the CDC, *S. aureus* was shown to have the lowest cheese-related outbreak frequency in comparison to *E. coli* O157:H7, *L. monocytogenes* and *Salmonella* spp., causing only one outbreak during a 13-year period of 1998-2011(38). The low number of staphylococcal food poisoning outbreak could be a result of good manufacturing and hygiene practices, and also a result of under-reporting. It is suggested that the mild symptoms of Staphylococcal food poisoning in many cases may lead to patients not seeking medical attention, plus the lack of routine clinical testing for this pathogen and toxin may contribute to under-reporting (11, 28).

Recalls of cheeses have occurred in the past due to high level of *S. aureus* in the product (101). To account for potential *S. aureus* contamination, proper time and temperature control is needed to prevent the pathogenic bacteria from growing and producing toxin in cheeses. Thus, information regarding the influence of intrinsic and extrinsic factors of cheeses upon *S. aureus* growth would be helpful in determining cheese safety under non-refrigerated holding conditions.

Table 1-9: *Staphylococcus aureus* poisoning outbreaks linked to cheeses from 1998 to 2015. Data were sourced from CDC Foodborne outbreaks online database (FOOD).

Year	State	Etiology Status	Illnesses	Hospitalizations	Food Vehicle
2002	Washington DC	Confirmed	8	0	other cheese, pasteurized; honeydew melon; potato, fried

1.5 *Listeria monocytogenes* on fresh Hispanic style cheese - Queso Fresco

Queso Fresco is a Hispanic-style unripened soft cheese with crumbly texture and nonmelting properties. This rennet-coagulated cheese is usually made without starter culture, but organic acids such as citric, acetic, or lactic may be added for curd formation. The growing Hispanic population in the U.S. has led to growing demand for Hispanic style cheeses, and Hispanic cheese production has increased from 76 million lbs in 2000 to 249 million lbs in 2015, with Queso Fresco one of the most popular varieties (96). Queso Fresco is a relatively bland-tasting cheese that serves as core ingredient for many Hispanic dishes. In the U.S., the FDA prohibits interstate sale or distribution of dairy products made with unpasteurized milk (21 CFR 1240.61), with the exception of aged cheeses that are subjected to a minimum of 60-day aging at ≥35°F (21 CFR 133) (98). Under this rule, fresh cheeses such as Queso Fresco made in the U.S. should be manufactured and commercialized using only pasteurized milk, with refrigerated shelf life typically ≤ 60 days.

Queso Fresco is a cheese type highly associated with foodborne illness outbreaks, causing 26% (19 outbreaks) of the total outbreaks linked to cheese during 1998-2011 in the U.S. (38). The largest listeriosis outbreak linked to Queso Fresco happened in 1985, in which there were 142 listeriosis cases, and 28 deaths (18 adults and 10 infants), and an additional 20 fetal deaths (57). Since pasteurization can kill *L. monocytogenes* effectively, outbreaks linked to soft cheeses were attributed to either post-processing contamination or the lack of standard

pasteurization. In previous investigations, *L. monocytogenes* has been isolated from dairy manufacturing plants (46, 52, 77). Particularly in a surveillance study of these 3 Latin-style fresh-cheese processing plants, *L .monocytogenes* was found in 6.3% of cheese samples and 11% of the environmental samples, with crates, drains, and floor found to be highly contaminated (48). Since there is no kill step after milk pasteurization, fresh cheeses such as Queso Fresco are highly susceptible to environmental contamination given the labor-intensive manufacturing procedure.

Once present on the cheese, *L. monocytogenes* in Queso Fresco is very likely to grow, due to the high pH and high moisture content of the cheese, and also the ability of psychrotrophic *L. monocytogenes* to grow at ≤4°C. Previous publications reported >4 log growth of *L. monocytogenes* on Queso Fresco stored at 4°C, with the storage length in these studies ranging from 28-40 days. Queso Fresco in these studies had a pH and salt content ranging from 6.08-6.75, and 0.8-1.67%, respectively; moisture content was not reported in all studies but the typical range for this cheese is 45-55% (55, 86, 87). Based on these results, Legget et al. suggested that the addition of antimicrobial compounds and/or the use of other post-processing intervention may be necessary to control the growth of *L. monocytogenes* on Queso Fresco (55).

Generally recognized as safe (GRAS) antimicrobials have been tested for their bactericidal and bacteriostatic effect on *L. monocytogenes*. Lauric alginate (LAE) was able to reduce the initial *L. monocytogenes* population on Queso Fresco, however *L. monocytogenes* grew rapidly afterwards and achieved ≥ 4-log growth within 28-days storage at 4°C. This result was not only observed at the FDA-approved level of LAE (200ppm), but also at a much higher concentration (800ppm) (86). The regrowth issue was also observed in a more recent study, in which bacteriophage P100, LAE, and potassium lactate-sodium diacetate mixture (PL-SD) were

tested singly and combined. The combination of two antimicrobials worked more effectively in reducing the initial *L. monocytogenes* population, however, a slow regrowth was observed during the 28-day storage (87). Given that Queso Fresco typically has a shelf life of up to 60 days, a longer storage time may allow *L. monocytogenes* to grow to higher concentrations despite the slower regrowth under these treatments.

Other GRAS antimicrobial ingredients that have been tested include nisin, caprylic acid and trans-cinnamaldehyde (35). These compounds were found effective in reducing the growth of *L. monocytogenes* on Queso Fresco at 4°C in comparison to the control. However, the majority of the strains still exhibited a 1-4 log growth at the end of 20-days of storage even with the antimicrobial treatments. The authors suggest that *L. monocytogenes* may be able to adapt to certain antimicrobials and regain the ability to grow to a high level (35). Another post-processing intervention that has been tested for Queso Fresco is high-hydrostatic pressure processing (HPP). Tomasula et al. found that a 20-min 600MPa HPP treatment was able to reduce the population of *L. monocytogenes* to below the detection limit (0.91 log CFU/g), however they observed significant regrowth of *L. monocytogenes* after a lag time that ranged from 7-28 days at 4°C storage. At the end of 60-days storage, the final population of *L. monocytogenes* ranged from 4-8 logs CFU/g, depending on the length of the HPP treatment and the location of *L. monocytogenes* (within the cheese matrix or on the surface). Based on these findings, the authors suggested that a combination of HPP and antimicrobial compounds might be effective in controlling the growth of *L. monocytogenes* in or on cheese during storage (92).

In summary, good manufacturing and good hygiene practices should be strictly followed to prevent pathogen contamination of cheese during manufacturing. However, recognizing the ubiquitous presence of the environmental pathogen *L. monocytogenes*, application of post-

processing interventions is highly recommended. Due to regrowth issue that have been observed in all studies, further research is needed to find antimicrobial treatments that are effective in either killing *L. monocytogenes* and suppressing re-growth of injured *L. monocytogenes* in Queso Fresco.

1.6 TRANSCRIPTOMICS STUDY OF PATHOGENIC BACTERIA IN FOOD

A better understanding of the physiology and survival mechanisms of pathogenic bacteria in food could help develop more effective antimicrobial strategies. Compositional factors of cheese such as acid, salt, and the presence of native microflora can influence the physiology and behavior of pathogenic bacteria. Studies have shown that exposure of pathogenic bacteria to certain stresses in food matrices, such as acid and osmotic stresses, can trigger cross-protection against certain antimicrobial treatments (14, 83, 105). Upon exposing *L. monocytogenes* to 6% salt stress, Bergholz et al. observed up-regulation of LiaFSR, a cell envelope stress response system, leading to cross-protection against the bactericidal effect of nisin (14). In comparison to non-adapted cells, Shen reported that *L. monocytogenes* that had been acid-adapted at pH 5.0 and 37°C exhibited a 2 log CFU/ml greater survival in milk and carrot juice with an added level of lauric alginate that would normally be lethal to the cells (83). Another study found that cells of *L. monocytogenes* that were acid-adapted at pH 5.5 for 1 h survived 10-fold greater under nisin treatment, when compared to non-adapted cells (105).

These studies above have pointed out the importance of understanding the stress responses of pathogenic bacteria in food matrices, an understanding that can be achieved through whole-genome gene expression studies, or transcriptomics studies. This information could help researchers to develop targeted antimicrobial approaches that circumvent the cross-protection mechanisms of pathogenic bacteria (104). Importantly, most studies to date have investigated the

cross-protection effect under a controlled environment, such as laboratory broth. However, food matrices present multiple stress factors simultaneously, and an in-situ study could provide a more accurate depiction about bacterial stress responses in food (88). And whole-genome gene expression studies can reveal the major metabolic pathways employed by pathogenic bacteria to survive and grow in food matrices. With this information, researchers could pursue a rational-design approach to finding bacterial inhibitors, instead of relying solely on the traditional trial and error approach (15).

Lastly, there is a lack of information on how food composition affects pathogenicity of foodborne bacteria. Hence, understanding the pathogenicity or virulence expression of pathogenic bacteria that have survived and grown on or in food matrices could enhance risk assessments (6, 70). In summary, the wealth of information provided by transcriptomics studies could provide information on the metabolic activity, adaptation mechanism and pathogenicity of foodborne bacteria in food. The improvement of sequencing technologies, advancement of bioinformatics software and the reduction of the experimental costs have made RNA-sequencing a favorable technique for microbiologists to study bacterial whole-genome gene expression (70). Nevertheless, the study of bacterial activity in situ can be complicated by technical limitations such as the low yield and purity issues during RNA procurement, as well as the contamination with food compounds and genetic materials of native microflora (104). Hence, a well-optimized RNA extraction protocol and the use of appropriate bioinformatics software are critical in ensuring a successful transcriptomics study of bacteria in food.

1.7 CONCLUSIONS

Cheese, given its wide number of varieties and possibly significant compositional diversity within the same variety, presents significant challenges for accurate safety assessment.

Efforts have been made in the past to assess bacterial survival in cheeses; however, there is still a need for reliable guidelines that can be used by cheese industry personnel and regulators to assess the safety of a variety of cheeses under extended non-refrigerated holding. Among all the cheese varieties, Queso Fresco has the highest outbreak frequency, mostly associated with *L. monocytogenes* contamination. Queso Fresco can be made safer with targeted post-processing antimicrobial strategies. One way to achieve this goal is by understanding the metabolic and adaptation activities of *L. monocytogenes* growing in Queso Fresco through whole-genome gene expression study using RNA-sequencing.

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CHAPTER 2:

GROWTH OF *LISTERIA MONOCYTOGENES*, *SALMONELLA SPP*., *ESCHERICHIA COLI* O157:H7, AND *STAPHYLOCOCCUS AUREUS* ON CHEESE DURING EXTENDED STORAGE AT 25°C

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2.1 ABSTRACT

Potentially hazardous foods require time/temperature control for safety. According to the U.S. Food and Drug Administration Food Code, most cheeses are potentially hazardous foods based on pH and water activity, and a product assessment is required to evaluate safety of storage >6 h at 21°C. We tested the ability of 67 market cheeses to support growth of *Listeria monocytogenes* (LM), *Salmonella* spp. (SALM), *Escherichia coli* O157:H7 (EC), and *Staphylococcus aureus* (SA) over 15 days at 25°C. Hard (Asiago and Cheddar), semi-hard (Colby and Havarti), and soft cheeses (mozzarella and Mexican-style), and reduced-sodium or reduced-fat types were tested. Single-pathogen cocktails were prepared and individually inoculated onto cheese slices (~10^5 CFU/g). Cocktails were 10 strains of *L. monocytogenes*, 6 of *Salmonella* spp., or 5 of *E. coli* O157:H7 or *S. aureus*. Inoculated slices were vacuum packaged and stored at 25°C for ≤15 days, with surviving inocula enumerated every 3 days. Percent salt-in-the-moisture phase, percent titratable acidity, pH, water activity, and levels of indigenous/starter bacteria were measured. Pathogens did not grow on 53 cheeses, while 14 cheeses supported growth of SA, 6 of SALM, 4 of LM, and 3 of EC. Of the cheeses supporting pathogen growth, all supported growth of SA, ranging from 0.57 to 3.08 log CFU/g (average 1.70 log CFU/g). Growth of SALM, LM, and EC ranged from 1.01 to 3.02 log CFU/g (average 2.05 log CFU/g), 0.60 to 2.68 log CFU/g (average 1.60 log CFU/g), and 0.41 to 2.90 log CFU/g (average 1.69 log CFU/g), respectively. Pathogen growth varied within cheese types or lots. Pathogen growth was influenced by pH and percent salt-in-the-moisture phase, and these two factors were used to establish growth/no-growth boundary conditions for safe, extended storage (≤25°C) of pasteurized milk cheeses. Pathogen growth/no-growth could not be predicted for Swiss-style cheeses, mold-ripened or bacterial surface–ripened cheeses, and cheeses made with nonbovine milk, as insufficient data were

gathered. This challenge study data can support science-based decision making in a regulatory framework.

2.2 INTRODUCTION

Temperature-dependent storage of most cheeses has three major roles—to allow for curing/ripening of cheeses that contain added or indigenous bacteria and enzymes, to prevent quality defects, and to control pathogen growth (3). The U.S. Food and Drug Administration (FDA) Food Code (41) defines a potentially hazardous food as one that requires time/temperature control to limit the growth of pathogenic microorganisms or toxin formation. According to the Food Code, foods with a pH of <4.2 and any water activity (aw) or a_w of <0.88 and any pH are not considered potentially hazardous. Foods considered potentially hazardous, also known as time/temperature control for safety (TCS) foods, fall into one of the following categories: $a_w \geq 0.88$ and pH > 5.0, $a_w > 0.90$ to 0.92 and pH > 4.6, or $a_w > 0.92$ and pH > 4.2. The Food Code indicates that TCS foods must be maintained at $\leq 5^{o}$C, or, if placed outside refrigeration, can be stored for up to 6 h at a temperature no greater than 21^{o}C, after which the product must be discarded (45).

The composition of many cheeses, when evaluated using the Food Code criteria, places them into the category of TCS foods, thus limiting the ability of retailers to market the cheeses under room-temperature conditions that could enhance cheese flavor and aroma (12). The Food Code– mandated time and temperature control may also limit industry flexibility in the transportation, handling, and storage of cheeses. It has, however, been suggested that the biochemical changes that occur during cheese ripening create an environment hostile for pathogen growth and that time/temperature control of some cheese is primarily needed to maintain the organoleptic quality of cheese, not to maintain safety (3). Bishop and Smukowski

(3) conducted a thorough review of the literature available up until 2006 and recommended that cheeses meeting certain criteria, e.g., cheeses manufactured in the United States with pasteurized or heat-treated milk (≥63°C for ≥16 s), cheeses manufactured following good manufacturing practices and under the principles of hazard analysis and critical control points (HACCP), and cheeses manufactured meeting standards of identity outlined in 21 Code of Federal Regulations part 133 (43), should be exempted from refrigeration requirements during ripening, storage, shipping, and display. Bishop and Smukowski recommended that the following cheeses could meet these criteria: Asiago (medium and old), Cheddar, Colby, Feta, Monterey Jack, Muenster, Parmesan, Pasteurized process, Provolone, Romano, and Swiss/Emmentaler.

To establish whether a particular food, e.g., cheese, can be exempt from TCS requirements, the Food Code allows processors or retailers to conduct a microbial challenge study to assess the ability of a food product to inhibit pathogenic bacterial growth or inactivate these microorganisms. The FDA has outlined parameters for conducting such challenge studies (44).

When experts consider the major microbiological hazards across the food supply, the risk of bacterial illness from dairy products, such as milk and cheese, can be attributed primarily to *Listeria monocytogenes*, *Yersinia entercolitica*, *Campylobacter* spp., and nontyphoidal *Salmonella* spp. (2). Between 1990 and 2011, there were 105 reported foodborne illness outbreaks in the United States, with over 2,000 illnesses, linked to cheese/cheese products (7). Major pathogens linked to these cheese-related outbreaks included *Salmonella* spp. (37 outbreaks), *L. monocytogenes* (16 outbreaks), pathogenic *Escherichia coli* (6 outbreaks), *Staphylococcus aureus* (4 outbreaks), norovirus (21 outbreaks), *Campylobacter* spp. (9 outbreaks), and *Brucella* spp. (5 outbreaks) (7). Among the 105 outbreaks, 17 were linked to

cheeses made with pasteurized milk, 30 were linked to cheese made with raw milk, and the pasteurization status of cheeses involved in the remaining 58 outbreaks was unspecified. The pathogenic bacteria primarily responsible for foodborne illness outbreaks linked to cheese manufactured with pasteurized milk were *L. monocytogenes*, *Salmonella* spp., and *E. coli* O157:H7. Cheeses implicated in these outbreaks included processed cheese and Mexican-style cheeses (7, 9). The low incidence of *S. aureus*–linked outbreaks related to cheese is presumed to be due to the low incidence of this pathogen in pasteurized milk and the growth characteristics of this bacterium (21). However, *S. aureus* is commonly carried by humans and thus could contaminate cheese during post-pasteurization handling (16). *S. aureus* is also the bacterial pathogen considered to have the highest tolerance to reduced-moisture conditions or increased salt concentration (22) and therefore could be considered a target pathogen in determining the safety of cheese contaminated post-processing and stored for extended periods of time at room temperature.

The goal of this project was to evaluate survival of strains of *L. monocytogenes*, *Salmonella* spp., *E. coli* O157:H7, and *S. aureus* on natural market cheeses during extended storage at 25°C and to determine the effect of cheese compositional factors such as pH, a_w, and salt on pathogen survival. Pathogen survival data from laboratory research and data from published literature were then combined to establish the boundary conditions for pathogen growth/no-growth during storage of cheese at room temperature.

2.3 MATERIALS AND METHODS

2.3.1 Cheeses

Sixty-seven cheeses were purchased from local retail establishments or obtained directly from the manufacturer and stored at 4°C. Cheeses studied were Asiago (aged, young), Brick (two brands), Cheddar (mild, regular, and sharp), Cheddar-mozzarella, Colby, Colby Jack, Farmer's, Feta, Gouda, Gruyere, Havarti (two brands), Jack (goat's milk), Monterey Jack, Muenster (two brands), Parmesan, Pepper Jack (two brands), Provolone (mild and regular; two brands of sharp), Provolone-mozzarella, Queso Blanco, Queso Fresco, Queso Quesadilla, String cheese (two brands), Swiss (Baby, two brands; Lacey, regular), reduced-fat cheeses (Cheddar, Colby Jack, and Provolone), and reduced-sodium cheeses (Colby Jack and Provolone). Where a type of cheese was tested more than once, replicates were from different brands and/or from different production dates of the same brand. All cheeses were manufactured in the U.S. from pasteurized milk (Tables 1 and 2, Cheese).

2.3.2 Proximate analysis

Cheeses were characterized by percentage of moisture, percentage of salt, and a_w at the beginning of each trial. The pH was measured at each sampling time on pathogen inoculated cheeses (days 0, 3, 6, 9, 12, and 15). To discern the impact of acid production by indigenous or starter bacteria on the microenvironment, the percentage of titratable acidity (%TA) was measured on uninoculated cheeses on days 0, 6, and 15. Uninoculated cheeses were handled the same as inoculated cheeses (see the following), except that 0.1 ml of Butterfield's phosphate diluent (Nelson Jameson, Marshfield, WI) replaced the inoculum. Duplicate trials were performed for each compositional analysis, and average values were reported. The percentage of moisture was determined using a standard method (4) by drying a representative 3-g sample at

100°C for 5 h in a vacuum oven maintained at 298 kPa throughout the drying process (M.D.O. Vacuum Oven, Model 3623, Lab-Line Instrument Inc., Melrose Park, IL). The percentage of salt was determined by titration of chloride using the silver titration standard method (4). For each trial, a representative 5-g sample was diluted with distilled water 1:20 (wt/vol) and the percentage of chloride was determined according to the standard method using a Model M926 Chloride Analyzer (Nelson Jameson). The percent chloride content was automatically calculated by the analyzer and expressed as milligrams percent of sodium chloride per liter, which was converted to percent salt by multiplying the appropriate dilution and conversion factors. Salt (percent) and moisture (percent) of an individual cheese sample were used to calculate percent salt-in-the-moisture phase (%SMP) using equation 1:

$$\%SMP = (\%\ salt \times 100) / (\%\ salt + \%\ moisture) \quad (1)$$

The a_w was determined for each cheese at the beginning of each trial using an AquaLab LITE water activity meter (Decagon Devices Inc., Pullman, WA) according to a standard method (1). Titratable acidity (%) was monitored during storage (days 0, 6, and 15) according to a standard method (4). Briefly, for each cheese/ trial, one sample (10.0 ± 0.5 g) that had been manually crumbled was automatically blended with 50 ml of distilled water and titrated using a Model DL22 Automatic Titrator (Mettler Toledo, Schwerzenbach, Switzerland), which was set to calculate %TA using the molecular weight of lactic acid. To determine the impact if any, of the presence of inoculum bacteria or growth of indigenous bacteria on cheese pH, the surface pH was measured for individual inoculated cheese slices at each sampling time (days 0, 3, 6, 9, 12, and 15) using an Accumet AB15 pH Meter equipped with a flat surface combination electrode (Fisher Scientific, Itasca, IL).

2.3.3 Inoculum preparation

Ten strains of *L. monocytogenes*, six strains of *Salmonella* spp., five strains of *E. coli* O157:H7, and five strains of *S. aureus*, representing a wide variety of sources and serotypes, were used in this study (Table 2-3). Stock cultures were maintained at -20°C in brain heart infusion broth (BHIB; Difco, BD, Sparks, MD) with 10% (wt/vol) added glycerol (Fisher Scientific). Fresh working cultures were prepared monthly by thawing stock cultures and streaking for isolation as follows: *L. monocytogenes* on Listeria selective agar (Oxoid, Ogdensburg, NY) with added Listeria selective supplement (Oxford formulation, Oxoid); *Salmonella* spp. and *E. coli* O157:H7 on modified Levine's eosin methylene blue agar (m-LEMB), prepared from lactose-free LEMB agar (Difco) with the addition of 10 g/liter D-sorbitol (Fisher Scientific) and 5 g/liter NaCl (Fisher Scientific); and *S. aureus* on Baird-Parker agar (Difco) with added egg yolk tellurite enrichment (Difco). Working culture plates were incubated for 24 h at 35°C for *Salmonella* spp. and *E. coli* O157:H7 and 48 h at 35°C for *L. monocytogenes* and *S. aureus*, whereupon all cultures were observed for consistent colony morphology and stored at 4°C for 40 days. Inoculation cultures were prepared for individual strains by transferring a single colony of each strain into a separate tube containing 9 ml of nutrient broth (Difco) for *L. monocytogenes* or BHIB for *Salmonella* spp., *E. coli* O157:H7, and *S. aureus*. Preliminary studies showed better survival of *L. monocytogenes* over 15 days at 25°C on Cheddar and Swiss cheeses when inocula had been grown in nutrient broth, while the other three pathogens survived better on cheeses when inocula had been grown in BHIB (n=2, data not shown). Following incubation for 20 to 24 h at 35°C, 1 ml of stationary-phase culture of each strain for a designated pathogen (10^8 CFU/ml for *L. monocytogenes* and 10^9 CFU/ml for *Salmonella* spp., *E. coli* O157:H7, and *S. aureus* was transferred to a sterile 9-ml tube to produce

a single-pathogen, multistrain cocktail. Each pathogen cocktail was mixed by vortexing and diluted, as necessary, to produce a starting inoculum cocktail of 10^7 CFU/ml. Pathogen levels in the cocktails were estimated by plating the inocula on brain heart infusion agar (Difco) and incubating at 35°C for 24 h.

2.3.4 Sample inoculation

The working surface of a biosafety cabinet was sterilized with 70% (vol/vol) ethanol and covered with aluminum foil prior to cheese inoculation. Cheese slices (approximately 25 to 30 g and approximately 70 to 80 cm^2) were placed on the aluminum foil aseptically, six cheese slices per trial. An aliquot (0.1 ml) of a single-pathogen cocktail (10^7 CFU/ml) was pipetted onto each of the six cheese slices. An L-shaped spreader was used to evenly distribute the inoculum over the surface of the six slices, then samples were left to air dry under the hood for 15 min to allow bacterial attachment and evaporation of excess liquid. The a_w values of control and air-dried inoculated samples were not significantly different (n=3; P>0.05; data not shown). Inoculated cheese slices were folded into half with the inoculated cheese surfaces facing inward to avoid inaccuracies due to pathogen adherence to packaging film. Folded cheese samples were weighed, then individually vacuum packaged in standard retail barrier bags (B-2175, Cryovac Food Packaging and Food Solutions, Duncan, SC) and stored at 25°C for up to 15 days. Oxygen transmission rate for the bags was 3 to 6 cm^3/m^2 at 40°F in 24 h. The initial inoculum level on each cheese slice was ~10^5 CFU/g.

2.3.5 Sampling and enumeration

Packaged cheese samples were analyzed following inoculation (time 0) and throughout storage for up to 15 days. Every 3 days, one cheese slice per pathogen was removed from incubation, the storage/barrier bag was aseptically opened, and Butterfield's phosphate diluent

was added to create a 1:10 (wt/wt) dilution. The cheese-diluent mixture was stomached in the bag (AES Smasher, AES Chemunex, Bruz, France) for 2 min at high speed. Stomached samples were serially diluted in Butterfield's phosphate diluent, and 0.1-ml portions were spread plated onto Listeria selective agar, m-LEMB, m-LEMB, and Baird Parker agar for cheeses inoculated with *L. monocytogenes, Salmonella* spp., *E. coli* O157:H7, and *S. aureus*, respectively. A preliminary trial confirmed better recovery of *Salmonella* spp. by plating on m-LEMB rather than on xylose lysine desoxycholate agar (Difco) and better recovery of *E. coli* O157:H7 by plating on m-LEMB agar rather than on sorbitol MacConkey agar (Difco). Inoculated samples were also spread plated on deMan Rogosa Sharpe agar (MRS; Difco) at 0, 6, and 15 days to monitor changes in lactic acid bacteria (LAB) populations during storage and to thereby investigate the impact, if any, of indigenous, starter, or adjunct bacterial growth on inoculum survival. The m-LEMB spread plates were incubated 24 h at 35°C, Listeria selective agar and Baird-Parker agar plates 48 h at 35°C, and MRS plates 72 h at 35°C, after which time counts were recorded for each plate, with countable plate converted to log CFU per gram. On m-LEMB agar, typical colonies of *E. coli* O157:H7 appear colorless to pink, while colonies of *Salmonella* spp. are dark red-black with a metallic green sheen. Colonies of *S. aureus* are typically shiny black and surrounded with clear zone on Baird-Parker agar. *L. monocytogenes* colonies are normally grey in color surrounded by a black halo on Listeria selective agar. On MRS agar, lactobacilli appear as medium to large white colonies. Data were used to calculate Delta log CFU per gram, relative to time 0, over the 15-day storage period for each bacterium-cheese combination.

2.3.6 Literature data search and selection

To provide additional data to augment our product assessment, data from published literature were combined with data from this study. In searching for relevant published studies, keywords including, but not limited to, ‘‘pathogen, survival, cheeses, temperature, pH, salt’’ were entered into online scientific databases. Reference lists of publications were also screened for relevant studies with appropriate data. Published challenge studies that met the following criteria were selected: (i) the inoculated cheeses were made with pasteurized cow’s milk, (ii) the cheeses were inoculated with at least one of the pathogens, *L. monocytogenes*, *Salmonella* spp., *E. coli* O157:H7, or *S. aureus*, (iii) the pathogen(s) was inoculated on the finished cheese, not into the milk, and (iv) inoculated cheeses were stored at 20 to 30°C. Studies with surface-ripened, mold-ripened, Swiss, or processed cheeses or cheese made with non-bovine milk were excluded. Of 155 studies published between 1959 and 2012 and which investigated pathogen behavior in or on cheeses, six published studies met the criteria (14, 24, 25, 33, 34, 39). From each publication, the following information was extracted (Table 4): type of cheese, temperature and length of storage, type and number of pathogen strains, composition (all available information for pH, a_w, percentage of moisture, %SMP, %TA) of cheeses and behavior (growth versus no growth) of pathogen(s).

2.3.7 Evaluating compositional characteristics affecting pathogen growth

The relationship between compositional factors and behavior of pathogens on cheeses was explored. Compositional factors of cheese, the percentage of moisture, initial (day 0) pH, %SMP, a_w, and initial %TA, were paired, i.e., one compositional factor as x and one as y and a growth versus no-growth outcome was plotted for each cheese as a function of the x and y values to analyze the influences of the paired compositional factors on pathogen growth. Values of

compositional factors were normalized to a 100-point scale before plotting as follows: for each compositional factor, the minimum value of the data set was subtracted from the observed value and the total was divided by the range of the values and multiplied by 100 to obtain the normalized value, as shown in equation 2.

$$\text{Normalized value} = [(\text{value-minimum value})/\text{range}] \times 100 \quad (2)$$

In this analysis, a growth result was indicated for a cheese when the Delta log CFU/g for any cheese-pathogen combination over the 15-day storage period was a positive value that exceeded the pathogen-specific plating variability: 0.39, 0.41, 0.27, and 0.25 log CFU/g for *L. monocytogenes*, *Salmonella* spp., *E. coli* O157:H7, and *S. aureus*, respectively. The growth/no-growth outcome plot from each pair of compositional factors was inspected and compared with predictions from a logistic regression equation (SAS 9.2, SAS Institute, Cary, NC). A model at $P = 0.05$ based on the variables initial pH and %SMP was generated, according to the method of McMeekin et al. (29) (Fig. 2-1).

2.4 RESULTS AND DISCUSSION

In this study, 67 cheese samples, representing a variety of national brands, were tested for their ability to support pathogen growth during extended storage at 25°C (Tables 2-1 and 2-2). Cheeses were manufactured using pasteurized milk in facilities meeting applicable federal and state food safety regulatory requirements. Cheeses met a standard of identity, where applicable. Among the 67 cheese samples tested, 52 were duplicate samples of cheeses from different lots or production dates of the same brand. The majority of cheeses that were tested in this study would be labeled as hard or semi-hard cheeses, according to FDA classification (43), and were expected to be safe for extended room-temperature storage due to reduced moisture level and low pH. Soft cheeses with higher moisture were also included to clarify compositional differences affecting

pathogen growth/no-growth outcomes. Inoculated cheeses were vacuum packaged to prevent moisture loss, delay mold growth, and to allow for pathogen growth, if any.

Salmonella spp., *L. monocytogenes*, and *E. coli* O157:H7 have, in recent years, been implicated in foodborne illness outbreaks linked to cheeses made with pasteurized milk (7, 9). *S. aureus* has not often been associated with foodborne illness outbreaks linked to cheese, even though this pathogen is generally linked to foods, such as cheese, which are often hand manipulated during processing and packaging (8, 40). We included *S. aureus* in the study design not only because of its link to poor sanitation and post-processing contamination but also because it is the pathogen most likely to grow in or on foods with reduced moisture and/or low a_w (21). For ready-to-eat food products, the FDA has established a zero-tolerance policy for *L. monocytogenes*, *Salmonella* spp., and *E. coli* O157:H7, due to the potentially low infectious dose of *E. coli* O157:H7 and *Salmonella* spp. and the high mortality rate (15 to 30%) associated with *L. monocytogenes* infections (42). Although none of these pathogens should be present in finished cheeses made from pasteurized or heat-treated milk, the composition of a cheese supporting growth of any of these bacteria during extended room temperature storage presents an unacceptable risk. A zero-tolerance policy is not in place for *S. aureus* in ready-to-eat foods because staphylococcal food poisoning occurs as a result of ingestion of a preformed enterotoxin, which is only produced in amounts sufficient to cause illness as a result of extended temperature abuse and growth of the pathogen to a high concentration (~10^5 CFU) (30). Thus, a cheese with compositional characteristics allowing growth of *S. aureus* during storage is also an unacceptable risk. For these reasons, the growth of four target pathogens, *L. monocytogenes*, *Salmonella* spp., *E. coli* O157:H7, and *S. aureus*, as post-processing contaminants on cheeses was investigated.

Pathogen strains used in this study represented a variety of sources and serotypes (Table 2-3). The strains of *L. monocytogenes* and *Salmonella* spp. had been screened in previous research in our laboratory to confirm tolerance to salt and pH conditions typical of cheese (13). Strains of *E. coli* O157:H7, *Salmonella* spp., and *S. aureus* were exposed to some acid during inoculum preparation in BHIB, as a pH drop of 1 unit was observed during overnight incubation. *L. monocytogenes* was grown in nutrient broth, with no pH drop during inoculum preparation. Where it occurred, the limited exposure to acid during inoculum preparation was unlikely to have led to acid adaptation of strains. Therefore, the key characteristic of strains selected for use in this study was their human or animal–animal product origin, making these strains perhaps representative of organisms to be found in a food processing or handling environment.

The FDA, in its guide to microbial challenge testing, notes that it can be important to evaluate a range of intrinsic factors that can influence the safety of a food during its intended shelf life (44). Compositional factors in cheese that could influence pathogen behavior were analyzed: surface pH (days 0, 3, 6, 9, 12, 15); percentage of moisture, percentage of salt, and a_w (day 0); and %TA (days 0, 6, 15). Change in LAB count was determined on days 0, 6, and 15. Across all cheese samples, moisture content ranged from a low of 32.07% to a high of 57.64%, for one lot of Gruyere and Feta cheese, respectively. Salt content ranged from 0.33% for one lot of Lacey Swiss to 3.30% for Queso Blanco. Salt-in-the-moisture phase was calculated from percentage of moisture and percentage of salt (equation 1), with values ranging from 0.73% for one lot of Lacey Swiss to 7.21% for one lot of Parmesan. The a_w varied little across the cheese samples tested, ranging from 0.96 to 0.99, except for Parmesan (average a_w= 0.93; Table 2-1).

Cheese pH on day 0 ranged from 4.33 to 6.49 for Feta (average of two lots) and Queso Fresco, respectively (Tables 2-1 and 2-2). Over the 15-day storage period, change in pH ranged

from -1.44 to +0.53 pH units for Queso Fresco and Baby Swiss, respectively, with most cheeses exhibiting only slight change in pH. To quantify the amount of lactic acid present in each cheese at the beginning of storage and to determine the effect, if any, of storage on changes in lactic acid level, %TA was measured (Tables 2-1 and 2-2). The %TA across the cheeses tested ranged from 0.26 to 2.83% for Queso Blanco and Feta, respectively, at the beginning of storage. Change in %TA over storage was not clearly linked with change in pH and bacterial survival (data not shown). Change in LAB count in cheese samples was estimated during extended storage at 25°C (Tables 2-1 and 2-2). LAB count on day 0 across the cheeses ranged from 2.00 to 8.08 log CFU/g for one lot of Pepper Jack and Monterey Jack, respectively. Initial LAB counts on similar cheese samples from different brands, or different lots of the same brand, could vary widely. The day 0 count for LAB on different lots of Provolone (reduced fat; brand 3) varied by 3.25 log CFU/g between purchase dates. Similarly, one sample of Provolone (brand 3) had one of the lowest day 0 LAB counts, 2.70 log CFU/g, while another sample of a different brand of Provolone (brand 4) had one of the highest initial LAB counts, 7.70 log CFU/g. The day 0 LAB counts for the two samples of brand 3 Provolone were 2.70 log CFU/g and 3.78 log CFU/g, and these rose to 5.40 log CFU/g and 7.19 log CFU/g, respectively, equivalent to a Δ log CFU/g of 2.70 and 3.41, respectively. The LAB count for the one lot of brand 4 Provolone increased by one order of magnitude, from 7.70 log CFU/g (day 0) to 8.70 log CFU/g (day 15). Throughout the storage period and across all cheese samples tested, changes in LAB count ranged from -2.92 log CFU/g for one lot of Parmesan to +5.66 log CFU/g for one lot of Pepper Jack (brand 4). Of the 67 cheese samples tested, LAB population increased on storage in 47 cheese samples tested. LAB count was relatively constant ($0 < \Delta\log \leq 0.3$ log CFU/g) in 7 cheese samples tested and declined (Δ log $\geq$ -0.3 log CFU/g) in 13 other cheese samples during storage.

Pathogens did not grow on 53 cheese samples over the 15 days (Table 2-1), while 14 cheese samples supported growth of *S. aureus*, 6 of *Salmonella* spp., 4 of *L. monocytogenes*, and 3 of *E. coli* O157:H7 (Table 2-2). The pattern of pathogen survival for each cheese lot was consistent over storage except for Queso Quesadilla (Table 2-2). We observed growth of *S. aureus* (+0.57 log CFU/g) on day 6 on Queso Quesadilla; however, by day 15, we noted an overall decrease in pathogen population (of -0.40 log CFU/g). Of the cheese samples that did support pathogen growth, all supported growth of *S. aureus*, ranging from 0.57 to 3.08 log CFU/g (average 1.62 log CFU/g across all 14 cheeses). Growth of *L. monocytogenes, Salmonella* spp., and *E. coli* O157:H7, ranged from 0.60 to 2.68 log CFU/g (average 1.60 log CFU/g), 1.01 to 3.02 log CFU/g (average 2.05 log CFU/g), and 0.41 to 2.90 log CFU/g (average 1.69 log CFU/g), respectively.

Growth of *L. monocytogenes*, which exceeded the plating variability, was observed on four cheese samples: Gruyere (one lot), Queso Blanco, Queso Fresco, and String cheese (brand 14; Table 2-2). Genigeorgis et al. (14) studied the survival of *L. monocytogenes* on market cheeses stored at 30°C and observed pathogen growth only on Hispanic style cheeses: Queso Fresco, Queso Ranchero, and Queso Panela, ranging from 0.38 to 3.18 log CFU/g. Uhlich et al. (39) observed an increase of more than 5 log CFU/g of *L. monocytogenes* on Queso Blanco stored at 25°C for up to 6.25 days. Genigeorgis et al. (14) did not observe growth of *L. monocytogenes* on String cheese, instead noting a drop in *L. monocytogenes* population of 2.36 log CFU/g over 9 days at 30°C. The String cheese that Genigeorgis et al. tested had similar pH and %SMP values to the cheese sample that we evaluated but an unknown level of LAB. The String cheese sample in our study that allowed some growth of pathogen simultaneously

supported a dramatic increase in LAB population, from 4.87 log CFU/g on day 0 to 8.86 log CFU/g by day 15 (Table 2-2).

Growth of *L. monocytogenes* was not observed on 63 samples of cheese tested (Tables 2-1 and 2-2). Many of the cheeses that did not support pathogen growth would be classified as hard or semi-hard cheeses based on FDA classification (43) and may be suitable for extended room temperature storage. Shrestha et al. (33) did not observe growth of *L. monocytogenes* on a range of Cheddar-type cheeses stored at 21°C for 30 days, with counts of *L. monocytogenes* dropping by ≤1.1 log CFU/g during storage. We observed a slight decrease in the population of *L. monocytogenes* on mild, reduced-fat, and sharp Cheddar cheeses during storage at 25°C (Table 1). Genigeorgis et al. (14) also reported a decrease of *L. monocytogenes* population on mild Cheddar cheeses during storage. Genigeorgis et al. evaluated the growth of *L. monocytogenes* on Monterey Jack, Colby, Provolone, Muenster, and Feta cheeses during storage and observed a decrease in pathogen population of > 1 to 2 log CFU/g in all cases. In our study, we noted an average decrease in pathogen population ranging from 0.20 log CFU/ g for Colby to 4.74 log CFU/g for Feta (Tables 2-1 and 2-2). Growth of *Salmonella* spp. was observed on six cheeses: Gruyere (two lots), Jack (goat's milk), Muenster (brand 6), Queso Fresco, and String (brand 14). Kasrazadeh and Genigeorgis (24) studied the growth of *Salmonella* inoculated onto sliced Queso Fresco stored at 20°C. They noted rapid growth, a lag time of 2.5 to 3.5 h, and a generation time of 1.65 to 2.17 h for *Salmonella* on Queso Fresco. We observed an increase in *Salmonella* spp. concentration of 3.02 log CFU/g on Queso Fresco stored at 25°C over 15 days. This was the highest level of *Salmonella* growth observed over all 67 cheese samples tested.

There were 61 cheeses that did not support the growth of *Salmonella* spp. in this study. Shrestha et al. (34) examined the survival of *Salmonella* on a range of Cheddar type cheeses

stored for up to 30 days at 21°C. Cheddar cheese manufactured to standards of pH and salt was comminuted, inoculated with *Salmonella* spp., and stored at 21°C for up to 30 days. *Salmonella* spp. counts decreased significantly at 21°C for all cheese types. We evaluated the survival of *Salmonella* spp. on mild, reduced-fat, and sharp Cheddar cheeses and observed average decreases of 0.3, 1.12, and 1.26 log CFU/g, respectively, for the brands tested.

Three cheeses supported growth of *E. coli* O157:H7, Muenster (brand 6), Queso Fresco, and String (brand 14). Kasrazadeh and Genigeorgis (25) reported rapid growth of *E. coli* O157:H7 on Queso Fresco stored at 20 and 30°C, with a lag time of 3 to 3.45 h and a generation time of 2.33 to 2.56 h at 20°C. The authors attributed the fast growth rate of *E. coli* to the lack of starter culture, near neutral pH (6.6), and low %SMP (1.61%). The Queso Fresco that we studied had similar pH (6.49) but higher %SMP (3.49), supporting the assertion that cheese pH has a dominant effect on pathogen growth.

Of the 14 cheeses that supported pathogen growth, all supported the growth of *S. aureus*. Cheese samples that supported growth of *S. aureus* included Farmer's, Gruyere (two lots), Jack (goat's milk), Muenster (brand 6), Provolone (brand 3; two lots), reduced-sodium Provolone (two lots), Queso Blanco, Queso Fresco, Queso Quesadilla, and two brands of String cheese. There are no reports of prior research evaluating the survival of *S. aureus* as a postprocessing contaminant on cheese made from pasteurized milk. Levels of *S. aureus* on Queso Quesadilla increased by 0.57 log CFU/g on day 6 of storage but decreased by 0.40 log CFU/g relative to the time-zero level by day 15. In all other cases, pathogen growth/no growth displayed a consistent increase or decrease over the 15-day storage period.

LAB count increased in 47 of 67 cheeses tested in this study. With one exception, cheeses that supported pathogen growth also supported LAB growth. LAB count decreased in

Jack (goat's milk) cheese that supported growth of *Salmonella* spp. and *S. aureus*; otherwise, LAB count increased from 1.54 to 4.47 log CFU/g in cheeses that supported pathogen growth. The level of inoculum on each cheese slice at time 0 averaged 4.7 log CFU/g (n =268). This level allowed for accurate enumeration of growth or death without reaching the limits of research methodology. This inoculum level could have placed pathogens at a level to effectively compete with active indigeneous organisms. LAB count on day 0 averaged 5.03 log CFU/g for cheeses that supported pathogen growth (n=14, Table 2-2). Although previous studies have shown that initial inoculum level does not affect the survivability or growth kinetics of pathogens (6, 26, 46), a higher proportion of *S. aureus* compared with LAB may aid in the survival or growth of this particular pathogen (17). Although growth of *S. aureus* is reported to be weak when a high load of competitive bacteria, e.g., LAB, is present, increasing the proportion of *S. aureus* to LAB has been shown to improve survival of this pathogen (17, 23).

The change in pH on storage among cheeses that supported pathogen growth showed no clear trend, remaining the same (ΔpH $\leq$0.3 units) in seven samples and increasing in six samples (Table 2-2). Change in %TA over storage (data not shown) had no apparent relationship with the change of pH and LAB count. Correlation between changes in pH and LAB count in cheeses that supported pathogen growth was weak (r^2=0.15).

The 14 cheeses that supported pathogen growth were characterized by relatively high pH. When cheese samples were separated into roughly equal groups by initial pH value, 4.29 to 5.20 (29 cheeses), 5.21 to 5.40 (18 cheeses), and 5.41 to 6.50 (20 cheeses), it was apparent that pathogen growth was better supported on higher pH cheeses. With the exception of brand 3 Provolone and reduced-sodium Provolone, cheeses with day 0 pH ranging from 4.8 to 5.2 did not support growth of any pathogen (Table 2-1). Feta was the most acidic cheese tested (average pH

4.33, n=two lots), and pathogen viability on this cheese type decreased over time more than for any other cheese (Table 2-1). As pH increased to 5.21 to 5.40, 4 of 18 cheeses supported growth: Provolone (brand 3; one lot), reduced-sodium Provolone (one lot), String cheese (brand 6), and Queso Quesadilla, all supporting the growth of *S. aureus* but no other pathogen (Table 2-2). In the pH range 5.41 to 6.50, eight cheeses supported pathogen growth: Jack (goat's milk), String (brand 14), Farmer's, Muenster, Gruyere (two lots), Queso Blanco, and Queso Fresco. Pathogen growth on Queso Fresco was the greatest across all cheeses tested; this was also the cheese with the highest initial pH (pH 6.49). Generally, cheeses with an initial pH ≥5.46 supported growth of at least one pathogen, with the exception of Swiss-style cheeses (Baby Swiss, Swiss, Lacey Swiss) and one lot of Havarti, which did not support growth. Optimal pH for growth of *S. aureus* is between pH 6.0 and 7.0, with pH 4.0 as the reported minimum for growth (20). Minimum pH values that have been reported for growth of *L. monocytogenes, Salmonella* spp., *E. coli* O157:H7 are 4.39, 4.20, and 4.40, respectively (20). Only Feta cheese (pH 4.29 and 4.38) was below the reported minimum pH for growth of any of the pathogens tested.

The %SMP for cheeses that supported pathogen growth ranged from 2.26 to 6.56% and from 0.73 to 7.21 %SMP for cheese samples that did not support growth. The greater growth potential that we observed for *S. aureus* on cheeses could be attributed, in part, to the high salt-tolerance of this pathogen. Nunheimer and Fabian (31) reported that some strains of *S. aureus* are able to tolerate up to 20% NaCl. Sutherland et al. (36) reported growth of *S. aureus* in BHIB with pH 4.48 and 8.5% NaCl at 25°C. Ingham et al. (19) reported greater tolerance of *S. aureus* than of *L. monocytogenes* to high salt and low aw in meat products stored at 21°C.

