AN ABSTRACT OF THE THESIS OF

Brandon Joseph Selover for the degree of Master of Science in Food Science and Technology presented on September 14 2020.

Title: Investigating Potential Environmental Sources for Coliforms in Cheddar Cheese Production

Abstract approved:

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Joy Waite-Cusic

The production of Cheddar cheese is a complex process with multiple potential sources of undesirable bacteria, including those that have negative impacts on product quality (spoilage organisms) and those that are used to evaluate sanitary conditions in the production environment (indicator organisms). The dairy industry commonly uses coliform bacteria as indicators of potential fecal contamination or unsanitary conditions.

The aim of this study is to identify potential sources and conditions in a commercial Cheddar cheese production facility that contribute to intermittent coliform detection in finished cheese. To approach this goal, we worked closely with an industry partner to analyze product and surfaces of the commercial processing facility to identify locations and conditions that promoted the growth of coliforms during Cheddar production. Historical commercial production data was analyzed to formulate initial hypotheses for potential coliform sources. A series of investigative sampling events were completed to confirm sources and locations as conditions that promote coliform growth. A second lab-scale study was conducted as a “proof-of-concept” to isolate

the contribution of coliforms from raw product that lead to finished product contamination.

Historical production data indicated a higher likelihood of coliform detection in finished cheeses that were produced later in the production day. Samples of raw milk, heat treated milk, whey, curd, and surface swabs were collected at different points in the production cycle and enumerated for coliforms using MacConkey agar incubated at 37°C for 24 hrs. Results confirmed the efficacy of heat treatment to reduce (to < 1 CFU/ml), but not eliminate, coliforms in milk. Coliform levels remained low in the vat and upon entry via the weir of the draining and matting conveyor (DMC). At the beginning of the production day, coliform levels remained low in all parts of the cheese production process. However, as the production day extended (18 hrs since sanitation), coliform counts in the DMC increased to 5.04 Log CFU/mL in whey collected below the drain belt and 2.20 Log CFU/g in curd collected just before the mill. Surface swabs of belts inside the DMC indicated that coliform subpopulations were increasing in each section of the DMC throughout the production day; however, the largest increased were found on the drain belt (5.53 ± 0.10 log CFU/swab) and belt 1 (5.46 ± 0.63 log CFU/swab). Pre- and post-sanitation swab results suggested that low levels of coliforms may be surviving on DMC belt surfaces. During the annual replacement of the draining belt, belt sections were collected and evaluated for post-sanitation survival using traditional cultural (enrichment-isolation-identification) and visualization (scanning electron microscopy; SEM) methods. Cultural methods confirmed that sanitized belt pieces (7/32; 22%) harbored low levels of *Enterobacter* sp., *Escherichia fergusonii, Klebsiella pneumoniae,* and *K. variicola.* SEM provided

evidence of large clusters of bacteria within belt cracks after sanitation. Taken collectively, in-plant sampling demonstrated two sources coliforms (low levels surviving heat treatment and harborage sites in belt pieces in the DMC) that serve as the seeds for coliform growth in early stages of the DMC during the production day. A lab-scale single-pass continuous flow system was designed to model the beginning section of the DMC to evaluate the potential growth of low levels of coliforms entering the DMC from naturally contaminated whey. Cheddar whey was sourced from the OSU Arbuthnot Dairy and tempered to 35°C for flow through a CDC bioreactor containing stainless steel and polypropylene coupons. Whey and coupons were enumerated for various subpopulations (coliforms, lactic acid bacteria, and pseudomonads) of bacteria after 0, 12, and 18 h of continuous flow and select isolates were identified by 16S rDNA sequencing. Non-starter bacteria present in the whey at 0 h included coliforms (*Enterobacter*), *Pseudomonas,* and *Acinetobacter* (0.80, 2.55, 2.32 log CFU/mL respectively), with each increasing significantly in whey (6.18, 7.00. 5.89 log CFU/mL) and on coupons (5.20, 6.85, 5.29 log CFU/cm^2, respectively) after 18 hrs of flow. Results from the lab-scale study demonstrated that naturally low levels of coliforms entering the DMC in the whey could replicate within the conditions of the draining section of the DMC to the levels found in the commercial production environment.

Continuous environmental conditions (pH, temperature, moisture and nutrients) within the DMC support the growth of various subpopulations of non-starter bacteria, including coliforms. Food contact surfaces, including conveyor belts can harbor bacteria in cracks and defects that survive routine sanitation. Our lab-scale model

system demonstrated that low levels of coliforms in incoming product entering the DMC can increase on surfaces exposed to a continuous flow of whey. Low level coliforms in incoming product as well as surviving bacteria on belt surfaces could serve as the seed for high levels of coliforms in the draining section of the DMC and could lead to finished production contamination. Production schedule, sanitation frequency, and the age or condition of conveyor belts are factors that contribute to intermittent coliform contamination in Cheddar cheese.