Recognizing the potential for compositional variability in cheeses across type, age, and manufacturer, we tested cheeses from different brands or from different lots within the same

brand. Along with observed variation in pH and a range of %SMP, the presence of inhibitory compounds in cheese, such as metabolites of LAB and the presence of free fatty acids, may have varied from lot to lot, brand to brand, and between cheese types, resulting in differences in pathogen growth during non-refrigerated storage. The effect of these factors on microbial survival has been shown to be highly dependent on the concentration of inhibitory compounds and the species and strain of both LAB and pathogen (11, 15, 17, 35). The apparent inconsistencies in pathogen growth patterns observed for cheeses of a similar type supports the assertion that compositional characteristics, more than cheese type, determine the likelihood of pathogen growth on a sample of cheese

The compositional factors of pH, %SMP, aw, and %TA were paired in all combinations and a pathogen growth/no-growth outcome for each cheese was plotted as a function of each pair of factors. Plotting growth/no-growth outcome as a function of pH and %SMP, combined with logistic regression, created a growth/no-growth interface that could be used to differentiate cheeses that inhibited pathogen growth from those that allowed pathogen growth (Fig. 2-1). These results are consistent with those of Oh et al. (32) who evaluated the effect of compositional factors of low-sodium Cheddar cheeses on the growth of strains of *Salmonella* spp., *L. monocytogenes*, *S. aureus*, and Shiga toxin–producing *E. coli*. In a model low-sodium Cheddar cheese extract, Shiga toxin– producing *E. coli* survived significantly better than the other three pathogens. Principal component analysis indicated that Shiga toxin–producing *E. coli* survival was primarily determined by pH, and not by percentage of salt or percentage of lactate (32).

The eight Swiss-style cheese samples tested did not fit the pattern established by data from the other cheeses tested. These Swiss-style cheeses had the lowest %SMP of all cheeses

tested, a relatively high pH, and a high a_w. Despite these compositional factors seeming to be permissive for growth, none of the Swiss-style cheeses supported pathogen growth. Leyer and Johnson (27) reported poorer survival of *Salmonella* spp. on Swiss cheeses than on Cheddar and mozzarella. Swiss-style cheeses are unique among the types of cheeses that we tested due to the addition of propionic acid bacteria as an adjunct culture in cheese manufacture. The added propionic acid bacteria can produce metabolites with antimicrobial properties, such as propionic acid, acetic acid, and diacetyl (10). Studies have shown greater antimicrobial properties linked to propionic acid (pKa = 4.87) as compared with lactic acid (pKa = 3.86) (37). The results of our study would suggest that target pathogens will not grow on Swiss-style cheeses during extended storage at 25°C, but the safety of such cheeses should be evaluated independently from cheeses that are fermented using only lactic acid–producing bacteria.

Further, our research suggests that the ability of pathogens to grow on bacterial surface–ripened or mold-ripened cheeses should be evaluated independently from cheeses manufactured without these ripening adjuncts. Bacterial surface–ripened and mold-ripened cheeses have added cultures that are capable of growing and altering the environment for pathogen growth. Growth of added bacterial and/or mold cultures can result in the production of antimicrobial compounds (e.g., bacteriocins) that could hinder pathogen growth but can also lead to lactate metabolism, which can subsequently increase cheese pH and enhance pathogen growth (5). Genigeorgis et al. (14) found a significant reduction of *L. monocytogenes* (>-2.36 log CFU/g) when inoculated onto Limburger, a bacterial surface–ripened cheese. While the high pH of Limburger (pH 7.2) would suggest that this cheese could support pathogen growth, the growth of smear bacteria results in extensive lipolysis that produces a high concentration of free fatty acids, which are compounds known to have antimicrobial activity (35). Goat's milk cheese may also contain high levels of

free fatty acids. Woo et al. (47) evaluated the free fatty acid content in a variety of cheeses and concluded that Blue, Swiss, Limburger, and goats' milk cheeses contained high concentrations of free fatty acids. Thus, we conclude that the safety of surface-ripened cheeses, mold-ripened cheeses, and non-cow's milk cheeses, along with Swiss-style cheeses, cannot be effectively evaluated using the logistic equation we developed to establish the pathogen growth/no-growth boundary for other cheeses in this study.

The a_w and pH are the two criteria used in the FDA Food Code to determine the shelf stability of food products (45). However, %SMP is an appropriate factor in assessing the likelihood of pathogen survival on cheese. In addition to salt, other solutes in cheese, such as nonprotein nitrogen-containing compounds and products released during proteolysis, could contribute to the reduction of a_w, yet these compounds may not play a role in inhibiting pathogen growth (28). Tapia et al. (38) suggested that the usefulness of measured aw as an indicator of microbial safety or stability is diminished by the ‘‘specific solute effect’’; that is, that the solute in the food matrix dramatically alters the minimum aw for microbial growth. Hilderbrand (18) supported %SMP as a more reliable factor than a_w in determining bacterial growth in smoked fish. In addition, %SMP is routinely determined and has historically been used in the cheese industry as a measure of product quality. Our search of published literature indicated that other researchers investigating survival of pathogens as postprocessing contaminants on cheese routinely monitored %SMP (14, 24, 25, 33, 34, 39), while only a few studies investigating pathogen survival on cheese considered the impact of product a_w (33, 34, 39). Furthermore we identified that pH and %SMP were the two compositional factors that could be used to differentiate cheeses that supported pathogen growth from those that inhibited growth (Fig. 2-1). More data are needed to confirm the use of aw in predicting growth of pathogens in cheese.

Of the 67 market cheeses studied, 53 did not support the growth of *L. monocytogenes*, *Salmonella* spp., *E. coli* O157:H7, or *S. aureus* and could safely be kept at ≤25°C for an extended period of time. The risk of pathogen growth for those cheeses that supported growth can be characterized as follows: *S. aureus* (growth on 14 of 14 cheeses supporting pathogen growth)>> *Salmonella* spp. (growth on 6 of 14)> *L. monocytogenes* (growth on 4 of 14) > *E. coli* O157:H7 (growth on 3 of 14). None of the cheeses supported growth of *S. aureus* to an extent that would be expected to result in sufficient enterotoxin production to present a food safety hazard. As noted by the National Advisory Committee on Microbiological Criteria for Foods, when growth of *S. aureus* is limited to less than 3 log CFU/g and the initial population of the pathogen does not exceed 3 log CFU/g, production of enterotoxin sufficient to cause illness does not occur (30). The presence of appropriate food safety monitoring programs during cheese manufacture, e.g., HACCP and good manufacturing practices, and sanitation programs in place during postprocessing handling, transportation, and storage would further ensure that the risk associated with *S. aureus* is mitigated. Protection of public health is reinforced by the selection of appropriate cheeses for extended room-temperature storage.

2.5 CONCLUSIONS

We used data for pH and %SMP from laboratory research and relevant published research from a total of 82 cheeses to establish the boundary conditions for pathogen growth/no-growth during extended room temperature storage of cheese ($P = 0.05$). Based on a search of the literature available at the time, Bishop and Smukowski (3) recommended that certain cheeses could be stored for extended periods without refrigeration: Asiago (medium and old), Cheddar, Colby, Feta, Monterey Jack, Muenster, Parmesan, pasteurized process, Provolone, Romano, and Swiss/Emmentaler. Our research does not support this conclusion. Rather, we suggest that

cheeses, regardless of type or brand, which meet specific compositional requirements for pH and %SMP may be safely stored at ≤25°C for extended periods of time. Data from Swiss-type cheeses, mold-ripened or bacterial surface–ripened cheeses, or cheeses made with nonbovine milk were excluded from this analysis due to insufficient data or lack of fit. The growth/no-growth interface established by the logistic regression line shows that many common cheese types, if made from pasteurized cow's milk in compliance with U.S. regulatory standards, can safely be considered non-TCS foods. Non-TCS cheeses should be described in terms of pH and %SMP, rather than cheese type or brand. Further research is underway to develop a model that will allow regulators and cheese industry personnel to predict the likelihood of pathogen growth on cheeses prior to extended room temperature storage. The data generated in this research will serve as supporting documentation for science-based decision making for the cheese industry.

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Figure 2-1. Growth (▲) or No-Growth (X) of *Listeria monocytogenes*, *Salmonella* spp., *Escherichia coli* O157:H7, and *Staphylococcus aureus* on cheeses stored at 20-30˚C based on cheese pH (Day 0) and %SMP (salt-in-moisture-phase). Data from published research (n=26; Table 4) and this study (n=55). Solid line represents the growth/no-growth interface (P=0.05).

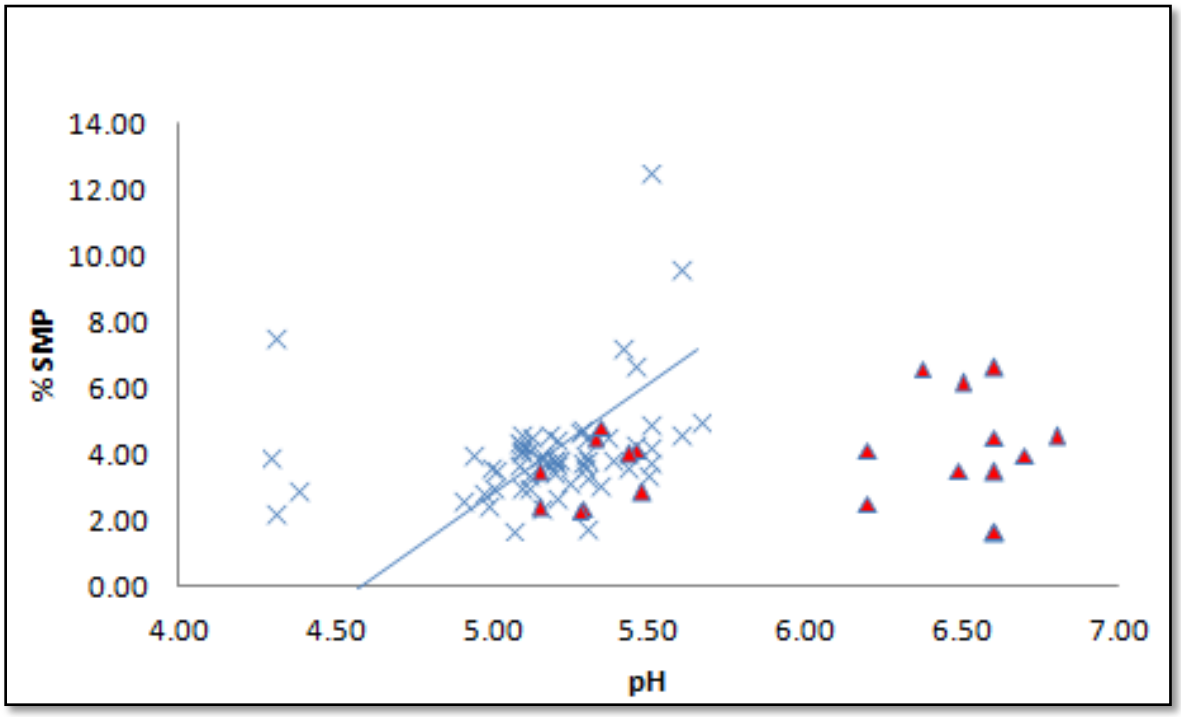

Table 2-1. Composition of natural cheeses that did not support growth of *L. monocytogenes*, *Salmonella* spp., *E. coli* O157:H7, and *S. aureus* and pathogen survival (Δlog CFU/g) during storage for 15-days at 25°C.

Cheese[a]	Brand	% Moisture[b]	% Salt[c]	%SMP[d]	a_w[e]	pH[f]		%TA[g]	LAB count[h]		Pathogen survival (Δlog CFU/g)[i]			
						0 d	15 d	0 d	0 d	15 d	LM	SALM	EC	SA
Asiago (Young)	1	36.00	1.71	4.53	0.96	5.36	5.21	2.16	7.36	6.87	-2.05	-3.74	-2.12	-1.13
Asiago (Young)	1	38.63	1.83	4.52	0.96	5.12	5.01	2.82	7.79	7.40	-2.26	-2.12	-0.68	-1.07
Asiago (Aged)	10	38.84	0.96	2.41	0.97	5.15	4.98	1.78	6.22	7.16	-2.92	-2.79	-3.70	-3.53
Asiago (Aged)	10	43.30	1.94	4.29	0.97	5.09	5.06	2.02	6.02	6.94	-3.84	-3.63	-1.59	-2.67
Baby Swiss	5	38.36	0.61	1.57	0.98	5.77	6.28	-[j]	7.07	7.50	-0.71	-1.38	-1.98	-0.62
Baby Swiss	5	36.25	0.69	1.87	0.98	5.79	6.32	-	6.94	8.05	-0.67	-0.76	-0.75	-1.15
Baby Swiss	9	37.21	0.62	1.64	0.99	5.55	6.04	-	7.19	7.72	-1.00	-2.43	-1.27	-0.79
Baby Swiss	9	35.58	0.65	1.79	0.99	5.71	6.27	-	7.35	7.72	-0.39	-1.45	-0.61	-1.02
Brick	11	40.39	1.52	3.63	0.96	5.43	4.90	1.29	7.23	7.66	-0.40	-0.71	-0.38	-0.74
Brick	11	41.21	1.95	4.52	0.97	5.30	4.98	0.90	7.19	7.82	-0.32	-0.70	-0.40	-0.98
Brick	2	38.28	1.52	3.82	-	5.25	5.37	1.07	6.33	8.08	-0.09	-0.22	-0.42	-0.79
Cheddar (Mild)	3	37.34	1.57	4.04	0.96	5.09	5.00	1.89	7.41	6.78	-0.70	-0.88	-0.30	-0.43
Cheddar (Mild)	3	36.59	1.77	4.61	0.97	5.09	5.06	1.44	7.39	6.81	-0.76	-1.00	-0.80	-0.17
Cheddar (Reduced-Fat)	6	40.26	1.60	3.82	0.97	5.19	5.11	1.15	5.35	6.21	-0.13	-0.65	-0.43	-1.28
Cheddar (Reduced-Fat)	6	44.00	1.66	3.64	0.98	4.99	5.27	0.90	5.52	5.79	-0.69	-0.57	-0.55	-0.97
Cheddar (Sharp)	3	36.34	1.78	4.67	0.96	5.27	5.27	1.69	4.30	6.39	-0.35	-0.75	-0.96	-1.19
Cheddar (Sharp)	3	36.57	1.32	3.48	0.97	5.19	5.28	1.71	4.63	5.84	0.00	-1.03	-0.59	-1.34
Cheddar-Mozzarella	6	40.09	1.62	3.88	-	5.19	5.33	1.42	6.99	6.24	-0.09	-0.27	-0.31	-0.48
Colby	4	35.96	1.61	4.28	0.96	5.45	5.61	1.09	5.76	7.39	-0.39	-0.50	-0.21	-0.57
Colby	4	40.14	1.60	3.83	0.97	5.30	5.47	1.78	5.91	6.38	-0.11	-0.63	-0.24	-0.39
Colby Jack	5	36.13	1.42	3.78	0.96	5.17	5.10	1.26	7.19	7.19	-0.20	-0.97	-0.80	-0.46
Colby Jack	5	36.85	1.35	3.53	0.98	5.01	5.40	1.37	7.70	7.38	-0.44	-0.59	-0.08	-0.46
Colby Jack (Reduced-Fat)	6	43.96	1.64	3.60	0.97	5.29	5.00	1.09	5.79	7.68	0.02	-0.90	-0.76	-1.12
Colby Jack (Reduced-Fat)	6	46.00	1.76	3.69	0.97	5.08	5.11	1.39	4.52	6.91	-0.56	-0.74	-0.73	-1.05
Colby Jack (Reduced-Na)	6	36.30	1.26	3.35	0.97	5.11	5.03	1.48	4.52	6.91	-0.17	-0.46	-1.03	-1.09
Colby Jack (Reduced-Na)	6	36.45	1.13	3.01	0.98	5.09	5.17	0.89	4.12	5.40	-0.69	-0.96	-0.39	-0.64
Feta	3	57.10	2.35	3.95	0.99	4.29	4.60	2.80	4.80	6.57	-4.58	-4.71	-4.60	-2.93
Feta	3	57.64	1.72	2.90	0.98	4.38	4.53	2.86	3.30	3.40	-4.89	-4.94	-4.07	-4.74
Gouda	6	41.15	1.62	3.79	0.97	5.28	5.25	0.88	7.29	7.38	-0.51	-0.32	-0.23	-0.83

Gouda	6	41.08	1.39	3.27	0.97	5.30	5.28	1.24	7.40	7.48	-0.44	-0.46	-0.34	-0.79
Havarti	3	37.79	1.33	3.40	0.97	5.49	5.52	1.08	6.88	7.26	-0.25	-0.61	-0.21	-0.73
Havarti	3	38.17	1.20	3.05	0.98	5.34	5.59	0.66	6.88	7.20	-0.51	+0.21 [k]	-0.29	+0.01 [k]
Havarti	6	41.32	1.27	2.98	-	5.11	5.26	1.40	8.28	7.75	-0.16	-0.61	-0.37	-0.70
Monterey Jack	5	45.10	1.87	3.98	0.98	5.15	5.20	2.41	8.08	8.16	-1.03	-0.91	-0.33	-0.37
Monterey Jack	5	35.45	1.64	4.42	0.97	5.08	5.11	2.28	8.06	7.98	-2.63	-1.17	-0.91	-0.66
Muenster	3	42.20	1.63	3.72	0.97	5.20	5.28	1.27	6.90	7.80	-0.49	-0.25	-0.24	0.00
Muenster	3	41.94	1.75	4.01	0.98	5.29	5.12	0.74	7.11	6.26	-0.10	-0.75	-0.45	-0.46
Parmesan	8	32.44	2.52	7.21	0.93	5.41	5.36	1.40	6.92	4.00	-0.88	-1.45	-1.25	-0.59
Parmesan	8	32.70	2.35	6.70	0.92	5.45	5.40	1.48	5.31	7.23	-1.51	-1.66	-1.86	-1.80
Pepper Jack	4	36.13	1.58	4.19	0.98	5.11	4.76	2.12	2.00	7.66	-0.85	-3.87	-0.81	-1.09
Pepper Jack	4	38.69	1.60	3.97	0.97	4.93	5.12	1.94	4.69	7.14	-2.86	-3.40	-3.25	-3.58
Pepper Jack	3	40.42	1.64	3.90	0.97	5.14	5.12	1.53	4.65	7.30	-2.39	-2.32	-2.10	-0.72
Pepper Jack	3	38.27	1.54	3.87	0.97	5.21	5.15	1.45	8.25	7.39	-0.62	-0.73	-0.35	-0.30
Provolone	4	42.15	1.38	3.17	-	5.24	4.97	1.81	7.70	8.70	-1.34	-0.97	-0.16	-0.72
Provolone (Mild)	8	43.05	2.08	4.61	-	5.18	5.22	1.80	5.53	6.70	-0.50	-1.84	-0.57	-0.71
Provolone (Sharp)	10	40.02	1.72	4.12	-	5.09	5.17	2.20	6.43	7.45	-1.59	-2.83	-1.27	-1.73
Provolone (Reduced-Fat)	3	48.98	1.43	2.84	0.97	4.97	4.67	1.83	6.95	7.98	-2.80	-2.23	-0.62	-1.55
Provolone (Reduced-Fat)	3	52.71	1.35	2.50	0.98	4.98	4.94	-	3.70	7.94	-0.56	-0.95	-0.24	-0.97
Provolone-Mozzarella	6	42.26	1.68	3.82	-	5.38	5.33	1.61	7.67	7.28	-0.25	-0.19	-0.17	-0.68
Swiss	6	38.57	0.52	1.33	0.98	5.36	5.50	-	5.95	6.59	-1.20	-1.11	-0.73	-2.32
Swiss	6	36.91	0.64	1.70	0.99	5.50	5.80	-	5.28	6.19	-0.93	-1.30	-0.36	-1.20
Swiss (Lacey)	5	45.17	0.33	0.73	0.99	6.02	5.87	-	7.00	8.18	-0.43	-1.19	-0.46	-1.02
Swiss (Lacey)	5	45.92	0.37	0.80	0.99	5.65	5.94	-	7.92	5.70	-1.83	-1.21	-0.31	-1.06

[a] Cheeses were national brands obtained from local retail outlets or directly from manufacturers. Qualifying descriptive information, e.g. ‘mild,’ ‘sharp’ is reproduced where provided on the package.

[b] Moisture content (%) of cheese sample on Day 0, n=2.

[c] % salt of cheese sample on Day 0, n=2.

[d] % salt-in-moisture phase (%SMP) of cheese sample on Day 0. Calculated from % moisture and % salt of the same cheese.

[e] Water activity (a_w) of cheese sample on Day 0.

[f] pH of cheese slice surface on Day 0 and Day 15, n=2.

[g] % titratable acidity (%TA) of cheese sample on Day 0 and Day 15, n=2.

[h] DeMan-Rogosa-Sharpe (MRS) agar count for lactic acid bacteria (LAB) on Day 0 and Day 15 (log CFU/g), n=2.

[i] Survival of pathogen LM=*L. monocytogenes*, SALM=*Salmonella* spp., EC=*E. coli* O157:H7, and SA=*S. aureus*, log CFU/g Day 15 – Day 0. (+) indicates growth, (-) indicates no-growth.

[j] not determined.

[k] Growth of pathogen did not exceed plating variability: 0.39, 0.41, 0.27, 0.25 log CFU/g for *L. monocytogenes*, *Salmonella* spp., *E. coli* O157:H7and S. *aureus*, respectively.

Table 2-2. Composition of natural cheeses that supported growth of *L. monocytogenes*, *Salmonella* spp., *E. coli* O157:H7, and/or *S. aureus* and pathogen survival (Δ log CFU/g) during storage for 15-days at 25°C.

Cheese[a]	Brand	% Moisture[b]	% Salt[c]	% SMP[d]	a_w[e]	pH[f]		% TA[g]	LAB count[h]		Pathogen survival (Δlog CFU/g)[i]			
						0 d	15 d	0 d	0 d	15 d	LM	SALM	EC	SA
Farmer's	12	39.85	1.71	4.11	-[j]	5.46	4.99	1.14	4.63	9.10	-0.41	-0.10	-0.39	**+1.48**[l]
Gruyere	7	34.25	1.01	2.86	0.97	5.68	5.74	1.04	5.70	7.40	**+1.01**	**+1.01**	-0.40	**+3.08**
Gruyere	7	32.07	1.41	4.21	0.98	6.28	5.78	1.55	5.04	6.70	-0.54	**+2.13**	-0.67	**+2.19**
Jack (goats' milk)	13	45.20	2.33	4.90	-	5.41	5.24	1.44	7.74	6.88	-0.40	**+2.50**	-0.62	**+1.62**
Muenster	6	41.58	1.21	2.83	-	5.48	5.53	0.66	4.85	7.67	+0.17[k]	**+1.65**	**+0.41**	**+1.77**
Provolone	3	43.17	1.03	2.33	0.97	5.29	4.78	1.36	2.70	5.40	-1.10	-0.40	-0.88	**+0.80**
Provolone	3	44.08	1.58	3.46	0.98	5.15	5.19	1.55	3.78	7.19	-0.40	-0.80	-0.52	**+0.81**
Provolone (Reduced-Na)	6	42.93	1.05	2.39	0.98	5.15	4.95	1.24	6.25	7.79	-1.20	-0.31	-0.30	**+0.62**
Provolone (Reduced-Na)	6	44.09	1.02	2.26	0.98	5.28	5.12	1.62	5.73	7.39	-0.29	-0.27	-0.63	**+1.59**
Queso Blanco	7	47.02	3.30	6.56	0.96	6.37	6.11	0.26	4.38	6.78	**+2.68**	-1.07	-2.11	**+2.57**
Queso Fresco	7	51.19	1.85	3.49	0.98	6.49	5.05	0.31	4.86	8.68	**+2.09**	**+3.02**	**+2.90**	**+1.55**
String	14	47.91	1.98	3.97	-	5.44	4.96	1.59	4.87	8.86	**+0.60**	**+2.00**	**+1.75**	**+2.39**
String	6	47.07	2.18	4.43	-	5.33	5.02	1.67	4.85	8.65	+0.22[k]	+0.39[k]	-0.38	**+1.58**
Queso Quesadilla	7	43.10	2.18	4.81	0.97	5.35	5.39	1.21	4.57	6.29	-0.01	-0.57	-0.48	**-0.40**[m]

[a] Cheeses were national brands obtained from local retail outlets or directly from manufacturers. Qualifying descriptive information, e.g. 'mild,' 'sharp' is reproduced where provided on the package.

[b] Moisture content (%) of cheese sample on Day 0, n=2.

[c] % salt of cheese sample on Day 0, n=2.

[d] % salt-in-moisture phase (%SMP) of cheese sample on Day 0. Calculated from % moisture and % salt of the same cheese.

[e] Water activity (a_w) of cheese sample on Day 0.

[f] pH of cheese slice surface on Day 0 and Day 15, n=2.

[g] % titratable acidity (%TA) of cheese sample on Day 0 and Day 15, n=2.

[h] DeMan-Rogosa-Sharpe (MRS) agar count for lactic acid bacteria (LAB) on Day 0 and Day 15 (log CFU/g), n=2.

[i] Survival of pathogen LM=*L. monocytogenes*, SALM=*Salmonella* spp., EC=*E. coli* O157:H7, and SA=*S.* aureus, log CFU/g Day 15 – Day 0. (+) indicates growth, (-) indicates no-growth.

[j] not determined.

[k] Growth of pathogen did not exceed plating variability: 0.39, 0.41, 0.27, 0.25 log CFU/g for *L. monocytogenes*, *Salmonella* spp., *E. coli* O157:H7 and S. *aureus*, respectively

[l] Bolded numbers indicate growth beyond the pathogen-plating variability.

[m] Growth (+ 0.57 log CFU/g) at Day 6 sampling; no net growth over 15-day storage period

Table 2-3. Pathogen strains used in laboratory cheese challenge studies.

Inoculum	Serotype	Strain[a]	Collection[b]	Source[c]
Listeria monocytogenes	4b	LM 101	FRI	Hard salami
	4b	LM 310	FRI	Goat cheese
	4b	ATCC 43256	ATCC	Mexican-style cheese, Calif. (1985 outbreak strain)
	4b	ATCC 43257	ATCC	Mexican-style cheese, Calif. (1985 outbreak strain)
	4b	ATCC 51414	ATCC	Raw milk, Massachusetts
	4b	ATCC 51776	ATCC	Cheese, Belgium
	4b	ATCC 51777	ATCC	Cheese, Belgium
	4b	ATCC 51778	ATCC	Cheese, Belgium
	4b	Scott A	FRI	Clinical
	1/2a	V7	FRI	Raw milk
Salmonella spp.	Cerro	FSL R8-370	FSL	Bovine
	Typhimurium	FSL S5-433	FSL	Bovine
	Newport	FSL S5-436	FSL	Bovine
	Agona	FSL S5-517	FSL	Human
	Typhimurium	FSL S5-536	FSL	Human
	Newport	FSL S5-639	FSL	Human
Escherichia coli O157:H7	O157:H7	FRIK 22	FRI	Unknown
	O157:H7	FRIK 2000	FRI	Bovine
	O157:H7	F5854	FRI	Cheese curds (1998 outbreak strain)
	O157:H7	039732	NMDH	Gouda cheese (2010 outbreak strain)
	O157:H7	CWD EC1	VT	Farmstead goat cheese
Staphylococcus aureus		I	FPL	Raw milk
		J	FPL	Raw milk
		FRI 100	FRI	Cake
		FRI 1007	FRI	Genoa sausage
		ATCC 25923	ATCC	Clinical

[a]Strain designation provided by Collection.

[b]Collection: FRI = Food Research Institute, University of Wisconsin-Madison, Madison, Wisc.; ATCC = American Type Culture Collection, Manassas, Va.; FSL = Food Safety Laboratory, Dr. Katherine Boor, Cornell University, Ithaca, N.Y.; NMDH = New Mexico Department of Health, Santa Fe, N.M.; VT = Vermont Institute for Artisan Cheese, Dr. D.J. D'Amico, University of Vermont, Burlington, Vt.; FPL= Food Pathogen Laboratory, Dr. Barbara Ingham, University of Wisconsin-Madison, Madison, Wisc.

[c] Source provided by Collection.

Table 2-4. Data from published research selected to augment laboratory product assessment.

Reference	Pathogen	No. of strains	Cheese	Storage (days)	Temp (˚C)	pH[a]	%SMP[b]	a_w	Growth/Death[c]
25	*Salmonella*	9	Queso Fresco	-[d]	20	6.60	1.64	-	LT[e]:2.5 - 3.5h GT: 1.65 - 2.17 h
24	*E. coli* O157:H7	2	Queso Fresco	-	20	6.60	1.61	-	LT: 3 - 3.45 h GT: 2.33 - 2.56 h
33	*L. monocytogenes*	5	Cheddar	30	21	5.06	1.70	0.98	-1.11
						5.30	1.80	0.97	-0.48
						5.66	5.00	0.95	-0.14
						5.28	4.80	0.95	-0.96
34	*Salmonella* spp.	5	Cheddar	30	2 1	5.06	1.70	0.98	-3.2
						5.30	1.80	0.97	-3.9
						5.66	5.00	0.95	-3.8
						5.28	4.80	0.95	-3.5
39	*L. monocytogenes*	5	Queso Blanco	6.25	25	6.80	4.53	0.97	> 5.00
14	*L. monocytogenes*	5	Queso Fresco	3	30	6.60	6.60	-	+0.39
			Queso Fresco	6	30	6.60	4.50	-	+0.95
			Queso Fresco	3	30	6.50	6.15	-	+0.74
			Queso Ranchero	1	30	6.20	4.10	-	+2.60
			Queso Panela	3	30	6.20	2.50	-	+1.81
			Queso Panela	1	30	6.70	3.95	-	+3.18
			Queso Panela	3	30	6.60	3.48	-	+0.79
			Cotija	8	30	5.60	9.60	-	> -2.00
			Cotija	6	30	5.50	12.50	-	> -2.00
			Monterey Jack	4	30	5.00	3.00	-	> -1.40
			Monterey Jack	13	30	5.20	2.72	-	> - 2.09
			Mild Cheddar	4	30	4.90	2.60	-	> -1.26
			Mild Cheddar	7	30	5.20	4.49	-	> -2.09
			Colby	9	30	5.50	4.93	-	> -2.36
			String Cheese	9	30	5.50	4.24	-	> -2.36
			Provolone	9	30	5.60	4.62	-	> -2.36
			Muenster	9	30	5.50	3.80	-	> -2.36
			Domestic Feta	4	30	4.30	7.50	-	> -2.04
			Domestic Feta	4	30	4.30	2.20	-	> -2.04

[a] pH values of cheeses at initial sampling point of experiment

[b] Certain publications stated %SMP as % brine, which was calculated using the same equation as in this study (Equation 1). For publications that included both %moisture and % salt, % SMP was calculated using Equation 1.

[c] Behavior of pathogen over storage, expressed as Δlog CFU/g or LT/GT.

[d] Not specified.

[e] LT: Lag time (h); GT: generation time

CHAPTER 3:

A Decision-Making Framework to Evaluate the Safety of Extended Non-Refrigerated Holding of Natural Cheese

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3.1 ABSTRACT

Merchandizing and handling of most cheese at >5°C is limited over concern for the potential for growth or toxin formation by pathogenic microorganisms. Analysis of data from 250 challenge trials involving 81 cheeses indicated that *Listeria monocytogenes* (LM), *Salmonella* spp. (SALM), *Staphylococcus aureus* (SA), or *Escherichia coli* O157:H7 (EC) would not grow on vacuum-packaged cheese held up to 15 days out of refrigeration (≤25°C), as long as pH alone or pH and % salt-in-the-moisture phase (%SMP) did not exceed certain limits. Cheese safety decision-making is, however, currently based on product pH and a_w. Additional trials (n=187 involving 35 cheeses) were, therefore, conducted to evaluate pathogen survival on cheese under isothermal holding (25°C) vs temperature cycling (alternating 4° and 25°C in 12 h intervals), and under aerobic vs anaerobic holding, with additional product characteristics considered. Temperature cycling and holding cheese under aerobic conditions did not significantly affect pathogen survival compared to cheese held under anaerobic, isothermal conditions. Data from all 437 trials involving 116 cheeses were combined and the impact of pH, %SMP, a_w, % moisture, and cheese type on pathogen growth/no-growth was determined. Type was not a definitive predictor of pathogen growth, some cheese did not support growth under any experimental condition, e.g. hard Italian-style, and others supported growth in all trials and across all manufacturers and lots, e.g. fresh Hispanic-style cheese; while pathogen growth varied with manufacturer/lot and holding conditions for others, e.g. Muenster. Evaluating pathogen growth/no-growth data across all intrinsic factors revealed that cheese could be held for up to 15 days at up to 25°C when pH is ≤5.1 and any % moisture, or pH is >5.1 and moisture ≤39%. This decision-making framework could be used to accurately predict pathogen growth with cheese held under a variety of merchandising or food processing conditions.

3.2 INTRODUCTION

The US Food and Drug Administration (FDA) Food Code guides food safety practices within retail food establishments and has defined a "time/temperature control for safety (TCS) food" as one for which temperature control is necessary to prevent growth or toxin production by pathogenic microorganisms *(30)*. Some foods, due to pH and water activity, are directly considered to be non-TCS foods. These foods either have a pH of < 4.2 and any water activity (a_w), or a_w < 0.88 and any pH. Foods with pH and a_w outside these boundaries are considered to be TCS foods and require refrigeration at 5°C or colder, or must be discarded after non-refrigerated holding for more than 6 h at a temperature not greater than 21°C. Alternatively, a product assessment may be conducted on TCS foods to evaluate whether extended non-refrigerated holding will allow growth of, or toxin formation by, pathogenic microorganisms. Similarly, food manufacturers operating under preventive controls must have evidence of safety if TCS foods are held for extended periods of time outside of refrigeration in a processing operation.

Many cheeses, when evaluated against the FDA Food Code criteria, would be deemed TCS foods due to their relatively high a_w and/or pH, and retail food operators abiding by this designation must hold cheese below 5°C. It has been suggested, however, that the biochemical changes that occur during cheese manufacturing and ripening create conditions that would prevent the growth of pathogenic microorganisms, thereby supporting classification of some cheeses as non-TCS foods *(5)*. A review by Bishop and Smukowski *(5)* proposed that Asiago (medium and old), Cheddar, Colby, Feta, Monterey Jack, Muenster, Parmesan, Pasteurized Process, Provolone, Romano, and Swiss/Emmentaler cheeses, when manufactured in the United States from pasteurized or heat-treated milk, following good manufacturing practices and under

the principles of Hazard Analysis and Critical Control Points (HACCP), and meeting applicable federal standards of identity *(33)* should be exempt from refrigeration requirements during ripening, storage, shipping, and display.

Leong et al. *(20)* tested the ability of 67 natural cheeses to support growth of *Listeria monocytogenes*, *Salmonella* spp., *Escherichia coli* O157:H7, and *Staphylococcus aureus* over 15 days at 25°C. Pathogens did not grow on 53 cheeses, while 14 cheeses supported growth of *S. aureus* (SA), six of *Salmonella* (SALM), four of *L. monocytogenes* (LM), and three of *E. coli* O157:H7 (EC). Of the cheeses supporting any pathogen growth, all supported growth of SA, with an increase in pathogen numbers of up to 3.0 log CFU/g. Pathogen growth varied within cheese type and between lots of a given type of cheese. Insufficient data were gathered on Swiss-style or mold-ripened cheeses, or cheeses made with non-bovine milk to evaluate pathogen growth. Leong et al. then combined results from their challenge studies with research results from other laboratories *(13, 16, 17, 27, 28, 29)* and, from this combined dataset, Leong et al. reported that pathogen growth on natural cheeses could be predicted based on intrinsic factors of pH and % salt-in-the-moisture-phase (SMP), but more data were needed to confirm the significance of a_w as a factor in predicting pathogen growth.

The data of Leong et al. and others were used to support an issue statement which was presented to the 2014 Conference for Food Protection (CFP), reviewed, and referred to the FDA for consideration *(8)*. The issue statement requested that the FDA "evaluate, consider, and research the possibility of the following change to the 2013 Food Code: *Add (E) under 3-501.19 Time as a Public Health Control to read as follows:*

(E) Natural cheeses made from *pasteurized bovine milk, that are not ripened with mold, that are not surface-ripened with bacteria, that are not Swiss, Emmentaler, and related cheeses that are*

produced using propionic acid-producing bacterial cultures, may be stored for up to a maximum of 15 days at up to a maximum of 77°F when a written procedure in the form of an SOP exists to control for time and temperature, when the cheese is accompanied by a letter of guarantee from the manufacturer, and when labeled for the consumer with a 'use by' date and the statement 'Refrigerate for quality', and that have pH and % salt in the moisture phase (SMP) levels meeting one of the following requirements for food safety:

- *pH not greater than 4.80 and % SMP not less than 1.88*
- *pH not greater than 4.90 and % SMP not less than 2.61*
- *pH not greater than 5.00 and % SMP not less than 3.34*
- *pH not greater than 5.10 and % SMP not less than 4.07*
- *pH not greater than 5.20 and % SMP not less than 4.79*
- *pH not greater than 5.30 and % SMP not less than 5.52*
- *pH not greater than 5.40 and % SMP not less than 6.25*
- *pH not greater than 5.50 and % SMP not less than 6.98*
- *pH not greater than 5.60 and % SMP not less than 7.70"*

Even though the findings of Leong et al. provided compelling evidence that certain cheeses could be safely held for extended periods without refrigeration, discussions with regulators and the industry suggested that the wording of the issue statement might present obstacles to effective decision-making. Whereas pH is routinely measured during cheese manufacture and is an intrinsic factor familiar to regulatory agents, % SMP is a measurement specific to certain segments of the cheese manufacturing industry and, since it is not defined in the FDA Food Code, the term may be unfamiliar to regulators of retail establishments. Furthermore, decision-making matrices in the FDA Food Code instruct regulators to use pH and

a_w in evaluating shelf stability of foods at retail *(30)*, suggesting that sanitarians inspecting retail food establishments may be hesitant to evaluate the safety of holding cheese unrefrigerated using the pH and %SMP criteria in the 2014 CFP issue statement.

There exists a need for practical, science-based guidance that merchandizers and food establishment operators or regulators can use to evaluate the safety of holding a particular cheese out of refrigeration for an extended period of time. Our goal was therefore to investigate intrinsic factors, in addition to pH and %SMP, which could influence pathogen growth on natural cheeses under a wide variety of merchandizing and food processing conditions and to develop a research-based decision-making framework that could be easily used by regulators to evaluate the safety of extended non-refrigerated holding of natural cheeses.

3.3 MATERIALS & METHODS

3.3.1 Cheeses. Thirty-five cheeses representing types found to be most likely to support pathogen growth in previous work *(20)* were obtained from local retail markets and stored at 4°C. Cheese types included Brick, Cheddar, Cheddar-Mozzarella, Colby Jack, Farmer's, Havarti, Monterey Jack, Muenster, Provolone, Provolone-Mozzarella and String. All cheeses were manufactured in the United States using pasteurized bovine milk and active starter cultures.

3.3.2 Proximate analysis. Cheese was analyzed for pH, % moisture, % salt, and water activity (a_w) using standard methods *(1, 6)* at the beginning of experimental trials (Day 0), as described in Leong et al. *(20)*. The % salt-in-the-moisture phase, SMP, was calculated from % moisture and % salt using equation 1:

$$\%SMP = (\%\ salt \times 100)/(\%\ salt + \%\ moisture) \qquad \text{(Equation 1).}$$

At least two measurements were taken and averages calculated for each analyte.

3.3.3 Inoculum preparation. Ten strains of *L. monocytogenes*, six strains of *Salmonella* spp., five strains of *E. coli* O157:H7, and five strains of *S. aureus* previously used in challenge-study work with natural cheeses *(20)* were used in this study. Stock cultures, working cultures, and single-pathogen multi-strain cocktail inocula were prepared according to methods described in Leong et al. *(20)*.

3.3.4 Sample inoculation. Cheese slices (approximately 25 to 30 g and approximately 70 to 80 cm^2) were inoculated with bacterial concentrations of 10^7 CFU/ml, using procedures described previously *(20)* to yield a starting inoculum concentration of 10^5 CFU/g. Inoculated cheese slices were folded with the inoculated surfaces contacting each other, and individually packaged in standard cheese retail barrier bags (B-2175; Cryovac Food Packaging and Food Solutions, Duncan, SC) with oxygen transmission rate of 3 to 6 cm^3/m^2 at 4.4°C in 24 h. To achieve aerobic conditions, bags were left unsealed; for anaerobic storage, bags were vacuum-sealed using an industry-standard vacuum sealer (Setting 3, Model 250, Ultravac Koch Packaging, Koch Supplies, Inc., Kansas City, MO).

3.3.5 Environmental conditions during unrefrigerated hold. Three conditions for non-refrigerated holding of cheese were studied: (i) holding vacuum-packaged cheese under isothermal conditions at 25°C for up to 15 days; or (ii) with temperature cycling between 4°C and 25°C every 12h; and (iii) holding cheese under aerobic conditions at 25°C for up to 15 days. Comparative trials were run to evaluate the effect of holding atmosphere (aerobic conditions vs vacuum packaging) and temperature (isothermal at 25°C vs cycling of holding temperature between 4°C and 25°C).

3.3.6 Pathogen survival. The survival of all four pathogens on 12 cheeses was evaluated under isothermal/anaerobic conditions and under temperature cycling/anaerobic conditions for up to 15

days (n=96 trials). Pathogen survival on nine of the 12 cheeses was also evaluated under isothermal/aerobic conditions (n=36 trials). Growth of *S. aureus* and *L. monocytogenes* under isothermal/aerobic and isothermal/anaerobic conditions was evaluated in 11 additional cheeses (n=43 trials). In the final set of experiments, an additional 12 cheeses were inoculated with *S. aureus* and held under aerobic conditions at 25°C (isothermal) for up to 15 days (n=12 trials).

Sampling and analysis occurred every 3 days, up to 15 days, for each pathogen/trial. Sampling and enumeration procedures were described in Leong et al. *(20).* Bacterial counts were converted to log CFU/g, and Δ-log CFU/g relative to day 0 was determined for each cheese/pathogen/trial/sampling time combination. 'Growth' of a given pathogen was an increase in pathogen number at day 15, i.e. Δ-log CFU/g at day 15 relative to day 0, which exceeded the pathogen-specific plating variability limit established in previous work *(20)*: 0.39, 0.41 and 0.27 log CFU/g for *L. monocytogenes*, *Salmonella* spp., and *E. coli* O157:H7, respectively. While there is no set tolerance for *S. aureus* in food, the National Advisory Committee for Microbial Criteria in Foods (NACMCF) has suggested a 3-log growth limit for *S. aureus* to protect public health *(15)*. 'Growth' of *S. aureus* in our work was therefore declared when Δ-log CFU/g cheese, relative to day 0, was >1.0 log at 2 or more sampling points. Any cheese that supported growth of at least one of the four pathogens was noted as 'supporting growth.'

3.3.7 Statistical analysis of the impact of unrefrigerated holding conditions on pathogen growth. There were data from 115 trials that tested pathogen survival on cheeses held under aerobic and anaerobic conditions. Nine cheeses challenged with LM, SALM, EC and SA and held under both aerobic and anaerobic conditions at 25°C yielded 72 trials total, with 36 trials for each atmosphere. Eleven cheeses, challenged only with LM and SA and held under both aerobic and anaerobic conditions, yielded another 43 complete trials, with 22 trials investigating aerobic

holding, and 21 trials investigating anaerobic holding; data were not available from one trial. Data were paired according to pathogen/cheese/atmosphere, resulting in 57 pathogen/cheese pairs that consisted of data from both aerobic/anaerobic trials. Pathogen growth was observed in 10 of the 57 paired-trials, and paired t-test was used to analyze the impact of holding atmosphere on pathogen growth in these 10 paired-trials. The survival of SA was further evaluated on 12 cheeses held at 25°C under aerobic conditions.

There were 96 trials which tested pathogen survival on cheese held under isothermal conditions (25°C) and under temperature cycling (cheese transferred between 4° and 25°C every 12 h). Twelve cheeses were challenged with LM, SALM, EC and SA and held under each of the two temperature regimes, isothermal and temperature cycling, resulting in 48 paired trials. In nine of the 48 paired-trials, pathogen growth was observed in at least one of the temperature conditions, and differences in the extent of pathogen growth (Δ-log CFU/g at day 15 relative to time 0) based on holding temperature were analyzed using paired t-test.

In addition to analyzing differences in pathogen survival based on holding atmosphere (aerobic vs. anaerobic) or temperature (isothermal vs temperature cycling), overall pathogen growth/no-growth outcomes across different holding conditions were evaluated using Fisher's exact test *(21)*. Two 2x2 contingency tables were created with the two nominal variables being pathogen survival ('growth' or 'no growth') and holding condition ('aerobic'/'anaerobic' or 'isothermal'/'temperature cycling') in two separate tests.

3.3.8 Data consolidation. Data presented by Leong et al. *(20)* and other selected publications describing pathogen survival on natural cheeses *(13, 16, 17, 27, 28, 29)* were combined with laboratory data from this study to build an inclusive dataset for statistical analyses. In alignment with the objective of Leong et al. *(20)*, all selected data met each of the following criteria: (i) the

cheeses were made with pasteurized bovine milk and active starter cultures, (ii) challenged with at least one of four pathogens, *L. monocytogenes*, *Salmonella* spp., *E. coli* O157:H7, or *S. aureus*, (iii) inoculated post-processing, and (iv) held at 20-30°C after pathogen inoculation. Information pertaining to each challenge study was collected from the selected publications, including the type of cheese, the species and number of strains of pathogenic bacteria, temperature and length of unrefrigerated holding, composition of cheese, including pH data, and survival of pathogen. Where %SMP was not provided by publication authors, this information was calculated using data that was provided for % moisture and % salt (Eq. 1). The combined dataset was statistically analyzed as outlined below.