Investigating Potential Environmental Sources for Coliforms in Cheddar Cheese Production

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I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

Brandon Joseph Selover, Author

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CONTRIBUTION OF AUTHORS

Joy Waite-Cusic and Jared Johnson assisted with experiment design, data collection, data interpretation, and writing and editing. Jared also provided technical guidance on 16S rDNA sequencing and bioinformatics.

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CHAPTER 1 - INTRODUCTION

Commercial Cheddar cheese production is a careful choreography of timing and controlled conditions to produce a consistent cheese. Each variable that impacts final product must be carefully controlled, from milk quality, heat treatment, starter culture, rate of acid production, timing of cut and stir, Cheddaring, milling, to finally aging the cheese. During Cheddaring, moisture is reduced, and chemistry and texture are altered based on the rate of acidification and moisture loss. Slight changes in any step will have a cascading impact and will lead to the production of a significantly different finished product. In large-scale commercial Cheddar production, the entire process is continuous, and each step timed carefully to maximize product quality and production efficiency. The cadence of each step is critical to keep the entire process running at a steady rate and skilled calculations are made to adjust a few variables that steer the cheese toward performance criteria. Following is a detailed description of commercial-scale Cheddar cheese production from raw milk to aged cheese (McSweeney et al., 2017).

Commercial-scale Cheddar Cheese Production

Dairy farms

Milk quality and safety depends on maintaining a healthy herd and sanitary milking practices. Healthy dairy cows must be free from disease, especially infected udders (mastitis) which produce milk with compromised microbial quality (Nyman et al., 2014). During milking, sanitary practices to reduce bacterial contamination of milk include use of gloves during milking, pre- and post-milking teat disinfection, and sanitization of milking equipment (Belage et al., 2017). Rapid cooling of milk to prior to temporary on-farm silo storage is critical to maintain optimum milk quality by inhibiting the growth of microbes. Milk must be cooled to <10°C

within 4 hrs after milking begins, and <7°C within 2 hrs after milking ends (Food and Drug Administration, 2017).

Transport and storage

Milk is transported to the production facility from the dairy farm in bulk tank trucks. During the transport process, the temperature of the milk must be maintained below 7°C upon receipt at the processing facility. On arrival, the tanker load is tested for temperature and also the presence of antibiotics, specifically penicillin. Tankers are cleaned and sanitized at least every 24 hours or as soon as possible after the tanker is emptied after the 24-hour mark (Food and Drug Administration, 2017). Tankers often pick up from multiple farms to fill out a load and haul multiple loads of milk within a 24-hour window. This practice allows for more efficiency and shortens the window between milking and delivery to the processing facility. Since tankers are not refrigerated, there is the potential for microbial growth in a tanker that is empty-but-dirty for an extensive period of time (>10 hours). Due to typical industry practices, negative microbial outcomes due to tanker and route management are very rare (Kuhn et al., 2018). Milk arrives at the plant and is transferred to large, refrigerated silos until used for production. The milk from many farms and tankers is commingled in these silos which assists in normalizing milk quality prior to processing.

Milk Standardization and Thermization

Commercial cheese production begins with standardizing bulk milk to achieve a casein-to-fat ratio between 0.67-0.72. Bulk milk from the silo is pumped into the plant where the fat is removed and added back to the skim milk. Protein can also be standardized through ultrafiltration of skim milk to concentrate protein (mostly casein) and added back to reach the

desired target. Standardization of milk minimizes differences in seasonality and allows for better control over the expulsion of whey during production (Ong et al., 2017). The skim milk and fat or protein streams are mixed in a balance tank and the resulting mixture is generally called "cheesemilk".

Most cheesemilk is thermally processed after standardization and prior to culturing. The primary intent of the thermal treatment is to reduce the microbial loads, including pathogens, in raw milk. Most large-scale operations heat the milk using a plate heat exchanger. This step is continuous with raw milk from the balance tank in, and heat treated milk out which is pumped into the cheese vat. Heat treatment may occur at a variety of time-temperature combinations, including treatments that achieve pasteurization or may be less intense (i.e., sub-pasteurization). Pasteurization is considered any time-temperature combination that achieves an equivalent microbial reduction as 143°F/62°C for 30 min (Food and Drug Administration, 2019). Most commercial cheese operations will pasteurize using high temperature short time (HTST) pasteurization of 162°F/72°C for 15 s (Food and Drug Administration, 2017); however, there are numerous cheese processors that use sub-pasteurization in their process. These facilities choose sub-pasteurization to optimize properties in the milk that improve cheese production as well as potential preservation of native microbiota that can contribute to finished product quality.

Vat

Heat-treated milk is pumped into a large vat and tempered prior to starter culture addition. Starter cultures for Cheddar cheese generally include *Lactococcus lactis* strains and may include adjunct bacteria, such as *Lactobacillus* spp.. Combinations of *Lactococcus lactis* strains are often used as a cocktail and are rotated to minimize the potential for bacteriophage to infect the production

facility. Starter cultures produce acid by fermenting lactose to lactic acid, which is a main driver of curd and whey separation later in the process. Controlling acid production and timing almost all remaining steps of Cheddar production is based on the acidification rate of the starter culture and will have a significant influence on the quality characteristics of the final product. Stater cultures can be added to milk from bulk starter tanks or directly added to each vat in lyophilized form.

Once the starter is active, rennet is added to initiate coagulation of the milk curd. Rennet is an enzyme (chymosin) that cleaves casein proteins, resulting in a protein network that traps fat and water. Rennet activity will transform the vat milk into a gel in 35-45 minutes. The gel is then cut by blades inside the vat, heated to 100°F/38-39°C, and stirred for ~35 min. Heating the newly formed curds shrinks the casein network and expels moisture. The starter culture is actively acidifying the curd, which facilitates a loss of colloidal calcium phosphate from the casein network. The pH needs to reach ~6.3 before whey is separated from the curd, although this is generally a prescribed amount of time to keep production moving and on schedule. Seasonal differences in milk can alter the time required to reach the target pH so starter amount can be varied to adjust timing. In most commercial processes, multiple vats are in use and staggered to keep production constant throughout the day.

Cheddaring and milling

Whey and curd are pumped from the vat to either a specialized Cheddaring table or, more commonly, a draining and matting conveyor (DMC) system. The purpose of the next steps in the process is to drain all the whey and allow the curd to mat. Heat and acid production during this step promotes the fusion of curd under the weight of gravity. In the case of the DMC, the curd

moves down a series of three conveyors, flipping the curd mat from one conveyor to another. In small-scale production, curd mats will be stacked on top of each other and flipped periodically. This process is called “Cheddaring” and is accomplished using slightly different methods all over the world. The effect of the Cheddaring process is to allow the casein network to begin orienting in a fibrous structure while expelling whey. The Cheddaring process has been debated as to its necessity in producing Cheddar cheese, although it is required in part by the US standard of identity for Cheddar cheese (Food and Drug Administration, 2019). After Cheddaring, the curd will be milled, or cut, into smaller pieces. Milling is often accomplished by large blades, although it can be done by any mechanical cutting device. Although milled curd size is a variable that can be adjusted to support production schedule needs, the optimal milled curd size is 1 cm x 2 cm x 6 cm to maximize whey drainage, support salt distribution, and easy block formation (Bennett, Johnston, 2011; Guinee, Sutherland, 2011).

Salting and pressing

Milled Cheddar curds are “dry salted”. This means that salt crystals are added directly to the moist curds. Salt will dissolve into the curds and causes the curd to further shrink and expel more moisture. Large commercial processors generally use a conveyor system and a mechanical salt dispenser that spreads salt while the curd is mixed to support even distribution. Smaller operations simply salt curd manually. The salt content for Cheddar cheese is generally targeted at 4.5-5.5% salt-in-moisture. Salting will also control the activity of bacteria and is effective in slowing fermentation as well as reducing the activity of many contaminants. This is a critical step in controlling further acid production during curing and impacts flavor and texture of the finished cheese.

Warm, salted curd will be pressed or formed into large blocks. The pressing phase promotes further whey expulsion and assists curd fusion (or "knitting"). Modern commercial cheese production generally use a tower system (20-30 ft/6-10 m high) where salted curd is moved via suction and dropped in a tower that begins pressing curd under its own weight. The tower is under a partial vacuum to remove trapped air and prevent texture defects during aging. At the bottom of the tower, cheese blocks are cut and pushed out to be packaged. Block size varies depending on facility, but a common size is approximately 40 lbs/18 kg.

Aging

Cheddar cheese is stored ("aged") under refrigeration (>35°F/1.7°C) for a period of time (months to several years) to mature the texture and flavor. If raw or sub-pasteurized milk is used, cheese must me aged a minimum of 60 days at >35°F to reduce potential pathogens (Food and Drug Administration, 2019). The properties of Cheddar cheese change significantly in the first 30 days of aging. These changes are a result of proteolysis, solubilization of colloidal calcium phosphate, casein breakdown, changes in bacteria metabolism (both starter and non-starter), and migration of salt. The texture of the cheese changes from elastic (and "squeaky") to hard. Microbial and enzyme activity will develop a "sharper" flavor as the aging period is extended. At targeted time points during aging, the cheese will be evaluated for desired characteristics to determine when the cheese is ready for sale. The large blocks of cheese will be cut to the desirable size and repackaged for distribution and sale.