3.3.9 Statistical analyses of intrinsic factors as predictors of pathogen growth. The consolidated dataset was used to examine the significance of intrinsic factors as predictors of pathogen growth. Subsets of the consolidated dataset were created which included variables used in a given regression analysis; these data subsets were comprised of datalines. A dataline for a particular brand/lot of cheese could include data for pH, % salt, % moisture, %SMP, a_w, and pathogen growth outcomes, where appropriate. Pairwise comparisons of intrinsic factors as predictors of pathogen growth in cheeses were conducted: pH vs. % moisture (97 datalines*;* current study, *16, 17, 20, 27, 28, 29)*; pH vs. %SMP (116 datalines; current study, *13*, *16, 17*, *20, 27, 28, 29);* pH vs. % salt (95 datalines; current study, *20, 27, 28, 29)*; and pH vs. a_w (85 datalines; current study, *20, 27, 28, 29),* using logistic regression analysis *(7)*. To rank the significance of the intrinsic factors, datalines that contained the values of pH, a_w, % moisture, and % SMP were consolidated into one single dataset for logistic regression analysis. For each natural cheese so characterized, the pathogen growth/no-growth outcome at the end of the

holding period served as the response variable and logistic regression analysis was conducted for each pair of compositional factors as predictive variables (SAS 9.2, SAS Institute, Cary, NC).

3.3.10 Statistical analysis of cheese type as predictive variable of pathogen growth. Cheese type, along with pH and % moisture, was tested for significance as a predictive factor for pathogen growth using the consolidated dataset (97 datalines; current study, *16, 17, 20, 27, 28, 29).* Each market cheese was assigned a categorical variable according to marketing/label 'type.' Cheeses of the same type, e.g. Cheddar, from different manufacturers/brands were placed into one group; reduced-sodium and reduced-fat cheeses were categorized separately from the original (non-modified) type. Analysis was conducted using the result of trials with 97 cheeses grouped into 22 types: hard Italian (n=6), Cheddar (n=7), reduced-fat Cheddar (n=2), low-sodium Cheddar (n=2), Cheddar-Mozzarella (n=3), Colby (n=7), reduced-fat Colby (n=2), reduced-sodium Colby (n=2), Farmer's (n=5), Feta (n=2), Monterey Jack (n=9), Havarti (n=6), Muenster (n=7), Brick (n=6), Gouda (n=2), String (n=4), Provolone (n=9), reduced-fat Provolone (n=3), reduced-sodium Provolone (n=2), Provolone-Mozzarella (n=3), fresh Hispanic cheeses (n=5) and semi-hard Hispanic cheese (n=3). The significance of cheese type as a predictive variable was determined with ANOVA on two logistic models: (i) pH and % moisture, and (ii) pH, % moisture, and cheese type. This analysis was conducted in R 3.3.1 (R, Vienna, Austria) using the brglm package *(19)* for bias reduction in binomial-response generalized linear models.

3.4 RESULTS & DISCUSSION

In order for a particular cheese to be able to be safely held for an extended period of time outside of refrigeration at retail or in a further-processing environment, evidence must be presented that pathogen growth will not occur and toxin will not be formed. The aim of this project was to establish a science-based decision-making framework that could easily be used by

food retailers or inspectors to assess the potential for natural cheeses to be safely held for extended periods out of refrigeration. Experimentally, pathogen growth was defined as a change in pathogen number (Δ-log CFU/g) across the 15-day trial period that exceeded the plating variability established for that pathogen (LM, SALM, EC; *20*) or which exceeded 1-log for SA. Growth of any one pathogen in a given trial under a given set of experimental conditions meant that a particular cheese was noted as 'supported growth.'

The impact of atmosphere and temperature cycling on pathogen growth on natural cheeses had not been previously addressed and was evaluated in this study**.** The survival of LM, SALM, EC, and SA on 9 cheeses was evaluated under both aerobic and anaerobic conditions at 25°C for up to 15 days (72 trials; Table 3-1). Growth of SA was noted on Brick, Provolone, and String (brand 6) cheeses, and growth of LM and SALM occurred on String (brand 6) cheese under both aerobic and anaerobic conditions. Growth of EC was observed on String (brand 6) but only under aerobic conditions. Survival of LM and SA was further evaluated on 11 cheeses at 25°C under aerobic and anaerobic conditions (43 trials; Table 3-2). Growth of SA was observed on Brick and two brands of Muenster cheese under aerobic storage; and on one brand of Muenster (brand 17) under anaerobic conditions. Growth of LM was observed only on Muenster (brand 17) and only under aerobic storage conditions.

Overall, 14 of 20 cheeses did not support pathogen growth under both aerobic and anaerobic conditions (Table 3-1, 3-2). The six cheeses that supported pathogen growth were characterized by somewhat higher pH, % moisture and $a_{w,}$ but not significantly so ($p>0.05$). There was no observable difference between %SMP in those cheeses which supported pathogen growth and those which did not. Consistent with the findings of Leong et al. *(20)*, SA was the pathogen with the greatest growth potential on cheese during extended unrefrigerated holding

(25°C) regardless of atmosphere. Overall, storage of inoculated cheese in an aerobic environment allowed growth of pathogen in 10 of 57 cheese/pathogen combinations, and anaerobic storage allowed pathogen growth in 6 of 57 trials.

Because data suggested that storage of cheese under aerobic conditions was somewhat more likely to allow for pathogen growth in general and growth of SA in particular, an additional 12 cheeses were challenged with SA at 25°C for up to 15 days (n=12 trials) under aerobic conditions (Table 3-3). Growth of SA was noted in 8 of 12 cheeses, including Brick, Farmer's, Muenster, Provolone (2 brands), reduced-fat Provolone, a Provolone-Mozzarella blend, and Queso Quesadilla. Cheeses which supported growth of SA under anaerobic conditions in this or a previous study *(20)* were: Brick, Farmer's, Muenster, Provolone, reduced-sodium Provolone and Queso Blanco, Queso Fresco, Queso Quesadilla and String. Cheese type was not indicative of pathogen growth ($p>0.05$), as Provolone (brand 17) and Farmer's (brand 12) did not support SA growth under study conditions, while Provolone (brand 4, brand 19) and Farmer's (brand 5) did support growth of SA (Table 3-3).

Of 57 direct comparisons of the outcome of pathogen growth/no-growth on cheese under both aerobic and anaerobic holding atmosphere, there were 47 outcomes of 'no-growth' under either holding atmosphere, six outcomes of 'growth' under both holding atmospheres, and four instances where there was a difference in outcome as follows: EC in String cheese (brand 6), LM in Muenster (brand 17), and SA in Mild Brick and Muenster (brand 7) (Tables 3-1, 3-2). The outcome of pathogen growth/no-growth was not statistically different ($p >0.05$) for holding atmosphere, as evidenced by the high consistency in the outcomes of 53/57 trials under both holding conditions. In the four cases in which the outcome was different, bacterial growth was observed only under aerobic conditions. This observation is not surprising as facultative

anaerobes such as LM, SALM, EC and SA are able to produce more energy using aerobic respiration, leading to greater growth *(11)*.

Most pathogen challenge studies on sliced cheese have been conducted with vacuum-packaged cheeses *(13, 16, 17, 20, 29*). Placement of inoculated cheeses at temperatures conducive to microbial growth for extended periods of time in an aerobic environment can lead to proliferation of competitive spoilage microorganisms, especially yeast and mold, which may affect the ability of pathogens to grow *(2)*. In addition, the FDA Food Code requires careful control of temperature when vacuum-packaged or Reduced-Oxygen Packaging (ROP) foods are held at room temperature, noting that ROP may allow for an extended shelf-life while also suppressing the growth of spoilage microorganisms, thereby creating an environment favorable for the growth of some pathogenic bacteria, even those that are slow-growing *(32)*.

Belay et al. studied the growth of SA in broth at 14° to 37°C under both aerobic and anaerobic conditions, and concluded that SA grew faster in aerobic conditions at all temperatures tested, with a final cell density 2-3 times greater under aerobic incubation conditions *(3)*. The growth of LM *(23)* and EC *(10)* was evaluated in broth at 37°C under both aerobic and anaerobic conditions, and the rate of pathogen growth was found to be greater under aerobic conditions. Similarly, where experimental growth occurred in the present study, the Δ-log CFU/g from day 0 to day 15 was significantly greater under aerobic storage than under anaerobic storage ($p \leq 0.05$). Pathogen survival (Δ-log CFU pathogen/g from day 0 to day 15) ranged from 1.59 to 3.59 Δ-log CFU/g for aerobic storage, and from 0.0 to 2.65 Δ-log CFU/g for the same pathogen/cheese combinations stored anaerobically. Despite the significant difference in the extent of bacterial growth for cheeses that supported growth ($p \leq 0.05$), the overall growth/no-growth outcome was not significantly different under aerobic and anaerobic conditions ($p > 0.05$), suggesting that

holding atmosphere (aerobic/anaerobic) would not be a critical factor in establishing safety of cheeses held for an extended period outside of refrigeration.

Retail display of cheese at room temperature can enhance the organoleptic quality of the product and increase sales. However, holding some cheeses at 25°C for the length of the time employed in this and a previous study, 15 days, may reduce quality through oiling-off or leakage of butterfat at the cheese surface. Therefore, we compared the impact of temperature cycling vs isothermal holding on pathogen survival on cheese. Pathogen survival was tested on 12 vacuum-packaged cheeses stored for 15 days either at a constant 25°C (isothermal), or with temperature alternating between 4° and 25°C in 12-h periods (temperature cycling) (Table 3-1). Cheeses which did not support pathogen growth under either temperature condition were characterized by slightly, but not significantly, lower pH and % moisture, and higher %SMP compared to cheeses where growth of at least one pathogen was observed. The ranges of a_w that characterized cheeses supporting growth under at least one temperature condition vs cheese not supporting pathogen growth were the same, 0.968 to 0.974. Four cheeses supported pathogen growth under both isothermal and temperature cycling conditions: Muenster, Provolone, and String (brands 14 and 6). Brick cheese supported pathogen growth under isothermal holding, but not on temperature cycling.

There was no significant difference ($p > 0.05$) in the extent of pathogen growth, Δ-log CFU/g, in cheeses stored under isothermal conditions at 25°C and cheeses exposed to temperature cycling between 4° and 25°C in 12-h shifts. Growth of SA was most often noted, and was the only pathogen that grew on Muenster and Provolone cheeses under both temperature conditions. String cheeses (brand 14, 6) supported the growth of LM, SALM and SA, but not EC, under either temperature condition. Of 48 direct comparisons of the outcome of pathogen

growth/no-growth on cheese under isothermal holding or temperature cycling, there were 39 outcomes of 'no-growth' under both temperature regimes, 7 outcomes of 'growth' under both temperature regimes, and two instances where there was a difference in outcome; SA grew on Brick and on String (brand 14) cheeses under isothermal conditions, but not during temperature cycling (Table 3-1). The outcome of pathogen growth/no-growth was not statistically different ($p>0.05$) for the two temperature regimes on holding.

The slightly lower number of 'growth' outcomes under temperature cycling compared to isothermal holding was not unexpected due to the extension of lag phase which can occur when microbial cells are introduced to a new environment *(4)*. The extension of lag phase is attributed to the metabolic adaptation needed by bacterial cells to adjust to a new environment, e.g. synthesis of cold shock proteins and alteration in membrane lipids fluidity *(9, 22)* and is most noticeable when microorganisms are exposed to temperatures near the limit of growth for the organism *(35)*. Xanthiakos et al. reported an extension of lag phase when *L. monocytogenes* growing in pasteurized milk was moved from 12°C to -2°C *(35)*. Similarly, Panagou et al. compared the observed growth and predicted growth of *L. monocytogenes* in pasteurized vanilla cream that was stored under temperature-fluctuating conditions (12 h at 4°C followed by 12 h at 12°C), and noted that observed growth was delayed relative to what was predicted, and attributed an extension in the lag phase to the temperature fluctuation *(25)*. The minimum temperatures for growth of *L. monocytogenes*, *Salmonella* spp., *E. coli* O157:H7 and *S. aureus* are reported to be 0°C, 5°C, 7°C and 7°C, respectively *(26)*, suggesting that temperature cycling which includes refrigerated storage (below 5°C) could extend lag phase and decrease the potential for pathogen growth *(24)*.

In addition to an extension of lag phase, placement of an organism at a suboptimal temperature can impact the response of the organism to other stresses such as pH. Tienungoon et al. tested the growth limits of *L. monocytogenes* in laboratory broth under a wide range of pH (3.9-6.1) and temperature (4, 10, 20, 30°C) conditions. As temperature decreased from 30°C to 4°C, the researchers observed a shift of growth limits for pH from 4.5 to 5.5, and suggested that temperatures below the optimum can negatively affect the ability of *L. monocytogenes* to grow at a pH that might otherwise be supportive of growth *(34)*. Hwang et al. investigated the effect of temperature (4, 8, 12, 16°C) and lactic acid concentration (0, 0.5, 0.75, 1.0, 1.25, 1.5%) on the growth of *L. monocytogenes* on cooked ham surfaces *(14)*. Hwang and colleagues noted that, at all lactic acid concentrations, as the storage temperature decreased, the lag phase duration increased and the growth rate decreased. Under all storage temperatures, the researchers observed an increase in lag phase duration and a non-linear decrease in growth rate of *L. monocytogenes* in the presence of higher concentrations of lactic acid *(14)*.

Given the negative impact on pathogen growth of storage at suboptimal temperatures and the consistency in the growth/no-growth outcome of pathogen growth in cheese trials when isothermal holding (25°C) and cycling of temperature between 4 and 25°C in 12-h periods were directly compared, we concluded that cheeses which do not support pathogen growth at 25°C (isothermal) would not support pathogen growth when cycled between refrigerated holding and temperatures up to 25°C for up to 15 days.

Results from the current study were combined with findings from selected publications *(13, 16, 17, 20, 27, 28, 29)* to build a comprehensive dataset of intrinsic compositional factors which could be used to predict the growth/no growth outcome for pathogenic bacteria on cheese. Data were drawn from published studies that indicated the survival of at least one of the four

pathogens (LM, SALM, SA and EC) on cheeses made from pasteurized bovine milk with active starter cultures and inoculated post-processing. The dataset excluded mold- and surface-ripened cheeses, cheeses made from unpasteurized milk or raw milk, and cheeses made from non-bovine milk. Where certain data were not reported in a particular study, e.g. %SMP, the information was calculated from data which were reported (for %SMP see Equation 1), or omitted. Each dataset was comprised of datalines which included the variables modeled in the predictive equation. The size of the dataset ranged from 80 datalines (current study, *20*) for the statistical analysis which evaluated the significance of all intrinsic compositional factor as predictors for pathogen growth (Table 3-4), to 116 datalines when the significance of only pH and %SMP were considered as predictors of pathogen growth (Table 3-5).

The significance of pH in combination with one other intrinsic factor (% moisture, %SMP, a_w, and % salt) as predictors for pathogen growth on natural cheeses was evaluated (Table 3-4). The combination of pH and either % moisture, % SMP, or a_w were significant as predictors of pathogen growth on natural cheeses ($p \leq 0.05$), with the combination of pH and % moisture being the most significant (Table 3-4). The combination of pH and % salt was the only pair that was not significant as predictive of pathogen growth (p=0.7474, Table 3-4). Consistent with the results of Leong et al., the combination of pH and %SMP was found significant in predicting cheese safety. The lack of reported a_w data in previous studies which investigated pathogen survival on cheeses *(20)*, resulted in fewer datalines available to evaluate the significance of a_w in predicting cheese safety, 45 datalines included a_w vs 81 datalines available for evaluating %SMP in Leong et al. *(20)*. To rank the significance of all compositional variables, logistic regression was used to analyze the complete dataset (80 datalines; Table 3-5). Percent salt was not a significant predictive factor when coupled with pH and was not included

in this ranking analysis. The exclusion of % salt as a variable also reduced the effect of multi-collinearity among predictive factors in the analysis. In this comprehensive approach, pH and % moisture remained highly significant predictors of pathogen growth on natural cheeses held at 20-30°C (Table 3-5). In summary, statistical findings in this study suggest that pH in combination with either % moisture, %SMP, or a_w, could be used to assess cheese safety under extended non-refrigerated storage, with the combination of pH and % moisture creating the strongest prediction.

Data from extensive research trials were plotted to establish the boundaries for growth/no-growth of pathogens on natural cheeses using the combination of pH and % moisture, pH and a_w, and pH and %SMP (Figure 3-1A – Figure 3-1C). The boundary conditions which did not support growth of pathogenic bacteria during extended room temperature holding were pH $\leq$ 5.1, and either moisture $\leq$ 39%, $a_w \leq$0.95, or % SMP $\geq$7.20.

There were 22 different types of cheese tested in the present study, and in cited published work *(16, 17, 20, 27, 28, 29)*, encompassing various holding conditions and challenges with multiple pathogens. These trials included sampling of cheeses across different manufacturers and among lots of cheese from a single manufacturer, with some cheese types employed in as many as 9 trials. Among these 22 types of cheese, 12 cheese types did not support pathogen growth under any experimental conditions: hard Italian, Cheddar (original, reduced-sodium, reduced-fat), Cheddar-Mozzarella, Colby (original, reduced-sodium, reduced-fat), Feta, Gouda, Havarti and Monterey Jack *(16, 17, 20, 27, 28, 29)*. Cheeses that supported growth of pathogens in all trials and across manufacturers and lots were fresh Hispanic-style cheeses (Queso Fresco, Queso Blanco), reduced-sodium Provolone and String *(13, 16, 17, 20)*. Statistically, cheese type was not a significant predictor of pathogen growth ($p>0.05$; data not shown). Indeed, there was an

interrelationship between cheese type and moisture content, given the existence of a standard of identity or typical manufacturing steps for some cheeses. For example, the standard of identity indicates that Cheddar should have ≤39% moisture, whereas Queso Fresco, a soft unripened cheese is expected to have ≥50% moisture. While defining a cheese as suitable for extended room temperature holding based on type or standard of identity has been suggested *(5)*, our results suggest that this approach may be flawed. Even in our work, we noted variability among pH, % moisture, and a_w in an individual cheese type across manufacturer or within lots from a single manufacturer. Differences in cheese composition within the same type of cheese may result from the use of different starter-culture strains, variation in milk quality, variation in manufacturing practices, and the extent of ripening, among other factors. When cheese type was included as a predictor of pathogen growth along with pH and % moisture, cheese type was not significant ($p > 0.05$), while both pH and % moisture were found to be highly significant ($p < 0.05$).

Statistical analyses indicated that the combination of pH and % moisture could most reliably be used to assess the potential of a given lot of cheese to support the growth of pathogenic bacteria under extended non-refrigerated holding. As shown in Figure 1A, cheeses that had a pH ≤ 5.10 and % moisture ≤ 39 did not support pathogen growth in laboratory trials. Using these compositional limits, a decision-making guide can be created to assess the safety of extended room temperature holding of cheese. According to 21 Code of Federal Regulations Part 133, cheese types, when manufactured in compliance with the standard-of-identity, which have a pH of 5.1 and below and a moisture maximum limit of 39% include Asiago (medium and old), Cheddar, low-sodium Cheddar, hard-grating cheese, hard cheeses, Parmesan (or Reggiano),

Romano and Sap sago *(33)*. These cheeses, based on our findings, would be safe for extended room temperature holding due to low moisture content (≤ 39%).

During unrefrigerated holding, the moisture content in cheese has been shown not to change significantly if the product is packaged in a way to prevent significant moisture loss, e.g. retail vacuum packaged or waxed *(20)*. In cases where a wheel or block of cheese is displayed unpackaged, moisture may slowly migrate out of the product, but this will further decrease the likelihood that the product will support the growth of pathogenic bacteria *(2)*.

The pH of cheese has been shown not to change significantly during unrefrigerated holding ($p < 0.05$) *(20)*. Data of Leong et al. showed that 33 of 55 cheeses had an average pH reduction of 0.22 ± 0.26 on holding for 15 days at 25°C, while pH increased 0.13 ± 0.10 in 22 of 55 cheeses held under similar condition. Cheeses that showed a pH increase of more than 0.05 included Brick, Reduced-Fat Cheddar, Sharp Cheddar, Cheddar-Mozzarella, Colby, Colby Jack, Reduced-Sodium Colby Jack, Feta, Havarti, Monterey Jack, Muenster, Pepper Jack, and Sharp Provolone, and none of these cheeses supported growth *(20)*. Cheese pH can, however, change during ripening prior to merchandizing or further processing *(12)*. Dutch cheeses such as Gouda typically have a pH of 5.1 within 24 h after production, but this pH can increase by approximately 0.15 units during the first 2 weeks of ripening, followed by a slight increase and greater stabilization of pH values thereafter *(12)*. Kongo reported pH values of 5.20 and 5.10 for Gouda and Cheddar during the first week after manufacturing, with pH increasing to 5.60 and 5.30, respectively at 6 months post-production *(18)*. A decision-making framework which includes compositional pH and which reflects the composition of the product at the time it is received by the retailer for merchandizing or the processor for further processing, can be safely

and effectively applied without concern for any slight modifications to pH which may occur under various holding conditions.

DECISION-MAKING AND REGULATORY FRAMEWORK

This research therefore suggests the following framework for extended unrefrigerated holding of cheese: “Cheese that, because of a combination of intrinsic factors of pH and % moisture that precludes the growth or toxin formation of pathogenic microorganisms during unrefrigerated holding at up to 77°C for up to 15 days, can be designated as a non-TCS food. Combinations of pH and % moisture which support classification of cheese as a non-TCS food are:

- pH of 5.1 or less and any % moisture;
- pH of greater than 5.1 and moisture of 39% or less.”

‘Holding’ in this case means placement of natural cheese, packaged or not, outside of refrigeration for merchandizing in the retail environment or further processing in a food establishment. Cheeses excluded from this decision-making paradigm include mold- or surface-ripened cheeses, Swiss-type cheeses, cheeses made from non-bovine milk, processed cheese, and cheese made from unpasteurized milk; insufficient data have been collected in order to evaluate safety for these cheeses during non-refrigerated holding. Natural cheeses which fall outside the boundaries for safety would have to be evaluated with a product assessment (challenge study) in order to determine suitability for extended non-refrigerated holding.

Decision-making should be supported by a regulatory framework which may require record-keeping by the food establishment, verification of cheese composition, traceability, and compliance with specific handling guidelines. The FDA Food Code currently includes such

language for raw shellfish from harvest through sale to the end-user *(31)*. Language which could provide a regulatory framework for unrefrigerated holding of some natural cheeses which meet pH and % moisture criteria would include:

- A given lot of cheese may be held at a food establishment above 5°C, and up to a maximum of 77°F (25°), for up to 15 days, when a certificate of analysis provided to the food establishment indicates that the cheese lot falls safely within boundary conditions for pH and % moisture and when other conditions are met: the cheese must be manufactured from pasteurized or heat-treated milk following good manufacturing practices and under the principles of HACCP and with added starter bacteria. A merchandiser or food processor wishing to hold cheese, unrefrigerated, for an extended period of time should set buying standards to ensure compliance with these compositional factors and have a standard written procedure on file for handling of such product.
- Values for pH and % moisture for each lot of cheese under consideration should be obtained using standard methods of analysis for cheese product moisture and pH *(6)*. Research has shown that the limits for the combination of pH and % moisture in a given lot of finished cheese will not be exceeded on holding as long as standards are met on receipt of product and prior to removal from refrigeration at a retail establishment or in a food processing environment, including in a ‘cut-and-wrap’ operation.
- The ‘clock’ for extended non-refrigerated holding begins when a given lot of cheese is removed from refrigeration at the food establishment (>5°C). Cheese removed from refrigeration may be stored at up to 25°C for up to 15 days. Storage at up to 25°C may be continuous or intermittent, with the total time not exceeding 15 days, e.g. a ‘running

clock'. Careful record-keeping will ensure management of time and temperature; cheese lot information should be clearly identified on the corresponding time/temperature log.

- Cheese sold to the consumer that was displayed as a larger block or wheel (unpackaged) at room temperature as allowed under the FDA Food Code and subsequently packaged at retail for the consumer should be marked with 'Keep Refrigerated' and *date-marked in accordance with FDA Food Code section 3-501.17 (B) and (G) and 3-502.12 (E) (30).*
- Records for each lot of cheese that is stored within the time and temperature limits should be kept by the establishment for at least 90 days.

The decision-making framework presented in this manuscript was shared with eight cheese retailers or suppliers. These businesses currently merchandise certain cheeses out of refrigeration for extended periods, 6-12 h, with the product usually returned to refrigeration at the end of the display period. Businesses cited specifications within 21 CFR 133 *(33)* for the type of cheese selected for extended non-refrigerated display. In six of eight situations, the business had already developed a written procedure such as a Standard Operating Procedure for handling cheese during merchandising which included non-refrigerated display. Each of the businesses surveyed indicated the need to work closely with regulators in order to successfully implement any changes in cheese-handling practice.

3.5 CONCLUSION

This research, in combination with previously published studies, establishes the safety of extended unrefrigerated holding of certain natural cheeses and suggests a regulatory framework for implementation of recommended guidelines for holding cheese during merchandizing or in a food processing environment.

3.6 ACKNOWLEDGEMENTS.

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Figure 3-1. (A-C) Solid lines depict boundary conditions differentiating growth (▲) and no-growth (X) of *Listeria monocytogenes*, *Salmonella* spp., *Escherichia coli* O157:H7, and *Staphylococcus aureus* on cheeses held unrefrigerated based on the following compositional factors: (A) pH and % moisture, (B) pH and $a_{w,}$ and (C) pH and %SMP.

Figure 3-1 (A)

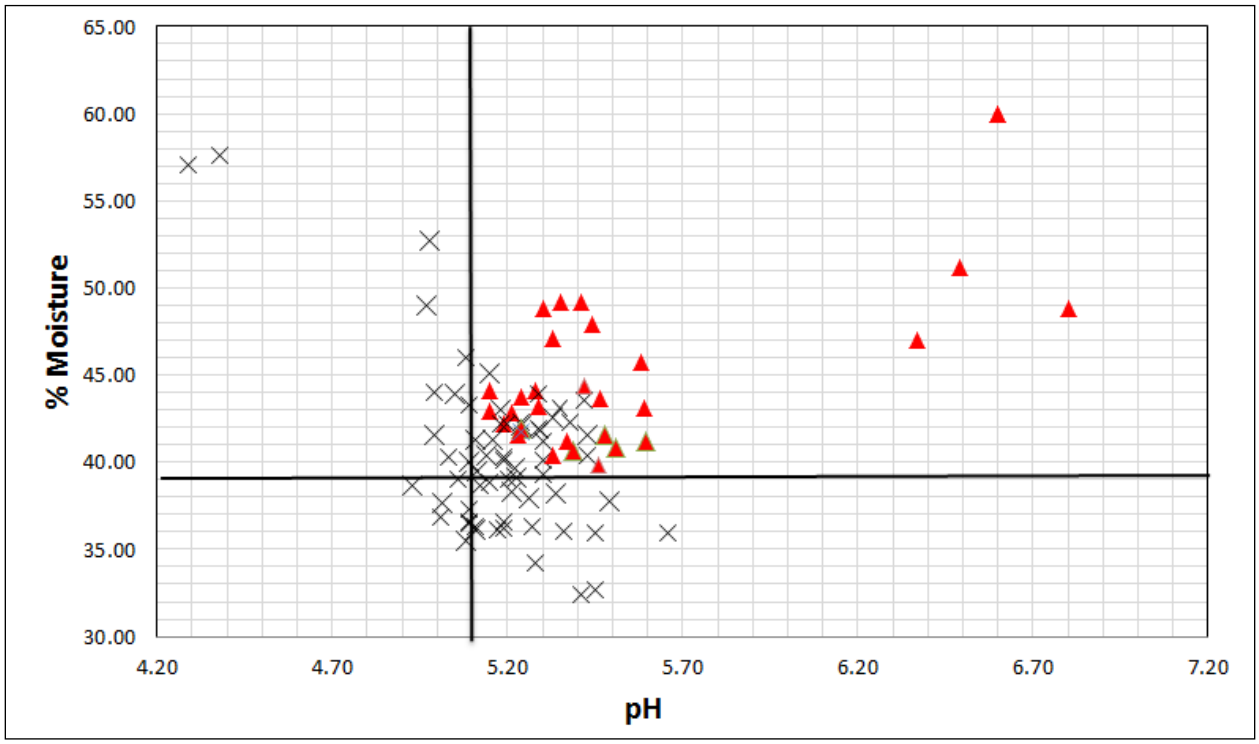

Figure 3-1(B)

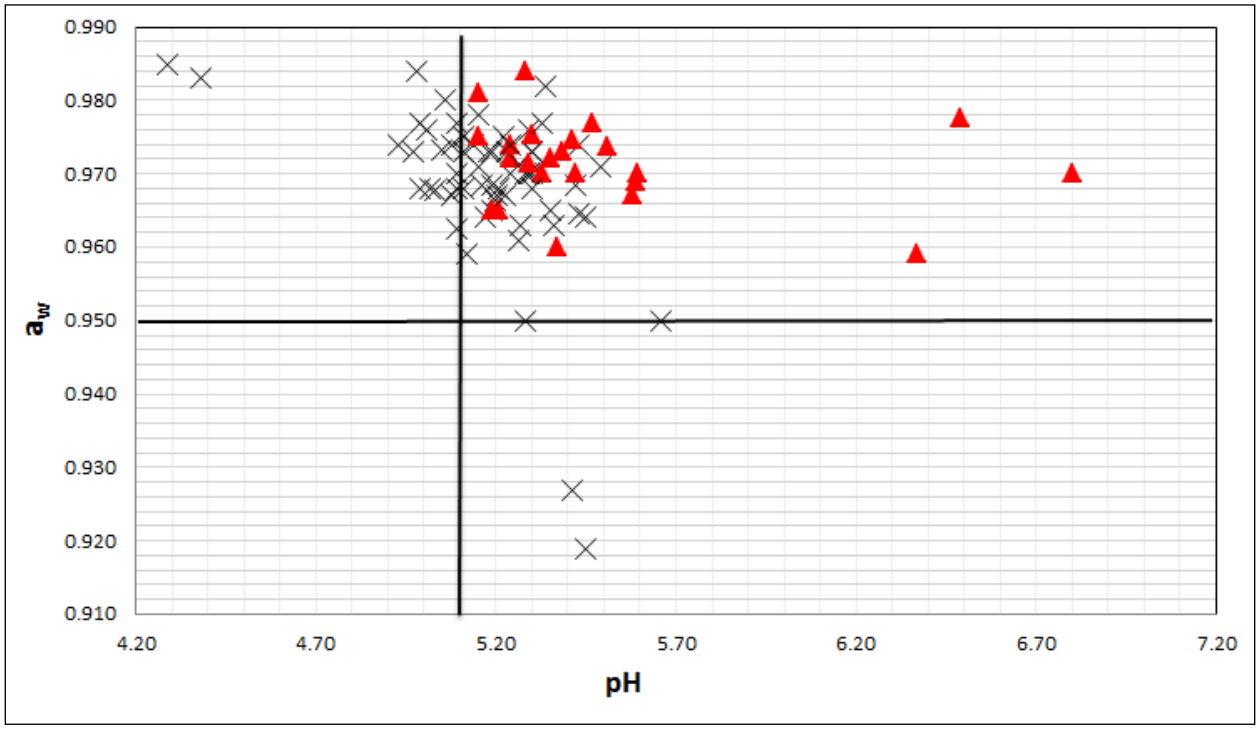

Figure 3-1(c)

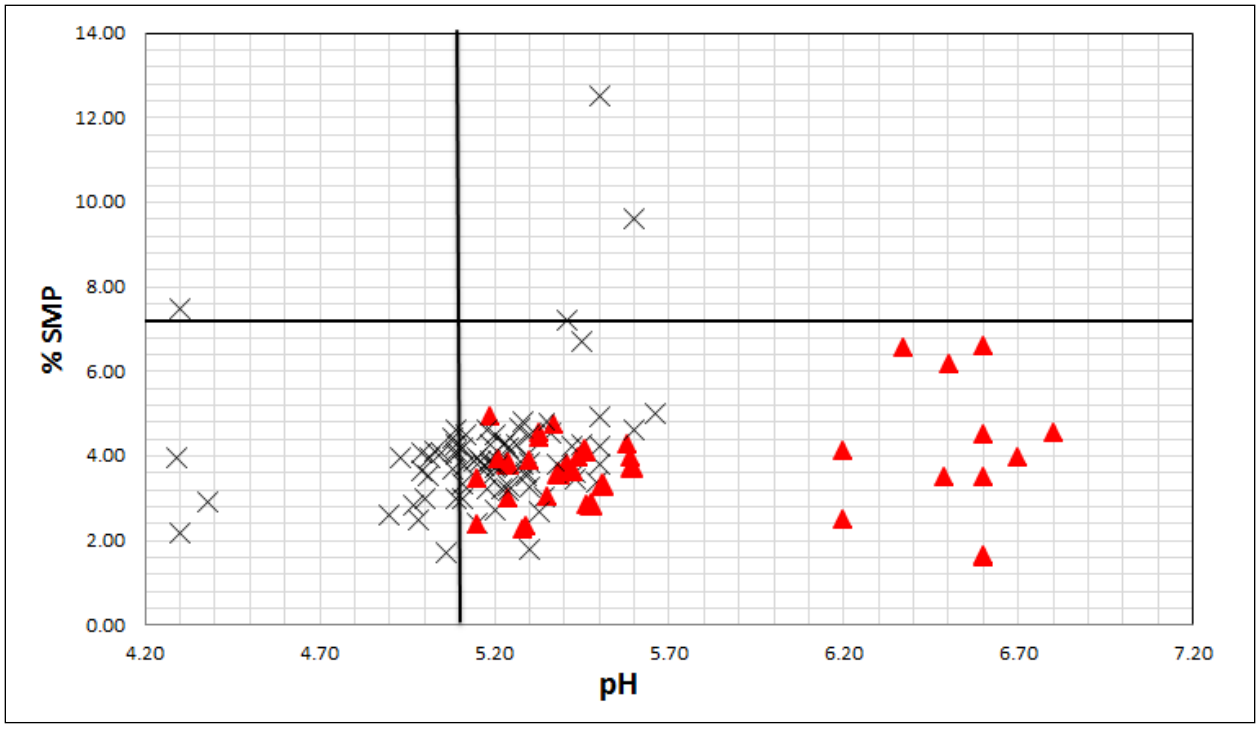

Table 3-1. Composition of natural cheeses and survival of *L. monocytogenes* (LM), *Salmonella* spp. (SALM), *E. coli* O157:H7 (EC) and *S. aureus* (SA) on natural cheeses during 15-days holding under various conditions.

Cheese	Brand	% Moisture	a_w	% Salt	%SMP[a]	pH	Storage treatment[b]			Pathogen survival (Δlog CFU/g)[c]			
							25AN	25A	4/25AN	LM	SALM	EC	SA
No Growth													
Cheddar	3	36.22	0.968	1.62	4.28	5.19	X			-0.52	-0.35	-0.38	-0.39
								X		-0.40	-0.55	-1.27	-0.46
									X	-0.65	-0.04	-0.29	-0.23
Cheddar Mozzarella	6	43.58	0.968	1.92	4.22	5.42	X			-0.33	0.05[e]	-0.16	0.00[e]
								X		ND[d]	ND	ND	ND
									X	-0.05	-0.28	-0.25	-0.98
Colby Jack	5	40.32	0.968	1.73	4.11	5.03	X			-0.95	-0.19	-0.21	-0.43
								X		-3.04	-2.43	-0.64	-1.04
									X	-2.16	-0.93	-0.48	-0.49
Farmer's	12	41.30	0.968	1.67	3.89	5.16	X			-0.44	-0.69	-0.38	-0.99
								X		-0.43	-1.32	-0.52	-1.20
									X	-0.16	-0.59	-0.22	-0.87
Havarti	3	41.58	0.974	1.49	3.46	5.43	X			-0.22	-0.50	-0.32	-0.36

							X		-0.38	-0.73	-0.01	-0.17
								X	-0.04	-0.23	-0.30	-0.59
Monterey Jack	5	43.91	0.973	1.86	4.06	5.05	X		-1.79	-0.73	-0.32	-0.85
							X		-1.92	-0.95	-0.64	-1.09
								X	-3.10	-0.45	-0.49	-1.45
Provolone Mozzarella	6	41.59	0.973	1.88	4.32	5.23	X		-1.15	-0.48	-0.17	-0.79
							X		-1.24	-0.80	-0.47	-2.22
								X	-0.44	-0.28	-0.44	-0.90
Growth												
Brick	11	40.36	0.970	1.92	4.54	5.33	X		-0.22	0.11[e]	-0.45	***1.56***[f]
							X		-0.27	-0.59	-0.25	***3.11***
								X	-0.04	-0.13	-0.21	0.21[e]
Muenster	6	40.87	0.974	1.39	3.29	5.51	X		0.27[e]	0.20[e]	-0.30	***1.56***
							X		ND	ND	ND	ND
								X	-0.45	0.18[e]	-0.23	***1.37***
Provolone	3	43.76	0.974	1.35	2.99	5.24	X		0.07[e]	-0.07	-0.08	***0.38***
							X		-0.12	-0.04	-0.10	***2.64***
								X	-0.27	-0.05	-0.23	***1.09***
String	14	49.19	0.975	1.95	3.81	5.41	X		***0.83***	***2.45***	-0.42	***2.19***
							X		ND	ND	ND	ND

									X	***0.89***	***1.41***	-0.26	0.24[e]
String	6	48.83	0.975	1.97	3.88	5.30	X			***1.66***	***1.83***	-0.46	***2.65***
								X		***4.05***	***1.86***	***3.53***	***3.59***
									X	***1.35***	***2.18***	-0.52	***2.17***

[a] % Salt-in-moisture phase, calculated: %SMP = (% salt x 100)/(% salt + % moisture)

[b] Storage treatment: 25AN = vacuum-sealed and held at 25°C (isothermal); 25A = unsealed retail bag held at 25°C (isothermal); 4/25 AN = vacuum-sealed and held with alternating 12-h periods at 4 and 25°C.

[c] Pathogen survival, change in log CFU/g from day 0 to day 15.

[d] ND=Not determined.

[e] Growth of pathogen did not exceed plating variability limit: 0.39, 0.41, 0.27 log CFU/g for LM, SALM., and EC respectively; or growth of SA did not surpass 1 log in $\geq$ 2 sampling points during the 15-day holding period.

[f] Bolded numbers indicate growth beyond the pathogen-plating variability limit, or 1.0 log CFU/g for SA in at least 2 sampling points.

Table 3-2. Composition of natural cheeses and survival (Δlog CFU/g) of *L. monocytogenes (*LM) and *S. aureus* (SA) on natural cheeses during 15-days holding at 25°C under anaerobic or aerobic conditions.

Cheese	Brand	% Moisture	a_w	% salt	%SMP[a]	pH	Pathogen survival (Δlog CFU/g)[c]			
							LM		SA	
							Anaerobic[b]	Aerobic[b]	Anaerobic	Aerobic
No Growth										
Brick	2	38.99	0.967	1.53	3.78	5.21	-1.13	-0.68	-0.62	-0.62
Colby Jack	12	42.32	0.970	1.85	4.19	5.24	-0.28	-0.59	-0.35	-0.28
Colby Jack	5	41.79	0.970	1.53	3.53	5.29	-0.26	-0.43	-0.30	-1.46
Farmer's	12	39.07	0.967	1.75	4.29	5.23	-0.58	-0.26	-0.44	-1.01
Havarti	10	39.67	0.975	1.32	3.22	5.22	ND[d]	-1.78	-0.89	-0.87
Havarti	15	37.93	0.961	1.69	4.27	5.26	-0.65	-0.37	-0.48	-0.11
Monterey Jack	3	39.49	0.968	1.54	3.75	5.11	-0.84	-3.11	-1.01	-0.74
Monterey Jack	5	37.66	0.968	1.55	3.95	5.02	-0.80	-2.70	-0.40	-0.78
Growth										
Brick (Mild)	16	43.68	0.977	1.27	2.83	5.47	-1.02	-0.83	-0.29	***1.59***[f]
Muenster	17	41.24	0.970	1.60	3.74	5.59	0.18[e]	***0.65***	***1.07***	***2.44***
Muenster	7	40.66	0.973	1.51	3.58	5.39	-0.24	-1.05	-0.31	***2.10***

[a] % Salt-in-moisture phase, calculated: %SMP = (% salt x 100)/(% salt + % moisture)

[b] Storage treatment: Anaerobic = vacuum-sealed and held at 25°C (isothermal); Aerobic = unsealed retail bag and held at 25°C (isothermal).

[c] Pathogen survival, change in log CFU/g from day 0 to day 15.

[d] ND=Not determined.

[e] Growth of pathogen did not exceed plating variability limit: 0.39 log CFU/g for; LM or growth of SA did not surpass 1 log in $\geq$ 2 sampling points during the 15-day holding period.

[f] Bolded numbers indicate growth of LM beyond the pathogen-plating variability limit; or growth of SA $\geq$ 1 log in $\geq$ 2 sampling points.

Table 3-3. Composition of natural cheeses and the survival of *S. aureus* at 25°C on natural cheeses during 15-day holding under aerobic conditions.

Cheese	Brand	% Moisture	a_w	% Salt	% SMP[a]	pH	Δlog CFU/g[b]
No Growth							
Cheddar Mozzarella	5	42.22	0.973	1.41	3.23	5.18	-1.18
Farmer's	12	41.57	0.968	1.76	4.06	4.99	0.21[c]
Provolone	17	42.57	0.977	1.18	2.70	5.33	-0.90
Queso Chihuahua	18	41.70	0.974	1.43	3.32	5.24	-0.21
Growth							
Brick	11	43.13	0.969	1.79	3.98	5.59	***0.22***[de]
Farmer's	5	44.40	0.97	1.68	3.65	5.42	***2.20***
Muenster	3	41.89	0.972	1.65	3.79	5.24	***0.29***
Provolone	4	42.83	0.965	1.75	3.93	5.21	***1.81***
Provolone	19	42.16	0.965	2.18	4.92	5.19	***1.40***
Provolone (Reduced Fat)	3	49.21	0.972	1.54	3.03	5.35	***2.58***
Provolone-Mozzarella	6	45.72	0.967	2.04	4.27	5.58	***-2.10***[df]
Queso Quesadilla	7	41.17	0.96	2.05	4.74	5.37	***1.51***

[a] % Salt-in-moisture phase, calculated: %SMP = (% salt x 100)/(% salt + % moisture)

[b] Pathogen survival, change in log CFU/g from day 0 to day 15.

[c] Growth of SA did not surpass 1 log in $\geq$ 2 sampling points on holding for 15-days

[d] Bolded numbers indicate growth of SA $\geq$ 1 log in $\geq$ 2 sampling points on holding for 15-days

[e] *Growth of* SA $\geq$ 1 log was observed at 3 sampling points (days 3, 6, 12), day-15 sampling point showed 0.22 Δlog CFU/g.

[f] Growth of SA≥ 1 log was observed at 2 sampling points (day 6, 12), day-15 sampling point showed -2.10 Δlog CFU/g.

Table 3-4. Significance of intrinsic factors in combination with pH as predictors for pathogen growth[a] on natural cheeses holding at 20-30°C.

Combination of Predictor variables	Datalines[b]	References	P values	
			pH	Associate predictor variable
pH | % Moisture	97	current study, *16, 17, 20, 27, 28, 29*	<0.0001	0.0002
pH | %SMP	116	current study, *13, 16, 17, 20, 27, 28, 29*	<0.0001	0.0168
pH | a_w	85	current study, *16, 17, 20, 29*	0.0002	0.0171
pH | % Salt	95	current study, *20, 27, 28, 29*	0.0002	0.7474

[a] Growth of pathogen exceeded plating variability limit: 0.39, 0.41, 0.27 log CFU/g for LM, SALM., and EC respectively; or growth of SA surpassed 1 log in $\geq$ 2 sampling points on holding for 15 days

[b]Each dataline includes the reported values of pH, % moisture, aw, % salt, and %SMP of the cheese sample and the growth or no-growth outcome of pathogenic bacteria after 15-day holding period.

Table 3-5. Significance of intrinsic compositional factors as predictor variables for pathogen growth[a] on natural cheeses held at 20-30°C.

Cheese compositional variables[b]	P values
pH	0.0003
% Moisture	0.0041
% SMP	0.5505
a_w	0.8499

[a] Growth of pathogen exceeded plating variability limit: 0.39, 0.41, 0.27 log CFU/g for LM, SALM., and EC respectively; or growth of SA surpassed 1 log in $\geq$ 2 sampling points during the 15-day holding period.

[b] The significance of compositional variables was analyzed using logistic regression (SAS 9.2, SAS Institute, Cary, NC). This analysis consisted of thirty-five datalines from this study (Tables 1-3) and 45 datalines from a previous publication *(20)*. Each dataline includes the values of pH, % moisture, aw, % salt, and %SMP of the cheese sample and the growth or no-growth outcome of pathogenic bacteria after 15-day holding period.

CHAPTER 4:

TRANSCRIPTOMIC ANALYSIS OF *LISTERIA MONOCYTOGENES* SCOTT A GROWN ON QUESO FRESCO, A FRESH HISPANIC-STYLE CHEESE THAT HAS BEEN IMPLICATED IN FOODBORNE ILLNESS OUTBREAKS

4.1 ABSTRACT

Listeria monocytogenes (LM), a ubiquitous pathogenic bacterium, is capable of adapting to environmental stresses and growing to a high concentration at standard refrigeration temperature (≤4°C). Queso Fresco (QF) is a fresh Hispanic-style cheese that has been frequently associated with listeriosis outbreaks, due to its near-to-neutral pH (6.2-6.5) and low salt content (<1.5%). Information about the molecular mechanisms and stress responses of LM growing in complex environment such as food is currently lacking, and such information could potentially facilitate the development of effective antilisterial strategies. This study aimed to understand the comparative whole-genome gene expression of LM-Scott A growing on QF and in tryptic soy broth (TSB) at 7°C. Compared to growth in TSB, a total of 703 genes were transcribed differentially (false discovery rate [FDR] ≤0.01; $\log_2$ fold change ≥1.5), with 494 genes significantly upregulated and 209 genes downregulated in LM-Scott A when grown on QF at 7°C. RNA extraction was performed when cultures were reaching late-log phase under both growing conditions, to ensure that the transcriptomics data reflect only the influence of growing environment, but not the effect of different growth phase. Results generated from gene ontology (GO) enrichment analyses of the differentially expressed genes suggest that LM-Scott A grown on Queso Fresco could be (i) diversifying its intake of carbohydrates, (ii) activating cobalamin and histidine biosynthesis, (iii) utilizing both ethanolamine and 1, 2-propanediol, (iv) increasing iron intake, and (v) activating prfA-dependent virulence genes, while decreasing its (vi) flagellar formation, (vii) putrescine/spermidine transport, (vii) synthesis of ribosomal proteins, peptidoglycan and pyrimidine ribonucleotide, when compared to the same strain grown in TSB. This study provides information about specific metabolic processes that are important for LM growth on QF, which could be taken into account when developing listerial control measures.

4.2 INTRODUCTION

Survival of *Listeria monocytogenes* in the food processing environment presents a challenge in the ready-to-eat foods, due to the ability of *L. monocytogenes* to grow under standard refrigeration temperatures (≤4°C). Immunocompromised individuals including pregnant women and the elderly are particularly susceptible to listeriosis, which can lead to meningitis, sepsis, miscarriages and neonatal deaths (69). The infection caused by *L. monocytogenes* is known to have high mortality rate, causing death in approximately 20-30% of the infected patients (57). One of the largest listeriosis outbreaks occurred in 1985 in Los Angeles. It was linked to contaminated Queso Fresco, a type of fresh unripened Hispanic-style cheese. In total, the 1985 outbreak resulted in 142 listeriosis cases and 28 deaths (18 adults, and 10 infants) (51). In comparison to other cheese varieties, Queso Fresco has been associated with listeriosis outbreaks at a greater frequency. During the period 1998-2011, Queso Fresco was linked to 19 listeriosis outbreaks in the United States. The majority of the outbreaks involved Queso Fresco that was made with unpasteurized milk; however fresh cheeses made with pasteurized milk are susceptible to *Listeria* contamination as well, given the ubiquitous nature of *L. monocytogenes*. A surveillance study conducted for 3 Latin-style fresh-cheese manufacturing plants showed that 6.3% of cheese samples and 11% of environmental samples were contaminated with *L. monocytogenes*, signaling that the manufacturing environment could be the source of *L. monocytogenes* contamination (38). Moreover, Queso Fresco, is likely to support growth of L. monocytogenes once contaminated, due to its high pH (~pH 6.5) and low salt content (≤1.5%)(48, 88, 89).

Results from previous studies indicated that Queso Fresco with pH and salt content ranging from 6.08-6.75, and 0.8-1.67% was able to supported >4 log growth of *L.*

monocytogenes, even when the inoculated cheeses were stored at 4°C (48, 88, 89). Recognizing the susceptibility of Queso Fresco to *L. monocytogenes* contamination and its ability to support extensive growth of the pathogen, Legget et al. recommended the addition of antimicrobial compounds or/and other post-processing control measures to kill and suppress the growth of *L. monocytogenes* (48). Generally recognized as safe (GRAS) antimicrobials including lauric alginate, bacteriophage P100 and potassium lactate-sodium diacetate mixtures have been tested for their bacteriacidal and bacteriostatic effect upon *L. monocytogenes* inoculated onto Queso Fresco. These antimicrobials were effective in reducing the initial population of *L. monocytogenes*, however regrowth of the pathogen up to 4-log CFU/g was observed during the 28-days storage at 4°C. A similar observation was reported for other antimicrobial ingredients as well, including nisin, caprylic acid and trans-cinnamaldehyde (24). The majority of the *L. monocytogenes* strains that survived the antimicrobial treatments on Queso Fresco exhibited 1-4 log CFU/g growth by the end of 20-days storage at 4°C. Based on these results, the authors suggested that adaptation to the antimicrobials may have helped the pathogen to survive and grow to a high concentration (24). Similar *L. monocytogenes* regrowth was also observed in Queso Fresco that was treated with high-hydrostatic pressure processing (HPP). A 20-min 600MPa HPP treatment was successful in reducing *L. monocytogenes* to below the detection limit (0.91 log CFU/g), while the surviving cells were able to grow up to 4-8 logs CFU/g at the end of the 60-day storage at 4°C (96). Overall, the outcomes of these studies indicate that there is still a need of a more effective antimicrobial treatment for Queso Fresco. A better understanding about *L. monocytogenes* physiology and its mechanisms to survive and grow in the food product could facilitate the development of effective antimicrobial strategies.

To understand the effect of a specific treatment on bacterial physiological responses, many studies have investigated the molecular mechanisms and stress responses of pathogenic bacteria in controlled environments such as laboratory media. While these approaches can be necessary first steps, investigation of the physiological responses of bacteria in complex food matrices is necessary to enhance our understanding of the simultaneous effect of multiple factors on bacterial survival in complex environment. This understanding can be achieved through whole-genome gene expression or transcriptomics studies, from which the global gene expression data could reveal the physiological responses and major metabolic pathways that are critical for bacterial survival/growth in specific food products. Bergholz and colleagues (8) argued that the information gleaned from transcriptomics analyses could potentially facilitate the development of solutions to improve food safety, e.g. identifying compounds that could hinder the critical metabolic processes. For example, *L. monocytogenes* grown on cold smoked salmon showed upregulation of genes related to metabolism of both 1,2-propanediol and ethanolamine, along with the induction of agmatine deiminase system, suggesting that *L. monocytogenes* were adapting to the acid by-products of 1,2-propanediol and ethanolamine metabolism (94). Based on this finding, Tang et al suggested that the use of antimicrobial treatment that incorporated acids similar to the by-products produced by the bacteria could be effective in controlling the pathogen on cold smoked salmon (94). Additionally, *L. monocytogenes* that was grown on cantaloupe and cold smoked salmon showed induction of PrfA-dependent virulence genes. The information regarding virulence gene expression of pathogenic bacteria as a result of growing on food matrices could further improve risk assessment.

To facilitate the development of effective antimicrobial strategies for Queso Fresco and to improve risk assessment, this study aimed to understand the metabolic activities and virulence

gene expression of *L. monocytogenes* Scott A grown on Queso Fresco, by generating whole-genome gene expression data using RNA-sequencing.

4.3 MATERIALS AND METHODS

4.3.1 Bacterial strain and inoculum preparation

L. monocytogenes Scott A (LM-Scott A), a widely investigated serovar 4b strain was used in this study. The stock culture of LM-Scott A (Food Research Institute, University of Wisconsin-Madison) was maintained at -20°C in tryptic soy broth (TSB) with added 0.6% yeast extract (TSBYE; Difco, Becton Dickinson, Sparks, MD) and 10% glycerol (wt/vol) (Fisher Scientific, Itasca, IL). Fresh working cultures were prepared by thawing an individual stock culture tube and streaking for isolation onto Listeria Selective agar (LSA, Oxoid, Ogdensburg, NY) with added Listeria selective supplement (Oxoid). The streaked culture plates were incubated at 35°C for 48 h, followed by immediate morphology and biochemical testing using Gram stain and API Listeria (API, bioMérieux, Durham, NC) respectively for identity verification. Upon verification, working culture plates were stored at 4°C for up to 1 month. Inoculum was prepared by transferring an isolated colony from a working culture plate into sterile 9 ml TSB, followed by incubation at 35°C for 20-22 h in a static condition. This culture was used to inoculate broth medium and Queso Fresco as described below.

4.3.2 Inoculation of TSB and determination of bacterial growth

The stationary-phase inoculum culture (~10^9 CFU/ml) was diluted 100-fold in TSB to a concentration of 10^7 CFU/ml. Three ml of the diluted culture were transferred to 27 ml sterile TSB in a 50 ml conical Falcon tube (Beckton Dickinson Labware, Franklin Lakes, NJ), which yielded approximately 10^6 CFU/ml as the starting concentration for bacterial growth. The inoculated broth tube was incubated at 7°C statically.

The cell numbers of LM-Scott A in TSB were determined by plating at every 24 h for up to 12 days. At each sampling point, TSB samples in the Falcon tube were vortexed for 5 s and 0.1 ml was transferred into 9.9 ml Butterfield's phosphate diluent (BPD, Nelson Jameson, Marshfield, WI) and serially diluted. From the final dilution tube, 0.1 ml of the sample was spread-plated onto LSA, which was then incubated at 35°C for 48 h. At every sampling point, the pH values of TSB samples were measured.

4.3.3 Inoculation of Queso Fresco and determination of bacterial growth

The stationary-phase inoculum culture (~10^9 CFU/ml) was diluted 10-fold in TSB to a concentration of 10^8 CFU/ml, which was then centrifuged at 4637 x g to pelletize the cells. The supernatant was removed and an equal volume of 0.8% saline solution was used to resuspend the pellet. Queso Fresco manufactured by a cheese company based in the U.S. was purchased from a local retail establishment, and aseptically sliced (2cm x 5cm, 10 ± 0.5 g) and inoculated for experimental study in less than 1 week after the purchase. Cheese slices were transferred to sterile petri dishes and inoculated on one-side with 100 µl of diluted inoculum (~10^8 CFU/ml) to reach approximately 10^6 CFU/g. Inoculated cheeses were left in the biosafety hood for 5 min to allow bacterial attachment. Then, the cheese slices were folded in half to ensure that as many of the inoculated bacterial cells as possible were in contact with the surface of Queso Fresco. The folded slices were subsequently transferred into standard retail barrier bags with an oxygen transmission rate of 3 to 6 cm^3/m^2 at 4°C in 24 h (B- 2175, Cryovac Food Packaging and Food Solutions, Duncan, SC). The bags with individual cheese slices were weighed and vacuum packaged using a commercial vacuum sealer (Setting 3, Model 250, Ultravac Koch Packaging, Koch Supplies Inc., Kansas City, MO). The packaged inoculated cheeses were incubated at 7°C for up to 12 days.

The population of LM-Scott A on Queso Fresco held at 7°C was determined every 24 h starting from day 0 through day 12. At every sampling point, the inoculated Queso Fresco was analyzed for pH value, followed by 1:10 (wt/wt) dilution using sterile trisodium citrate buffer (71.4 mmol/l), and stomaching (AES Smasher, AES Chemunex, Bruz, France) at high speed for 2 min. The samples were serially diluted in 9-ml BPD and 0.1 ml of the final dilution was spread-plated onto LSA. Colonies of LM-Scott A on agar plates were counted after 48 h incubation at 35°C. To determine the concentration of lactic acid bacteria in Queso Fresco, samples from Day 0, 6, and 12 were plated on de Man, Rogosa and Sharpe (MRS) agar by following the similar precedures mentioned previously, and the colonies of lactic acid bacteria on agar plates were counted after 72 h incubation at 35°C.

Three independent biological replicates (n=3) were conducted for the growth of LM-Scott A, both in TSB and on Queso Fresco. The cell density of LM-Scott A over time was plotted using the bacterial cell numbers obtained from the growth trials on Queso Fresco and in TSB (Figure 4-1). DMFit (ComBase online: http://browser.combase.cc/DMFit.aspx) was used to measure the growth parameters of LM-Scott A, including the lag phase duration, maximum growth rate and population. Paired t-test (Excel 2010) was used to statiscally compare these growth parameters of LM-Scott A under the two growing conditions.

4.3.4 RNA isolation, quantity and quality assessment

For each replicate of LM-Scott A grown in TSB, a total of 10 ml TSB that contained late-log phase LM- Scott A culture was transferred into a new 50-ml Falcon tube, and 2 volumes of RNAprotect bacterial reagent (Qiagen Inc., Valencia, CA) with added 100 µg/ml of rifampicin (Sigma, St. Louis, MO) were transferred to the same tube. The mixture was vortexed for 10 s, followed by incubation at room temperature (~21°C) for 10 mins.

For LM-Scott A grown to late-log phase on Queso Fresco, prior optimization work was conducted to ensure successful extraction of high quality RNA. The quality of RNA samples extracted using RNAprotect bacterial reagent (RNApro) and trisodium citrate buffer were compared in three independent biological trials. In each trial, the bacterial RNA was extracted from 6 slices of cheese (each ca. 10 g). To each 2 slices of cheese, 20 ml of trisodium citrate solution or RNApro was added, followed by 30 s hand stomach for each slice of cheese. The solution mixture was then filtered using the filter bag (B1348WA, Whirl-Pak bag; Nasco, Fort Atkinson, WI) to reduce cheese particles, and the filtered solution was transferred into a new 50-ml Falcon tube, followed by 10 min incubation at room temperature (~21°C). This procedure was repeated consistently for all 6 cheese slices. Qubit 2.0 Fluorometer (Life Technologies, Carlsbad, CA) was used to quantify the RNA samples, while RNA PicoChip of Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA) was used to assess the integrity of the RNA samples.

Based on results from the optimization work, trisodium citrate buffer was subsequently used to extract RNA samples for transcriptomics analysis, and the extraction procedures mentioned above were strictly followed. However, to increase the RNA yield, a total of 14 cheese slices was used in the extraction procedures.

Upon harvesting the cells of LM-Scott A from TSB or Queso Fresco, centrifugation at 4637 x g for 10 min was conducted to pelletize the bacterial cells. After removing the supernatant, 3 ml and 6 ml of pre-chilled Tri Reagent solution (AM9738; Ambion, Austin, TX) were used to dissolve the cell pellets from TSB and Queso Fresco samples, respectively. The suspension was distributed into 1.5-ml sterile screw-capped vials that contained 200 mg of zirconia beads (0.1 mm diameter; Biospec products, Bartlesville, OK) and 200 mg of glass beads

(0.1 mm diameter; Biospec products), with each tube containing 1 ml of the dissolved cell pellet. The bacterial cells in the suspension were subjected to 5 x 45 s mechanical lysis using a MiniBeadbeater-16 (Biospec products), with 3 min cooling on ice between each round of lysis. After the lysis step, to each tube was added 200 µl of chloroform, and each tube was subjected to 30 s beadbeating, 3 min incubation at room temperature (~21°C) and 2 min on ice. The samples were centrifuged at 14,100 x g at 4°C for 15 min. The aqueous phase of the lysate was carefully transferred into a new sterile 1.5-ml vial, extra care was taken to avoid transferring the interphase which typically contains DNA molecules. An equal volume of 70 % ethanol was added to the aqueous phase, and the mixture was transferred to an RNeasy mini spin column, which was then subjected to 15 s centrifugation at 14,100 x g at room temperature (~21°C). Subsequently, the standard protocol of RNeasy mini kit (Qiagen, Germantown, MD) was followed for the rest of the RNA extraction procedures. Purified RNA samples were kept at -80°C until use in next steps.

In addition to using Qubit 2.0 and Agilent Bioanalyzer, Nanodrop ND1000 (Thermo Fisher Scientific) was also utilized to assess the purity of all RNA samples. RNA samples with a minimum concentration of 30 ng/µl, RIN (RNA Integrity Number) ≥7.0, A260/A280 at 2.0 ± 0.1, and A260/230 at 2.1 ± 0.1 were qualified for downstream procedures.

4.3.5 Reduction of rRNA, preparation of cDNA fragment libraries and RNA-sequencing

Ribo-Zero rRNA Removal kit (Epicentre, an Illumina company, Madison, WI) was used to remove rRNA from the extracted RNA samples, by following the manufacturer's protocols. Briefly, magnetic beads were batch-washed using provided resuspension solution. The removal solution containing probes for rRNA hybridization was combined with 1.5 µg of each of the RNA samples. To remove the probe-hybridized rRNA, the washed magnetic beads and the RNA samples were combined, heat treated (50°C for 5 min) and placed on a magnetic stand until the

liquid became clear (~1 min). The supernatant that contained the depleted RNA was obtained and further purified using Agencourt RNAClean XP kit (Beckman Coulter Genomics, Danvers, MA), for removing the remaining salt and buffer, and for concentrating the depleted RNA samples. To assess the effectiveness of the rRNA depletion procedure, 1 µl of each RNA sample was transferred onto a RNA PicoChip (Agilent Technologies, Santa Clara, CA) and loaded into the Agilent 2100 Bioanalyzer (Agilent Technologies).

The following procedures convert RNA to cDNA, which can be processed by the sequencing machine for downstream data analysis. The cDNA fragment libraries were prepared using TruSeq RNA Sample Preparation Kit v2 (Illumina, San Diego, CA, USA), by following the manufacturer's protocol. The depleted RNA samples were concentrated using the Vacufuge concentrator (Eppendorf, Hauppauge, NY), and subsequently fragmented. Reverse transcriptase and random primers were utilized to copy the resulting RNA fragments into first strand cDNA, while DNA Polymerase I and RNase H were used to synthesize the second strand cDNA. The last steps involve repairing the ends of fragments, adenylating the 3' end and ligating adapters. Lastly, the cDNA libraries were purified using Agencourt AMPure XP beads (Beckman Coulter Genomics), and enriched with PCR.

Prior to sequencing, the cDNA libraries were assessed for quantity using Qubit dsDNA HS (Invitrogen, Waltham, MA), and for quality using Agilent DNA1000 chip (Agilent Technologies). A total of six libraries (three replicates each for Queso Fresco and TSB, respectively) were loaded on a single lane of a flow cell, and sequenced using HiSeq 2500 (Illumina) to generate single-end 100bp reads.

4.3.6 Alignment of RNA-sequencing data

Quality of sequence data was analyzed using FastQC (Barbraham Bioinformatics, Barbraham, Cambridge, UK). The adapter sequences and low quality reads were trimmed using Skewer 0.2.2 (34). The annotated genome of LM-Scott A was downloaded from NCBI (GenBank accession number: CM001159). The trimmed sequences from this experiment were aligned to the indexed reference genome using STAR (Spliced Transcripts Alignment to a Reference; 21) with the default mode.

4.3.7 Expression estimation and differential expression analysis

RSEM (RNA-Seq by Expectation Maximization; 50) was used to quantify the relative transcript abundances for each sample. In RSEM, the uncertainty resulting from read alignment ambiguity is accounted for by using the Bayesian model, which produces the maximum likelihood abundances, posterior mean estimate and 95% credibility interval of gene abundances. RSEM provided the RPKM (Reads Per Kilobase of transcript per Million mapped reads) and TPM (Transcript Per Million) values, which normalized the gene expression read counts for the biases resulted from length of transcript and sequencing depth. Statistical significance of differential gene expression under the two growing conditions (Queso Fresco versus TSB) was determined using EdgeR (Empirical analysis of Digital Gene Expression in R; 75), which is a Bioconductor package that controls the extent of overdispersion across transcripts using an empirical Bayes method, and takes into account both technical and biological variability using the overdispersed Poisson model. For each gene, EdgeR returned the fold-change values and the FDR (false discovery rate) values, which are the adjusted p-values that can inform the statistical significance of differential expression. Genes that were considered as differentially expressed fulfilled the following criteria: FDR ≤ 0.01 and $\log_2$ fold-change ≥ 1.5. The annotation of

differentially expressed genes was confirmed by comparing the amino acid sequence of LM-Scott A against the sequences of *L. monocytogenes* F2365 (GenBank accession number: NC_002973) using BLAST (56).

4.3.8 Gene ontology enrichment analyses

To understand the physiological state of LM-Scott A grown on Queso Fresco in comparison to LM-Scott A grown in TSB, Gene Ontology (GO) enrichment analysis was conducted using BiNGO (54), a Cytoscape plugin, to identify metabolic pathways or biological functions in which a significant number of the genes was differentially expressed. The hypergeometric statistical test and Benjamini & Hocberg FDR correction were selected for the GO enrichment analyses, and the analyses were carried out separately for up- and down-regulated genes.

4.4 RESULTS

Growth parameters of LM-Scott A in TSB and on Queso Fresco at 7°C.

The growth parameters of LM-Scott A on Queso Fresco and in TSB at 7°C were determined (n=3). Under 7°C storage temperature, the average lag phase durations of LM-Scott A on Queso Fresco and in TSB were not significantly different (2.01 ± 0.15 vs. 2.29 ± 0.37 days; $p > 0.05$). The average maximum growth rate of LM-Scott A grown on Queso Fresco was significantly lower than culture grown on TSB (0.43 ± 0.05 log CFU/g/day vs. 0.71 ± 0.03 log CFU/ml/day; $p < 0.05$). Similarly, the maximum population achieved by LM-Scott A on Queso Fresco was 7.31 ± 0.08 log CFU/g, which is significantly lower ($p < 0.05$) than the maximum population achieved in TSB, 9.14 ± 0.06 log CFU/ml. At late-log phase of LM-Scott A growth, the pH values of inoculated Queso Fresco and TSB were pH 6.34 ± 0.03 and 7.09 ± 0.03, respectively (Figure 4-1). The pH of inoculated TSB decreased from pH 7.20 ± 0.02 on day 0 to

pH 6.70 ± 0.05 on day 12; whereas the pH of inoculated Queso Fresco stayed relatively constant throughout the 12-day storage period, with pH 6.20 ± 0.03 on day 0 and pH 6.27 ± 0.01 on day 12. Additionally, the concentration of native microflora in Queso Fresco as indicated by the colonies count on MRS agar were 6.33 ± 0.54 log CFU/g (Day 0), 7.25 ± 0.27 log CFU/g (Day 6), and 7.73 ± 0.22 log CFU/g (Day 12).

RNA samples extracted with trisodium citrate solution had lower quantity but greater quality, when compared to RNA samples extracted using RNAprotect bacterial reagent.

Three independent biological replicates were conducted to compare the quantity and quality of RNA samples that were extracted from Queso Fresco using RNAprotect bacterial reagent and trisodium citrate solution. Results showed that extraction using RNAprotect reagent yielded higher RNA quantity (65.2-90.6 µg/ml), compared to the use of trisodium citrate solution (5.3-20.5 µg/ml). However, intregrity of RNA samples extracted using trisodium citrate solution was greater (RIN 8.4-9.3; Figure 4-2), in comparison to RNA samples that were extracted using RNAprotect reagent (RIN 2.2-4.8; Figure 4-3).

Late-log phase LM-Scott A has 494 up- and 209 down-regulated genes when grown on Queso Fresco compared to TSB.

Whole-genome gene expression was conducted using RNA-sequencing for three biological replicates each of LM-Scott A grown in TSB and on Queso Fresco. Bacterial cells were harvested at late-log phase (Figure 4-1) with population of 6.83 ± 0.12 log CFU/g on Queso Fresco and 8.19 ± 0.29 log CFU/ml in TSB. RNA-sequencing generated 31 million to 49.5 million reads per sample (Table 4-1). For TSB samples, 97.8-98.3% of the reads were mapped successfully to the protein coding sequences of LM-Scott A. However, for Queso Fresco samples, only 3.5-14.98% of the reads were mapped to protein coding sequences, and the

majority of the reads (67.1%-78.1%) were unmapped due to short sequence length. The total number of genes with coverage (transcripts per million (TPM) > 0.1) was 2725 for LM-Scott A grown on Queso Fresco, versus 2886 genes for cultures that was grown in TSB.

Of 2725 genes that were expressed under both growing conditions, 494 genes were found to be significantly upregulated, and 209 genes were downregulated (FDR $\leq$0.01; $\log_2$FC $\geq$1.5). Gene ontology (GO) enrichment analysis of the upregulated genes showed that 62 GO terms were overrepresented (FDR <0.05; Table 4-2). The overrepresented GO terms indicated that a majority of the upregulated genes are related to the following biological processes and molecular functions: carbohydrate transport and metabolism, cobalamin biosynthesis, ethanolamine and 1, 2-propanediol metabolism, histidine biosynthesis, iron utilization and pathogenesis/virulence. For the down-regulated genes, GO enrichment analysis revealed majority of the genes were involved in flagellar biosynthesis, putrescine/spermidine transport, ribosomal protein synthesis, peptidoglycan biosynthesis and pyrimidine ribonucleotide biosynthesis.

Genes related to cobalamin biosynthesis, ethanolamine and 1,2-propanediol metabolic processes were upregulated in LM-Scott A that was grown on Queso Fresco.

For LM-Scott A that was grown on Queso Fresco, genes related to cobalamin biosynthesis and two other metabolic processes that require cobalamin as cofactor: metabolism of ethanolamine and 1,2-propanediol, were upregulated. Among the 28 genes related to cobalamin metabolic processes, 16 genes were significantly upregulated (FC: 2.76-12.89; FDR$\leq$0.01) (Table 4-3). Additionally, the GO enrichment analysis indicated that “Cobalamin biosynthetic process”, “Porphyrin bionsynthetic process”, and “Tertrapyrrole biosynthetic process” were overrepresented (FDR <0.05) (Table 4-2) among the upregulated genes of LM-Scott A grown on Queso Fresco. Based on information from the BioCyc database (40), KEGG database (65), and

published literature (76, 94), these upregulated genes were mapped onto the cobalamin metabolic pathways (Figure 4-4). Among the 16 upregulated genes that are related to cobalamin biosynthesis, fourteen genes encode the enzymes that are directly involved in cobalamin metabolic pathways, and the other 2 genes are related to ATP-dependent transport system CBiMNQO for cobalt, which is an essential component of cobalamin (79).

Among the upregulated genes, the GO term "ethanolamine metabolic process" was overrepresented (FDR<0.05) (Table 4-2). Based on gene annotations and data from published literature (59, 94), 15 genes of LM-Scott A were categorized under ethanolamine metabolism, and all these 15 genes were significantly upregulated (FC: 8.82 -51.78; FDR ≤0.01; Table 4-4) when LM-Scott A was growing on Queso Fresco. To further understand the gene functions, these upregulated genes were mapped onto the ethanolamine utilization pathway (Figure 4-5) that was constructed based on information from the BioCyc database (40) and published literature (94). Results showed that nine out of the 15 genes were directly involved in the ethanolamine degradation process, and another five genes were linked to shell proteins for the carboxysomes, an organelle-like structure in which the ethanolamine pathways unfold (26).

Another cobalamin-dependent metabolic process: "Propanediol metabolic process" was among the overrepresented GO terms (FDR <0.05) (Table 4-2) in the analysis that involved the upregulated genes. Gene annotations showed that LM-Scott A has 22 genes that are potentially related to the propanediol utilization pathway, and 19 of these genes were significantly upregulated for LM-Scott A that was grown on Queso Fresco (FC: 2.53-22.61; FDR ≤0.01; Table 4-5). Based on the pathway information in the BioCyc database (40), 8 of the 19 upregulated genes of Scott A were directly related to the 1,2-propanediol degradation pathway (Figure 4-5). According to information about the Pdu operon provided by Bobik et al. (11), two

of the 19 upregulated genes were identified to encode proteins that reactivate adenosylcobalamin-dependent diol dehydratase, and three other upregulated genes are related to the formation of carboxysomes, and six remaining genes have yet unknown functions in the propanediol metabolic process (Table 4-5).

Genes involved in sugar transport and utilization were upregulated in LM-Scott A growing on Queso Fresco.

Fifty-two genes related to carbohydrate transport were upregulated when LM-Scott A was growing on Queso Fresco, with 40 of these genes directly related to phosphoenolpyruvate (PEP) dependent phosphotransferase system (PTS), and 12 other genes annotated as sugar ATP-binding cassette (ABC) transporter. Upper level carbohydrate-related GO terms including "carbohydrate transport/metabolic process", "disaccharide and oligosaccharide transporting ATPase activity" and "PEP-dependent sugar PTS system" were overrepresented (FDR <0.05; Table 4-2) among the upregulated genes. The more specific carbohydrate-related GO terms that were overrepresented are "Glucoside/Beta-glucoside transport" and "Maltose transmembrane activity" (FDR <0.05). Transporter genes that have been annotated with specific carbohydrates were found associated with the transfer of lactose**,** fructose/mannitol, galactitol, maltose/maltodextrin, mannose and sucrose (Table 4-6). Significant upregulation was observed for genes that encode all three PTS components (IIA, IIB, IIC) of lactose-specific PTS system (FC: 2.73-51.48; FDR ≤0.01), galactitol-specific PTS system (FC: 3.15-9.25; FDR ≤0.01), and fructose/mannitol-specific PTS system (FC: 2.94-5.92; FDR≤0.01). For the mannose-specific PTS system, genes encoding all four components (IIA, IIB, IIC, and IID) were significantly upregulated (FC: 2.97-21.75; FDR ≤0.01). For maltose/maltodextrin-specific ABC transport systems, genes encoding all three components including the extracellular protein (MdxE), and the membrane bound ABC

transporter subunits (MdxF and MdxG) (28) were significantly upregulated (FC: 31.02-38.90; FDR≤0.01). Additionally, gene encoding the IIBC component of sucrose-specific PTS system was upregulated (FC: 3.75; FDR ≤0.01).

Genes encoding the proteins for transport and metabolism of glycerol (sugar alcohol) were upregulated in LM-Scott A that was grown on Queso Fresco at 7°C, in comparison to LM-Scott A grown in TSB. Glycerol uptake facilitator protein (glpF) was significantly upregulated (FC: 8.25, FDR ≤0.01), along with another eight genes (FC: 3.77-19.48; FDR ≤0.01; Table 4-7) that were linked to glycerol metabolism based on gene annotations. Among the upregulated genes, the GO terms including "Alcohol/Glycerol metabolic process", "Alcohol/Polyol catabolic process" and "Glycerol dehydratase activity" were overrepresented (FDR <0.05; Table 4-2). Additionally, all four key reactions in the nonoxidative branch of pentose phosphate pathway were likely activated in LM-Scott A that was grown on Queso Fresco, as the genes encoding the necessary enzymes were significantly upregulated (FC:3.45-9.63; FDR ≤0.01; Table 4-8).

Gene related to iron transport and utilization were upregulated in LM-Scott A grown on Queso Fresco

Based on the information provided by Lechowicz et al. (47) and gene annotations, it was recognized that 12 upregulated genes (FC: 3.26-92.09; FDR ≤0.01; Table 4-9) were related to the iron transport mechanisms of LM-Scott A. In addition, "iron ion transport" appeared as one of the significant GO terms (FDR <0.05; Table 4-2) among the upregulated genes.

Upregulation of genes related to histidine biosynthesis in LM-Scott A grown on Queso Fresco.

"Histidine biosynthetic process" was one of the overrepresented GO terms (FDR <0.05. Table 4-2) among the upregulated genes of LM-Scott A grown on Queso Fresco. Using the pathway of *L. monocytogenes* F2365 in the BioCyc database as guidance, the histidine biosynthesis pathways were constructed to further explore the function of the upregulated genes (Figure 4-6), it was observed that 8 of the 11 key enzymes for histidine biosynthesis process were significantly upregulated (FC: 2.89-4.69; FDR ≤0.01; Table 4-10).

Upregulation of genes related to agmatine deminase system in LM-Scott A grown on Queso Fresco

Four genes related to agmatine deiminase system were found highly expressed (FC: 5.38-16.34; FDR≤0.01; Table 4-11) in LM-Scott A that was grown on Queso Fresco, in comparison to cultures grown in TSB. Based on findings from previous studies (16, 53, 94), these 4 genes were mapped onto the agmatine deiminase pathway (Figure 4-7) in order to gain further understanding about the function of each gene.

Induction of virulence genes in PrfA regulon in LM-Scott A grown on Queso Fresco

Among the upregulated genes, GO terms including "pathogenesis" and "entry into host cell" were overrepresented (FDR <0.05; Table 4-2). Virulence genes in the LM pathogenicity island-1 (LIPI-1) that were significantly upregulated (FDR ≤0.01) include hly (FC: 3.22), mpl (FC: 3.16), actA (FC: 2.37), plcB (FC: 4.48), and the master transcriptional factor of virulence genes, prfA (FC: 3.91). The prfA-dependent virulence genes that encode for Internalin A (FC: 54.44) and Internalin B (FC: 10.20) were significantly upregulated as well (FDR ≤0.01) (Table 4-12).

Prophage genes were upregulated in LM-Scott A grown on Queso Fresco

Fifty-four genes related to prophage proteins were significantly upregulated (FDR ≤0.01; Table 4-13) in LM-Scott A that was grown on Queso Fresco, in comparison to LM-Scott A grown in TSB at 7°C. The upregulated genes include uncharacterized phage protein and structural phage proteins, such as the major tail shaft protein (FC: 12.49); minor capsid protein (FC: 12.49), tail protein (FC: 3.24), head-tail connector (FC: 9.88), head-tail adaptor (FC: 8.61), and major tail protein B (FC: 4.18). Additionally, gene encoding the functional phage proteins such as the phage holin proteins (FC: 3.73-6.32); phage portal protein (FC: 4.68-6.13), transcriptional activator (FC: 2.91), and antirepressor (FC: 4.44) were also high expressed (FDR ≤0.01).

Gene related to the flagellum organization and assembly activities were downregulated in LM-Scott A growing on Queso Fresco.

Among the downregulated genes, the GO term "flagellum organization" was found significant (FDR <0.05). Nine genes related to flagellar biosynthesis were significantly downregulated (FC:-2.79 to -4.05; FDR ≤0.01; Table 4-14) in LM-Scott A that was grown on Queso Fresco, in comparison to LM-Scott A grown in rich liquid medium (TSB).

Genes related to putrescine/spermidine transport were downregulated in LM-Scott A growing on Queso Fresco.

"Putrescine/Spermidine transport" was one of the overrepresented (FDR <0.05) GO terms among the downregulated genes. In comparison to similar strain grown in TSB, LM-Scott A grown on Queso Fresco significantly downregulated (FDR ≤0.01; Table 4-15) all three genes related to putrescine/spermidine transport.

Genes related to ribosomal proteins were significantly downregulated in LM-Scott A grown on Queso Fresco.

Among the downregulated genes of LM-Scott A grown on Queso Fresco, GO terms including “Ribosome”, “Ribonucleoprotein complex”, Ribosomal subunit” were overrepresented (FDR <0.05). In total, twenty-two genes related to ribosomal proteins were significantly downregulated (FDR ≤0.01; Table 4-16) in LM-Scott A that was grown on Queso Fresco, in comparison to similar strain grown in TSB at 7°C.

Gene related to peptidoglycan biosynthesis were significantly downregulated in LM-Scott A grown on Queso Fresco.

GO terms including “peptidoglycan metabolic process” and “glycosaminoglycan metabolic process” were found significant (FDR <0.05) among the downregulated genes. In LM-Scott A that was grown on Queso Fresco, seven genes related to peptidoglycan synthesis were downregulated (FDR ≤0.01; Table 4-17), when compared to the gene expression of similar strain grown in TSB at 7°C.

Gene related to pyrimidine ribonucleotide biosynthesis were downregulated in LM-Scott A grown on Queso Fresco.

Among the downregulated genes, GO term “pyrimidine ribonucleotide biosynthetic process” was overrepresented (FDR <0.05). LM-Scott A grown on Queso Fresco significantly downregulated (FDR ≤0.01; Table 4-18) seven genes related to pyrimidine biosynthesis in comparison to LM-Scott A grown in TSB.

4.5 DISCUSSION

In this study, the physiology and molecular mechanisms of LM-Scott A growing on Queso Fresco and in TSB at 7°C were defined based on whole-genome gene expression data that were generated using RNA-sequencing. The storage temperature 7°C was chosen because it is the average temperature of most refrigerators in retail markets (25). The selection of TSB as reference condition was supported by the fact that other studies (5, 87, 94) have effectively used rich broth medium including TSB, brain heart infusion broth (BHIB) and deMan, Rogosa and Sharpe (MRS) as reference conditions to investigate the bacterial gene expression in food. Similarly, for studies that examined bacterial gene expression in animal hosts or human cells (44, 61), in which a reference condition is necessary, a rich broth medium such as TSB has been chosen as reference to facilitate the gene expression characterization. TSB as the reference condition in this work was able to facilitate the understanding about the influence of Queso Fresco and TSB upon the gene expression of LM-Scott A.

The growth parameters showed that LM-Scott A had relatively similar lag phase durations in TSB and on Queso Fresco, however LM-Scott A had significantly greater ($p < 0.05$) maximum growth rate and greater final population (Figure 4-1) when grown in TSB, in comparison to growth on Queso Fresco. This result is not surprising as TSB is a rich laboratory medium that provides good support for *L. monocytogenes* growth, and the lack of native microflora in the liquid broth means less competition over nutrients. Additionally, the higher pH of TSB (7.20) in comparison to Queso Fresco (pH 6.20), as well as the aerobic storage of inoculated TSB (static) may have promoted the greater growth of LM-Scott A in TSB, as compared to the inoculated Queso Fresco slices that were vacuum packaged. Aerobic respirataion generally provides greater amount of energy, hence bacterial growth under aerobic condition is usually greater when compared to growth under anaerobic condition (23).

Late-log phase as the optimal growth phase for RNA extraction in this study

Pilot experiments in this study (data not shown) showed that a minimum bacterial concentration of approximately 7 log CFU/g on cheese is necessary to obtain sufficient quantity of RNA. Based on the growth data (Figure 4-1), LM-Scott A grown on Queso Fresco at 7°C was in late-log phase at this cell density. Previous study (94) has applied a similar strategy to maximize RNA yield. Tang et al. (94) harvested RNA of *L. monocytogenes* from cold smoked salmon at late-log phase, with bacterial concentration achieving 8 log CFU/g. Sirsat et al. (86) experimented with concentrations of *Salmonella* at 8, 7, 6 and 5 log CFU/g on raw chicken breast, and reported that 8 log CFU/g is the concentration for the extraction of RNA yielding optimal quantity and quality. In comparison to previous study, LM-Scott A achieved late log phase on Queso Fresco at a lower concentration, 7 log CFU/g versus 8 log CFU/g on cold-smoked salmon (94). As a result, a greater volume of inoculated cheeses was required during the RNA extraction to collect sufficient amount of RNA, 14 slices of 10g Queso Fresco were used for one biological replicate in this study, versus one piece of 10 g cold smoked salmon in the study by Tang et al. (94). Given that the bacterial RNA was extracted consistently at the late log phase in both growing conditions (on day 5 for Queso Fresco samples and day 6 for TSB samples), it is assumed that the observed transcript differences were not likely the results of different growth phases.

Trisodium citrate solution is effective for isolating high quality bacterial RNA from cheese

RNA extraction procedures that were performed using trisodium citrate solution consistently yielded lower quantity but greater quality of RNA (RIN: 8.4-9.3; Figures 4-2), whereas RNA samples that were extracted using RNAprotect bacterial reagent (Qiagen Inc.) consistently showed greater quantity but lower quality (RIN 2.2-4.8; Figure 4-3). Consistent with

this study, Sirsat et al. (86) also found greater RNA yield when using RNAprotect bacterial reagent to extract the RNA of *Salmonella* from inoculated raw chicken breast, while they observed 10-30 fold less in RNA quantity when using 0.1% peptone water during the extraction procedures. The authors explained that the inhibition of the RNA degrading nucleases by RNAprotect bacterial reagent helped preserve the RNA, leading to greater RNA yield. However, the claim on RNA preservation is not well supported as Sirsat et al. only assessed the RNA quantity using Nanodrop 8000, an absorbance spectrophotometry method that is useful in revealing organic/protein contamination, but ineffective in revealing RNA degradation (84). In another study that analyzed a RNA sample with severe degradation (RIN 2.4), Nanodrop provided a quantity almost doubles the amount that was provided by Agilent 2100 Bioanalyzer (84). This study showed that Nanodrop is prone to overestimation when quantifying RNA samples in which degradation has occured. Moreover, the quality of RNA samples of Sirsat et al. was not analyzed using Agilent Bioanalyzer 2100, a standard method for assessing the integrity or the degree of RNA degradation, hence no direct comparison can be made on the aspect of RNA integrity. Recognizing the shortcoming of Nanodrop in quantification, Qubit 2.0 fluorometer was used to quantify the RNA samples in our study, as it is a more sensitive and precise fluorescence-based quantification tool that can also minimize the influence of degraded RNA upon quantification. Along with Qubit 2.0, the RNA samples in this study were also analyzed by Nanodrop 2000 for organic/protein contamination and Agilent Bioanalyzer 2100 for RNA integrity. To ensure successful downstream applications including the library preparation and RNA-sequencing, multiple assessment methods are necessary to confirm the quantity and quality of RNA.

In our study, the greater RNA yield (quantified by Qubit 2.0) and the low RNA quality that resulted from RNAprotect reagent in comparison to trisodium citrate solution seemed to be partly due to the solubility properties of the reagent. The chemical composition of RNAprotect reagent isn't clear, however this reagent seems to have surfactant activity, as substantial amount of foam was observed during stomaching and vortexing during RNA extraction procedures in our study. When hand-stomaching the inoculated cheeses using RNAprotect reagent, it was apparent that the stomached solution was much more turbid compared to when trisodium citrate solution was used as stomaching solution. After the centrifugation step, a greater amount of fat and cheese solids were observed (data not shown) for the samples that were extracted using RNAprotect reagent. Based on these observations, it is speculated that RNAprotect reagent may be more effective in extracting cells from the cheese slices, which leads to greater RNA yield, but the substantial carry-over of fat and cheese solids may have affected its efficiency in protecting the RNA from degradation. Prior to our study, Duquenne and colleagues (22) have shown a successful use of trisodium citrate solution in isolating RNA of *Staphylococcus aureus* from semi-hard cheeses. By using the high-quality RNA (RIN >7) samples in RT-qPCR analysis, Duquenee et al. (22) were able to show the correlation between the expression of enterotoxin genes and the production of staphylococcal exterotoxin in semi-hard cheeses. Nevertheless, RNAprotect reagent seemed to work effectively in protecting the RNA of *L. monocytogenes* that was isolated from inoculated cold smoked salmon, with RIN >7 (94). Collectively, these results suggest that the food composition should be taken into account when considering the choice of reagent/solution for isolating bacterial cells for RNA extraction. Since the RNA samples that were extracted using trisodium citrate solution consistently achieved the quality threshold (RIN >7), this buffer was chosen to isolate RNA for this transcriptomics study to ensure a successful

study outcome. To compensate for the low RNA yield, a greater number of inoculated cheeses (14 slices of 10 g cheeses) was used in the RNA extraction procedures.

High coverage of bacterial transcriptome was achieved despite large percentages of unmapped short sequences for LM-Scott A grown on Queso Fresco

Optimization works as mentioned in the previous paragraphs were done to ensure extraction of high quality RNA from Queso Fresco, and only RNA samples with RIN >7 were selected for library preparation and RNA-sequencing. However, degradation of RNA samples isolated from Queso Fresco may have happened during downstream procedures such as the cDNA library preparation, as evidenced by the large percentages of unmapped reads due to short sequence length (Table 4-1) (102). Under this situation, it is important to assess the complexity of the cDNA libraries to evaluate if the RNA samples in this study were good samplings of the transcription activities of LM-Scott A grown on Queso Fresco. One way to evaluate the library complexity is by comparing the number of expressed genes (1). Based on the genome assembly work conducted by Briers et al. (12), the chromosome of LM-Scott A contains 2953 predicted protein coding sequences. Our study showed that 2886 and 2725 genes in LM-Scott A were expressed (transcripts per million (TPM) > 0.1) when the cultures were grown in TSB and on Queso Fresco respectively. The large numbers of expressed genes suggest that the cDNA libraries contained good samplings of the RNA of LM-Scott A grown on Queso Fresco, despite the high percentage of unmapped short reads. Additionally, the sequencing depth used in this study has facilitated good coverage of the bacterial transcriptome. Although the % of reads mapped to LM-Scott A coding sequences for the RNA samples extracted from Queso Fresco were low (3.5%-14.98%), the total number of mapped reads (1.6 – 4.6 millions) was still comparable to the total number of mapped reads (1.31-2.83 millions) achieved by Tang et al.

(94) for their RNA samples isolated from cold smoked salmon, while the % reads mapped in their study were significantly higher (80-82%) than ours. This suggests that increasing the sequencing depth could be worthwhile when conducting RNA-sequencing for bacterial cells that are isolated from complex environment, in addition to proper optimization of the RNA extraction procedures.

Overall, the transcriptomics data from our study suggest that exposing LM-Scott A to Queso Fresco such that he cell populations reach late log phase leads to (i) activation of the cobalamin and histidine biosynthesis pathways (ii) utilization of ethanolamine and 1, 2-propanediol as carbon and nitrogen sources (iii) transport and utilization of multiple types of carbohydrates to improve survival/growth, (iv) upregulation of agmatine deiminase system to improve tolerance against organic acid, (v) increases in iron intake and (vi) decreased flagella formation/motility activity (vii) transport of putrescine/spermidine, and (viii) reduction in synthesis of ribosomal proteins, peptidoglycan and pyrimidine ribonucleotides, when compared to LM-Scott A grown in TSB at 7°C.

Genes encoding enzymes for cobalamin biosynthesis were upregulated in LM-Scott A that was grown on Queso Fresco

Cobalamin, or coenzyme B_{12} and its derivatives serve as critical cofactors for enzymes that catalyze transmethylation and rearrangement reactions. Ethanolamine and 1,2-propanediol metabolic processes, which were found significant (FDR <0.05; Table 2) in this study, are examples of reactions that require cobalamin and its derivatives as essential cofactors (76, 78). According to the USDA Food Composition database (99), Queso Fresco has an estimated concentration of 1.68 µg vitamin B_{12} per 100 g. *L. monocytogenes* grown on cold smoked salmon, which has higher level of cobalamin (18.10 µg/100 g) compared to Queso Fresco,

showed induction of genes related to the cobalamin biosynthesis pathway (94). Perhaps due to low level of cobalamin in Queso Fresco, LM-Scott A was activating its cobalamin biosynthesis pathway to sustain the metabolic processes that are necessary to facilitate the utilization of 1,2-propandeiol and ethanolamine.

Genes related to ethanolamine and 1, 2-propanediol metabolism were upregulated in LM-Scott A grown on Queso Fresco, in comparison to LM-Scott A grown in TSB.