Cheese making at any scale is a complex mixture of microbiology, chemistry, and physics, with a bit of art. Commercial Cheddar cheese processors uses automation to maximize efficient production; however, this automation reduces the flexibility to adjust processing parameters to

maximize quality of such a dynamic system. It is impressive that commercial operations can standardize the production process and make such a complex, but consistent product. As automation has increased the production potential of cheese making facilities, it also led to new challenges for sanitation of production equipment and controlling contamination of cheese by undesirable and pathogenic bacteria.

Coliforms

What are coliforms?

Coliforms are a non-taxonomic group of bacteria that mostly belong to the family *Enterobacteriaceae* (with the exception of *Aeromonas*). They are Gram-negative, rod shaped, facultative anaerobes that ferment lactose to produce acid and gas at 32-37°C within 48 hrs (Food and Drug Administration, 2018). Coliforms represent 19 genera, with *Escherichia, Citrobacter, Klebsiella, Enterobacter,* and *Serratia* being the most common in dairy products (Martin et al., 2016). *Escherichia coli* is often given specific attention among coliforms and is often considered a reliable indicator of fecal contamination (Food and Drug Administration, 2018).

Why are coliforms important?

The coliform group as a whole are not considered pathogens, with the exception of Shiga toxin-producing *E. coli* (STEC). Coliforms are used by the dairy industry as "indicator" organisms. Their presence and/or quality in pasteurized dairy products is interpreted as the product being processed under insanitary conditions. Coliforms have also been used as "index" organism (predictor of pathogen presence); however, the correlation is very low (Martin et al 2016). The

FDA set limits on the presence of non-toxigenic *E. coli* in all cheese, whether made from pasteurized or raw milk as: "levels exceeding 10 MPN/g but less than 100 MPN/g in three or more subsamples of the five examined; or levels at or above 100 MPN/g in one or more subsamples of the five examined" (Food and Drug Administration, 2020). The Pasteurized Milk Ordinance (PMO) limits coliforms to <10 CFU/g for pasteurized milk or milk products, including cheese (Food and Drug Administration, 2017). The PMO only applies to pasteurized milk products, leaving cheese made from raw or sub-pasteurized milk with no federal limit for coliforms. States have the choice to impose regulatory limits on coliforms for raw or sub-pasteurized milk products; however, Oregon has no coliform limits. Cheese producers often set their own limits for coliforms in finished cheese to meet internal quality goals. Pathogens, including STEC, *Salmonella*, and *Listeria monocytogenes*, are not allowed in finished cheese (Food and Drug Administration, 2020).

Coliforms in commercial cheese production

Commercial cheese production is susceptible to coliform contamination due to the presence of coliforms in raw milk entering the plant. The pH (6.9-5.5), temperature (36°C), availability of nutrients, and sufficient moisture of various steps of production could support coliform growth. The continuous production schedule is designed to maximize production time and limit non-production time (i.e., sanitation time). Large, automated equipment requires an extensive sanitation procedure that lasts several hours, so the amount of in-production and out-of-production time must be balanced.

Raw milk and thermization failure

Raw milk coming from the dairy farm has a diverse microbial ecology that includes *Streptococcus*, *Pseudomonas*, *Enterobacteriaceae* (including coliforms), and potentially 20-30 other genera (Kable et al., 2019). The microbial community of milk is influenced by cow health and can be negatively impacted if the cow is suffering from mastitis (Mbuk et al., 2016). The udder is unfortunately located below the rectum of the cow and will be contaminated with feces. Bacterial contamination is reduced by thermal treatments; however, these treatments are not intended to sterilize the product and low levels of survivors (1.69-2.18 log CFU/ml) remain present in the milk through further processing (Ranieri et al., 2009).

Sanitation and biofilm potential

Poor sanitation practices can contribute to coliform contamination during production and allow for the persistence of coliforms in production equipment. Production schedules that perform sanitation once in a 24-hour day can allow bacteria to increase throughout the day in production equipment (Kable et al., 2019). Bacterial attachment and biofilms in cheese production equipment has not been studied. Related areas of dairy processing have been researched, including whey concentration membranes used on whey processing. Several studies have found biofilm development on membranes (Hassan et al., 2010; Tang et al., 2009). Other studies have documented the increase in microbial community over a production day in dairy processing environments (Kable et al., 2019; O'Sullivan et al., 2015). There is a gap in our knowledge related to the microbial ecology within cheese production equipment and how production length influences the potential development of biofilms and/or finished product contamination.