The precursor of ethanolamine is phosphatidylethanolamine, which is a major type of polar phospholipid found in the milk fat globule membrane (20, 100). Through the action of phospholipases, phosphatidylethanolamine can be broken down to glycerol and ethanolamine (46, 77). Given the enzymatic activities of microbial cultures and background microflora, a high level ethanolamine is commonly found in fermented products such as cheeses, fermented fish and meat products (66, 82). On the other hand, upregulation of genes related to 1,2-propanediol metabolism in LM-Scott A grown on Queso Fresco indicate the presence of L-fucose or L-rhamnose in cheese milk, as 1,2-propanediol is the breakdown product of these two deoxyhexoses under anaerobic conditions (6, 17). Fucose-containing oligosaccharides in bovine milk could be the source of L-fucose (93), while exopolysaccharide produced by some lactic acid bacteria strains in cheeses could contain L-rhamnose (14).

Studies have shown that *L. monocytogenes*, along with *Salmonella enterica* and *Clostridium perfringens*, is one of the three pathogenic bacteria that are capable of utilizing both ethanolamine and 1, 2-propanediol as carbon and nitrogen sources (43, 80). Ethanolamine-related genes in LM-Scott A grown on Queso Fresco were significantly induced, and a similar observation was reported for *S. enterica* that was grown in food with a high lipid content, such as egg yolk and milk (90). Consistent with the results of this study, both ethanolamine and 1,2-

propanediol metabolic processes were also found significant in *L. monocytogenes* H7858 grown on cold smoked salmon, which has high content of lipid and glycoconjugates that contain L-fucose and L-rhamnose (94). The expression of genes required for ethanolamine and 1, 2-propanediol metabolic processes (Figure 4-5) is affected by the presence of cobalamin, which is a critical cofactor. This has been confirmed by research studies that found regulation activities of cobalamin-binding riboswitches that are located upstream of both eut (*e*thanolamine *ut*ilization) and pdu (*p*ropane*d*iol *u*tilization) loci (58, 59).

Previous studies (4, 30) have indicated the importance of ethanolamine and 1, 2-propanediol metabolism for pathogenic bacterial survival and growth in food and host environments. For example, *S. enterica* serovar *Typhimurium* with a deletion on pocR (regulate propanediol utilizing operon) and eutR had significant growth reduction in egg yolk and milk (90) when compared to the wild type strain. Bertin et al. (9) suggested that ethanolamine metabolism by enterohaemorrhagic *E. coli* improve its survival in the bovine intestine, in which the majority of the resident microorganisms were lacking the eut operon. A similar finding was observed for *S. Typhimurium*, which is capable of eliciting gut inflammation and production of tertrathionate (a respiratory electron acceptor) that support ethanolamine metabolism by *S. Typhimurium* under anaerobic conditions. The ability to utilize ethanolamine provides a significant growth advantage to *S. Typhimurium* in an inflamed gut (95), suggesting the importance of ethanolamine utilization for pathogenic bacteria survival in a competitive environment. In a study that investigated the influence of *lactobacillus* species upon *L. monocytogenes* survival in a gnotobiotic humanized mouse model, upregulation of genes related to cobalamin biosynthesis, 1, 2-propanediol and ethanolamine utilization were observed in *L. monocytogenes* when the gnotobiotic mice were treated with *Lactobacillus* species prior to

Listeria infection. Although the induction of genes related to ethanolamine and 1,2-propanediol metabolism did not seem to improve survival of *L. monocytogenes*, Archambaud et al. (4) suggested that the presence of *lactobacilli* forces *L. monocytogenes* to utilize ethanolamine that is not usable by *lactobacilli*, in order to compete for the limiting carbon and nitrogen sources. In summary, results from this study along with previous investigations indicate that pathogenic bacteria including *L. monocytogenes* may take advantage of existing ethanolamine and 1, 2-propanediol to survive and grow in environments that are occupied with competing microflora.

Adaptation processes of LM-Scott A to the end products of ethanolamine and 1, 2-propanediol provides a potential target for growth inhibition.

Acetate and propionate are two end products of ethanolamine and 1,2-propanediol metabolism under anaerobic conditions (72, 78, 97). Upregulation of genes related to agmatine deiminase system (Table 4-11; Figure 4-4) suggest that LM-Scott A grown on Queso Fresco was working to elevate the cytoplasmic pH by neutralizing intracellular protons (18). Consistent with this study, *L. monocytogenes* grown on cold smoked salmon highly expressed genes related to ethanolamine and 1,2-propanediol metabolism, as well as the agmatine deiminase system (94). Tang et al. suggested that the NH_3 molecules generated from arginine deamination potentially buffer the acid products (acetate/propionate) of ethanolamine and 1, 2-propanediol metabolism. Therefore, Tang et al. (94) proposed that antimicrobial strategies that incorporate the two organic acids (acetate and propionate) could be effective in inhibiting the growth of *L. monocytogenes.* This hypothesis is supported by a study, which shows that the cells of *L. monocytogenes* exposed to acetate and lactate shifted from acetate- and lactate-producing pathways to a less efficient pathway that generates acetoin (91).

LM-Scott A grown on Queso Fresco significantly upregulated genes related to transport of multiple types of carbohydrates

L. monocytogenes, a ubiquitous pathogenic bacterium, is capable of utilizing variety of carbohydrates (92) as energy sources. Barabote and Saier (7) analyzed the genome sequences of 174 bacterial species from the following phyla: Actinobacteria, Firmicutes, Proteobacteria and Spirochetes, and found that *L. monocytogenes* EGD-e has the highest number of pts gene (91 genes) and 30 complete PTS permeases. The large number of pts genes may partially explain the ubiquitous nature of *L. monocytogenes*, as the ability to transport a variety of carbohydrates as energy sources would improve the survival and growth of this pathogenic bacterium. As lactose is the dominant carbohydrate in fresh, unripened cheese such as Queso Freso and would, therefore, serve as a primary energy source for LM-Scott A, the upregulation of genes related to transport of other types of carbohydrates suggests that additional carbohydrate sources in Queso Fresco were used by LM-Scott A for survival and growth. Similarly, Tang et al. (94) observed that *L. monocytogenes* growing on cold smoked salmon activated genes related to transport of multiple carbohydrates, including galactitol, mannose and maltose, when compared to culture grown in TSB. Galactitol may be present in Queso Fresco due to the breakdown of lactose into glucose and galactose, followed by the reduction of galactose to galactitol by aldolase reductase (55, 71). Mannose as a sugar monomer is commonly found in glycoconjugates in milk (64). Although maltose and fructose are not the appararent carbohydrates sources in milk/cheese, genes that encode transporters for these carbohydrates were also upregulated in LM-Scott A that was grown on Queso Fresco. Consistent with our study, Tang et al also observed upregulation of genes related to maltose-transport in *L. monocytogenes* grown on cold smoked-salmon, despite the fact that salmon is not an apparent source of of maltose (94). The presence of fructose may

result from the isomerization of lactose to lactulose during the heat treatment of cheese milk, a process in which the glucose is converted to a fructose moiety (107). The upregulation of genes involved in glycerol (sugar alcohol) intake and metabolism indicate the presence of glycerol in Queso Fresco, which is likely resulted from the breakdown of phospholipids in milk/cheese (46, 77). Similarly, *Lactobacillus helveticus* that was grown in milk showed upregulation of genes related to glycerol uptake, when compared to culture grown in liquid MRS (de Man, Rogosa and Sharpe) broth (87).

Consistent with our study, genes encoding carbohydrates intake were more expressed in *L. monocytogenes* strains that were grown on cold-smoked salmon (94), when compared to cultures grown in TSB and BHI broth, respectively. In contrast to out study, genes related to carbohydrate transport/metabolism were not upregulated in *L. monocytogenes* F2365 that was grown in UHT skim milk (52), which could be partly be attributed to the strain differences, e.g. the inability of some *L. monocytogenes* strains to ferment lactose (71). In a murine macrophage infection study, *L. monocytogenes* had an increased expression of genes related to uptake and metabolism of glycerol, which served as an alternative carbon source for the bacteria during intracellular growth (15). The diversification of carbohydrates/sugar consumption serves to improve the survival and growth of *L. monocytogenes* in competitive environments.

Genes encoding enzymes for the pentose phosphate pathways were significantly upregulated in LM-Scott A grown on Queso Fresco

This study observed significant upregulation of genes encoding key enzymes for the nonoxidative branch of the pentose phosphate pathway (PPP), in comparison to LM-Scott A grown in TSB. Activation of this pathway is plausible for *L. monocytogenes* growing on Queso Fresco because PPP is important in yielding xylulose- and ribose-5-phosphate, the precursors for

DNA/RNA synthesis (106). For *L. monocytogenes* that was grown in defined media with glycerol as the sole carbon source, Joseph et al. (36) reported upregulation of genes related to PPP, and downregulation of genes involved in earlier part of glycolysis. In our study, genes related to glycerol metabolism were upregulated in LM-Scott A growing on Queso Fresco as well. Under these scenarios, the product of glycerol degradation, dihydroxyacetone phosphate was likely be isomerized to D-glyceraldehyde-3-phosphate, which can then participate in the PPP. Consistent with or study, genes related to PPP were also activated in *L. monocytogenes* that was grown on cold smoked salmon (94), ready-to-eat turkey deli meat (5) and inside murine macrophages (15). Collectively, results from all these studies have indicated the importance of the pentose phosphate pathway for *L. monocytogenes* growing under various types of environment

Genes related to iron transport were significantly upregulated in LM-Scott A grown on Queso Fresco

Bacteria require iron as a cofactor for the proper function of basic metabolic systems, including DNA synthesis, glycolysis, energy generation and detoxification of oxygen radicals (63). Queso Fresco is a poor source of iron, with an estimated amount of 0.20 mg Fe/kg (99). Upregulation of genes related to the ABC (ATP-binding cassette transporter) -type Fe3+-sideorophore transport system suggest that hydroxamate siderophores are some of the iron sources in Queso Fresco for *L. monocytogenes*. Siderophore is a type of secondary metabolite produced by numerous bacteria under low iron environment, and the binding of siderophores to Fe3+ makes the iron complex transportable into bacterial cells (47, 67). Despite lacking biosynthetic genes for sideorophores (27), *L. monocytogenes* is able to use siderophores produced by other species (85). More than 500 varieties of siderophores produced by bacteria

have been recognized (103), and the ability of *L. monocytogenes* to acquire iron from various types of siderophores improves its survival in diverse environments including soil, and mammalian intestine (35, 47). A study by Jin et al. (35) found that *L. monocytogenes* in iron-restricted media had a slower growth rate and lower cell density compared to culture grown in similar media that were added with iron sources, emphasizing the importance of iron for the optimal growth of *L. monocytogenes*. Information provided by previous publications and the result from our study suggest that native microflora in Queso Fresco could have produced siderophores in Queso Fresco due to the restricted amount of iron, and the siderophores may have been taken up by *L. monocytogenes* to improved its survival and growth.

Although dairy products are not apparent sources of heme iron, significant upregulation of genes that are related to sortase-B dependent heme transport was observed in LM-Scott A that was grown on Queso Fresco. Research by Klebba et al. (41) showed that *L. monocytogenes* transported heme through peptidoglycan-anchored proteins and sortase B-dependent mechanism under low concentration (<50 nM) of iron. Using this mechanism, the heme was transferred from the outer environment to the ABC-transporters situated at the cytoplasmic membrane. On the other hand, the transfer mechanism becomes sortase B-independent when iron level is higher (>50nM) (41). In our study, significant induction of the gene encoding Sortase B in LM-Scott A suggested that heme iron level in Queso Fresco is low; hence LM-Scott A was utilizing the sortase B-dependent heme transfer mechanism while growing on the cheese.

Virulence gene in LM-Scott A were highly expressed when growing on Queso Fresco at 7°C

Virulence gene expression of *L. monocytogenes* could be influenced by the type of strains, composition of the food matrix, and packaging environment. Vacuum packaging or oxygen restriction might induce the expression of virulence genes. Andersen et al. (3) reported that *L.*

monocytogenes that was grown under a low oxygen environment was more invasive towards Caco-2 cells, and resulted in a greater number of pathogen cells in invaded organs, in comparison to the bacterial cells that were grown without oxygen restriction. Similar results were also observed in another study, *L. monocytogenes* that were grown on modified-atmosphere packaged ham for 2 and 4 weeks showed greater invasion towards Caco-2 cells as compared to growth in BHIB (45). In studies that compared virulence gene expression of *L. monocytogenes* under aerobic and anaerobic condition using controlled laboratory media, some prfA-dependent virulence genes were significantly induced, specifically genes that encode internalin (37, 62).

In this study, inoculated Queso Fresco slices were vacuum packaged throughout storage, the oxygen-restricted condition might have induced the prfA-dependent virulence genes of LM-Scott A. Consistent with our study, *L. monocytogenes* grown on vacuum-packaged cold smoked salmon (94) also significantly upregulated the prfA-dependent virulence genes, when compared to similar strain grown in TSB. On the other hand, there was no significant change in prfA-dependent virulence gene expression in cells of *L. monocytogenes* that were grown on turkey deli slices and UHT milk, when compared to *L. monocytogenes* growtn on BHI agar and broth, respectively (5, 52). Both the inoculated turkey slices and UHT milk were stored without oxygen restriction. Nevertheless, for *L. monocytogenes* that was grown on cantaloupe pieces without oxygen restriction, upregulation of some prfA-dependent virulence genes were observed (39). Collectively, results from multiple studies strongly suggest that the oxygen availability has a significant influence upon virulence gene expression. However, the discrepancies that were observed among these studies, including the type of virulence genes that were expressed and the magnitude of gene expression, indicate the presence of other influencing factors. In addition to

the type of strains and experimental methods, storage temperature and food composition could have significant influence on virulence gene expression as well (74).

Upregulation of prophage genes in LM-Scott A grown on Queso Fresco

In comparison to gene expression during growth in TSB, significant induction of 54 prophage genes in LM-Scott A grown on Queso Fresco was observed (Table 4-13). In contrast to our study, downregulation of large number of prophage genes (31 of 32) was observed in *Staphylococcus aureus* that was exposed to mild acidic condition (pH 5.5), suggesting that expression of prophage genes could be suppressed by low pH. (105). Similarly, *L. monocytogenes* that was exposed to a quaternary ammonium compound, benzethonium chloride (BZT), showed downregulation of prophage genes, which was attributed to the low pH of BZT solution (4.8-5.5) (13). In our study, the pH of Queso Fresco (6.3) is lower than the pH of TSB (7.0), and yet upregulation of prophage genes was observed in LM-Scott A that was grown on Queso Fresco, suggesting the presence of influencing factors other than pH. Consistent with this study, Smeianov et al. (87) showed that prophage genes of *Lactobacillus helveticus* grown in milk were significantly upregulated, when compared to culture grown in rich MRS broth. Lysis of *L. helveticus* is known to happen during molding and ripening of cheese, and the release of intracellular peptidases facilitates the proteolytic reactions in cheeses. However, the phage lysin gene in *L. helveticus* grown in milk was not upregulated, despite the fact that other 18 prophage genes were significantly upregulated. Although the cause of prophage gene induction remains unclear, the results from the research by Smeianov et al. (87), and our study suggest that composition of milk and cheese may stimulate the induction of prophage genes. Additionally, results from previous studies (32, 73) suggest a relationship between prophage gene and virulence of *L. monocytogenes*. Induction of prophage genes was observed in *L. monocytogenes*

grown in host cells (32), and *L. monocytogenes* prophage genes was shown to act as genetic switch that regulate virulence (73). Further research is needed to understand the trigger of phage gene induction and the role of those genes for the survival and growth of *L. monocytogenes*.

Downregulation of flagella organization/assembly genes

Flagella are important for *L. monocytogenes* mobility and have been shown to mediate adhesion and biofilm formation (49). Flagellar synthesis is affected by environmental temperature, and significant downregulation of related genes was observed for most strains at the physiologic temperature of a mammalian host, 37°C; whereas upregulation of those genes was observed at 30°C (10, 68, 104). Although a storage temperature of 7°C was used in this study, genes related to flagellar biosynthesis were significantly downregulated in LM-Scott A grown on Queso Fresco. Previous studies have shown that flagella are involved in attachment and colonization of solid surfaces including stainless steel, (101) and surfaces of plants such as alfalfa, radish and broccoli sprouts (29). In contrast to our study, the flagellar biosynthesis genes of *L. monocytogenes* were upregulated during growth on cantaloupe pieces, in comparison to growth in rich liquid broth BHIB (39). Overall, these findings suggest that the downregulation of flagellar biosynthesis gene in LM-Scott A grown on Queso Fresco was not mainly due to the solid or liquid state of the growth medium.

As flagella synthesis and utilization is an energy-consuming process, the downregulation of flagellar formation genes might be an energy-saving strategy of *L. monocytogenes* under stressed condition, possibly conserving energy for more important metabolic processes to help with survival and growth (33). The energy conservation hypothesis seems to be supported by the study of Cordero et al. (19), which showed that fast-growing strains of *L. monocytogenes* had repressed transcription of genes related to flagellar formation at low temperature (8°C), in

comparison to slow-growing strains. In another study, 7 out of 15 *L. monocytogenes* strains that showed greater survival under desiccation stress had mutations in genes related to flagellar biosynthesis, and three of the same strains showed greater osmotic tolerance as well (33). In comparison to TSB which is a rich medium, Queso Fresco has lower pH (6.3 versus pH 7.0 of TSB) and slightly greater salt content (1.5% versus 0.5% of TSB), Queso Fresco also presents a more competitive environment with the presence of background microflora. Hence, it is likely that LM- Scott A was reducing the formation and utilization of flagella to conserve energy for other metabolic processes that improve its survival and growth on Queso Fresco. Additionally, the potential influence of vacuum packaging and associated oxygen limitation upon flagella synthesis of *L. monocytogenes* remains to be elucidated.

Production of putrescine in agmatine deiminase pathway may have influenced the downregulation of putrescine/spermidine transport in LM-Scott A grown on Queso Fresco, in comparison to LM-Scott A grown in TSB.

The concentration of polyamine compounds in bacterial cells is strictly regulated to ensure optimal conformation of DNA and RNA molecules (83). In comparison to fresh cheeses, ripened cheeses generally have greater concentration of polyamine compound such as putrescine and spermidine due to the fermentation activities of starter cultures and native microflora (2). LM-Scott A grown on Queso Fresco shown significant upregulation of genes related to agmatine deiminase pathway, and this metabolic process is known to produce putrescine, a polyamine compound that is also a precursor to spermidine (Figure 4-7). Perhaps due to the intracellular production of putrescine, the bacterial cells were downregulating the polyamine transporter to achieve an optimal concentration of intracellular polyamine.

Greater expression of genes encoding genes related to ribosomal proteins and peptidoglycan biosynthesis in LM-Scott A grown in TSB may be resulted from the greater growth rate, in comparison to culture grown on Queso Fresco

The growth rate of bacterial cells can have significant influence on the gene expression. Given that the amount of RNA polymerases and ribosomes is positively correlated to the bacterial growth rate (42), culture with greater growth rate is likely to show greater expression of genes encoding ribosomal proteins and/or other proteins that are necessary for growth and replications. Similar finding was found in our study, LM-Scott A grown in TSB had significantly greater maximum growth rate (0.71 versus 0.43 log CFU/g/day) as compared to similar culture grown on Queso Fresco, and the TSB culture with greater growth rate showed increased expression of genes encoding ribosomal proteins (22 genes, Table 4-16) and peptidoglycan synthesis proteins (7 genes, Table 4-17), when compared to LM-Scott A grown on Queso Fresco. In addition to ribosomal proteins, bacterial cells are known to regulate the synthesis of peptidoglycan in proportion to the cellular growth rate, as peptidoglycan is the essential component of cell wall (98).

Downregulation of Pyrimidine ribonucleotide biosynthesis in LM-Scott A grown on Queso Fresco may be resulted from the growth rate differences, presence of sufficient pyrimidine in Queso Fresco and/or lactose metabolism by LM-Scott A.

Pyrmidines are essential molecules for the synthesis of nucleic acids. Samant et al.observed that the mutants of *E. coli, S. enterica* and *B. anthracis* with deletion of genes related to purine or pyrmidine synthesis showed a 20- to 20000-fold reduction in cell numbers, in comparison to the respective wild-type strains that were grown in human serum for 24 h (81). The findings of Samant et al. emphasized that de novo synthesis of purines and pyrimidine are critical for the

optimal growth of bacteria (81). The greater growth rate of LM-Scott A in TSB may be one cause for the downregulation of genes related to pyrimidine biosynthesis in LM-Scott A grown on Queso Fresco. Additionally, the concentration of pyrimidine molecules in the growth environment may also affect the pyrimidine biosynthesis activity in bacteria. Smeianov et al. found that *L. helveticus* grown in milk upregulated genes related to pyrimidine salvage pathway in comparison to growth in MRS broth, and suggested that *L. helveticus* may focus on the use of pre-existing pyrimidine molecules in milk instead of de novo synthesis (87). In our study, LM-Scott A grown on Queso Fresco upregulated genes related to cytosine (pyrimidine) permease whereas LM-Scott A grown in TSB upregulated genes related to uracil permease (pyrimidine) and pyrimidine biosynthesis. The results suggest that LM-Scott A may have utilized the pre-existing pyrimidine molecules in both Queso Fresco and TSB, while the greater demand for pyrimidine may have prompted LM-Scott A grown in TSB to activate the pyrimidine synthesis activities. On the other hand, a proteomics study (31) suggested an unexpected link between lactose metabolism and pyrimidine biosynthesis. Guillot et al. observed that *L. lactis* grown in broth containing lactose had significantly lower expression of five enzymes related to pyrimidine biosynthesis, in comparison to similar strain grown in broth that contained glucose (31). Our results seemed to be consistent with the findings of Guillot et al. (31), as LM-Scott A grown on Queso Fresco, in which lactose is the pre-dominant carbohydrate, showed downregulation of genes related to pyrimidine biosynthesis, when compared to LM-Scott A grown in TSB, in which glucose was the major carbohydrate.

4.6 CONCLUSIONS

Components of food matrices can have significant influence on bacterial survival/growth. A whole-genome gene expression study using RNA-sequencing can inform our understanding as to the behavior and survival mechanisms of pathogenic bacteria in a complex food environment. Information from these studies may facilitate the development of novel microbial control measures. In addition, understanding on influence of food matrices and packaging condition upon bacterial virulence expression could better inform decisions related to storage and processing strategies. Importantly, the virulence gene expression information could potentially be taken into account to improve industry or regulatory risk assessment study.

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Figure 4-1. Growth of *Listeria monocytogenes* Scott A on Queso Fresco (closed triangle) and in TSB (closed square) at 7°C was determined by plating on Listeria Selective Agar, and data were obtained from three independent biological replicates. The pH values of the inoculated Queso Fresco (opened triangle) and TSB (opened square) at each sampling point were measured before the plating of samples (n=3). RNA extraction was performed on day 5 for the inoculated Queso Fresco, and day 6 for the inoculated TSB (marked by circle).

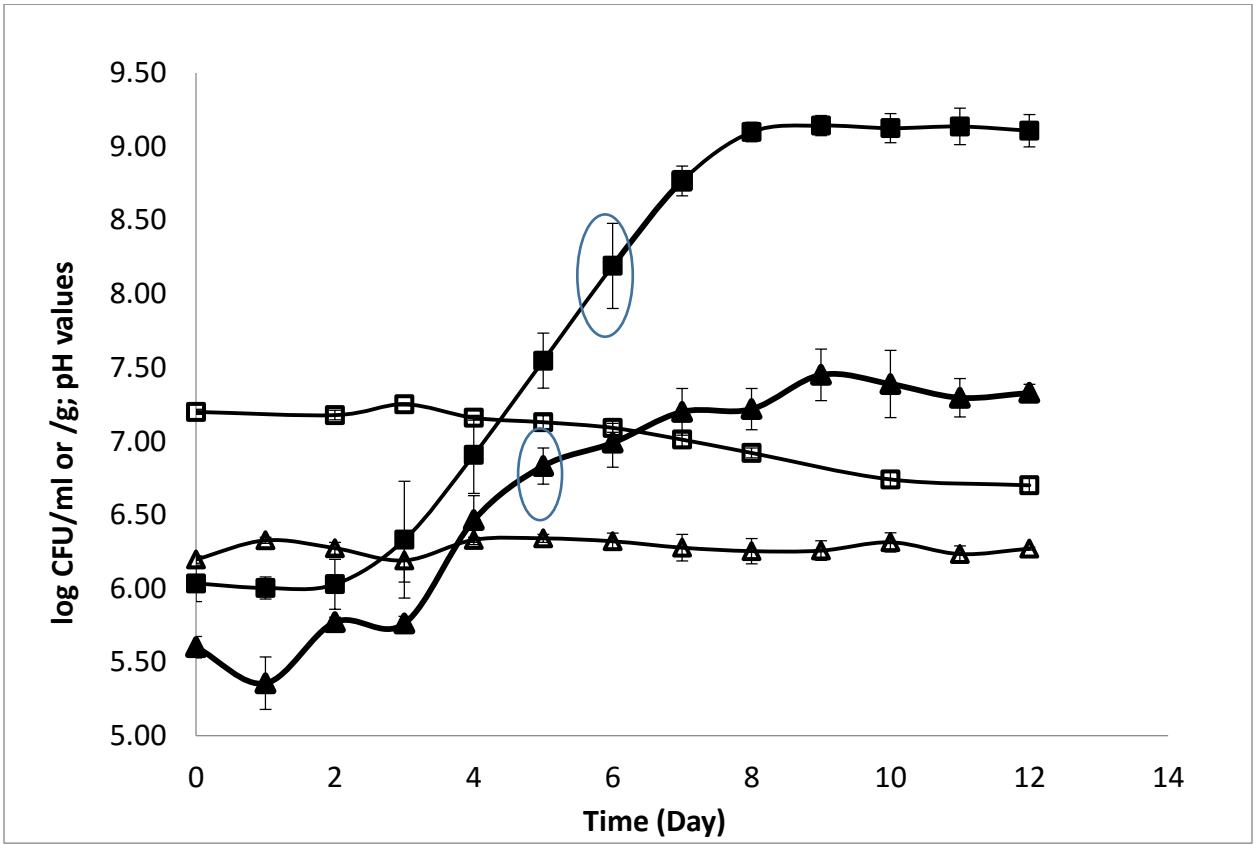
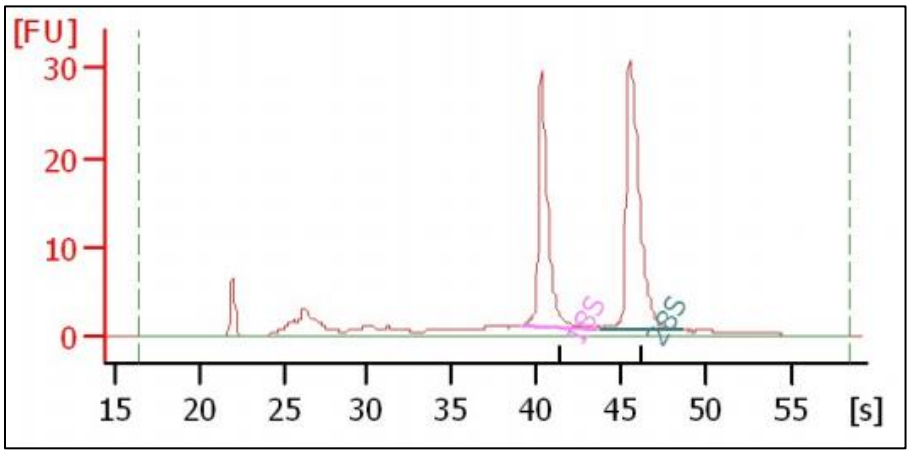
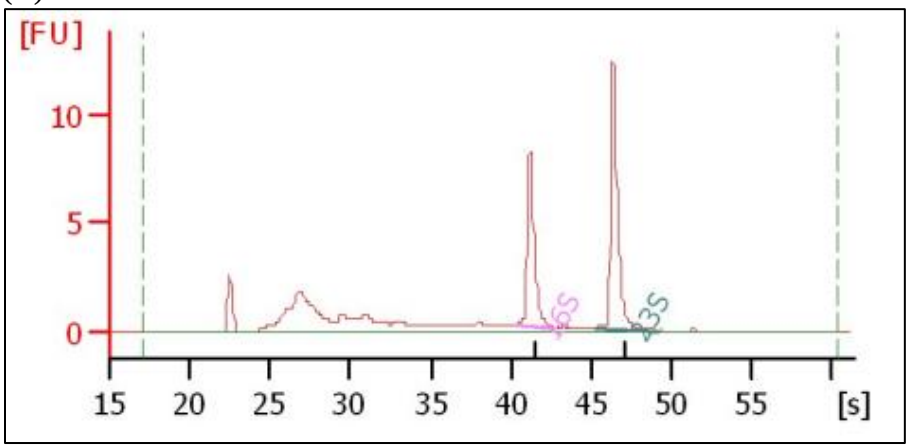


Figure 4-2 (A-C) Assessment of RNA quality using Agilent 2100 Bioanalyzer, which provides RIN (RNA Intregrity Number) ranges from 1 (degraded) to 10 (intact). Electropherograms of RNA samples (n=3) extracted from Queso Fresco by using trisodium citrate solution.

(A) RIN: 9.30



(B) RIN: 8.4



(C) RIN: 9.1

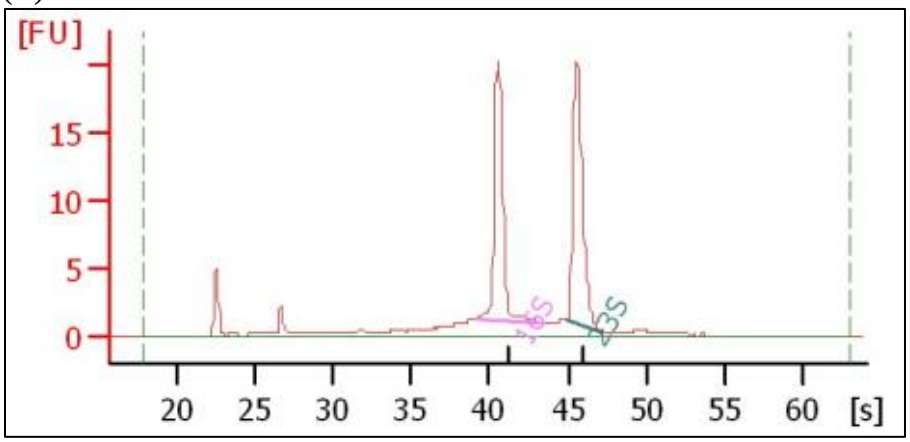
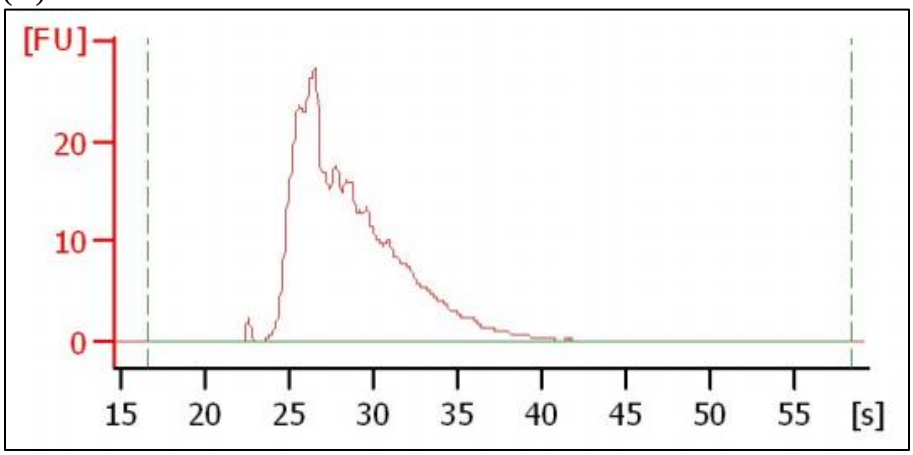
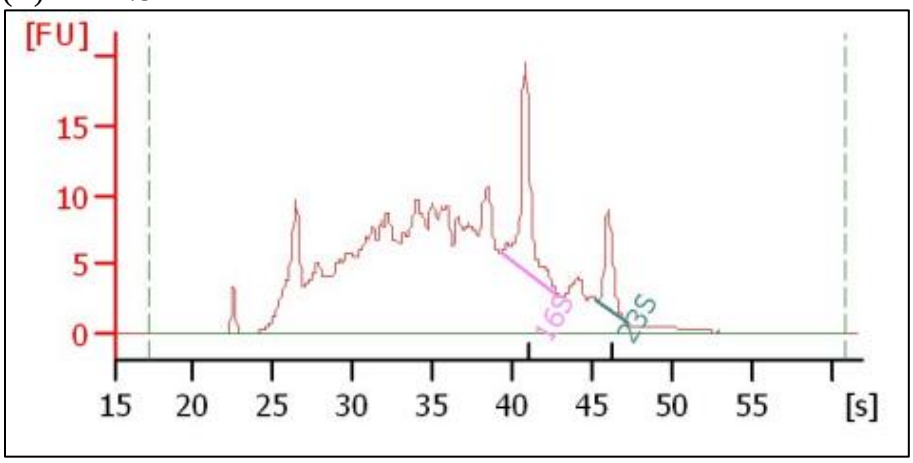


Figure 4-3 (A-C) Assessment of RNA quality using Agilent 2100 Bioanalyzer, which provides RIN (RNA Intregrity Number) ranges from 1 (degraded) to 10 (intact). Electropherograms of RNA samples (n=3) extracted from Queso Fresco by using RNAprotect bacterial reagent.

(A) RIN: 2.2



(B) RIN 4.8



(C) RIN 2.50

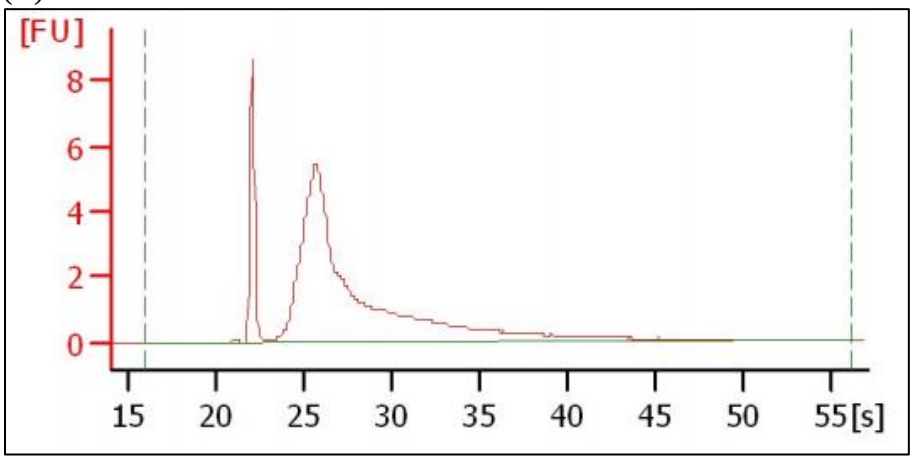


Figure 4-4. Cobalamin biosynthesis pathways that were constructed based on information from the BioCyc database (40), KEGG database (65), and published literature (76, 94). Genes in the solid-line boxes were significantly upregulated (FDR ≤0.01; Log_2FC ≥1.5) in LM-Scott A that was grown on Queso Fresco when compared to LM-Scott A grown in TSB at 7°C. Genes in the dotted line box were not significantly expressed according to statistical analyses. Solid and dotted lines indicate enzymatic reactions that happen in aerobic and anaerobic conditions, respectively.

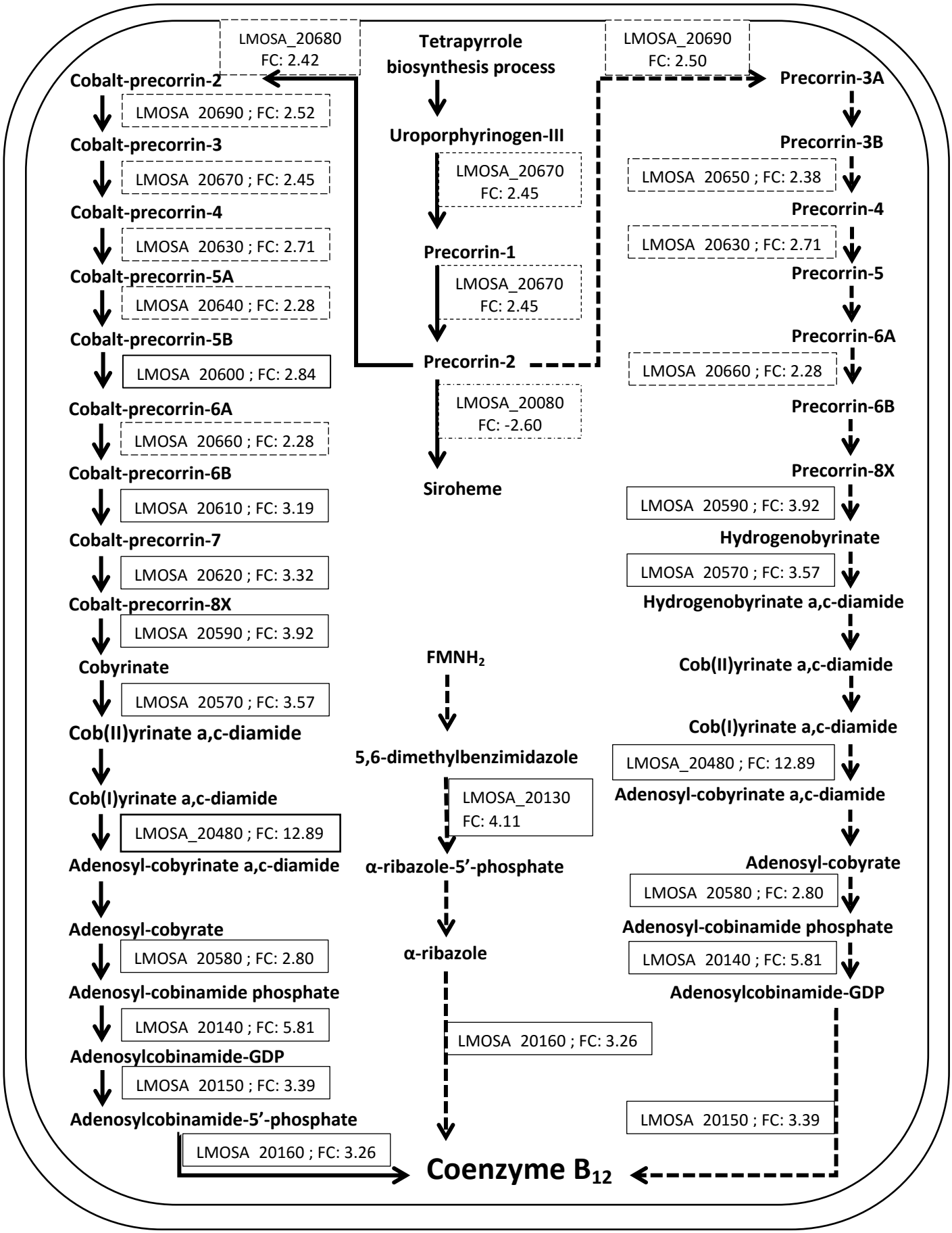

LMOSA_20680
FC: 2.42
Tetrapyrrole
biosynthesis process
LMOSA_20690
FC: 2.50
Cobalt-precorrin-2
LMOSA 20690 ; FC: 2.52
Cobalt-precorrin-3
LMOSA 20670 ; FC: 2.45
Cobalt-precorrin-4
LMOSA 20630 ; FC: 2.71
Cobalt-precorrin-5A
LMOSA 20640 ; FC: 2.28
Cobalt-precorrin-5B
LMOSA 20600 ; FC: 2.84
Cobalt-precorrin-6A
LMOSA 20660 ; FC: 2.28
Cobalt-precorrin-6B
LMOSA 20610 ; FC: 3.19
Cobalt-precorrin-7
LMOSA 20620 ; FC: 3.32
Cobalt-precorrin-8X
LMOSA 20590 ; FC: 3.92
Cobyrinate
LMOSA 20570 ; FC: 3.57
Cob(II)yrinate a,c-diamide
Cob(I)yrinate a,c-diamide
LMOSA_20480 ; FC: 12.89
Adenosyl-cobyrinate a,c-diamide
Adenosyl-cobyrate
LMOSA 20580 ; FC: 2.80
Adenosyl-cobinamide phosphate
LMOSA 20140 ; FC: 5.81
Adenosylcobinamide-GDP
LMOSA 20150 ; FC: 3.39
Adenosylcobinamide-5'-phosphate
LMOSA 20160 ; FC: 3.26
Uroporphyrinogen-III
LMOSA_20670
FC: 2.45
Precorrin-1
LMOSA_20670
FC: 2.45
Precorrin-2
LMOSA_20080
FC: -2.60
Siroheme
FMNH2
5,6-dimethylbenzimidazole
LMOSA_20130
FC: 4.11
α-ribazole-5'-phosphate
α-ribazole
LMOSA 20160 ; FC: 3.26
Precorrin-3A
Precorrin-3B
LMOSA 20650 ; FC: 2.38
Precorrin-4
LMOSA 20630 ; FC: 2.71
Precorrin-5
Precorrin-6A
LMOSA 20660 ; FC: 2.28
Precorrin-6B
Precorrin-8X
LMOSA 20590 ; FC: 3.92
Hydrogenobyrinate
LMOSA 20570 ; FC: 3.57
Hydrogenobyrinate a,c-diamide
Cob(II)yrinate a,c-diamide
Cob(I)yrinate a,c-diamide
LMOSA_20480 ; FC: 12.89
Adenosyl-cobyrinate a,c-diamide
Adenosyl-cobyrate
LMOSA 20580 ; FC: 2.80
Adenosyl-cobinamide phosphate
LMOSA 20140 ; FC: 5.81
Adenosylcobinamide-GDP
LMOSA 20150 ; FC: 3.39
Coenzyme B12

Figure 4-5. Ethanolamine and 1,2-Propanediol utilization pathways. Pathways were constructed based on information from BioCyc database (40; *L. monocytogenes* strain F2365 as reference), KEGG database (65), and published literature (94). Genes listed in boxes were significantly upregulated (FDR≤0.01; $Log_2FC \geq 1.5$) in LM-Scott A that was grown on Queso Fresco when compared to LM-Scott A grown in TSB at 7°C. Microcompartments or carboxysomes are represented by dash lines. Genes listed in boxes that lie above the dash-lined boxes (microcompartments) encode proteins of microcompartments.

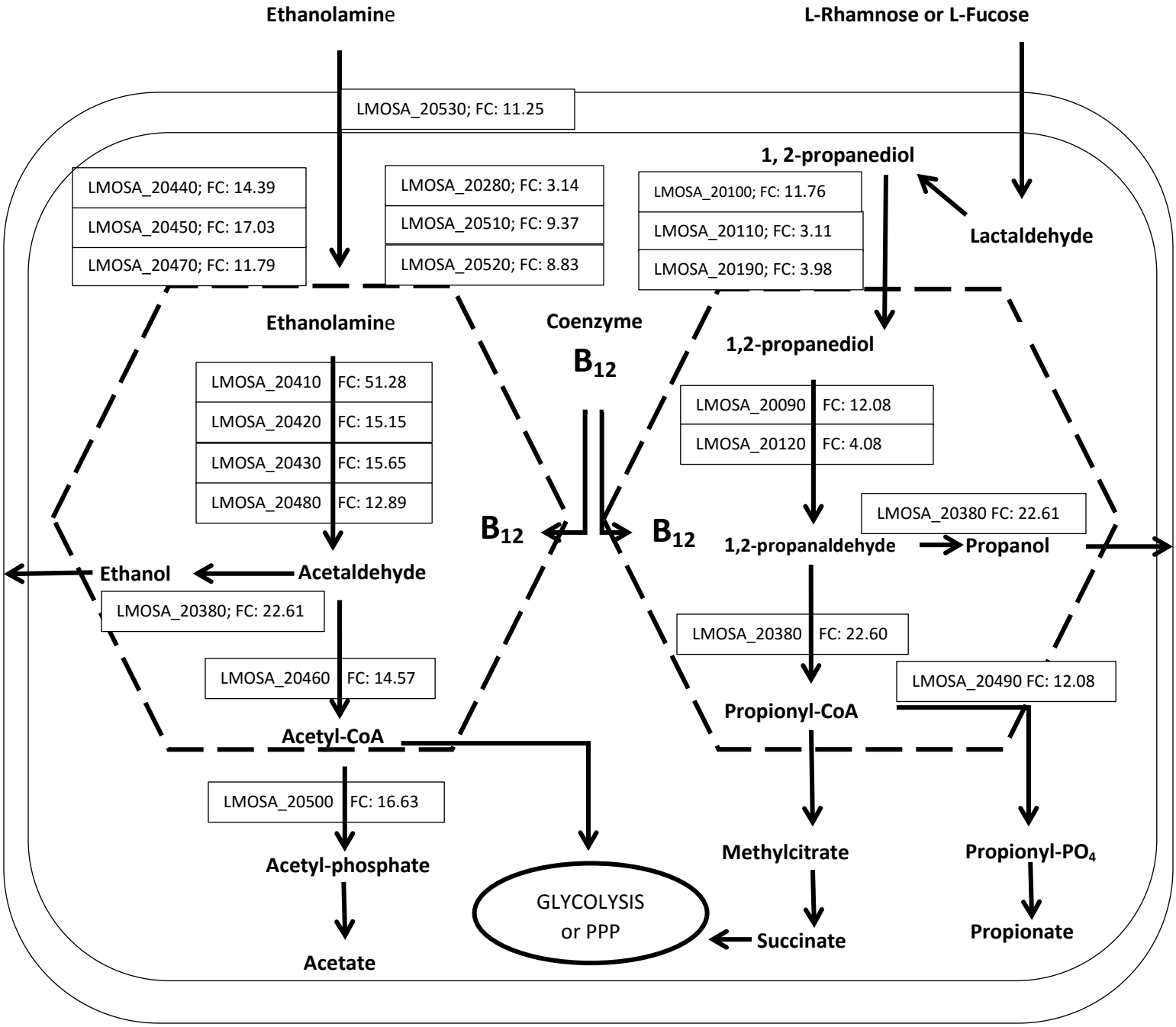

Ethanolamine
L-Rhamnose or L-Fucose
LMOSA_20530; FC: 11.25
1, 2-propanediol
LMOSA_20440; FC: 14.39
LMOSA_20450; FC: 17.03
LMOSA_20470; FC: 11.79
LMOSA_20280; FC: 3.14
LMOSA_20510; FC: 9.37
LMOSA_20520; FC: 8.83
LMOSA_20100; FC: 11.76
LMOSA_20110; FC: 3.11
LMOSA_20190; FC: 3.98
Lactaldehyde
Ethanolamine
Coenzyme
B12
1,2-propanediol
LMOSA_20410 FC: 51.28
LMOSA_20420 FC: 15.15
LMOSA_20430 FC: 15.65
LMOSA_20480 FC: 12.89
LMOSA_20090 FC: 12.08
LMOSA_20120 FC: 4.08
LMOSA_20380 FC: 22.61
B12
B12
1,2-propanaldehyde
Propanol
Ethanol
Acetaldehyde
LMOSA_20380; FC: 22.61
LMOSA_20380 FC: 22.60
LMOSA_20460 FC: 14.57
LMOSA_20490 FC: 12.08
Propionyl-CoA
Acetyl-CoA
LMOSA_20500 FC: 16.63
Methylcitrate
Propionyl-PO4
Acetyl-phosphate
GLYCOLYSIS
or PPP
Succinate
Propionate
Acetate

Figure 4-6. Histidine biosynthesis in LM-Scott A. Pathway was constructed based on information from Biocyc database (40; *L. monocytogenes* strain F2365 as reference). Genes listed in solid-lined boxes were significantly upregulated (FDR≤0.01; $Log_2FC \geq 1.5$) in LM-Scott A that was grown on Queso Fresco when compared to LM-Scott A grown in TSB at 7°C.

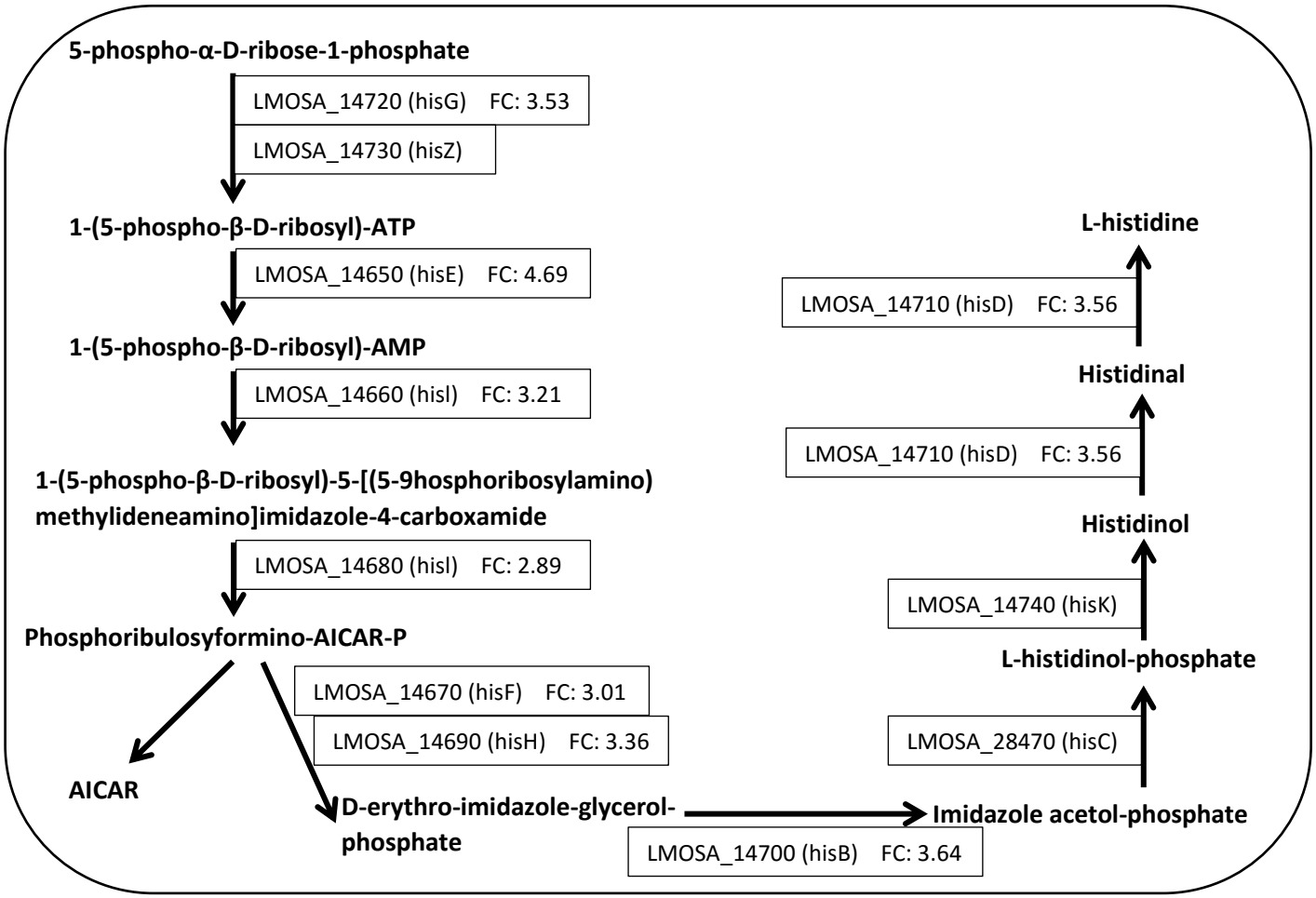


Figure 4-7. Agmatine deiminase system. The pathway was constructed based on information provided by previous studies (16, 53, 94). Genes listed in solid-lined boxes were significantly upregulated (FDR ≤0.01; Log_2FC ≥1.5) in LM-Scott A that was grown on Queso Fresco when compared to LM-Scott A grown in TSB at 7°C.

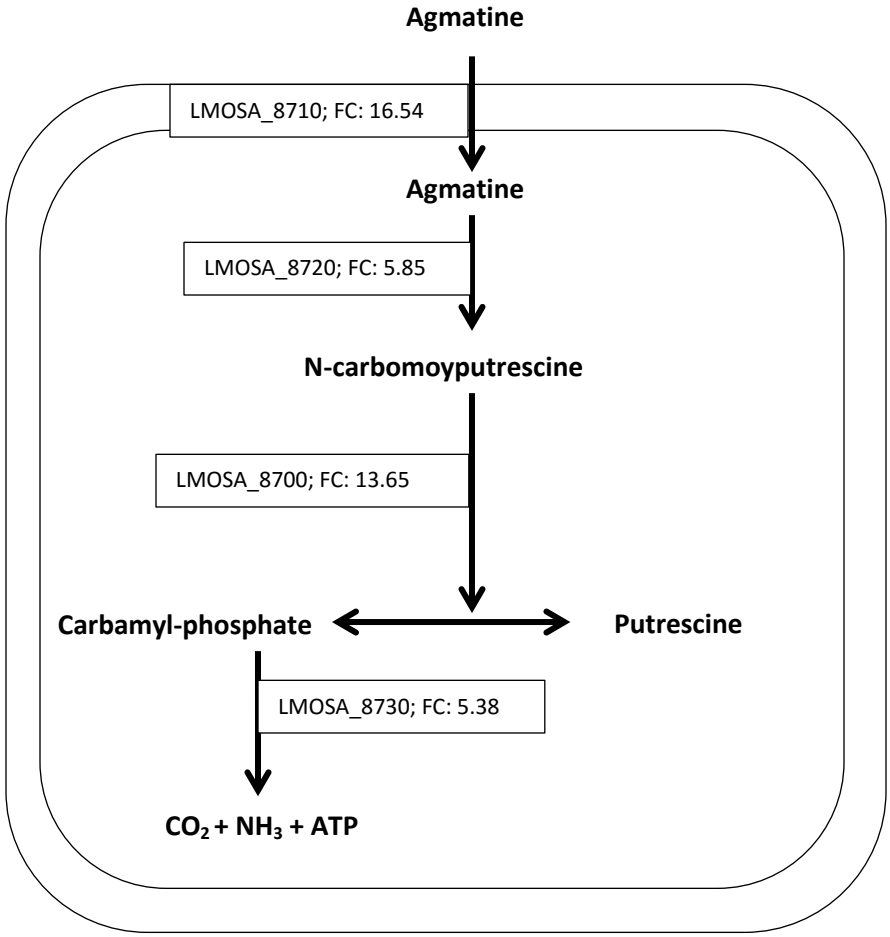


Figure 4-8. Non-oxidative branch of pentose phosphate pathway. The pathway was constructed based on information obtained from Biocyc database (40). Genes listed in solid-lined boxes were significantly upregulated (FDR ≤0.01; Log_2FC ≥1.5) in LM-Scott A that was grown on Queso Fresco when compared to LM-Scott A grown in TSB at 7°C.

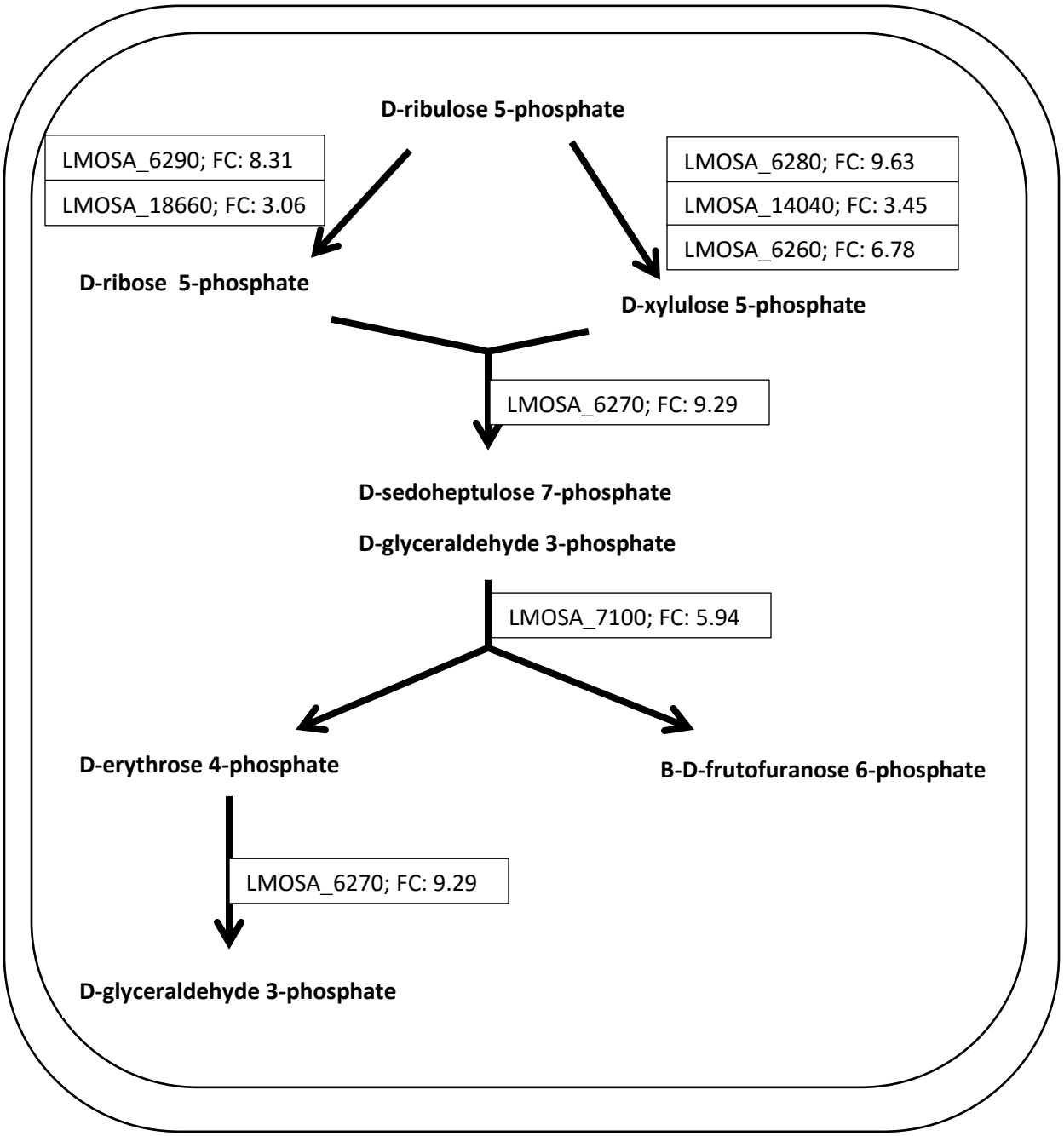


Table 4-1. Summary of RNA-sequencing data for LM-Scott A grown in TSB and on Queso Fresco at 7°C.

Matrix and sample ID[a]	No. of input reads	No. of reads mapped to Scott A CDS[b]	% of reads mapped to Scott A CDS	% reads unmapped: too short
TSB				
TSB-1	36,224,858	35,424,777	97.79%	0.53%
TSB-2	33,750,275	33,057,943	97.95%	0.53%
TSB-3	40,707,005	40,040,091	98.36%	0.49%
Queso Fresco				
Cheese-1	31,017,290	4,645,513	14.98%	67.08%
Cheese-2	49,567,604	5,311,495	10.72%	72.50%
Cheese-3	47,038,718	1,644,863	3.50%	78.09%

[a] TSB 1-3: 3 biological replicates of RNA samples extracted from LM-Scott A that was grown in TSB (Tryptic Soy Broth) at 7°C. Cheese 1-3: 3 biological replicates of RNA samples extracted from LM-Scott A that was grown on Queso Fresco.

[b]CDS: Coding DNA sequence for protein.

Table 4-2. GO terms that are overrepresented (FDR <0.05) among upregulated genes of LM-Scott A grown on Queso Fresco, in comparison to LM-Scott A grown in TSB at 7°C.

GO Terms[a]	FDR[b]	Carbohydrate	Cobalamin	Ethanolamine/ Propanediol	Histidine	Iron Utilization
Transition metal ion transport	8.09E-04					Y[a]
Histidine biosynthetic process	1.64E-03				Y	
Maltose transmembrane transporter activity	3.53E-02	Y				
Iron ion transmembrane transporter activity	5.89E-03					Y
carbohydrate metabolic process	2.25E-03	Y				
Alcohol Metabolic process	7.01E-07	Y				
Glycerol metabolic process	5.13E-03	Y				
Histidine metabolic process	1.64E-03				Y	
Cellular biogenic amine metabolic process	1.37E-02			Y		
Ethanolamine metabolic process	1.71E-05			Y		
Porphyrin metabolic process	4.14E-02		Y			
Porphyrin biosythetic process	4.14E-02		Y			
Cation transport	8.71E-03					Y
Iron ion transport	2.77E-04					Y
Carbohydrate transport	2.53E-08	Y				
Protein-N(PI)-Phosphohistidine-sugar phosphotransferase activity	1.71E-05	Y				
Catabolic process	7.20E-03	Y		Y		
Histidine family amino acid metabolic process	1.64E-03				Y	
Histidine family amino acid biosynthetic process	1.64E-03				Y	
Cobalamin metabolic process	1.04E-03		Y			
Transition metal ion transport	5.47E-03					Y
Histidine biosynthetic process	5.18E-03				Y	
Maltose transmembrane transporter activity	4.89E-03	Y				
Iron ion transmembrane transporter activity	4.61E-03					Y
carbohydrate metabolic process	4.32E-03	Y				
Alcohol Metabolic process	4.03E-03	Y				
Glycerol metabolic process	3.75E-03	Y				
Primary active transmembrane transporter	2.14E-02	Y				Y

activity						
P-P-bond-hydrolysis-driven transmembrane transporter activity	2.14E-02	Y				Y
Oligosaccharide-transporting ATPase activity	3.53E-02	Y				
Maltose-transporting ATPase activity	3.53E-02	Y				
di-, trivalent inorganic cation transport	4.17E-03					Y
Beta-glucoside transport	4.95E-03	Y				
Maltose transport	3.53E-02	Y				
Phosphotransferase activity, alcohol group as acceptor	1.04E-03	Y				
Hydrolase activity, acting on acid anhydrides, catalyzing transmembrane movement of substance	8.93E-03	Y				Y
Alditol metabolic process	5.13E-03	Y				
Polyol Metabolic process	5.65E-04	Y				
Active transmembrane transporter actvity	1.71E-05	Y				Y
Trasnmembrane transporter activity	5.66E-07	Y				Y
Substrate-specific transmembrane transporter activity	1.71E-05					Y
Substrate-Specific transporter activity	2.02E-05					Y
Metal ion transport	5.19E-04					Y
entry into host cell	4.17E-03					
Tertrapyrrole metabolic process	4.14E-02		Y			
Tetrapyrrole biosynthetic process	4.14E-02		Y			
Diol metabolic process	3.38E-04			Y		
Diol Catabolic process	3.38E-04			Y		
Ethanolamine and derivative metabolic process	1.71E-05			Y		
ATPase activity, coupled	3.39E-02	Y				Y
ATPase activity, coupled to transmembrane movement of substances	8.93E-03	Y				Y
Glucoside transport	6.73E-04	Y				
ATPase activity, coupled to movement of substances	8.93E-03	Y				Y
Cellular carbohydrate metabolic process	1.23E-02	Y				
Alcohol catabolic process	1.06E-03	Y				

Polyol catabolic process	5.89E-03	Y				
Glycerol dehydratase activity	3.53E-02	Y				
Metal ion transmembrane transporter activity	1.90E-02					Y
Trasition metal ion transmembrane transporter activity	8.71E-03					Y
Sugar transmembrane transporter activity	4.24E-03	Y				
Propanediol metabolic process	3.38E-04			Y		
Propanediol catabolic process	3.38E-04			Y		

[a] GO terms: Gene Ontology terms

[b]FDR: False Discovery rate, an adjusted P-value, indicating the type I error rate, or the possibility of getting false positive.

[c]Y: yes, this gene ontology term is related to the corresponding processes or function.

Table 4-3. Upregulated genes related to cobalamin biosynthesis, observed in LM-Scott A that was grown on Queso Fresco, in comparison to LM-Scott A grown in TSB at 7°C.

Scott A Locus Tag	F2365[a] Locus Tag	Gene Product	FDR[s]	Log_2FC[c]	FC[d]
LMOSA_20090	LMOf2365_1149	PduS type ferredoxin (Cobalamin reductase)	5.01E-09	3.06	8.37
LMOSA_20130	LMOf2365_1153	hypothetical protein (H7858: Predicted alpha-ribazole-5-phosphate synthase CblS for cobalamin biosynthesis	2.41E-07	2.04	4.12
LMOSA_20140	LMOf2365_1154	Cobinamide kinase/cobinamide phosphate	7.20E-12	2.55	5.85
LMOSA_20150	LMOf2365_1155	Cobalamin synthase	6.16E-06	1.76	3.39
LMOSA_20160	LMOf2365_1156	Alpha-ribazole-5'-phosphate phosphatase	3.32E-06	1.71	3.26
LMOSA_20360	LMOf2365_1177	L-threonine-O-3-phosphate decarboxylase	2.42E-03	1.46	2.76
LMOSA_20570	LMOf2365_1200	Cobyrinic acid A,C-diamide synthase	4.75E-06	1.84	3.57
LMOSA_20580	LMOf2365_1201	Cobalamin biosynthesis protein CobD	2.99E-05	1.49	2.80
LMOSA_20590	LMOf2365_1202	Precorrin-8X methylmutase / precorrin isomerase	5.93E-08	1.97	3.93
LMOSA_20600	LMOf2365_1203	Putative cobalt-precorrin-6A synthase	2.25E-04	1.51	2.84
LMOSA_20610	LMOf2365_1204	cobalt-precorrin-6Y C(5)-methyltransferase	2.55E-06	1.68	3.19
LMOSA_20620	LMOf2365_1205	Precorrin-6B methylase 2	1.66E-06	1.74	3.33
LMOSA_20480	LMOf2365_1191	similar to Ethanolamine pathway cobalamin adenosyltransferase COG4812	3.33E-15	3.69	12.89
LMOSA_20310	LMOf2365_1172	similar to cob(I)alamin adenosyltransferase COG2096	5.41E-05	1.99	3.96
LMOSA_20560	LMOf2365_1199	Membrane protein (H7858: Substrate-specific component CblT of predicted B-12 regulated ECF transporter	5.91E-09	3.02	8.12
LMOSA_20720	LMOf2365_1215	Cobalt ABC transporter	1.60E-04	1.47	2.76

[a] *Listeria monocytogenes* F2365 (GenBank accession number: NC_002973.6)

[b]FDR: False Discovery rate, an adjusted P-value, indicating the type I error rate, or the possibility of getting false positive.

[c]Log_2FC: Log_2 Fold Change, FC= average normalized read count (NRC) (Queso Fresco)/ average NRC (TSB).

[d]FC: Fold change, , FC= average normalized read count (NRC) (Queso Fresco)/ average NRC(TSB).

Table 4-4. Upregulated genes related to ethanolamine metabolism, observed in LM-Scott A that was grown on Queso Fresco, in comparison to LM-Scott A grown in TSB at 7°C.

Scott A Locus Tag	F2365[a] Locus Tag	Gene Product	FDR[s]	Log_2FC[c]	FC[d]
LMOSA_20280	LMOf2365_1169	Ethanolamine pathway protein	2.46E-04	1.65	3.15
LMOSA_20380	LMOf2365_1181	Alcohol dehydrogenase	7.83E-13	4.50	22.61
LMOSA_20410	LMOf2365_1184	Ethanolamine utilization EutA	1.22E-18	5.69	51.79
LMOSA_20420	LMOf2365_1185	Ethanolamine ammonia-lyase	8.28E-14	3.92	15.15
LMOSA_20430	LMOf2365_1186	Ethanolamine ammonia-lyase	1.53E-14	3.97	15.65
LMOSA_20460	LMOf2365_1189	Acetaldehyde dehydrogenase	1.61E-12	3.87	14.58
LMOSA_20480	LMOf2365_1191	Ethanolamine pathway cobalamin adenosyltransferase (UniProt:EutT)	3.33E-15	3.69	12.89
LMOSA_20500	LMOf2365_1193	Ethanolamine utilization protein	1.41E-19	4.06	16.63
LMOSA_20530	LMOf2365_1196	Ethanolamine pathway protein (Permease)	5.73E-12	3.49	11.25
LMOSA_20540	LMOf2365_1197	Ethanolamine pathway protein EutQ	1.99E-22	4.25	19.01
LMOSA_20470	LMOf2365_1190	Propanediol utilization protein	6.25E-11	3.56	11.80
LMOSA_20520	LMOf2365_1195	Ethanolamine/propanediol pathway protein	1.67E-09	3.14	8.83
LMOSA_20440	LMOf2365_1187	Microcompartments protein	6.25E-13	3.85	14.39
LMOSA_20450	LMOf2365_1188	Microcompartment protein family	3.62E-17	4.11	17.30
LMOSA_20510	LMOf2365_1194	Carbon dioxide concentrating protein CcmL	5.03E-10	3.23	9.37

[a] *Listeria monocytogenes* F2365 (GenBank accession number: NC_002973.6)

[b]FDR: False Discovery rate, an adjusted P-value, indicating the type I error rate, or the possibility of getting false positive.

[c]Log_2FC: Log_2 Fold Change, FC= average normalized read count (NRC) (Queso Fresco)/ average NRC (TSB).

[d]FC: Fold change, , FC= average normalized read count (NRC) (Queso Fresco)/ average NRC(TSB).

Table 4-5. Upregulated genes related to 1,2-propanediol metabolism, observed in LM-Scott A that was grown on Queso Fresco, in comparison to LM-Scott A grown in TSB at 7°C.

Scott A Locus tag	F2365[a] Locus Tag	Gene Product	FDR[b]	Log_2FC[c]	FC[d]
LMOSA_20090	LMOf2365_1149	PduS type ferredoxin (Cobalamin reductase)	0.00	3.06	8.37
LMOSA_20200	LMOf2365_1161	Glycerol dehydratase large subunit	0.00	1.91	3.77
LMOSA_20210	LMOf2365_1162	Propanediol utilization protein	0.00	1.91	3.77
LMOSA_20220	LMOf2365_1163	Propanediol dehydratase small subunit	0.00	2.15	4.43
LMOSA_20270	LMOf2365_1168	Propanediol pathway protein PduL	0.00	1.71	3.28
LMOSA_20320	LMOf2365_1173	CoA-dependent propionaldehyde dehydrogenase	0.00	1.88	3.69
LMOSA_20330	LMOf2365_1174	Propanol dehydrogenase	0.00	1.61	3.04
LMOSA_20350	LMOf2365_1176	Acetate/Propionate kinase	0.01	1.34	2.53
LMOSA_20260	LMOf2365_1167	Propanediol utilization protein	0.01	1.74	3.35
LMOSA_20310	LMOf2365_1172	ATP:cob (PduO)	0.00	1.99	3.96
LMOSA_20380	LMOf2365_1181	Alcohol dehydrogenase	0.00	4.50	22.61
LMOSA_20230	LMOf2365_1164	PduG protein	0.00	2.00	4.01
LMOSA_20240	LMOf2365_1165	PduH protein	0.00	2.30	4.93
LMOSA_20100	LMOf2365_1150	Microcompartments protein	0.00	3.56	11.76
LMOSA_20110	LMOf2365_1151	Propanediol utilization protein PduU(polyhedral)	0.00	1.64	3.11
LMOSA_20190	LMOf2365_1160	Propanediol utilization protein PduB(polyhedral)	0.00	2.00	3.99
LMOSA_20120	LMOf2365_1152	PduV protein	0.00	2.03	4.09
LMOSA_20180	LMOf2365_1159	Propanediol utilization protein	0.00	2.29	4.91
LMOSA_20370	LMOf2365_1178	Propanediol utilization protein PduX	0.00	1.38	2.61

[a] *Listeria monocytogenes* F2365 (GenBank accession number: NC_002973.6)

[b]FDR: False Discovery rate, an adjusted P-value, indicating the type I error rate, or the possibility of getting false positive.

[c]Log_2FC: Log_2 Fold Change, FC= average normalized read count (NRC) (Queso Fresco)/ average NRC (TSB).

[d]FC: Fold change, , FC= average normalized read count (NRC) (Queso Fresco)/ average NRC(TSB).

Table 4-6. Upregulated genes related to carbohydrates transport, observed in LM-Scott A that was grown on Queso Fresco, in comparison to LM-Scott A grown in TSB at 7°C.

Scott A Locus Tag	F2365[a] Locus Tag	Gene Product	FDR[s]	Log_2FC[c]	FC[d]
Lactose					
LMOSA_12730	LMOf2365_0390	PTS system, Lactose class of PTS, component IIB Type 3,	4.91E-03	1.45	2.73
LMOSA_17930	LMOf2365_0922	Oligo-beta-mannoside permease	5.45E-06	1.97	3.92
LMOSA_26410	LMOf2365_1743	PTS system, lactose/cellobiose-type IIA subunit	5.71E-06	1.74	3.33
LMOSA_26420	LMOf2365_1744	PTS system, Lactose class of PTS, component IIB Type 3,	1.10E-06	1.97	3.91
LMOSA_6160	LMOf2365_2622	PTS system, lactose/cellobiose-specific IIB subunit	1.46E-03	2.05	4.14
LMOSA_6500	LMOf2365_2663	PTS system, Lactose class of PTS, component IIB Type 3,	2.01E-28	5.69	51.48
LMOSA_6510	LMOf2365_2664	PTS system, lactose/cellobiose-specific component IIC	1.61E-29	5.44	43.30
LMOSA_6520	LMOf2365_2665	PTS system, lactose/cellobiose-type IIA subunit	3.47E-25	5.20	36.77
LMOSA_6760	LMOf2365_2688	PTS system, lactose/cellobiose-specific component IIC	5.87E-23	4.12	17.44
LMOSA_7500	LMOf2365_2771	PTS system, lactose/cellobiose-type IIA subunit	7.64E-03	1.85	3.62
LMOSA_7520	LMOf2365_2773	PTS system, Lactose class of PTS, component IIB Type 3,	1.01E-03	2.52	5.74
LMOSA_7530	LMOf2365_2774	PTS system, lactose/cellobiose-specific component IIC	4.63E-05	1.74	3.34
Galactitol					
LMOSA_6320	LMOf2365_2645	Galactitol-specific IIc component	1.17E-11	3.21	9.25
LMOSA_6330	LMOf2365_2646	Galactitol PTS, EIIB	4.37E-05	2.84	7.15
LMOSA_850	LMOf2365_2130	PTS system galactitol-specific enzyme IIA component	1.57E-07	3.03	8.18
LMOSA_14130	LMOf2365_0537	PTS system, galactitol-specific	1.18E-04	1.65	3.15
LMOSA_6150	LMOf2365_2621	Galactitol permease IIC component	1.50E-06	2.14	4.40
Maltose					
LMOSA_1140	LMOf2365_2159	Maltodextrin-binding protein mdxE	1.09E-32	5.28	38.90
LMOSA_1130	LMOf2365_2158	Permease protein mdxF	2.45E-40	5.09	33.96
LMOSA_1120	LMOf2365_2157	Permease protein mdxG	1.11E-34	4.96	31.02
LMOSA_1100	LMOf2365_2155	hypothetical glycosyl hydrolase yvdK	1.33E-34	4.84	28.61
LMOSA_10760	LMOf2365_0194	Alpha-glucosidase	3.49E-12	2.82	7.07
Fructose/Mannitol					
LMOSA_7640	LMOf2365_2785	PTS system, IIA component	6.04E-05	1.77	3.40
LMOSA_12620	LMOf2365_0378	PTS system fructose-specific EIIC component	9.08E-04	1.58	2.98
LMOSA_6340	LMOf2365_2647	PTS system, IIA component	2.70E-05	2.27	4.83

LMOSA_14080	LMOf2365_0532	PTS system, IIA component	9.58E-04	1.89	3.70
LMOSA_13020	LMOf2365_0420	PTS family fructose/mannitol (Fru) porter	2.35E-04	1.76	3.38
LMOSA_14060	LMOf2365_0530	PTS system,EIIB component, type 2 , Mannitol and Fructose class of PTS	1.37E-04	2.35	5.09
LMOSA_7660	LMOf2365_2787	PTS system protein	1.83E-07	1.96	3.88
LMOSA_15320	LMOf2365_0659	EIIB component, type 2, Mannitol and Fructose class of PTS	5.32E-04	2.57	5.92
LMOSA_17640	LMOf2365_0892	EIIB component, type 2, Mannitol and Fructose class of PTS	1.12E-02	1.55	2.94
LMOSA_6170	LMOf2365_2623	PTS system IIA, mannitol-specific	2.25E-04	1.77	3.41
LMOSA_6350	LMOf2365_2648	PTS EIIB type 2 domain, Mannitol and Fructose class of PTS	1.49E-05	2.06	4.16
Mannose					
LMOSA_16700	LMOf2365_0801	Pts system mannose-specific component IIA	5.24E-25	4.44	21.75
LMOSA_8490	LMOf2365_0024	PTS system,mannose-specific component IIA	6.61E-03	2.04	4.12
LMOSA_16690	LMOf2365_0800	PTS system mannose-specific component IIB	5.87E-23	4.44	21.70
LMOSA_8500	LMOf2365_0025	PTS system, ,mannose-specific component IIB	5.57E-05	3.31	9.91
LMOSA_16680	LMOf2365_0799	Mannose permease IIC component	1.06E-26	4.24	18.87
LMOSA_8510	LMOf2365_0026	PTS system, IIC component	4.94E-03	1.86	3.64
LMOSA_16670	LMOf2365_0798	PTS system, mannose-specific IID component	1.64E-27	4.19	18.32
LMOSA_8520	LMOf2365_0027	PTS system, mannose/fructose/sorbose family component IID	2.89E-04	2.33	5.04
LMOSA_9970	LMOf2365_0115	PTS system protein, mannose/fructose/sorbose family, IID component	5.84E-03	1.57	2.97
Sucrose					
LMOSA_25790	LMOf2365_1681	PTS system, sucrose-specific, IIBC component	8.69E-06	1.91	3.75

[a] *Listeria monocytogenes* F2365 (GenBank accession number: NC_002973.6)

[b]FDR: False Discovery rate, an adjusted P-value, indicating the type I error rate, or the possibility of getting false positive.

[c]Log_2FC: Log_2 Fold Change, FC= average normalized read count (NRC) (Queso Fresco)/ average NRC (TSB).

[d]FC: Fold change, , FC= average normalized read count (NRC) (Queso Fresco)/ average NRC(TSB).

Table 4-7. Upregulated genes related to glycerol intake and metabolism, observed in LM-Scott A that was grown on Queso Fresco, in comparison to LM-Scott A grown in TSB at 7°C.

Scott A Locus Tag	F2365[a] Locus Tag	Gene Product	FDR[b]	Log_2FC[c]	FC[d]
LMOSA_24610	LMOf2365_1558	Glycerol uptake facilitator protein	1.95E-16	3.05	8.26
LMOSA_26520	LMOf2365_1754	ABC-type glycerol-3-phosphate transport system	4.66E-10	2.74	6.68
LMOSA_22160	LMOf2365_1310	Aerobic glycerol-3-phosphate dehydrogenase	1.34E-26	3.91	15.05
LMOSA_20200	LMOf2365_1161	Glycerol dehydratase large subunit	1.39E-04	1.91	3.77
LMOSA_20210	LMOf2365_1162	Propanediol utilization protein	1.40E-03	1.91	3.77
LMOSA_20220	LMOf2365_1163	Propanediol dehydratase small subunit	5.16E-04	2.15	4.43
LMOSA_6630	LMOf2365_2675	similar to BH3396 protein	5.05E-23	4.28	19.48
LMOSA_24600	LMOf2365_1557	Glycerol kinase	3.71E-10	2.30	4.92
LMOSA_24610	LMOf2365_1558	Glycerol uptake facilitator protein	1.95E-16	3.05	8.26

[a] *Listeria monocytogenes* F2365 (GenBank accession number: NC_002973.6)

[b]FDR: False Discovery rate, an adjusted P-value, indicating the type I error rate, or the possibility of getting false positive.

[c]Log_2FC: Log_2 Fold Change, FC= average normalized read count (NRC) (Queso Fresco)/ average NRC (TSB).

[d]FC: Fold change, , FC= average normalized read count (NRC) (Queso Fresco)/ average NRC(TSB).

Table 4-8. Upregulated genes related to nonoxidative branch of pentose phosphate pathway, observed in LM-Scott A that was grown on Queso Fresco, in comparison to LM-Scott A grown in TSB at 7°C.

Scott A Locus Tag	F2365[a] Locus Tag	Gene Product	FDR[s]	Log_2FC[c]	FC[d]
LMOSA_6280	LMOf2365_2641	Ribulose-phosphate 3-epimerase	7.09E-14	3.3	9.63
LMOSA_6260	LMOf2365_2639	Ribulose-phosphate 3-epimerase	4.17E-07	2.8	6.78
LMOSA_14040	LMOf2365_0528	Ribulose-phosphate 3-epimerase	1.54E-03	1.8	3.45
LMOSA_18660	LMOf2365_0996	Ribose-5-phosphate isomerase A	5.69E-04	1.6	3.06
LMOSA_6290	LMOf2365_2642	Ribose-5-phosphate isomerase B	2.62E-08	3.1	8.31
LMOSA_6270	LMOf2365_2640	Transketolase	7.94E-11	3.2	9.29
LMOSA_7100	LMOf2365_2730	Transaldolase	6.68E-11	2.6	5.94

[a] *Listeria monocytogenes* F2365 (GenBank accession number: NC_002973.6)

[b]FDR: False Discovery rate, an adjusted P-value, indicating the type I error rate, or the possibility of getting false positive.

[c]Log_2FC: Log_2 Fold Change, FC= average normalized read count (NRC) (Queso Fresco)/ average NRC (TSB).

[d]FC: Fold change, , FC= average normalized read count (NRC) (Queso Fresco)/ average NRC(TSB).

Table 4-9. Upregulated genes related to iron intake, observed in LM-Scott A that was grown on Queso Fresco, in comparison to LM-Scott A grown in TSB at 7°C.

Scott A Locus Tag	F2365[a] Locus Tag	Gene Product	FDR[s]	Log_2FC[c]	FC[d]
LMOSA_910	LMOf2365_2136	Ferrous iron transport protein	2.27E-16	3.65	12.59
LMOSA_920	LMOf2365_2137	Ferrous iron transport protein	1.99E-22	3.74	13.32
LMOSA_1710	LMOf2365_2214	Sortase, SrtB family	1.24E-13	6.53	92.09
LMOSA_1720	LMOf2365_2215	ATP-binding protein	1.36E-17	5.12	34.81
LMOSA_1730	LMOf2365_2216	ABC-type Fe3+-siderophore transport system, permease component	3.27E-16	5.38	41.72
LMOSA_1740	LMOf2365_2217	Periplasmic binding protein	3.62E-21	5.70	51.93
LMOSA_1750	LMOf2365_2218	Iron Transport-associated domain family	1.45E-15	6.40	84.45
LMOSA_1760	LMOf2365_2219	Iron Transport-associated domain family	4.02E-13	6.43	86.21
LMOSA_28800	LMOf2365_1987	ABC-type Fe3+-siderophore transport system, permease component PhuB	5.16E-06	1.98	3.96
LMOSA_28810	LMOf2365_1988	ABC-type Fe3+-siderophore transport system, permease component PhuG	4.08E-06	2.05	4.15
LMOSA_28820	LMOf2365_1989	Iron compound ABC transporter	6.93E-09	2.71	6.54
LMOSA_28830	LMOf2365_1990	ATP-binding protein fhuC	2.64E-05	1.70	3.26

[a] *Listeria monocytogenes* F2365 (GenBank accession number: NC_002973.6)

[b]FDR: False Discovery rate, an adjusted P-value, indicating the type I error rate, or the possibility of getting false positive.

[c]Log_2FC: Log_2 Fold Change, FC= average normalized read count (NRC) (Queso Fresco)/ average NRC (TSB).

[d]FC: Fold change, , FC= average normalized read count (NRC) (Queso Fresco)/ average NRC(TSB).

Table 4-10. Upregulated genes related to histidine biosynthesis, observed in LM-Scott A that was grown on Queso Fresco, in comparison to LM-Scott A grown in TSB at 7°C.

Scott A Locus tag	F2365[a] Locus Tag	Gene Product	FDR[s]	Log_2FC[c]	FC[d]
LMOSA_14720	LMOf2365_0597	ATP phosphoribosyltransferase catalytic subunit	1.45E-06	1.82	3.53
LMOSA_14650	LMOf2365_0590	Phosphoribosyl-ATP pyrophosphatase	7.86E-09	2.23	4.69
LMOSA_14660	LMOf2365_0591	Phosphoribosyl-AMP cyclohydrolase	3.18E-06	1.68	3.21
LMOSA_14680	LMOf2365_0593	1-(5-phosphoribosyl)-5-[(5-phosphoribosylamino)methylideneamino] imidazole-4-carboxamide isomerase	1.96E-05	1.53	2.89
LMOSA_14670	LMOf2365_0592	Imidazole glycerol phosphate synthase	5.70E-06	1.59	3.01
LMOSA_14690	LMOf2365_0594	Imidazole glycerol phosphate synthase subunit hisH	1.06E-06	1.75	3.37
LMOSA_14700	LMOf2365_0595	Imidazoleglycerol-phosphate dehydratase	1.26E-07	1.87	3.65
LMOSA_14710	LMOf2365_0596	Histidinol dehydrogenase	6.09E-08	1.84	3.57

[a] *Listeria monocytogenes* F2365 (GenBank accession number: NC_002973.6)

[b]FDR: False Discovery rate, an adjusted P-value, indicating the type I error rate, or the possibility of getting false positive.

[c]Log_2FC: Log_2 Fold Change, FC= average normalized read count (NRC) (Queso Fresco)/ average NRC (TSB).

[d]FC: Fold change, , FC= average normalized read count (NRC) (Queso Fresco)/ average NRC(TSB).

Table 4-11. Upregulated genes related to agmatine deiminase system, observed in LM-Scott A that was grown on Queso Fresco, in comparison to LM-Scott A in TSB at 7°C.

Scott A Locus Tag	F2365[a] Locus Tag	Gene Product	FDR[s]	Log_2FC[c]	FC[d]
LMOSA_8700	LMOf2365_0045	Putrescine carbamoyltransferase	1.97E-09	3.77	13.65
LMOSA_8710	LMOf2365_0046	Agmatine/putrescine antiporter, associated with agmatine catabolism (Amino acid permease family protein)	4.66E-10	4.03	16.34
LMOSA_8720	LMOf2365_0047	Agmatine deiminase 1	4.11E-08	2.55	5.85
LMOSA_8730	LMOf2365_0048	Carbamate kinase	1.16E-07	2.43	5.38

[a] *Listeria monocytogenes* F2365 (GenBank accession number: NC_002973.6)

[b]FDR: False Discovery rate, an adjusted P-value, indicating the type I error rate, or the possibility of getting false positive.

[c]Log_2FC: Log_2 Fold Change, FC= average normalized read count (NRC) (Queso Fresco)/ average NRC (TSB).

[d]FC: Fold change, , FC= average normalized read count (NRC) (Queso Fresco)/ average NRC(TSB).

Table 4-12. Upregulated genes related to virulence, observed in LM-Scott A that was grown on Queso Fresco, in comparison to LM-Scott A in TSB at 7°C.

Scott A Locus Tag	F2365[a] Locus Tag	Gene Product	FDR[s]	Log_2FC[c]	FC[d]
LMOSA_10930	prfA	Listeriolysin regulatory protein	1.64E-05	1.97	3.91
LMOSA_10950	hly	Listeriolysin O	1.13E-05	1.69	3.22
LMOSA_10960	mpl	Zinc metalloproteinase	1.69E-03	1.66	3.16
LMOSA_10970	actA	Actin-assembly inducing protein	7.13E-03	1.24	2.37
LMOSA_10980	plcB	Phospholipase C	2.12E-04	2.16	4.48
LMOSA_13470	inlA	Internalin-A	1.17E-26	5.77	54.44
LMOSA_13480	inlB	Internalin B	2.90E-14	3.35	10.20

[a] *Listeria monocytogenes* F2365 (GenBank accession number: NC_002973.6)

[b]FDR: False Discovery rate, an adjusted P-value, indicating the type I error rate, or the possibility of getting false positive.

[c]Log_2FC: Log_2 Fold Change, FC= average normalized read count (NRC) (Queso Fresco)/ average NRC (TSB).

[d]FC: Fold change, , FC= average normalized read count (NRC) (Queso Fresco)/ average NRC(TSB).

Table 4-13. Upregulated genes related to prophage, observed in LM-Scott A that was grown on Queso Fresco, in comparison to LM-Scott A grown in TSB at 7°C.

Scott A Locus tag	F2365[a] Locus Tag	Gene Product	FDR[s]	Log_2FC[c]	FC[d]
LMOSA_930	LMOf2365_2138	hypothetical protein (hypothetical protein)(Virus attachment-like protein)	3.32E-23	4.21	18.47
LMOSA_9270	NA	Phage antirepressor family protein	1.86E-05	2.15	4.44
LMOSA_9280	NA	Gp43 phage protein	2.11E-03	1.60	3.03
LMOSA_9340	NA	Gp44 phage protein	1.21E-03	1.84	3.59
LMOSA_9350	NA	Phage protein	9.37E-05	2.52	5.72
LMOSA_9380	NA	Gp35 phage protein	4.50E-03	1.62	3.08
LMOSA_9390	NA	Conserved phage protein	1.16E-03	2.07	4.19
LMOSA_9440	NA	Gp91 phage protein	7.94E-06	3.98	15.83
LMOSA_9550	NA	Phage portal protein	7.79E-08	2.23	4.68
LMOSA_9560	NA	Gp4 protein	8.44E-10	2.47	5.55
LMOSA_9570	NA	Phage protein Gp5	2.50E-08	2.98	7.91
LMOSA_9580	NA	Phage coat protein	3.41E-13	3.14	8.84
LMOSA_9590	NA	Phage protein gp8	7.20E-12	3.57	11.86
LMOSA_9600	NA	Phage protein gp9	1.06E-09	4.07	16.82
LMOSA_9610	NA	Phage protein gp10	1.19E-11	3.78	13.78
LMOSA_9620	NA	Phage protein gp11	7.56E-10	4.07	16.74
LMOSA_9630	NA	Major tail shaft protein	7.21E-14	3.64	12.49
LMOSA_9640	NA	Phage minor capsid protein	4.48E-09	3.64	12.49
LMOSA_9660	NA	Gp15 phage protein	5.11E-06	2.43	5.40
LMOSA_9670	NA	Phage-related tail protein	8.97E-06	1.69	3.24
LMOSA_9680	NA	Gp17 phage protein	7.21E-08	2.43	5.38
LMOSA_9690	NA	Gp18 phage protein	4.00E-08	2.42	5.34
LMOSA_9700	NA	Gp19 phage protein	1.08E-07	2.08	4.24
LMOSA_9710	NA	hypothetical protein (putative gp20)(putative long tail fibre protein)	9.88E-07	2.09	4.27
LMOSA_9740	NA	Phage Holin Protein	8.47E-03	1.90	3.73
LMOSA_10120	LMOf2365_0131	Bacteriophage gp35-type protein	3.13E-06	2.36	5.12
LMOSA_10140	LMOf2365_0133	Prophage LambdaLm01, antigen D	2.69E-03	1.61	3.05
LMOSA_10150	LMOf2365_0134	Phage transcriptional activator, antigen C	1.28E-03	1.54	2.91
LMOSA_10160	LMOf2365_0135	Antigen B	4.65E-12	2.92	7.57
LMOSA_10170	LMOf2365_0136	Antigen A	2.29E-09	2.62	6.15
LMOSA_10190	LMOf2365_0138	Phage tail length tape-measure protein	1.10E-15	3.27	9.68
LMOSA_10200	LMOf2365_0139	Phage tail length tape-measure protein	2.78E-12	2.58	5.98
LMOSA_10210	LMOf2365_0140	Phage tail fiber	1.47E-15	2.96	7.79
LMOSA_10220	LMOf2365_0141	Putative tail or base plate protein gp18	1.11E-19	3.64	12.44
LMOSA_10270	LMOf2365_0146	Phage-related holin (Lysis protein)	3.59E-11	2.66	6.32
LMOSA_10280	LMOf2365_0147	Prophage LambdaLm01 amidase	4.67E-14	2.78	6.86

LMOSA_21080	NA	Integrase	4.36E-04	1.82	3.53
LMOSA_21090	NA	Gp25 protein	1.68E-03	1.77	3.41
LMOSA_21280	NA	Conserved phage-related protein	2.70E-05	2.36	5.12
LMOSA_21290	NA	Phage-related protein	3.35E-05	2.71	6.56
LMOSA_21300	NA	Phage nucleotide-binding protein	1.76E-05	2.31	4.94
LMOSA_21310	NA	Phage DEAH-family helicase	1.60E-06	1.86	3.64
LMOSA_21320	NA	Gp59 phage protein	2.73E-06	3.06	8.36
LMOSA_21340	NA	Conserved phage-related protein	5.52E-04	2.17	4.51
LMOSA_21350	NA	Gp62 phage protein	3.18E-04	2.76	6.76
LMOSA_21400	NA	Phage portal protein	3.02E-05	2.62	6.13
LMOSA_21430	NA	Gp6 protein, head-tail connector	2.33E-08	3.30	9.88
LMOSA_21431	NA	Gp7 protein, head-tail adaptor	1.07E-05	3.11	8.61
LMOSA_21440	NA	Gp8 phage protein	1.60E-06	3.20	9.17
LMOSA_21450	NA	Gp9 phage protein	3.77E-09	4.03	16.36
LMOSA_21460	NA	Major tail protein B	3.35E-05	2.06	4.18
LMOSA_21500	NA	Gp13 phage protein	1.09E-09	3.88	14.73
LMOSA_21510	NA	Phage-related structural protein	7.55E-07	2.10	4.28
LMOSA_21530	NA	hypothetical protein (Gp16 protein)(Gp17 protein)	5.60E-03	1.93	3.81

[a] *Listeria monocytogenes* F2365 (GenBank accession number: NC_002973.6)

[b]FDR: False Discovery rate, an adjusted P-value, indicating the type I error rate, or the possibility of getting false positive.