Industry partner production process and schedule

Production Process

Our industry partner produces Cheddar cheese in a continuous production system using the general steps as detailed above. Milk is sourced from numerous small farms and stored in silos at the plant. Standardization of milk is followed by sub-pasteurization (153-158°F/67-70°C for 29 sec) before cheesemilk is pumped into one of 8 vats. Separation of whey and curd and Cheddaring is accomplished using a custom-manufactured DMC. Their DMC includes a weir that acts as a transitional container for whey and curd before it spills onto the drain belt conveyor (pH 6.3, 98°F/36°C). Curd is flipped as it falls onto the next belt (cheddar belt 1, pH 5.7, 98°F/36°C), and again on the last belt (cheddar belt 2, pH 5.2, 99°C/37°C) which ends at a mill that cuts the curd into small pieces. The entire transit time inside the DMC is 90 min. Milled curd is salted on another belt before being vacuum pumped into 9 m/30 ft towers for pressing, block formation, and wrapping before being aged in the refrigerated aging warehouse. The entire cheese production process runs continuously from approximately 11:00 PM to 10:00 PM the following day; however, each section will be in production for around 18 hours. It takes about 5 hours to produce a single batch of cheese, so there is a window for staggered sanitation in different parts of the facility from about 4:00 PM to 11:00 PM.

Plant Sanitation

The sanitation of the production plant begins as soon as product is past each section of the process at the end of the day. Workers spray water to remove large pieces of product from the interior of the vats, DMC, salting belt and any other accessible equipment. The exterior of all equipment is washed and sanitized, as well as the floors, tables and other surfaces. Production equipment (raw milk lines, pasteurizer, milk-to-vat lines, DMC, salter belt) is sanitized by an automated clean-in-place (CIP) system and program. CIP consists of the succession of 70°C

caustic, acid, and detergent, followed by a hot water rinse. Delivery of these reagents in sections with pipes is accomplished by cycling each reagent through the section. The DMC and salter belt contain sprayers located throughout the interior where CIP reagents are delivered. This form of CIP relies on consistent water pressure and functioning sprayers located in areas that reach all surfaces inside equipment. If even one of these fails, the efficacy of the sanitation can be compromised. CIP is performed on each fermentation vat every time whey and curd is pumped out all throughout the production day. A shortened sanitation cycle (30 min) is performed midway through the production day on the milk lines prior to the vat. The careful timing and utilization of 8 fermentation vats allows for production to continue in the DMC during midday wash of milk lines. The amount of time required for CIP in milk lines is significantly less than the nearly 2 hour sanitation process for the DMC and salter belts.

Industry partner's coliform problems

Our industry partner has detected intermittent low coliform levels in young cheese (before aging) for the last several years. Occasionally, high coliform counts (3.00 log CFU/g) including *E. coli* have been detected. They have been unable to identify the cause or pattern of this contamination. Routine testing of coliforms is only done for cheese before aging and not at any point during production; therefore, they had limited information on sources or causes in the facility. The company asked for our assistance in determining sources and potential harborage sites of coliforms in their commercial operation.

Research approach

Aim 1: Investigate intermittent coliform detection in finished cheese using historical production data, in-process product sampling, and collection and evaluation of food contact surfaces in the commercial production environment.

Our approach included:

Review plant historical data to identify trends in time-of-day coliform counts.

Design a sampling plan to assess the coliform contribution from each section of production including frequency throughout the day and across several days.

Perform detailed sampling, swabbing and analysis of problem areas, isolate and identify bacteria.

Identify possible sources of coliforms and suggest mitigation steps to control coliforms during production.

Aim 2: Determine if naturally, low level coliform contaminants in whey can increase under the conditions (time, temperature, nutrient availability, pH) present in the drain belt section of the DMC.

Our approach included:

Design a lab-scale model system that mimics the continuous flow conditions of the drain belt section of the DMC (pH 6.3-5.8, 95-98°F) with naturally low-level bacterial contamination in whey.

Assess the ability of various subpopulations of bacteria to grow in whey and attach to surfaces in conditions similar to a DMC over a typical 18-hr production schedule.

Overall Goal

Identify locations within the production environment that support or encourage the growth of coliforms during production to support mitigation strategies that would reduce the likelihood of coliforms in finished Cheddar cheese.

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CHAPTER 2 – POPULATION DYNAMICS OF COLIFORMS IN A COMMERCIAL CHEDDAR CHEESE PRODUCTION FACILITY

Title: Population dynamics of coliforms in a commercial Cheddar cheese production facility

Running head: Coliform dynamics in cheese production

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ABSTRACT

The aim of this study was to investigate sources and conditions that lead to the intermittent detection of coliforms in finished Cheddar cheese at a single commercial facility. Historical production data was analyzed, and cheese produced later in the production day (≥16 hrs) was significantly more likely to test positive for coliforms than cheese made earlier in the production day (<12 hrs). An investigative sampling approach was performed to determine coliform levels in milk, whey, curd, and surfaces at the beginning, middle and end of the production day. After sanitation, conveyor belt pieces from the draining and matting conveyor (DMC) were collected and evaluated for bacterial survivors using culture-based methods and Scanning Electron Microscopy (SEM). The enumeration of coliforms in raw and heat-treated milk demonstrated that the sub-pasteurization thermal treatment was effective at reducing, but not eliminating coliforms. Repeated sampling identified the DMC as a critical area that supported coliform growth during the production day. Coliform levels in whey entering the weir maintained a level of <1 CFU/mL throughout production; however, coliform levels in whey below the drain belt increased from <1 CFU/mL at midday (8 hrs) to 5.04 log CFU/mL by the end of the production day (~18 hrs). Routine sanitation inside the DMC resulted in undetectable coliform levels on easily accessible surfaces. However, enrichment and SEM of belt sections revealed pockets of viable coliforms and other bacteria in cracks and defects in conveyor belts, indicating that sanitation did not eliminate viable bacteria. This study identified the drain belt and belt 1 having conditions that support the growth of coliforms throughout the production day. Low levels of coliforms are present in heat-treated milk as well as surviving sanitation in the DMC and could serve as the initial seed for high levels of coliforms at the end of the production day.

Keywords: Coliforms, cheese, facility, whey, conveyor belt

INTRODUCTION

Commercial-scale Cheddar cheese production is accomplished through a complex choreography of continuous, automated processing stages to optimize the consistent quality of the finished cheese. Large-scale cheese production requires extensive networks of containers, pipes, equipment, and conveyors to move ingredients, in-process product, byproducts and finished product throughout the facility. Cheesemakers are constantly making predictive decisions from normalizing incoming raw milk quality to analyzing sensory scores from young cheese to predict aged product quality. During the production day, decisions are made to maximize product throughput to maximize daily output, while also balancing with adequate cleaning and sanitation schedules to create products free from microbial threats to consumer safety and spoilage losses.

One strategy that dairy processors use to evaluate the microbial quality of their products and/or the production environment are microbiological tests for "indicator organisms" (Chapin et al., 2014). Common microbial indicator tests used in the food industry include aerobic plate count, yeast and mold, *Enterobacteriaceae,* and coliforms. Coliforms are a sub-group of the Gram-negative *Enterobacteriaceae* that ferment lactose and produce acid and gas within 24-48 hrs when incubated at 37°C. The coliform group includes *Citrobacter, Escherichia, Enterobacter, Klebsiella,* and others. Coliforms are naturally found at low levels (<1000 CFU/ml) in raw milk. Higher levels of coliforms in raw milk may indicate significant problems on the farm, including mastitic cows, sanitation problems, or a lack of adequate refrigeration (Martin et al., 2016). Pasteurization is an effective approach to reduce coliforms in milk; therefore, the Pasteurized Milk Ordinance (PMO) sets the standard for no detectable coliforms (<10 CFU/ml or g) in all Grade A pasteurized milk products (Food and Drug Administration, 2017). If coliforms are detected in pasteurized dairy products, it serves as an indicator of post-pasteurization contamination and insanitary conditions occurring in the processing facility.

In the US, Cheddar cheese may be made from raw or sub-pasteurized milk and aging at greater than 35°F/1.7°C for at least 60 days is an alternative mitigation strategy to reduce undesirable bacterial groups, including coliforms however, the efficacy of this approach is controversial (CFR Title 21). Because these cheese products are unpasteurized, they are not subject to the PMO and do not have a specified coliform requirement; however, some states have implemented coliforms standards (Martin et al., 2016). In the US, the Food and Drug Administration (FDA) requires all Cheddar cheese (domestic and imported; pasteurized and unpasteurized) must be free from bacterial pathogens, but low levels of non-toxigenic (generic) *E. coli* are allowable (Food and Drug Administration, 2020). Regardless of the regulatory requirements, cheesemakers use routine coliform testing as an indication of microbial product quality and environmental conditions in the facility.

Post-pasteurization or environmental contamination of dairy products is often traced to a harborage site for a significant number of bacteria, usually as a biofilm, in equipment defects (e.g., cracks), wear points (e.g., gaskets), or poorly designed piping systems (e.g., dead ends) (Martin et al., 2016). Effective sanitary design of equipment, implementation of effective cleaning and sanitation programs, and a robust preventive maintenance program should prevent significant contamination problems. However, effective implementation of these programs in a production facility is challenging and timelines and resources for these activities are often in direct opposition to maximizing throughput and efficiency.