[c]Log_2FC: Log_2 Fold Change, FC= average normalized read count (NRC) (Queso Fresco)/ average NRC (TSB).

[d]FC: Fold change, , FC= average normalized read count (NRC) (Queso Fresco)/ average NRC(TSB).

Table 4-14. Downregulated genes related to flagellar formation/assembly, observed in LM-Scott A that was grown on Queso Fresco, in comparison to LM-Scott A grown in TSB at 7°C.

Scott A Locus Tag	F2365[a] Locus Tag	Gene Product	FDR[s]	Log_2FC[c]	FC[d]
LMOSA_15820	LMOf2365_0712	Flagellar biosynthesis protein FliP	1.10E-08	-1.84	-3.57
LMOSA_15830	LMOf2365_0713	Export protein FliQ family 3	1.47E-06	-1.98	-3.94
LMOSA_15840	LMOf2365_0714	Flagellar biosynthetic protein FliR	2.35E-08	-2.02	-4.05
LMOSA_15850	LMOf2365_0715	Flagellar biosynthesis protein	2.62E-05	-1.69	-3.22
LMOSA_15860	LMOf2365_0716	Flagellar biosynthesis protein FlhA	7.64E-05	-1.48	-2.79
LMOSA_15990	LMOf2365_0729	Flagellar motor switch protein FliN	6.82E-05	-1.53	-2.89
LMOSA_16000	LMOf2365_0730	Flagellar biosynthesis chaperone COG2882 FliJ	1.03E-07	-1.96	-3.90
LMOSA_16010	LMOf2365_0731	Flagellar hook-associated protein FliK	5.53E-05	-1.70	-3.26
LMOSA_16180	LMOf2365_0749	Flagellar M-ring protein FliF	1.12E-06	-1.76	-3.40

[a] *Listeria monocytogenes* F2365 (GenBank accession number: NC_002973.6)

[b]FDR: False Discovery rate, an adjusted P-value, indicating the type I error rate, or the possibility of getting false positive.

[c]Log_2FC: Log_2 Fold Change, FC= - average normalized read count (NRC) (TSB)/ average NRC (Queso Fresco).

[d]FC: Fold change, , FC= - average normalized read count (NRC) (TSB)/ average NRC(Queso Fresco).

Table 4-15. Downregulated genes related to putrescine/spermidine transport, observed in LM-Scott A that was grown on Queso Fresco, in comparison to LM-Scott A grown in TSB at 7°C.

Scott A Locus Tag	F2365[a] Locus Tag	Gene Product	FDR[s]	Log_2FC[c]	FC[d]
LMOSA_16960	LMOf2365_0824	permease protein	8.67E-05	-1.73	-3.32
LMOSA_16950	LMOf2365_0823	ATP-binding protein PotA	9.60E-08	-2.24	-4.73
LMOSA_16970	LMOf2365_0825	permease protein	1.04E-03	-1.57	-2.97

[a] *Listeria monocytogenes* F2365 (GenBank accession number: NC_002973.6)

[b]FDR: False Discovery rate, an adjusted P-value, indicating the type I error rate, or the possibility of getting false positive.

[c]Log_2FC: Log_2 Fold Change, FC= - average normalized read count (NRC) (TSB)/ average NRC (Queso Fresco).

[d]FC: Fold change, , FC= - average normalized read count (NRC) (TSB)/ average NRC(Queso Fresco).

Table 4-16. Downregulated genes related to ribosomal protein synthesis, observed in LM-Scott A that was grown on Queso Fresco, in comparison to LM-Scott A grown in TSB at 7°C.

Scott A Locus Tag	F2365[a] Locus Tag	Gene Product	FDR[b]	Log_2FC[c]	FC[d]
LMOSA_5140	LMOf2365_2521	50S ribosomal protein L31 type B	4.90E-03	-1.62	-3.07
LMOSA_5630	LMOf2365_2569	30S ribosomal protein S9	2.41E-03	-1.49	-2.81
LMOSA_5640	LMOf2365_2570	50S ribosomal protein L13	1.20E-04	-1.50	-2.82
LMOSA_5990	LMOf2365_2605	50S ribosomal protein L3	6.31E-05	-1.71	-3.28
LMOSA_6000	LMOf2365_2606	30S ribosomal protein S10	7.93E-06	-2.11	-4.32
LMOSA_6210	LMOf2365_2634	30S ribosomal protein S7	7.74E-05	-1.47	-2.78
LMOSA_6220	LMOf2365_2635	30S ribosomal protein S12	4.24E-06	-1.68	-3.21
LMOSA_7690	LMOf2365_2790	Ribosomal RNA small subunit methyltransferase G	2.20E-08	-2.42	-5.36
LMOSA_8220	LMOf2365_2846	50S ribosomal protein L34	1.25E-03	-1.52	-2.87
LMOSA_11110	LMOf2365_0229	S1 RNA binding domain protein	4.48E-09	-2.30	-4.94
LMOSA_11450	LMOf2365_0260	50S ribosomal protein L11	1.56E-03	-1.59	-3.00
LMOSA_13850	LMOf2365_0509	Ribosomal RNA large subunit methyltransferase N	1.05E-09	-2.42	-5.37
LMOSA_22190	LMOf2365_1313	GTP-binding protein HflX	9.96E-09	-2.03	-4.08
LMOSA_22440	LMOf2365_1338	Ribosome maturation factor rimP	2.68E-06	-1.62	-3.07
LMOSA_24020	LMOf2365_1499	30S ribosomal protein S20	8.60E-03	-1.68	-3.22
LMOSA_24130	LMOf2365_1510	GTP-binding protein (participate in biosynthesis of 30S ribosome)	3.43E-06	-1.52	-2.87
LMOSA_24590	LMOf2365_1556	GTPase obg (KEGG: Ribosome biogenesis)	6.68E-11	-2.72	-6.57
LMOSA_25180	LMOf2365_1618	30S ribosomal protein S4	3.36E-04	-1.67	-3.19
LMOSA_25780	LMOf2365_1679	30S ribosomal protein S2	1.25E-04	-1.73	-3.31
LMOSA_27060	LMOf2365_1809	Ribosomal protein L35	2.64E-04	-1.45	-2.74
LMOSA_27100	LMOf2365_1814	50S ribosomal protein L19	7.52E-03	-1.53	-2.88
LMOSA_27200	LMOf2365_1824	30S ribosomal protein S16	7.34E-05	-1.92	-3.79

[a] *Listeria monocytogenes* F2365 (GenBank accession number: NC_002973.6)

[b]FDR: False Discovery rate, an adjusted P-value, indicating the type I error rate, or the possibility of getting false positive.

[c]Log_2FC: Log_2 Fold Change, FC= - average normalized read count (NRC) (TSB)/ average NRC(Queso Fresco).

[d]FC: Fold change, , FC= - average normalized read count (NRC) (TSB)/ average NRC(Queso Fresco).

Table 4-17. Downregulated genes related to peptidogylcan synthesis, observed in LM-Scott A that was grown on Queso Fresco, in comparison to LM-Scott A grown in TSB at 7°C.

Scott A Locus Tag	F2365[a] Locus Tag	Gene Product	FDR[s]	Log_2FC[c]	FC[d]
LMOSA_7220	LMOf2365_2742	D-alanyl-D-alanine carboxypeptidase	4.49E-08	-1.92	-3.79
LMOSA_4920	LMOf2365_2499	UDP-N-acetylglucosamine 1-carboxyvinyltransferase 1	9.05E-07	-1.78	-3.45
LMOSA_22380	LMOf2365_1332	Undecaprenyl pyrophosphate synthase	1.02E-05	-1.53	-2.90
LMOSA_17460	LMOf2365_0872	D-alanine--D-alanine ligase	1.10E-05	-1.50	-2.83
LMOSA_16220	LMOf2365_0753	Transglycosylase, SLT family	1.36E-05	-1.54	-2.92
LMOSA_14440	LMOf2365_0569	Beta-lactamase	2.31E-05	-1.51	-2.84
LMOSA_23410	LMOf2365_1439	UDP-N-acetylenolpyruvoylglucosamine reductase	1.06E-06	-1.70	-3.25

[a] *Listeria monocytogenes* F2365 (GenBank accession number: NC_002973.6)

[b]FDR: False Discovery rate, an adjusted P-value, indicating the type I error rate, or the possibility of getting false positive.

[c]Log_2FC: Log_2 Fold Change, FC= - average normalized read count (NRC) (TSB)/ average NRC (Queso Fresco).

[d]FC: Fold change, , FC= - average normalized read count (NRC) (TSB)/ average NRC(Queso Fresco).

Table 4-18. Downregulated genes related to pyrimidine biosynthesis, observed in LM-Scott A that was grown on Queso Fresco, in comparison to LM-Scott A grown in TSB at 7°C.

Scott A Locus Tag	F2365[a] Locus Tag	Gene Product	FDR[s]	Log_2FC[c]	FC[d]
LMOSA_5240	LMOf2365_2531	CTP synthase	1.06E-05	-1.59	-3.01
LMOSA_22540	LMOf2365_1348	Polyribonucleotide nucleotidyltransferase	1.87E-04	-1.46	-2.74
LMOSA_27580	LMOf2365_1863	Carbamoyl-phosphate synthase	4.76E-04	-1.60	-3.04
LMOSA_27590	LMOf2365_1864	Carbamoyl-phosphate synthase	3.71E-05	-2.40	-5.28
LMOSA_27600	LMOf2365_1865	Dihydroorotase	7.99E-06	-2.77	-6.82
LMOSA_27610	LMOf2365_1866	Aspartate carbamoyltransferase	1.60E-06	-2.81	-7.01
LMOSA_27630	LMOf2365_1868	Bifunctional protein pyrR	3.46E-15	-3.49	-11.25

[a] *Listeria monocytogenes* F2365 (GenBank accession number: NC_002973.6)

[b]FDR: False Discovery rate, an adjusted P-value, indicating the type I error rate, or the possibility of getting false positive.

[c]Log_2FC: Log_2 Fold Change, FC= - average normalized read count (NRC) (TSB)/ average NRC (Queso Fresco).

[d]FC: Fold change, , FC= - average normalized read count (NRC) (TSB)/ average NRC(Queso Fresco).

CHAPTER 5:

CONCLUSIONS AND FUTURE WORK

Our research investigated the influence of compositional factors and storage environment upon pathogenic bacterial growth on cheeses. Combining data from our studies and results from selected publications, we have established a framework that could be used to predict pathogen growth on cheeses held under variety of retail and food processing conditions. In the final project, we explored the use of next-generation sequencing to understand the specific metabolic pathways and physiological responses critical for *L. monocytogenes* growth on Queso Fresco, a fresh Hispanic-style cheese that had been implicated in foodborne illness outbreaks at a greater frequency compared to other cheeses. Our study provided information that could potentially be useful in facilitating the development of effective antimicrobial strategies. Additionally, the understanding about virulence gene expression of pathogenic bacteria grown in a food environment could further enhance risk assessment.

L. monocytogenes, along with *Salmonella* spp. and *Clostridium perfringens,* were the only three pathogenic bacteria that are able to utilize both ethanolamine and 1, 2-propanediol as carbon and nitrogen sources (3, 4). In this dissertation, transcriptomics analysis of *L. monocytogenes* Scott A showed that the pathogen was likely utilizing both ethanolamine and 1, 2-propanediol to survive and grow on Queso Fresco. Consistently, *L. monocytogenes* grown on cold smoked salmon was also activating the genes related to metabolism of ethanolamine and 1, 2-propanediol (6). It has been suggested that the ability to utilize both of these compounds provides significant advantages towards the pathogenic bacteria to survive and grow in an environment with competing microflora. However, the majority of studies have been focusing on the importance of ethanolamine and/or 1, 2-propanediol metabolisms in Gram-negative pathogenic bacteria such as pathogenic *E. coli* and *Salmonella* spp (1, 5, 8); and in mammalian cell and gut environment (1, 2, 8). To understand and confirm the significance of ethanolamine

and 1, 2-propanediol metabolisms for the survival and growth of *L. monocytogenes* in competitive food environment such as cheese, experiments using mutant strains that carry deletions of genes related to these metabolic pathways should be conducted.

Additionlly, the transcriptomic work in this dissertation also revealed that addition of acetate and/or propionate to Queso Fresco could potentially shift *L. monocytogenes* away from metabolizing ethanolamine and/or 1, 2-propanediol. Further work is needed to test this hypothesis, and to investigate if the proposed treatment would reduce the competitive advantage of *L. monocytogenes*. Potentially, transcriptomics analyses along with plating method can be used to investigate the survival/growth and gene expression of *L. monocytogenes* on Queso Frseco treated with acetate/propionate in combination with or without other antimicrobial compound. In fact, the study elaborated in Chapter 2 of this dissertation showed that Swiss-type cheeses, in which propionate and acetate are the major form of acid instead of lactate, did not support growth of all four pathogenic bacteria (*E. coli* O157:H7, *L. monocytogenes*, *Salmonella* spp., and *S. aureus*), despite the Swiss-type cheese having greater pH values (5.36-6.02) compared to some cheeses that had lactate as major form of acid and supported pathogen growth. Previous publications have indicated the greater antimicrobial activity of propionate (pKa = 4.87) in comparison to lactate (pKa = 3.86), likely due to the greater amount of undissociated form of acids for propionate (7). It could also be interesting to test the hypothesis if *L. monocytogenes* that was exposed to Swiss-type cheese has lower gene expression for ethanolamine and 1, 2-propanediol activity, in comparison to *L. monocytogenes* that was exposed to cheeses with the same pH value, but having lactate as the major form of acid.

5.1 REFERENCES

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APPENDIX 1: Genes that were significantly upregulated (FDR ≤ 0.01; $Log_2FC \geq 1.50$ in LM-Scott A grown on Queso Fresco at 7°C, in comparison to the similar strain grown in TSB.

Scott A Locus tag	F2365[a] Locus Tag	Gene Product	FDR[s]	Log_2FC[c]	FC[d]
LMOSA_80	NA	Oxidoreductase	1.30E-03	1.95	3.87
LMOSA_130	LMOf2365_2057	hypothetical protein	4.36E-05	2.84	7.14
LMOSA_140	LMOf2365_2058	Methyltransferase	5.57E-05	2.20	4.58
LMOSA_500	LMOf2365_2094	Secreted protein	3.38E-03	1.58	2.99
LMOSA_540	LMOf2365_2098	Choloylglycine hydrolase	7.20E-12	3.06	8.36
LMOSA_800	LMOf2365_2125	Secreted protein	2.39E-04	2.59	6.00
LMOSA_810	LMOf2365_2126	Class II aldolase/adducin domain protein	2.23E-04	1.58	3.00
LMOSA_820	LMOf2365_2127	hypothetical protein	2.29E-03	2.02	4.05
LMOSA_850	LMOf2365_2130	PTS system galactitol-specific enzyme IIA component	1.57E-07	3.03	8.18
LMOSA_860	LMOf2365_2131	M protein trans-acting positive regulator	2.22E-08	2.42	5.36
LMOSA_910	LMOf2365_2136	Ferrous iron transport protein	2.27E-16	3.65	12.59
LMOSA_920	LMOf2365_2137	Ferrous iron transport protein	1.99E-22	3.74	13.32
LMOSA_930	LMOf2365_2138	hypothetical protein	3.32E-23	4.21	18.47
LMOSA_950	LMOf2365_2140	Transcriptional regulator	1.22E-07	1.95	3.86
LMOSA_960	LMOf2365_2141	N-acetylglucosamine-6-phosphate deacetylase	1.22E-07	1.74	3.35
LMOSA_970	LMOf2365_2142	Hydrolase	2.04E-07	1.81	3.50
LMOSA_1030	LMOf2365_2148	Permease	1.10E-06	2.42	5.34
LMOSA_1090	LMOf2365_2154	teichoic acid biosynthesis domain protein	7.41E-15	3.60	12.12
LMOSA_1100	LMOf2365_2155	hypothetical glycosyl hydrolase yvdK	1.33E-34	4.84	28.61
LMOSA_1110	LMOf2365_2156	Maltodextrose utilization protein MalA	1.10E-35	4.62	24.67
LMOSA_1120	LMOf2365_2157	Permease protein mdxG	1.11E-34	4.96	31.02
LMOSA_1130	LMOf2365_2158	Permease protein mdxF	2.45E-40	5.09	33.96
LMOSA_1140	LMOf2365_2159	Maltodextrin-binding protein mdxE	1.09E-32	5.28	38.90
LMOSA_1150	LMOf2365_2160	Intracellular maltogenic amylase	3.63E-13	2.60	6.06
LMOSA_1210	LMOf2365_2166	hypothetical protein	2.55E-06	2.48	5.58
LMOSA_1460	LMOf2365_2189	Metallo-beta-lactamase protein	2.29E-23	4.15	17.75
LMOSA_1470	LMOf2365_2190	hypothetical protein	1.24E-18	6.38	83.37
LMOSA_1480	LMOf2365_2191	Oxidoreductase domain protein	2.14E-19	3.90	14.94
LMOSA_1490	LMOf2365_2192	Sugar phosphate isomerase/epimerase	3.32E-23	4.16	17.88
LMOSA_1500	LMOf2365_2193	Trehalosemaltose utilization protein	7.22E-14	3.42	10.74
LMOSA_1510	LMOf2365_2194	Sugar phosphate isomerase/epimerase	6.95E-30	4.63	24.74

LMOSA_1520	LMOf2365_2195	NADH-dependent dehydrogenase	7.71E-17	3.92	15.13
LMOSA_1540	LMOf2365_2197	hypothetical protein	4.85E-04	2.10	4.30
LMOSA_1580	LMOf2365_2201	hypothetical protein	2.65E-04	2.39	5.24
LMOSA_1640	LMOf2365_2207	Oxidoreductase, short-chain dehydrogenase/reductase	2.07E-03	1.76	3.38
LMOSA_1660	LMOf2365_2209	Membrane protein	4.92E-04	1.55	2.94
LMOSA_1690	LMOf2365_2212	Peptidoglycan binding protein	6.78E-05	1.96	3.89
LMOSA_1710	LMOf2365_2214	Sortase, SrtB family	1.24E-13	6.53	92.09
LMOSA_1720	LMOf2365_2215	ATP-binding protein	1.36E-17	5.12	34.81
LMOSA_1730	LMOf2365_2216	Permease protein	3.27E-16	5.38	41.72
LMOSA_1740	LMOf2365_2217	Periplasmic binding protein	3.62E-21	5.70	51.93
LMOSA_1750	LMOf2365_2218	Iron Transport-associated domain family	1.45E-15	6.40	84.45
LMOSA_1760	LMOf2365_2219	Iron Transport-associated domain family	4.02E-13	6.43	86.21
LMOSA_1930	LMOf2365_2237	hypothetical protein	3.10E-03	1.74	3.34
LMOSA_1940	LMOf2365_2238	2,3-bisphosphoglycerate-dependent phosphoglycerate mutase	2.98E-08	2.25	4.75
LMOSA_2020	LMOf2365_2246	hypothetical protein	1.53E-13	5.03	32.66
LMOSA_2110	LMOf2365_2254	hypothetical protein	1.68E-04	1.51	2.84
LMOSA_2220	NA	Arsenical pump-driving ATPase	4.57E-03	1.62	3.08
LMOSA_2230	NA	Arsenical resistance repressor ArsR	7.05E-05	2.88	7.35
LMOSA_2240	NA	Trans-acting repressor ArsD	9.17E-05	2.16	4.46
LMOSA_2540	LMOf2365_2263	Arsenate reductase	3.15E-20	5.41	42.48
LMOSA_2550	LMOf2365_2264	Cation efflux family protein	2.41E-10	2.94	7.70
LMOSA_2630	LMOf2365_2272	hypothetical protein	4.28E-04	1.87	3.66
LMOSA_2780	LMOf2365_2287	Guanine/hypoxanthine permease pbuO	2.31E-05	1.80	3.48
LMOSA_2801	LMOf2365_2290	hypothetical protein	2.64E-11	4.89	29.56
LMOSA_2820	LMOf2365_2293	Acetyltransferase	8.22E-04	1.98	3.93
LMOSA_2870	LMOf2365_2298	hypothetical protein	1.90E-03	1.88	3.68
LMOSA_2900	LMOf2365_2301	ATP-dependent helicase/deoxyribonuclease	2.34E-04	1.56	2.96
LMOSA_2910	LMOf2365_2302	YhzC protein	1.81E-14	5.35	40.71
LMOSA_2970	LMOf2365_2308	Aminopeptidase C	3.03E-08	2.39	5.25
LMOSA_2980	LMOf2365_2310	Pseudouridine-5'-phosphate glycosidase	1.67E-07	1.89	3.72
LMOSA_2990	LMOf2365_2311	PfkB family carbohydrate kinase	3.47E-10	2.40	5.27
LMOSA_3010	LMOf2365_2313	Putative monooxygenase moxC	3.57E-29	5.66	50.43
LMOSA_3020	LMOf2365_2315	Bacterial luciferase protein	3.57E-29	6.06	66.73
LMOSA_3030	LMOf2365_2316	L-cystine import ATP-binding protein TcyN	2.31E-28	5.78	54.92
LMOSA_3040	LMOf2365_2317	L-cystine transport system permease protein tcyM	3.63E-28	5.57	47.46
LMOSA_3050	LMOf2365_2318	L-cystine transport system permease	1.61E-24	5.83	57.07

		protein tcyL			
LMOSA_3060	LMOf2365_2319	L-cystine-binding protein tcyK	2.10E-21	5.79	55.34
LMOSA_3070	LMOf2365_2320	hypothetical N-acetyltransferase YtmI	3.62E-21	5.75	53.86
LMOSA_3080	LMOf2365_2321	FMN reductase, NADPH-dependent	5.84E-15	4.28	19.45
LMOSA_3150	LMOf2365_2327	Membrane protein	2.30E-03	1.80	3.49
LMOSA_3350	NA	hypothetical protein	1.46E-02	1.50	2.83
LMOSA_3370	NA	Class I glutamine amidotransferase	1.38E-04	2.60	6.07
LMOSA_3380	NA	Putative ATPase-like protein	7.85E-05	2.72	6.58
LMOSA_3390	NA	Putative argininosuccinate-like protein	7.59E-03	1.56	2.95
LMOSA_3410	NA	Peptidase U32	7.47E-03	1.56	2.95
LMOSA_3420	NA	Solute-binding family 5 protein	5.36E-03	1.55	2.93
LMOSA_3430	NA	Putative solute-binding protein	6.07E-03	1.86	3.63
LMOSA_3550	LMOf2365_2360	Membrane protein	9.78E-06	1.79	3.45
LMOSA_3600	LMOf2365_2365	NAD-dependent epimerase/dehydratase	4.95E-05	2.11	4.31
LMOSA_3740	LMOf2365_2381	hypothetical protein	5.54E-03	1.55	2.93
LMOSA_3990	LMOf2365_2405	Glutamate decarboxylase	1.30E-20	5.02	32.40
LMOSA_4040	LMOf2365_2411	hypothetical protein	1.21E-04	1.81	3.51
LMOSA_4050	LMOf2365_2412	Transcription activator effector binding	1.95E-03	1.59	3.01
LMOSA_4090	LMOf2365_2416	Leucine rich repeat protein	7.36E-03	1.89	3.71
LMOSA_4200	LMOf2365_2427	hypothetical protein	3.26E-08	4.74	26.71
LMOSA_4360	LMOf2365_2443	Leucine rich repeat protein	3.17E-05	2.78	6.85
LMOSA_4500	LMOf2365_2457	Integral membrane protein	9.30E-06	2.51	5.68
LMOSA_4510	LMOf2365_2458	Phage shock protein PspC	3.12E-06	1.79	3.46
LMOSA_4770	LMOf2365_2484	Sigma 54 modulation protein	5.48E-08	2.95	7.73
LMOSA_4880	LMOf2365_2495	LysM domain protein	1.33E-04	1.46	2.74
LMOSA_4890	LMOf2365_2496	Single-strand binding protein	5.24E-03	1.50	2.83
LMOSA_5360	LMOf2365_2543	Membrane protein	8.34E-10	3.20	9.18
LMOSA_5370	LMOf2365_2544	Nicotinamidase	1.31E-10	2.61	6.10
LMOSA_5380	LMOf2365_2545	Riboflavin biosynthesis protein	2.72E-11	2.79	6.92
LMOSA_5390	LMOf2365_2546	Zinc-binding alcohol dehydrogenase protein	1.67E-08	2.23	4.69
LMOSA_5690	LMOf2365_2575	MgtC family protein	7.76E-04	1.77	3.40
LMOSA_6110	LMOf2365_2617	hypothetical protein	4.66E-06	1.82	3.54
LMOSA_6120	LMOf2365_2618	hypothetical protein	9.31E-08	2.09	4.26
LMOSA_6130	LMOf2365_2619	Creatinine amidohydrolase	7.97E-07	1.99	3.98
LMOSA_6140	LMOf2365_2620	Aryldialkylphosphatase	3.48E-08	2.27	4.82
LMOSA_6150	LMOf2365_2621	Membrane protein	1.50E-06	2.14	4.40
LMOSA_6160	LMOf2365_2622	Phosphotransferase system	1.46E-03	2.05	4.14
LMOSA_6170	LMOf2365_2623	PTS system IIA	2.25E-04	1.77	3.41
LMOSA_6250	LMOf2365_2638	Cell wall surface anchor	1.32E-14	6.80	111.34
LMOSA_6260	LMOf2365_2639	Ribulose-phosphate 3-epimerase	4.17E-07	2.76	6.78

LMOSA_6270	LMOf2365_2640	Tkt	7.94E-11	3.22	9.29
LMOSA_6280	LMOf2365_2641	Ribulose-phosphate 3-epimerase	7.09E-14	3.27	9.63
LMOSA_6290	LMOf2365_2642	Sugar-phosphate isomerase	2.62E-08	3.05	8.31
LMOSA_6300	LMOf2365_2643	L-iditol 2-dehydrogenase	4.31E-14	3.95	15.49
LMOSA_6310	LMOf2365_2644	Alcohol dehydrogenase	1.00E-12	3.59	12.01
LMOSA_6320	LMOf2365_2645	Galactitol-specific IIc component	1.17E-11	3.21	9.25
LMOSA_6330	LMOf2365_2646	Galactitol PTS, EIIB	4.37E-05	2.84	7.15
LMOSA_6340	LMOf2365_2647	PTS system, IIA component	2.70E-05	2.27	4.83
LMOSA_6350	LMOf2365_2648	PTS system IIA 2 domain protein	1.49E-05	2.06	4.16
LMOSA_6400	LMOf2365_2653	Universal stress protein	4.82E-15	4.24	18.89
LMOSA_6420	LMOf2365_2655	hypothetical protein	2.01E-06	2.11	4.32
LMOSA_6430	LMOf2365_2656	ImpB/MucB/SamB family protein	7.21E-08	1.93	3.82
LMOSA_6460	LMOf2365_2659	Sensor protein KdpD	3.17E-05	1.53	2.88
LMOSA_6480	LMOf2365_2661	ATPase, B subunit	9.42E-07	2.02	4.07
LMOSA_6500	LMOf2365_2663	PTS system, IIB component	2.01E-28	5.69	51.48
LMOSA_6510	LMOf2365_2664	PTS system, beta-glucoside-specific, IIC component	1.61E-29	5.44	43.30
LMOSA_6520	LMOf2365_2665	cellobiose phosphotransferase enzyme IIA component	3.47E-25	5.20	36.77
LMOSA_6620	LMOf2365_2674	Dihydroxyacetone kinase	5.95E-24	4.52	23.02
LMOSA_6630	LMOf2365_2675	hypothetical protein	5.05E-23	4.28	19.48
LMOSA_6640	LMOf2365_2676	Dihydroxyacetone kinase	7.61E-17	4.17	18.01
LMOSA_6740	LMOf2365_2686	hypothetical protein	1.13E-04	2.24	4.74
LMOSA_6750	LMOf2365_2687	hypothetical protein	2.33E-04	2.28	4.84
LMOSA_6760	LMOf2365_2688	PTS system, IIC component	5.87E-23	4.12	17.44
LMOSA_6970	LMOf2365_2717	HAD-superfamily hydrolase	4.17E-05	1.54	2.91
LMOSA_6980	LMOf2365_2718	Transcription regulator	5.41E-05	2.65	6.28
LMOSA_6990	LMOf2365_2719	Phosphosugar-binding protein	6.69E-03	1.60	3.04
LMOSA_7090	LMOf2365_2729	SH3 domain protein	2.64E-14	3.51	11.40
LMOSA_7100	LMOf2365_2730	Putative transaldolase	6.68E-11	2.57	5.94
LMOSA_7150	LMOf2365_2735	General stress protein 26	4.40E-13	4.77	27.34
LMOSA_7450	LMOf2365_2766	hypothetical protein	2.50E-05	3.02	8.12
LMOSA_7500	LMOf2365_2771	PTS system, beta-glucoside-specific	7.64E-03	1.85	3.62
LMOSA_7520	LMOf2365_2773	PTS system protein	1.01E-03	2.52	5.74
LMOSA_7530	LMOf2365_2774	PTS system protein	4.63E-05	1.74	3.34
LMOSA_7640	LMOf2365_2785	PTS system, IIA component	6.04E-05	1.77	3.40
LMOSA_7660	LMOf2365_2787	PTS system protein	1.83E-07	1.96	3.88
LMOSA_7670	LMOf2365_2788	Oxidoreductase	4.06E-08	2.29	4.88
LMOSA_7680	LMOf2365_2789	Putative N-acetylmannosamine-6-phosphate 2-epimerase	3.04E-06	1.88	3.69
LMOSA_7820	LMOf2365_2806	3-oxoacyl-[acyl-carrier-protein] reductase	1.95E-07	2.31	4.94
LMOSA_7830	LMOf2365_2807	Major facilitator family transporter	1.34E-22	3.61	12.19

LMOSA_7840	LMOf2365_2808	Thermostable carboxypeptidase 1	7.59E-45	6.17	72.05
LMOSA_7850	LMOf2365_2809	Quinolone resistance protein norB	1.38E-46	6.63	99.01
LMOSA_7860	LMOf2365_2810	Thermostable carboxypeptidase 1	3.27E-44	6.62	98.45
LMOSA_7950	LMOf2365_2819	hypothetical protein	8.47E-05	2.45	5.47
LMOSA_8050	LMOf2365_2829	Permease protein	9.44E-03	1.60	3.03
LMOSA_8070	LMOf2365_2831	Alpha amylase catalytic region	1.28E-03	1.54	2.92
LMOSA_8090	LMOf2365_2833	hypothetical protein	1.17E-04	2.12	4.33
LMOSA_8150	LMOf2365_2839	Rhamnulokinase	8.30E-04	2.59	6.01
LMOSA_8170	LMOf2365_2841	Transcriptional regulator	1.25E-06	3.49	11.22
LMOSA_8450	LMOf2365_0020	hypothetical protein	1.89E-04	1.84	3.57
LMOSA_8460	LMOf2365_0021	Glycoside hydrolase, family 1	2.64E-10	2.59	6.01
LMOSA_8470	LMOf2365_0022	Bacterial SH3 domain family	5.33E-24	4.40	21.11
LMOSA_8490	LMOf2365_0024	PTS system, fructose-specific	6.61E-03	2.04	4.12
LMOSA_8500	LMOf2365_0025	Phosphoenolpyruvate-dependent sugar phosphotransferase system	5.57E-05	3.31	9.91
LMOSA_8510	LMOf2365_0026	PTS system, IIC component	4.94E-03	1.86	3.64
LMOSA_8520	LMOf2365_0027	PTS system, mannose/fructose/sorbose family	2.89E-04	2.33	5.04
LMOSA_8530	LMOf2365_0028	hypothetical protein	1.36E-04	1.97	3.92
LMOSA_8700	LMOf2365_0045	Putrescine carbamoyltransferase	1.97E-09	3.77	13.65
LMOSA_8710	LMOf2365_0046	Amino acid permease family protein	4.66E-10	4.03	16.34
LMOSA_8720	LMOf2365_0047	Putative agmatine deiminase 1	4.11E-08	2.55	5.85
LMOSA_8730	LMOf2365_0048	Carbamate kinase	1.16E-07	2.43	5.38
LMOSA_8750	LMOf2365_0050	Transcriptional regulator	1.51E-03	2.05	4.13
LMOSA_9210	NA	Putative repressor protein	5.25E-03	1.86	3.63
LMOSA_9260	NA	hypothetical protein	9.59E-03	1.55	2.94
LMOSA_9270	NA	Phage antirepressor family protein	1.86E-05	2.15	4.44
LMOSA_9280	NA	Gp43	2.11E-03	1.60	3.03
LMOSA_9340	NA	Gp44	1.21E-03	1.84	3.59
LMOSA_9350	NA	Phage protein	9.37E-05	2.52	5.72
LMOSA_9380	NA	Gp35 protein	4.50E-03	1.62	3.08
LMOSA_9390	NA	Conserved phage protein	1.16E-03	2.07	4.19
LMOSA_9440	NA	Gp91	7.94E-06	3.98	15.83
LMOSA_9550	NA	Phage portal protein	7.79E-08	2.23	4.68
LMOSA_9560	NA	Protein gp4	8.44E-10	2.47	5.55
LMOSA_9570	NA	Phage protein Gp5	2.50E-08	2.98	7.91
LMOSA_9580	NA	Phage coat protein	3.41E-13	3.14	8.84
LMOSA_9590	NA	Phage protein gp8	7.20E-12	3.57	11.86
LMOSA_9600	NA	Phage protein gp9	1.06E-09	4.07	16.82
LMOSA_9610	NA	Phage protein gp10	1.19E-11	3.78	13.78
LMOSA_9620	NA	Phage protein gp11	7.56E-10	4.07	16.74
LMOSA_9630	NA	Major tail shaft protein	7.21E-14	3.64	12.49
LMOSA_9640	NA	Phage protein	4.48E-09	3.64	12.49

LMOSA_9660	NA	Phage protein gp15	5.11E-06	2.43	5.40
LMOSA_9670	NA	Tmp	8.97E-06	1.69	3.24
LMOSA_9680	NA	Phage protein gp17	7.21E-08	2.43	5.38
LMOSA_9690	NA	hypothetical protein	4.00E-08	2.42	5.34
LMOSA_9700	NA	Protein gp19	1.08E-07	2.08	4.24
LMOSA_9710	NA	hypothetical protein	9.88E-07	2.09	4.27
LMOSA_9740	NA	Gp18	8.47E-03	1.90	3.73
LMOSA_9880	LMOf2365_0106	ATP synthase F1, delta subunit	4.76E-03	1.80	3.48
LMOSA_9970	LMOf2365_0115	PTS system protein	5.84E-03	1.57	2.97
LMOSA_10050	LMOf2365_0123	Chitinase B	9.18E-13	3.63	12.39
LMOSA_10070	LMOf2365_0125	ABC transporter protein2	1.17E-05	1.66	3.15
LMOSA_10080	LMOf2365_0126	ABC transporter protein1	3.79E-06	1.78	3.44
LMOSA_10120	LMOf2365_0131	Bacteriophage gp35-type protein	3.13E-06	2.36	5.12
LMOSA_10140	LMOf2365_0133	Prophage LambdaLm01, antigen D	2.69E-03	1.61	3.05
LMOSA_10150	LMOf2365_0134	Phage transcriptional activator, antigen C	1.28E-03	1.54	2.91
LMOSA_10160	LMOf2365_0135	Antigen B	4.65E-12	2.92	7.57
LMOSA_10170	LMOf2365_0136	Antigen A	2.29E-09	2.62	6.15
LMOSA_10180	LMOf2365_0137	hypothetical protein	7.78E-16	3.31	9.90
LMOSA_10190	LMOf2365_0138	hypothetical protein	1.10E-15	3.27	9.68
LMOSA_10200	LMOf2365_0139	Membrane protein	2.78E-12	2.58	5.98
LMOSA_10210	LMOf2365_0140	hypothetical protein	1.47E-15	2.96	7.79
LMOSA_10220	LMOf2365_0141	Minor structural protein	1.11E-19	3.64	12.44
LMOSA_10230	LMOf2365_0142	hypothetical protein	4.40E-13	2.89	7.39
LMOSA_10240	LMOf2365_0143	hypothetical protein	2.10E-12	2.68	6.42
LMOSA_10250	LMOf2365_0144	hypothetical protein	1.04E-08	2.48	5.57
LMOSA_10260	LMOf2365_0145	hypothetical protein	4.96E-11	2.59	6.01
LMOSA_10270	LMOf2365_0146	hypothetical protein	3.59E-11	2.66	6.32
LMOSA_10280	LMOf2365_0147	Prophage LambdaLm01 amidase	4.67E-14	2.78	6.86
LMOSA_10330	LMOf2365_0152	Acetyltransferase, GNAT family	1.53E-09	3.53	11.58
LMOSA_10340	LMOf2365_0153	Oligopeptide ABC transporter	1.34E-03	1.58	2.99
LMOSA_10350	LMOf2365_0154	Oligopeptide ABC transporter	6.24E-07	2.49	5.63
LMOSA_10360	LMOf2365_0155	Oligopeptide ABC transporter	4.32E-07	2.52	5.75
LMOSA_10420	LMOf2365_0160	hypothetical protein	6.45E-03	1.99	3.97
LMOSA_10510	LMOf2365_0170	Putative zinc transport system	1.31E-02	1.77	3.41
LMOSA_10520	LMOf2365_0171	Cyclic nucleotide-binding protein	3.09E-03	2.06	4.18
LMOSA_10650	LMOf2365_0184	hypothetical protein	9.31E-11	3.68	12.80
LMOSA_10660	LMOf2365_0185	hypothetical protein	3.33E-05	1.69	3.23
LMOSA_10710	LMOf2365_0189	Xylose repressor protein	2.97E-03	1.81	3.51
LMOSA_10720	LMOf2365_0190	Sugar ABC transporter	9.05E-07	2.83	7.12
LMOSA_10730	LMOf2365_0191	Sugar ABC transporter	4.85E-08	3.31	9.88
LMOSA_10740	LMOf2365_0192	Sugar ABC transporter	2.64E-11	3.64	12.46
LMOSA_10750	LMOf2365_0193	Glycosyl hydrolase	4.57E-14	3.19	9.10

LMOSA_10760	LMOf2365_0194	Alpha-glucosidase	3.49E-12	2.82	7.07
LMOSA_10930	LMOf2365_0211	Listeriolysin regulatory protein	1.64E-05	1.97	3.91
LMOSA_10950	LMOf2365_0213	Listeriolysin O	1.13E-05	1.69	3.22
LMOSA_10960	LMOf2365_0214	Zinc metalloproteinase	1.69E-03	1.66	3.16
LMOSA_10980	LMOf2365_0216	Phospholipase C	2.12E-04	2.16	4.48
LMOSA_11170	LMOf2365_0235	hypothetical protein	1.17E-05	2.71	6.52
LMOSA_11270	LMOf2365_0241	Transcriptional regulator CtsR	2.92E-03	1.45	2.74
LMOSA_11520	LMOf2365_0267	Sugar ABC transporter	8.72E-03	1.89	3.72
LMOSA_11560	LMOf2365_0271	Sucrose phosphorylase	5.54E-03	2.00	4.01
LMOSA_11600	LMOf2365_0277	Glycoside hydrolase	3.60E-10	2.72	6.57
LMOSA_11620	LMOf2365_0279	ABC transporter	3.12E-03	2.48	5.57
LMOSA_11650	LMOf2365_0281	Internalin D	2.35E-10	3.25	9.49
LMOSA_11660	LMOf2365_0282	Internalin D	4.05E-09	2.41	5.32
LMOSA_11670	LMOf2365_0283	Internalin E	3.85E-04	1.62	3.08
LMOSA_11680	LMOf2365_0284	Peptidase, M20/M25/M40 family	2.62E-05	2.07	4.20
LMOSA_11730	LMOf2365_0289	Leucine rich repeat domain protein	1.36E-04	1.84	3.59
LMOSA_11740	LMOf2365_0290	hypothetical protein	1.23E-13	2.48	5.58
LMOSA_11750	LMOf2365_0291	6-phospho-beta-glucosidase	2.64E-10	2.18	4.54
LMOSA_11820	LMOf2365_0298	ABC transporter related protein	4.59E-05	1.81	3.51
LMOSA_12180	LMOf2365_0333	TENA/THI-4 family protein	3.07E-03	1.90	3.74
LMOSA_12270	LMOf2365_0339	Membrane protein	9.90E-12	2.75	6.73
LMOSA_12310	LMOf2365_0343	Transcriptional activator	6.56E-04	1.72	3.28
LMOSA_12380	LMOf2365_0351	hypothetical protein	3.22E-08	3.81	13.98
LMOSA_12570	LMOf2365_0374	Internalin	4.83E-07	2.07	4.19
LMOSA_12620	LMOf2365_0378	Pts system fructose-specific eiibbc component	9.08E-04	1.58	2.98
LMOSA_12710	LMOf2365_0388	6-phospho-beta-glucosidase gmuD	4.76E-04	1.51	2.85
LMOSA_12730	LMOf2365_0390	PTS system, beta-glucoside-specific	4.91E-03	1.45	2.73
LMOSA_12750	LMOf2365_0392	HTH domain family	2.39E-05	1.54	2.92
LMOSA_12780	LMOf2365_0395	Methylmalonate semialdehyde dehydrogenase	5.84E-09	2.42	5.37
LMOSA_12790	LMOf2365_0396	5-deoxy-glucuronate isomerase	3.38E-06	2.22	4.67
LMOSA_12800	LMOf2365_0397	5-dehydro-2-deoxygluconokinase	2.93E-04	1.59	3.01
LMOSA_12970	LMOf2365_0417	ABC transporter, permease protein	6.65E-04	2.11	4.32
LMOSA_13020	LMOf2365_0420	PTS family fructose/mannitol (Fru) porter	2.35E-04	1.76	3.38
LMOSA_13090	LMOf2365_0427	hypothetical protein	1.20E-03	2.02	4.05
LMOSA_13190	LMOf2365_0436	hypothetical protein	6.74E-03	1.84	3.59
LMOSA_13430	LMOf2365_0464	hypothetical protein	2.00E-03	2.25	4.75
LMOSA_13440	LMOf2365_0466	hypothetical protein	6.02E-05	2.91	7.51
LMOSA_13470	LMOf2365_0471	Internalin-A	1.17E-26	5.77	54.44
LMOSA_13480	LMOf2365_0472	Internalin B	2.90E-14	3.35	10.20
LMOSA_13500	LMOf2365_0474	hypothetical nucleoside-diphosphate-	3.48E-11	2.23	4.69

		sugar epimerase			
LMOSA_13510	LMOf2365_0475	hypothetical protein	9.71E-08	3.81	14.05
LMOSA_13520	LMOf2365_0476	hypothetical protein	2.62E-17	4.26	19.20
LMOSA_13600	LMOf2365_0484	hypothetical protein	8.30E-04	2.08	4.21
LMOSA_13650	LMOf2365_0489	permease family protein	3.86E-11	2.52	5.72
LMOSA_13660	LMOf2365_0490	hypothetical protein	8.23E-09	2.11	4.31
LMOSA_13670	LMOf2365_0491	Hydantoinase/oxoprolinase	7.68E-09	1.98	3.95
LMOSA_13680	LMOf2365_0493	hypothetical protein	7.45E-05	2.33	5.02
LMOSA_13690	LMOf2365_0493	hypothetical protein	6.78E-06	3.44	10.89
LMOSA_13870	LMOf2365_0511	Heme-degrading monooxygenase isdG	5.84E-06	2.50	5.67
LMOSA_13900	LMOf2365_0514	hypothetical protein	5.00E-03	1.74	3.35
LMOSA_14040	LMOf2365_0528	hypothetical protein	1.54E-03	1.79	3.45
LMOSA_14060	LMOf2365_0530	PTS system, IIA 2 domain protein	1.37E-04	2.35	5.09
LMOSA_14070	LMOf2365_0531	SIS domain protein	6.63E-05	2.23	4.68
LMOSA_14080	LMOf2365_0532	PTS system, IIA component	9.58E-04	1.89	3.70
LMOSA_14090	LMOf2365_0533	hypothetical protein	1.53E-03	2.02	4.06
LMOSA_14130	LMOf2365_0537	PTS system, galactitol-specific	1.18E-04	1.65	3.15
LMOSA_14150	LMOf2365_0539	Putative lipoprotein	3.72E-05	3.06	8.33
LMOSA_14200	LMOf2365_0544	Universal stress protein family	3.40E-16	6.20	73.47
LMOSA_14210	LMOf2365_0545	CapA domain protein	2.07E-06	2.44	5.44
LMOSA_14260	LMOf2365_0550	Glycoside hydrolase	1.13E-05	1.53	2.89
LMOSA_14400	LMOf2365_0565	Glycoside hydrolase, family 4 protein	5.90E-10	3.18	9.04
LMOSA_14430	LMOf2365_0568	Tagatose 1,6-diphosphate aldolase 2	7.48E-10	2.50	5.67
LMOSA_14450	LMOf2365_0570	ABC transporter, substrate-binding lipoprotein	5.06E-10	2.63	6.18
LMOSA_14530	LMOf2365_0578	Leucine rich repeat domain protein	1.71E-06	1.94	3.85
LMOSA_14540	LMOf2365_0579	Pepdidoglycan bound protein	3.15E-04	1.77	3.41
LMOSA_14560	LMOf2365_0581	Cell surface protein	1.51E-11	2.85	7.22
LMOSA_14650	LMOf2365_0590	Phosphoribosyl-ATP pyrophosphatase	7.86E-09	2.23	4.69
LMOSA_14660	LMOf2365_0591	Phosphoribosyl-AMP cyclohydrolase	3.18E-06	1.68	3.21
LMOSA_14670	LMOf2365_0592	Imidazole glycerol phosphate synthase	5.70E-06	1.59	3.01
LMOSA_14680	LMOf2365_0593	isomerase	1.96E-05	1.53	2.89
LMOSA_14690	LMOf2365_0594	Imidazole glycerol phosphate synthase subunit hisH	1.06E-06	1.75	3.37
LMOSA_14700	LMOf2365_0595	Imidazoleglycerol-phosphate dehydratase	1.26E-07	1.87	3.65
LMOSA_14710	LMOf2365_0596	Histidinol dehydrogenase	6.09E-08	1.84	3.57
LMOSA_14720	LMOf2365_0597	ATP phosphoribosyltransferase	1.45E-06	1.82	3.53
LMOSA_14770	LMOf2365_0603	6-phospho-beta-glucosidase gmuD	4.52E-49	5.90	59.63
LMOSA_14780	LMOf2365_0604	HTH-type transcriptional regulator gmuR	1.12E-19	3.15	8.85
LMOSA_14790	LMOf2365_0605	Secreted MUCin-binding domain protein	4.71E-26	5.44	43.54