Protecting dairy products from environmental contamination is difficult, as coliforms are commonly detected in pasteurized milk in the US (7.5-26.6%) (Martin et al., 2012). This indicates the significant challenge of protecting even the simplest of dairy products from post-pasteurization contamination. The detection of coliforms (>10 CFU/g) in cow milk cheese available in US retail stores occurs at a similar rate (27%; 34/125). Coliform detection is more frequent in raw milk cheeses (44%; 25/57); however, detection is not uncommon in cheese made with pasteurized milk (13%; 9/68). The separation between

the likelihood of detecting coliforms in raw and pasteurized cheeses increases as moisture/water activity decreases (i.e., soft vs. semihard vs. hard) (Trmčić et al., 2016).

Cheddar cheese production is especially prone to bacterial contamination because it is a fermented cheese, therefore conditions during parts of production are designed to facilitate bacterial growth of the lactic acid bacteria (LAB) starter culture used. These conditions of temperature, pH, moisture and nutrients may favor the growth of contaminant bacteria that may be present. Bacteria from the coliform group are a common member of the intestinal microbiota of dairy cows and of raw milk and have a high potential to contaminate cheese production.

This research project was initiated to assist a commercial cheese facility in identifying conditions that lead to intermittent coliform contamination of their finished Cheddar cheese. The approach was investigative and included assessment of historical production data, in-process product sampling, and collection and evaluation of food contact surfaces in the commercial production environment. Our overall goal was to identify potential sources of coliforms as well as conditions within the processing environment that could support the growth of coliforms during production.

MATERIALS AND METHODS

Commercial cheese production data and analysis

A commercial cheese production facility with intermittent detection of coliforms in finished product (>10 CFU/g cheese) approached our laboratory for help to identify potential sources of this problem. The facility shared production data for all cheese products produced over a two-month period in 2019. A total of 1699 cheese makes spanning all 60 production days were included in the data set. As part of their quality assurance program, cheese was periodically sampled from the block press and analyzed for various characteristics, including coliforms. Coliforms were enumerated using 3M E. coli/Coliform Count Petrifilm plates according to the manufacturer's instructions. The data set was analyzed by visualization

to identify events and patterns associated with increased coliform detection in finished product and elevated coliform levels. All analyses were conducted in R using base R and the tidyverse package (Wickham et al., 2019).

Sample collection from commercial cheese production facility

Samples were collected from the commercial cheese production facility mentioned above. Milk, whey, curd, and environmental swab samples were collected on 13 unique production days (3-4 consecutive production days) at various points within a 12-month period (2018-2019). Milk, whey, curd, and food contact surface (FCS) samples were collected throughout the production shift (beginning, middle (before and after midday wash), end of day). Additional FCS samples were collected after sanitation to verify efficacy of daily clean-in-place (CIP) protocol.

The overall generic processing scheme of the commercial production facilities and sample locations are shown in Figure 1. Milk samples (50 ml) were collected from the balance tank (raw) and from a sample port located after the pasteurizer (heat-treated). Whey samples (50 ml) were collected from the fermentation vats and from several access points within the draining and matting conveyor (DMC), including the weir and under the drain belts. Liquid samples (milk and whey) were collected into sterile 50 ml conical tubes using a syringe (raw milk, sample ports), sanitized ladle (vat, DMC weir), or allowing whey to drip into collection tubes (under DMC belts). Curds (100-300 g) were sampled from the fermentation vat and at several points during cheddaring in the DMC, including the weir and belt 2 (before milling). Cheese samples (100-300 g) were also collected from pressed cheese blocks (18 kg/40 lb). Solid samples (curd and cheese) were collected into Whirl-Pak bags (Nasco, Madison, WI) using a sanitized cheese trier. Environmental samples (FCS and non-FCS) were collected using sponge sticks (Hardy Diagnostics, Santa Maria, CA) or quick swabs (3M, Saint Paul, MN). A surface area of approximately 10 cm^2 was sampled for each location. Environmental sampling sites were selected to represent surfaces that were easily accessible during sanitation and surfaces that were less easily