LMOSA_14870	LMOf2365_0613	Membrane protein YubA	2.03E-05	2.35	5.11
LMOSA_14990	LMOf2365_0625	Membrane protein	7.75E-17	3.27	9.66
LMOSA_15050	LMOf2365_0631	Acetyltransferase	1.59E-05	2.19	4.57
LMOSA_15130	LMOf2365_0639	LRR-and LPXTG-motif protein	4.59E-16	3.97	15.64
LMOSA_15320	LMOf2365_0659	PTS system IIA 2 domain protein	5.32E-04	2.57	5.92
LMOSA_15360	LMOf2365_0663	hypothetical protein	2.34E-04	1.85	3.60
LMOSA_15510	LMOf2365_0679	Magnesium transporter	1.63E-04	2.10	4.29
LMOSA_15570	LMOf2365_0685	hypothetical protein	6.51E-03	1.65	3.15
LMOSA_15580	LMOf2365_0686	Serine/threonine protein phosphatase	9.37E-05	1.85	3.60
LMOSA_15630	LMOf2365_0693	Cell wall surface anchor family protein	2.59E-04	2.09	4.25
LMOSA_15640	LMOf2365_0693.1	hypothetical protein	1.09E-11	5.29	39.22
LMOSA_15650	LMOf2365_0694	Cell wall surface anchor family protein	1.80E-12	3.23	9.41
LMOSA_15660	LMOf2365_0695	Carboxymuconolactone decarboxylase	1.26E-04	1.82	3.52
LMOSA_15750	LMOf2365_0704	Oxidoreductase	1.33E-10	2.81	6.99
LMOSA_15760	LMOf2365_0705	hypothetical protein	2.14E-06	2.67	6.37
LMOSA_16270	LMOf2365_0758	Pyruvate oxidase	1.26E-13	3.14	8.80
LMOSA_16670	LMOf2365_0798	Mannose permease IID component	1.64E-27	4.19	18.32
LMOSA_16680	LMOf2365_0799	Mannose permease IIC component	1.06E-26	4.24	18.87
LMOSA_16690	LMOf2365_0800	PTS system mannose-specific	5.87E-23	4.44	21.70
LMOSA_16700	LMOf2365_0801	Pts system mannose-specific	5.24E-25	4.44	21.75
LMOSA_16790	LMOf2365_0808	Lipoprotein	4.36E-03	1.52	2.87
LMOSA_16820	LMOf2365_0811	YwnB	1.38E-04	1.74	3.34
LMOSA_17060	LMOf2365_0834	hypothetical protein	2.32E-04	2.70	6.52
LMOSA_17140	LMOf2365_0842	putative 2,5-didehydrogluconate reductase	6.78E-06	1.66	3.15
LMOSA_17230	LMOf2365_0851	hypothetical protein	4.00E-08	2.43	5.38
LMOSA_17240	LMOf2365_0852	Cell wall surface anchor family protein	2.67E-09	2.79	6.94
LMOSA_17520	LMOf2365_0878	Sugar ABC transporter permease protein	2.87E-03	1.52	2.86
LMOSA_17640	LMOf2365_0892	PTS system IIA 2 domain protein	1.12E-02	1.55	2.94
LMOSA_17710	LMOf2365_0899	Peptidoglycan-bound protein	4.05E-09	2.94	7.67
LMOSA_17851	LMOf2365_0913	hypothetical protein	2.04E-03	1.45	2.74
LMOSA_17930	LMOf2365_0922	Oligo-beta-mannoside permease	5.45E-06	1.97	3.92
LMOSA_18030	LMOf2365_0933	Bacterial transcription activator	1.45E-05	2.18	4.52
LMOSA_18050	LMOf2365_0935	Succinate-semialdehyde dehydrogenase	1.24E-16	5.02	32.36
LMOSA_18160	LMOf2365_0946	ABC transporter, permease protein	3.76E-05	1.51	2.85
LMOSA_18240	LMOf2365_0954	tRNA-Gly	2.59E-09	2.20	4.60
LMOSA_18330	LMOf2365_0963	DNA protection during starvation protein	1.21E-02	1.66	3.15
LMOSA_18460	LMOf2365_0976	N-acetylglucosamine 6-phosphate deacetylase	1.79E-05	1.59	3.00
LMOSA_18610	LMOf2365_0991	Protein DltD	1.65E-05	2.05	4.14
LMOSA_18620	LMOf2365_0992	D-alanine--poly(phosphoribitol) ligase	1.28E-04	2.18	4.54

LMOSA_18630	LMOf2365_0993	Membrane bound O-acyl transferase MBOAT family protein	2.24E-05	2.15	4.45
LMOSA_18640	LMOf2365_0994	D-alanine--poly(phosphoribitol) ligase	7.12E-05	1.85	3.61
LMOSA_18650	LMOf2365_0995	hypothetical protein	3.31E-06	2.84	7.14
LMOSA_18660	LMOf2365_0996	Ribose-5-phosphate isomerase A	5.69E-04	1.61	3.06
LMOSA_18750	LMOf2365_1005	LytTr DNA-binding domain family	6.92E-04	2.21	4.64
LMOSA_18850	LMOf2365_1015	hypothetical protein	1.35E-14	5.20	36.85
LMOSA_18860	LMOf2365_1016	Acyltransferases family protein	7.93E-10	2.86	7.28
LMOSA_18880	LMOf2365_1018	ATP-dependent Clp protease	1.08E-04	2.51	5.70
LMOSA_18980	LMOf2365_1028	hypothetical protein	1.26E-03	1.56	2.96
LMOSA_19370	LMOf2365_1066	Molybdopterin biosynthesis	1.64E-03	1.70	3.25
LMOSA_19690	LMOf2365_1098	hypothetical protein	7.66E-03	1.57	2.96
LMOSA_19760	LMOf2365_1105	Glycerol-3-phosphate cytidylyltransferase	3.26E-03	1.57	2.97
LMOSA_19950	LMOf2365_1135	Acetyltransferase, GNAT family	4.75E-03	1.88	3.68
LMOSA_19970	LMOf2365_1137	ATP-binding/permease protein	1.95E-16	3.51	11.42
LMOSA_19980	LMOf2365_1138	ATP-binding/permease protein	5.67E-25	4.29	19.57
LMOSA_20050	LMOf2365_1145	hypothetical protein	3.99E-04	2.71	6.54
LMOSA_20070	LMOf2365_1147	Methyltransferase domain protein	7.95E-05	2.45	5.46
LMOSA_20090	LMOf2365_1149	PduS type ferredoxin (Cobalamin reductase)	5.01E-09	3.06	8.37
LMOSA_20100	LMOf2365_1150	Microcompartments protein	4.29E-13	3.56	11.76
LMOSA_20110	LMOf2365_1151	Propanediol utilization protein PduU	1.98E-05	1.64	3.11
LMOSA_20120	LMOf2365_1152	PduV protein	2.53E-06	2.03	4.09
LMOSA_20130	LMOf2365_1153	hypothetical protein	2.41E-07	2.04	4.12
LMOSA_20140	LMOf2365_1154	Cobinamide kinase/cobinamide phosphate	7.20E-12	2.55	5.85
LMOSA_20150	LMOf2365_1155	Cobalamin synthase	6.16E-06	1.76	3.39
LMOSA_20160	LMOf2365_1156	Alpha-ribazole-5'-phosphate phosphatase	3.32E-06	1.71	3.26
LMOSA_20170	LMOf2365_1157	Regulatory protein	7.54E-05	1.84	3.58
LMOSA_20180	LMOf2365_1159	Propanediol utilization protein	1.94E-03	2.29	4.91
LMOSA_20190	LMOf2365_1160	Propanediol utilization protein PduB	2.79E-04	2.00	3.99
LMOSA_20200	LMOf2365_1161	Glycerol dehydratase large subunit	1.39E-04	1.91	3.77
LMOSA_20210	LMOf2365_1162	Propanediol utilization protein	1.40E-03	1.91	3.77
LMOSA_20220	LMOf2365_1163	Propanediol dehydratase small subunit	5.16E-04	2.15	4.43
LMOSA_20230	LMOf2365_1164	PduG protein	3.10E-06	2.00	4.01
LMOSA_20240	LMOf2365_1165	PduH protein	2.10E-04	2.30	4.93
LMOSA_20260	LMOf2365_1167	Propanediol utilization protein	6.08E-03	1.74	3.35
LMOSA_20270	LMOf2365_1168	Propanediol pathway protein PduL	3.61E-03	1.71	3.28
LMOSA_20280	LMOf2365_1169	Ethanolamine pathway protein	2.46E-04	1.65	3.15
LMOSA_20310	LMOf2365_1172	ATP:cob	5.41E-05	1.99	3.96
LMOSA_20320	LMOf2365_1173	CoA-dependent propionaldehyde	2.56E-05	1.88	3.69

		dehydrogenase			
LMOSA_20330	LMOf2365_1174	Propanol dehydrogenase	1.65E-03	1.61	3.04
LMOSA_20360	LMOf2365_1177	L-threonine-O-3-phosphate decarboxylase	2.42E-03	1.46	2.76
LMOSA_20380	LMOf2365_1181	Alcohol dehydrogenase	7.83E-13	4.50	22.61
LMOSA_20390	LMOf2365_1182	Response regulator	2.35E-08	3.53	11.53
LMOSA_20400	LMOf2365_1183	Sensor histidine kinase	3.22E-12	3.94	15.30
LMOSA_20410	LMOf2365_1184	Ethanolamine utilization EutA	1.22E-18	5.69	51.79
LMOSA_20420	LMOf2365_1185	Ethanolamine ammonia-lyase	8.28E-14	3.92	15.15
LMOSA_20430	LMOf2365_1186	Ethanolamine ammonia-lyase	1.53E-14	3.97	15.65
LMOSA_20440	LMOf2365_1187	Microcompartments protein	6.25E-13	3.85	14.39
LMOSA_20450	LMOf2365_1188	Microcompartment protein family	3.62E-17	4.11	17.30
LMOSA_20460	LMOf2365_1189	Acetaldehyde dehydrogenase	1.61E-12	3.87	14.58
LMOSA_20470	LMOf2365_1190	Propanediol utilization protein	6.25E-11	3.56	11.80
LMOSA_20480	LMOf2365_1191	Ethanolamine pathway cobalamin adenosyltransferase	3.33E-15	3.69	12.89
LMOSA_20490	LMOf2365_1192	Propanediol utilization protein	4.21E-13	3.60	12.09
LMOSA_20500	LMOf2365_1193	Ethanolamine utilization protein	1.41E-19	4.06	16.63
LMOSA_20510	LMOf2365_1194	Carbon dioxide concentrating protein CcmL	5.03E-10	3.23	9.37
LMOSA_20520	LMOf2365_1195	Ethanolamine/propanediol pathway protein	1.67E-09	3.14	8.83
LMOSA_20530	LMOf2365_1196	Ethanolamine pathway protein	5.73E-12	3.49	11.25
LMOSA_20540	LMOf2365_1197	Ethanolamine pathway protein EutQ	1.99E-22	4.25	19.01
LMOSA_20550	LMOf2365_1198	Transcriptional regulator, AraC family	2.15E-08	3.07	8.41
LMOSA_20560	LMOf2365_1199	Membrane protein	5.91E-09	3.02	8.12
LMOSA_20570	LMOf2365_1200	Cobyrinic acid A,C-diamide synthase	4.75E-06	1.84	3.57
LMOSA_20580	LMOf2365_1201	Cobalamin biosynthesis protein CobD	2.99E-05	1.49	2.80
LMOSA_20590	LMOf2365_1202	Precorrin-8X methylmutase / precorrin isomerase	5.93E-08	1.97	3.93
LMOSA_20600	LMOf2365_1203	Putative cobalt-precorrin-6A synthase	2.25E-04	1.51	2.84
LMOSA_20610	LMOf2365_1204	cobalt-precorrin-6Y C(5)-methyltransferase	2.55E-06	1.68	3.19
LMOSA_20620	LMOf2365_1205	Precorrin-6B methylase 2	1.66E-06	1.74	3.33
LMOSA_20720	LMOf2365_1215	Cobalt ABC transporter	1.60E-04	1.47	2.76
LMOSA_21080	NA	Integrase	4.36E-04	1.82	3.53
LMOSA_21090	NA	Gp25 protein	1.68E-03	1.77	3.41
LMOSA_21180	NA	D12 class N6 adenine-specific DNA methyltransferase	2.00E-04	1.72	3.30
LMOSA_21280	NA	Conserved phage-related protein	2.70E-05	2.36	5.12
LMOSA_21290	NA	Phage-related protein	3.35E-05	2.71	6.56
LMOSA_21300	NA	Phage nucleotide-binding protein	1.76E-05	2.31	4.94
LMOSA_21310	NA	Phage DEAH-family helicase	1.60E-06	1.86	3.64
LMOSA_21320	NA	Gp59	2.73E-06	3.06	8.36

LMOSA_21340	NA	Conserved phage-related protein	5.52E-04	2.17	4.51
LMOSA_21350	NA	Gp62	3.18E-04	2.76	6.76
LMOSA_21400	NA	hypothetical protein	3.02E-05	2.62	6.13
LMOSA_21410	NA	Clp protease	7.00E-06	1.86	3.62
LMOSA_21430	NA	Gp6 protein	2.33E-08	3.30	9.88
LMOSA_21431	NA	hypothetical protein	1.07E-05	3.11	8.61
LMOSA_21440	NA	Gp8 protein	1.60E-06	3.20	9.17
LMOSA_21450	NA	Gp9 protein	3.77E-09	4.03	16.36
LMOSA_21460	NA	Major tail protein B	3.35E-05	2.06	4.18
LMOSA_21500	NA	Gp13 protein	1.09E-09	3.88	14.73
LMOSA_21510	NA	Phage-related structural protein	7.55E-07	2.10	4.28
LMOSA_21530	NA	hypothetical protein	5.60E-03	1.93	3.81
LMOSA_21650	LMOf2365_1255	hypothetical protein	7.90E-27	5.02	32.36
LMOSA_21670	LMOf2365_1257	3-demethylubiquinone-9 3-methyltransferase protein	1.81E-06	1.86	3.62
LMOSA_21750	LMOf2365_1266	hypothetical protein	2.54E-04	1.94	3.83
LMOSA_21810	LMOf2365_1273	MutT/nudix family protein	1.73E-05	3.68	12.79
LMOSA_21820	LMOf2365_1274	hypothetical protein	5.60E-03	2.13	4.39
LMOSA_22160	LMOf2365_1310	Aerobic glycerol-3-phosphate dehydrogenase	1.34E-26	3.91	15.05
LMOSA_22240	LMOf2365_1318	Arsenic efflux pump	9.35E-12	2.50	5.64
LMOSA_22640	LMOf2365_1359	ComG operon protein	5.91E-06	2.96	7.76
LMOSA_22650	LMOf2365_1361	ComG operon protein	2.29E-08	3.39	10.47
LMOSA_23020	LMOf2365_1400	Acylphosphatase	2.76E-05	2.25	4.77
LMOSA_23440	LMOf2365_1442	hypothetical protein	1.76E-03	1.46	2.75
LMOSA_23460	LMOf2365_1444	Permease	1.18E-06	1.61	3.06
LMOSA_23470	LMOf2365_1445	ABC transport protein	4.27E-09	2.50	5.65
LMOSA_23480	LMOf2365_1446	Permease	2.97E-09	2.16	4.46
LMOSA_23490	LMOf2365_1447	Choline transport ATP-binding protein	6.53E-09	2.11	4.32
LMOSA_23540	LMOf2365_1452	Pyridine nucleotide-disulfide oxidoreductase family protein	5.37E-08	1.92	3.78
LMOSA_24230	LMOf2365_1520	hypothetical protein	1.14E-04	1.59	3.00
LMOSA_24600	LMOf2365_1557	Glycerol kinase	3.71E-10	2.30	4.92
LMOSA_24610	LMOf2365_1558	Glycerol uptake facilitator protein	1.95E-16	3.05	8.26
LMOSA_25010	LMOf2365_1601	Alanine dehydrogenase 2	1.37E-07	1.90	3.74
LMOSA_25020	LMOf2365_1602	Universal stress protein family	1.98E-14	4.34	20.30
LMOSA_25750	LMOf2365_1676	hypothetical protein	1.02E-02	1.78	3.43
LMOSA_25790	LMOf2365_1681	sucrose-specific PTS system IIBC component	8.69E-06	1.91	3.75
LMOSA_25800	LMOf2365_1682	N-acetylmuramic acid 6-phosphate etherase	8.67E-03	1.59	3.02
LMOSA_26170	LMOf2365_1718	NAD-dependent epimerase/dehydratase	1.70E-05	2.09	4.26
LMOSA_26380	LMOf2365_1740	Methyltransferase protein	7.01E-06	3.49	11.27

LMOSA_26400	LMOf2365_1742	Outer surface protein	9.31E-11	2.37	5.18
LMOSA_26410	LMOf2365_1743	PTS system protein	5.71E-06	1.74	3.33
LMOSA_26420	LMOf2365_1744	PTS system, IIB component	1.10E-06	1.97	3.91
LMOSA_26480	LMOf2365_1750	Oxidoreductase	2.20E-05	1.46	2.76
LMOSA_26520	LMOf2365_1754	ABC transporter, substrate-binding protein	4.66E-10	2.74	6.68
LMOSA_26530	LMOf2365_1755	Binding-protein-dependent transport systems inner membrane component	8.27E-10	2.63	6.19
LMOSA_26540	LMOf2365_1756	Binding-protein-dependent transport systems inner membrane component	7.02E-08	2.23	4.68
LMOSA_26550	LMOf2365_1757	GltB	1.58E-07	1.93	3.81
LMOSA_26560	LMOf2365_1758	Glutamate synthase	1.32E-07	2.05	4.15
LMOSA_26590	LMOf2365_1762	Amino acid binding protein	4.30E-07	1.83	3.55
LMOSA_26850	LMOf2365_1789	Phosphoribosylamine--glycine ligase	3.98E-07	1.84	3.59
LMOSA_26860	LMOf2365_1790	Bifunctional purine biosynthesis protein purH	1.48E-07	1.86	3.64
LMOSA_26870	LMOf2365_1791	Phosphoribosylglycinamide formyltransferase	7.52E-07	1.88	3.69
LMOSA_26880	LMOf2365_1792	Phosphoribosylformylglycinamidine cyclo-ligase	4.74E-07	1.86	3.64
LMOSA_26890	LMOf2365_1793	Amidophosphoribosyltransferase	2.89E-08	2.04	4.11
LMOSA_26900	LMOf2365_1794	Phosphoribosylformylglycinamidine synthase 2	1.19E-07	1.96	3.90
LMOSA_26910	LMOf2365_1795	Phosphoribosylformylglycinamidine synthase	1.63E-05	1.70	3.25
LMOSA_26920	LMOf2365_1796	Phosphoribosylformylglycinamidine synthase	9.71E-08	2.63	6.21
LMOSA_27220	LMOf2365_1826	Putative peptidoglycan bound protein	1.50E-04	2.30	4.94
LMOSA_27530	LMOf2365_1858	Dehydrogenase	3.18E-08	2.67	6.38
LMOSA_27700	LMOf2365_1875	Substrate-binding lipoprotein	5.97E-05	2.19	4.55
LMOSA_27710	LMOf2365_1876	Manganese transport system membrane protein mntC	1.14E-04	2.10	4.29
LMOSA_27750	LMOf2365_1880	mercuric ion binding protein	6.77E-04	2.04	4.11
LMOSA_27760	LMOf2365_1881	Copper-translocating P-type ATPase	8.45E-04	1.76	3.39
LMOSA_27770	LMOf2365_1882	YvgZ	2.39E-04	2.23	4.68
LMOSA_27910	LMOf2365_1897	Glyoxalase/bleomycin resistance protein/dioxygenase	7.46E-03	1.59	3.01
LMOSA_28050	LMOf2365_1911	30S ribosomal protein S14	5.20E-04	1.55	2.94
LMOSA_28800	LMOf2365_1987	Transport system permease protein PhuB	5.16E-06	1.98	3.96
LMOSA_28810	LMOf2365_1988	Transport system permease protein PhuG	4.08E-06	2.05	4.15
LMOSA_28820	LMOf2365_1989	Iron compound ABC transporter	6.93E-09	2.71	6.54
LMOSA_28830	LMOf2365_1990	ATP-binding protein fhuC	2.64E-05	1.70	3.26
LMOSA_28840	LMOf2365_1991	Ferredoxin--NADP reductase 1	1.12E-09	2.24	4.72

LMOSA_28850	LMOf2365_1992	Transcriptional regulator	5.34E-05	1.46	2.75
LMOSA_28860	LMOf2365_1993	Permease protein	3.61E-06	1.66	3.17
LMOSA_28870	LMOf2365_1994	ATP-binding protein	3.36E-05	1.71	3.27
LMOSA_28950	LMOf2365_2003	hypothetical protein	4.92E-03	1.52	2.86
LMOSA_29200	LMOf2365_2029	Oxidoreductase, aldo/keto reductase family	3.22E-10	2.65	6.27
LMOSA_29300	LMOf2365_2040	Alpha-mannosidase	3.32E-05	1.53	2.89

APPENDIX 2: Genes that were significantly upregulated (FDR ≤0.01; Log_2FC ≥1.50 in LM-Scott A grown on Queso Fresco at 7°C, in comparison to the similar strain grown in TSB.

Scott A Locus tag	F2365[a] Locus Tag	Gene Product	FDR[s]	Log_2FC[c]	FC[d]
LMOSA_330	LMOf2365_2077	Putative 2-dehydropantoate 2-reductase	1.43E-05	-1.69	-3.22
LMOSA_340	NA	hypothetical protein	1.36E-04	-2.93	-7.62
LMOSA_390	LMOf2365_2083	Phosphopantetheine adenylyltransferase	7.09E-05	-1.64	-3.12
LMOSA_400	LMOf2365_2084	Methyltransferase	3.48E-11	-2.47	-5.54
LMOSA_450	LMOf2365_2089	Heme A synthase	3.04E-06	-1.97	-3.92
LMOSA_490	LMOf2365_2093	Copper resistance domain protein	5.40E-05	-1.70	-3.25
LMOSA_520	LMOf2365_2096	Membrane protein	7.23E-04	-1.57	-2.97
LMOSA_650	LMOf2365_2110	ATP/GTP hydrolase	1.39E-04	-1.84	-3.59
LMOSA_670	LMOf2365_2112	Lipoprotein	1.44E-04	-1.73	-3.32
LMOSA_770	LMOf2365_2122	Argininosuccinate synthase	6.13E-06	-1.87	-3.66
LMOSA_780	LMOf2365_2123	Argininosuccinate lyase	2.51E-07	-2.11	-4.31
LMOSA_880	LMOf2365_2133	Pyridoxal biosynthesis protein PDX1.3	6.77E-14	-2.68	-6.39
LMOSA_890	LMOf2365_2134	Glutamine amidotransferase subunit pdxT	1.40E-16	-2.78	-6.86
LMOSA_1310	LMOf2365_2174	Transcriptional regulator	2.41E-05	-1.47	-2.77
LMOSA_2080	LMOf2365_2252	Foldase protein prsA 1	5.21E-09	-2.24	-4.72
LMOSA_1910	LMOf2365_2235	3-oxoacyl-[acyl-carrier-protein] synthase 3	1.39E-04	-1.58	-3.00
LMOSA_2670	LMOf2365_2276	Methylphosphotriester-DNA alkyltransferase	1.22E-04	-1.48	-2.79
LMOSA_3170	LMOf2365_2329	Cof-like hydrolase	1.38E-04	-1.45	-2.74
LMOSA_3180	LMOf2365_2330	Membrane protein	2.70E-10	-2.65	-6.28
LMOSA_3190	LMOf2365_2331	Rrf2 family protein	1.63E-13	-2.89	-7.40
LMOSA_3220	LMOf2365_2334	Glutamate decarboxylase	1.38E-03	-1.45	-2.73
LMOSA_3650	LMOf2365_2371	Nitrogen-fixing NifU protein	1.14E-05	-2.03	-4.07
LMOSA_3810	LMOf2365_2387	Lipoprotein	2.83E-05	-1.65	-3.14
LMOSA_4310	LMOf2365_2438	hypothetical protein	3.21E-04	-1.52	-2.86
LMOSA_4590	LMOf2365_2466	YozA	3.93E-08	-2.44	-5.42
LMOSA_4920	LMOf2365_2499	UDP-N-acetylglucosamine 1-carboxyvinyltransferase 1	9.05E-07	-1.78	-3.45
LMOSA_5240	LMOf2365_2531	CTP synthase	1.06E-05	-1.59	-3.01
LMOSA_6240	LMOf2365_2637	Acetyltransferase	1.13E-03	-1.51	-2.85
LMOSA_6570	LMOf2365_2669	Transcriptional regulator	1.26E-04	-1.60	-3.03
LMOSA_6930	LMOf2365_2713	Transcriptional regulator	2.54E-04	-1.92	-3.79
LMOSA_7110	LMOf2365_2731	Cyclic nucleotide-binding protein	4.27E-08	-2.26	-4.79
LMOSA_7160	LMOf2365_2736	Glutamine amidotransferase	1.73E-04	-1.58	-2.99
LMOSA_7220	LMOf2365_2742	D-alanyl-D-alanine carboxypeptidase	4.49E-08	-1.92	-3.79
LMOSA_7310	LMOf2365_2751	6-phospho-beta-glucosidase	9.36E-34	-4.54	-23.33
LMOSA_7320	LMOf2365_2752	PTS system, IIB component	6.81E-12	-3.14	-8.82
LMOSA_7330	LMOf2365_2753	hypothetical protein	8.42E-19	-3.13	-8.77

LMOSA_7340	LMOf2365_2754	Beta-glucoside kinase	3.84E-12	-2.55	-5.86
LMOSA_7350	LMOf2365_2755	PTS system, IIA component	9.25E-13	-3.14	-8.83
LMOSA_7370	LMOf2365_2757	hypothetical protein	1.50E-06	-1.74	-3.35
LMOSA_7490	LMOf2365_2770	GTP-dependent nucleic acid-binding protein engD	1.85E-05	-1.69	-3.23
LMOSA_7550	LMOf2365_2776	Catalase	1.07E-04	-1.67	-3.19
LMOSA_7600	LMOf2365_2781	Lipoprotein	1.60E-04	-1.49	-2.80
LMOSA_7610	LMOf2365_2782	Nucleoid occlusion protein	2.30E-04	-1.47	-2.77
LMOSA_8270	LMOf2365_0004	S4 domain protein YaaA	3.37E-03	-1.48	-2.78
LMOSA_8550	LMOf2365_0030	PTS system beta-glucoside-specific	2.04E-07	-3.87	-14.64
LMOSA_8610	LMOf2365_0037	Putative yybN protein	7.47E-03	-2.31	-4.95
LMOSA_8790	LMOf2365_0054	Single-stranded DNA-binding protein	1.69E-04	-1.51	-2.84
LMOSA_8820	LMOf2365_0057	Putative AgrB-like protein	1.07E-11	-2.71	-6.56
LMOSA_9980	LMOf2365_0116	hypothetical protein	1.13E-07	-2.24	-4.72
LMOSA_10830	LMOf2365_0201	4-diphosphocytidyl-2-C-methyl-D-erythritol kinase	4.11E-07	-2.14	-4.39
LMOSA_11110	LMOf2365_0229	S1 RNA binding domain protein	4.48E-09	-2.30	-4.94
LMOSA_11190	LMOf2365_0236	Dihydropteroate synthase	7.55E-05	-1.46	-2.75
LMOSA_11430	LMOf2365_0258	Transcription antitermination protein nusG	7.97E-04	-1.84	-3.57
LMOSA_11760	LMOf2365_0292	Hydrolase, haloacid dehalogenase-like family	1.86E-05	-1.79	-3.45
LMOSA_11880	LMOf2365_0304	Methionine import ATP-binding protein MetN 1	1.38E-04	-1.54	-2.90
LMOSA_11890	LMOf2365_0305	D-methionine ABC transporter, D-methionine-binding protein	6.64E-07	-1.74	-3.33
LMOSA_12250	LMOf2365_0337	6-phospho-beta-glucosidase	2.02E-12	-2.65	-6.29
LMOSA_12550	LMOf2365_0372	Transcriptional regulator	1.01E-06	-1.85	-3.61
LMOSA_12700	LMOf2365_0387	Transcriptional regulator, GntR family	1.37E-06	-2.12	-4.34
LMOSA_13050	LMOf2365_0423	hypothetical protein	3.35E-04	-1.67	-3.18
LMOSA_13130	LMOf2365_0431	hypothetical protein	6.20E-07	-2.32	-5.00
LMOSA_13810	LMOf2365_0435	hypothetical protein	1.53E-09	-2.74	-6.69
LMOSA_14220	LMOf2365_0546	Phosphoglycerate mutase family protein	3.44E-03	-1.49	-2.81
LMOSA_14440	LMOf2365_0569	Beta-lactamase	2.31E-05	-1.51	-2.84
LMOSA_14960	LMOf2365_0622	Formate/nitrite transporter family protein	3.19E-09	-2.43	-5.39
LMOSA_15010	LMOf2365_0627	BioY family membrane protein	1.18E-04	-1.78	-3.44
LMOSA_15030	LMOf2365_0629	hypothetical protein	1.95E-04	-1.50	-2.82
LMOSA_15120	LMOf2365_0638	Rhodanese-like domain protein	3.31E-03	-1.72	-3.29
LMOSA_15140	LMOf2365_0640	FMN-dependent NADH-azoreductase	6.31E-05	-2.19	-4.58
LMOSA_15370	LMOf2365_0664	HAD-superfamily hydrolase	1.15E-07	-2.18	-4.53
LMOSA_15480	LMOf2365_0676	Amino acid permease	7.49E-05	-1.57	-2.97
LMOSA_15590	LMOf2365_0688	hypothetical protein	1.66E-08	-2.59	-6.00
LMOSA_15600	LMOf2365_0689	hypothetical protein	1.27E-04	-1.55	-2.93
LMOSA_15780	LMOf2365_0708	hypothetical protein	8.23E-08	-2.40	-5.28
LMOSA_15810	LMOf2365_0711	hypothetical protein	7.87E-04	-1.47	-2.78

LMOSA_15820	LMOf2365_0712	Flagellar biosynthesis protein FliP	1.10E-08	-1.84	-3.57
LMOSA_15830	LMOf2365_0713	Export protein FliQ family 3	1.47E-06	-1.98	-3.94
LMOSA_15840	LMOf2365_0714	Flagellar biosynthetic protein FliR	2.35E-08	-2.02	-4.05
LMOSA_15850	LMOf2365_0715	Flagellar biosynthesis protein	2.62E-05	-1.69	-3.22
LMOSA_15860	LMOf2365_0716	Flagellar biosynthesis protein FlhA	7.64E-05	-1.48	-2.79
LMOSA_15970	LMOf2365_0727	Chemotaxis protein CheY	5.00E-09	-2.09	-4.27
LMOSA_15980	LMOf2365_0728	Chemotaxis protein CheA	4.29E-05	-1.49	-2.81
LMOSA_15990	LMOf2365_0729	Flagellar motor switch protein	6.82E-05	-1.53	-2.89
LMOSA_16000	LMOf2365_0730	hypothetical protein	1.03E-07	-1.96	-3.90
LMOSA_16010	LMOf2365_0731	Flagellar hook-associated protein	5.53E-05	-1.70	-3.26
LMOSA_16070	LMOf2365_0737	hypothetical protein	1.04E-04	-1.47	-2.77
LMOSA_16100	LMOf2365_0740	hypothetical protein	1.43E-05	-1.52	-2.86
LMOSA_16180	LMOf2365_0749	Flagellar M-ring protein FliF	1.12E-06	-1.76	-3.40
LMOSA_16220	LMOf2365_0753	Transglycosylase, SLT family	1.36E-05	-1.54	-2.92
LMOSA_16280	LMOf2365_0759	Methyl-accepting chemotaxis protein	6.84E-09	-2.21	-4.63
LMOSA_16290	LMOf2365_0760	hypothetical protein	1.62E-06	-1.79	-3.46
LMOSA_16320	LMOf2365_0763	Riboflavin kinase/FMN adenylyltransferase	2.42E-04	-1.72	-3.30
LMOSA_16640	LMOf2365_0795	hypothetical protein	3.46E-05	-2.10	-4.28
LMOSA_16660	LMOf2365_0797	hypothetical protein	2.75E-03	-1.59	-3.01
LMOSA_16810	LMOf2365_0810	Membrane protein	1.14E-06	-1.99	-3.97
LMOSA_16830	LMOf2365_0812	RarD protein	1.47E-04	-1.64	-3.12
LMOSA_16940	LMOf2365_0822	DNA-binding protein	1.14E-10	-2.90	-7.47
LMOSA_16950	LMOf2365_0823	ATP-binding protein PotA	9.60E-08	-2.24	-4.73
LMOSA_16960	LMOf2365_0824	permease protein	8.67E-05	-1.73	-3.32
LMOSA_16970	LMOf2365_0825	permease protein	1.04E-03	-1.57	-2.97
LMOSA_17000	LMOf2365_0828	HD domain protein	1.21E-03	-1.46	-2.75
LMOSA_17110	LMOf2365_0839	Acetyltransferase	1.46E-04	-1.49	-2.81
LMOSA_17250	LMOf2365_0853	psiE-like protein	3.32E-07	-2.45	-5.46
LMOSA_17460	LMOf2365_0872	D-alanine--D-alanine ligase	1.10E-05	-1.50	-2.83
LMOSA_17570	LMOf2365_0884	ATP-dependent RNA helicase	9.71E-05	-1.71	-3.27
LMOSA_17580	LMOf2365_0886	Conserved membrane protein	1.09E-15	-3.29	-9.80
LMOSA_18190	LMOf2365_0949	Putative 3-methyladenine DNA glycosylase	2.62E-05	-1.68	-3.19
LMOSA_18370	LMOf2365_0967	MFS family major facilitator transporter	7.83E-07	-1.78	-3.44
LMOSA_18380	LMOf2365_0968	Transcriptional regulator	8.12E-08	-2.09	-4.26
LMOSA_18410	LMOf2365_0971	hypothetical protein	1.17E-04	-1.74	-3.33
LMOSA_18500	LMOf2365_0980	Peptidase family protein	5.47E-08	-2.06	-4.17
LMOSA_18790	LMOf2365_1009	Peptide chain release factor 3	1.35E-05	-1.78	-3.43
LMOSA_18800	LMOf2365_1010	Transcriptional regulator	1.54E-04	-2.27	-4.84
LMOSA_18890	LMOf2365_1019	CAAX amino terminal membrane protease	4.36E-05	-2.03	-4.08
LMOSA_19060	LMOf2365_1035	Glycine betaine transport protein	1.36E-04	-1.61	-3.05
LMOSA_19190	LMOf2365_1048	Metallo-beta-lactamase superfamily protein	1.75E-05	-1.58	-2.99
LMOSA_19200	LMOf2365_1049	hypothetical protein	9.96E-09	-2.39	-5.26
LMOSA_19220	LMOf2365_1051	Transcriptional regulator, LacI family	5.61E-05	-1.57	-2.96

LMOSA_19320	LMOf2365_1061	Molybdenum ABC transporter	7.86E-09	-2.40	-5.29
LMOSA_19430	LMOf2365_1072	Peptide deformylase 1	1.30E-05	-2.02	-4.07
LMOSA_19440	LMOf2365_1073	Pyruvate dehydrogenase E1 component	4.86E-04	-1.54	-2.91
LMOSA_19550	LMOf2365_1084	GTP-binding protein TypA	4.88E-08	-2.51	-5.69
LMOSA_19810	LMOf2365_1110	GMP synthase [glutamine-hydrolyzing]	1.22E-06	-1.85	-3.61
LMOSA_21720	LMOf2365_1262	MutT/nudix family protein	1.10E-06	-2.11	-4.30
LMOSA_21790	LMOf2365_1271	Trehalose-6-phosphate hydrolase	5.13E-08	-2.30	-4.91
LMOSA_21800	LMOf2365_1272	Phosphotransferase system	7.12E-05	-1.77	-3.41
LMOSA_22080	LMOf2365_1302	Glycerol-3-phosphate acyltransferase	1.67E-04	-1.61	-3.06
LMOSA_22170	LMOf2365_1311	tRNA delta(2)-isopentenylpyrophosphate transferase	1.01E-04	-1.53	-2.90
LMOSA_22190	LMOf2365_1313	GTP-binding protein HflX	9.96E-09	-2.03	-4.08
LMOSA_22300	LMOf2365_1324	hypothetical protein	4.42E-04	-1.81	-3.50
LMOSA_22380	LMOf2365_1332	Undecaprenyl pyrophosphate synthase	1.02E-05	-1.53	-2.90
LMOSA_22540	LMOf2365_1348	Polyribonucleotide nucleotidyltransferase	1.87E-04	-1.46	-2.74
LMOSA_22740	LMOf2365_1370	YqhQ	4.60E-07	-1.95	-3.87
LMOSA_22770	LMOf2365_1373	Acetyl-CoA carboxylase	9.05E-07	-1.93	-3.80
LMOSA_22850	LMOf2365_1381	Cold shock-like protein CspLA	6.35E-05	-2.57	-5.95
LMOSA_22900	LMOf2365_1386	Phosphate acetyl/butyryltransferase family protein	2.23E-08	-2.11	-4.32
LMOSA_23350	LMOf2365_1433	Acetyl-CoA acetyltransferase/HMG-CoA reductase	3.24E-06	-1.66	-3.17
LMOSA_23410	LMOf2365_1439	UDP-N-acetylenolpyruvoylglucosamine reductase	1.06E-06	-1.70	-3.25
LMOSA_23500	LMOf2365_1448	Thiamine transporter family membrane protein	1.01E-04	-2.22	-4.66
LMOSA_23520	LMOf2365_1450	hypothetical ABC transporter ATP-binding protein YkpA	4.44E-09	-2.08	-4.22
LMOSA_23610	LMOf2365_1459	Membrane protein	7.98E-12	-2.89	-7.39
LMOSA_23710	LMOf2365_1469	ATP-dependent RNA helicase	1.58E-07	-1.80	-3.47
LMOSA_24130	LMOf2365_1510	GTP-binding protein	3.43E-06	-1.52	-2.87
LMOSA_24140	LMOf2365_1511	HAD-superfamily hydrolase protein	2.64E-10	-2.38	-5.22
LMOSA_24220	LMOf2365_1519	DedA family protein	2.69E-05	-1.68	-3.21
LMOSA_24580	LMOf2365_1555	Prephenate dehydratase	1.68E-07	-1.94	-3.84
LMOSA_24590	LMOf2365_1556	GTPase obg	6.68E-11	-2.72	-6.57
LMOSA_24900	LMOf2365_1590	membrane protein	8.69E-05	-1.57	-2.96
LMOSA_25170	LMOf2365_1617	GAF domain protein	3.34E-07	-2.06	-4.16
LMOSA_25250	LMOf2365_1625	Peroxiredoxin	5.50E-04	-1.46	-2.76
LMOSA_25680	LMOf2365_1668	1-acylglycerol-3-phosphate O-acyltransferase	1.09E-08	-2.33	-5.04
LMOSA_25820	LMOf2365_1684	Leucyl-tRNA synthetase	9.86E-05	-1.55	-2.92
LMOSA_25830	LMOf2365_1685	Putative protein ytqA	5.51E-05	-1.47	-2.76
LMOSA_25860	LMOf2365_1688	S-adenosylmethionine synthase	8.82E-06	-1.68	-3.21
LMOSA_25990	LMOf2365_1701	1,4-dihydroxy-2-naphthoate octaprenyltransferase	2.33E-07	-1.92	-3.78

LMOSA_26120	LMOf2365_1713	A/G-specific adenine glycosylase protein	1.36E-07	-2.16	-4.46
LMOSA_26320	LMOf2365_1733	Methionine aminopeptidase, type I	1.47E-05	-1.54	-2.90
LMOSA_26330	LMOf2365_1734	Flavodoxin	1.63E-04	-1.62	-3.07
LMOSA_26440	LMOf2365_1746	Helicase domain protein	3.27E-06	-1.83	-3.55
LMOSA_26810	LMOf2365_1785	Putative glycerol-1-phosphate prenyltransferase	2.59E-05	-1.68	-3.20
LMOSA_26820	LMOf2365_1786	hypothetical sodium-dependent transporter yhdH	1.08E-07	-1.99	-3.96
LMOSA_27180	LMOf2365_1822	Peptidase family protein	2.57E-05	-1.57	-2.97
LMOSA_27190	LMOf2365_1823	hypothetical protein	4.21E-04	-1.72	-3.29
LMOSA_27510	LMOf2365_1856	YicC protein	1.15E-08	-1.95	-3.85
LMOSA_27580	LMOf2365_1863	Carbamoyl-phosphate synthase	4.76E-04	-1.60	-3.04
LMOSA_27590	LMOf2365_1864	Carbamoyl-phosphate synthase	3.71E-05	-2.40	-5.28
LMOSA_27600	LMOf2365_1865	Dihydroorotase	7.99E-06	-2.77	-6.82
LMOSA_27610	LMOf2365_1866	Aspartate carbamoyltransferase	1.60E-06	-2.81	-7.01
LMOSA_27630	LMOf2365_1868	Bifunctional protein pyrR	3.46E-15	-3.49	-11.25
LMOSA_27740	LMOf2365_1879	Carboxy-terminal processing protease	5.53E-06	-1.52	-2.87
LMOSA_27860	LMOf2365_1892	hypothetical protein	5.14E-05	-1.47	-2.77
LMOSA_27880	LMOf2365_1894	CBS domain protein	2.77E-04	-1.52	-2.87
LMOSA_27930	LMOf2365_1899	Alkaline phosphatase	5.64E-12	-2.68	-6.43
LMOSA_28330	LMOf2365_1940	GGDEF domain protein	2.05E-06	-1.71	-3.26
LMOSA_28360	LMOf2365_1943	EAL domain protein	6.60E-07	-1.66	-3.15
LMOSA_28540	LMOf2365_1961	Heptaprenyl diphosphate synthase component I	2.14E-07	-1.75	-3.35
LMOSA_28550	LMOf2365_1962	GTP cyclohydrolase I	4.02E-13	-2.62	-6.16
LMOSA_28630	LMOf2365_1970	LysM domain protein	1.17E-05	-1.79	-3.47
LMOSA_28750	LMOf2365_1982	Diaminopimelate decarboxylase	6.99E-08	-1.94	-3.83
LMOSA_28970	LMOf2365_2005	Acetyltransferase	2.08E-03	-1.63	-3.09
LMOSA_29140	LMOf2365_2022	Glucosamine--fructose-6-phosphate aminotransferase	6.51E-03	-1.45	-2.73