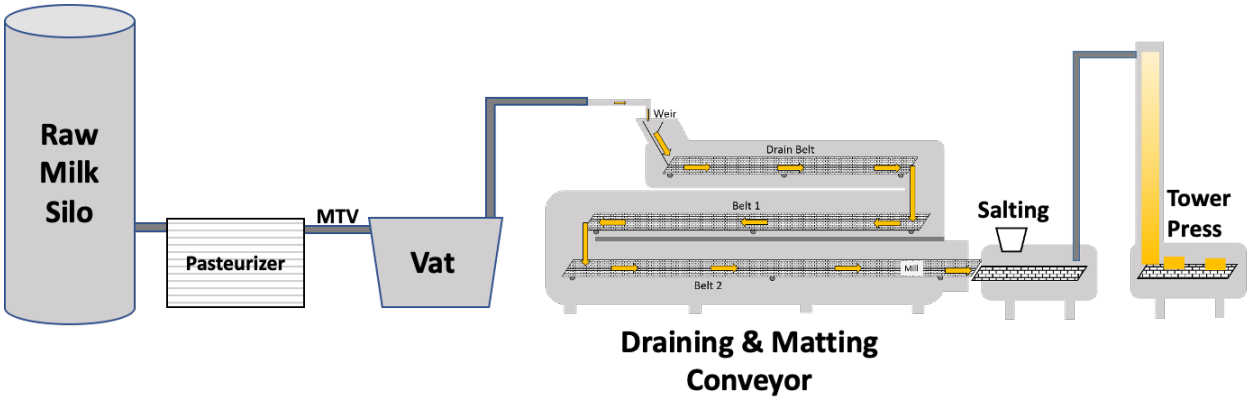

Figure 2.1. Overview of the Cheddar cheese production process. Samples were collected from five main sections of the process: raw milk, heat-treated milk, vat, draining and matting conveyor, and pressed cheese after exiting the tower press.

accessible during sanitation. All samples were immediately cooled to 4°C and transported to the Oregon State University Food Safety Systems Laboratory (Corvallis, OR) within 36 hours.

Sections of the polypropylene DMC drain belt (4 cm x 27 cm; 4 cm x 13.5 cm) were collected in coordination with our industry partner's preventive maintenance program for belt replacement. After a complete sanitation cycle, the drain belt was dismantled inside the DMC by removing the metal pins that connect the individual belt pieces. Individual belt sections (n = 38) were aseptically transferred from the DMC to WhirlPak bags via sampling windows. Belt sections representing the center of the belt surface (n = 28) and the edges (n = 10) spanning approximately 25% of the belt length were collected for analysis. Belt pieces were transported to the Oregon State University Food Safety Systems Laboratory (Corvallis, OR) within 6 hours of collection.

Microbial analysis

Milk, whey, curd, cheese, and swab samples were enumerated by serial dilution with spread plating on MacConkey agar (Neogen). Initial dilutions (1:10) of curd and cheese samples were created with 1.5% sodium citrate (Anachemia, Rouses PT, NY) and stomached for 2 min. Other sample types and serial dilutions were performed in 0.1% buffered peptone water (BPW, Neogen, Lansing, MI) and mixed by vortexing. MacConkey plates were incubated at 37°C for 24 hrs prior to enumeration. Detection limits for plating were 10 CFU/g for curd and cheese samples, 1 CFU/g for liquid samples, and 1 CFU/swab for environmental samples.

A most probable number method (MPN) approach was used to evaluate samples for low levels (<1 CFU/ml or g) of coliforms in milk, whey, and curd at the middle and end of a single production day. For the MPN method, 0.1, 1, 10, and/or 100 g or ml of sample was mixed at a 1:10 ratio with MacConkey broth and incubated at 37°C for 24 hrs. Incubated MacConkey broth cultures were streaked for isolation on MacConkey agar and incubated at 37°C for 24 hrs. Plates with at least one typical coliform colony on

MacConkey (red colonies) were considered positive for the respective subsample and survivors were calculated using the Thomas approximation of MPN/g (Swanson, KM, Petran. RL, Hanlin, 2001):

$$\text{MPN/g or ml} = P/\text{sqrt}(NT)$$

where P is the number of positive subsamples, N is the total g or ml of sample in all negative tubes, and T is the total g or ml of subsamples in all tubes. In the case where none of the subsamples were positive for coliforms, the result was reported as <1 per total g or ml of sample analyzed.

DMC drain belt sections (middle: n = 24; edge: n = 8) were enriched to recover bacteria remaining on the belts after sanitation. TSB (500 mL) was added to each WhirlPak bag containing an individual belt section and incubated at 37°C for 48 h. Enrichments were streaked for isolation on MacConkey agar and incubated at 37°C for 48 h.

Isolates representing typical, but unique colony morphology, from each sample type were transferred to tryptic soy broth (Neogen) with yeast extract (Neogen) (TSBYE) and incubated at 37°C for 24 hrs. The resulting liquid cultures were mixed with 35% glycerol (Fisher Scientific, Hampton, NH), transferred to cryogenic tubes, and stored at -80°C for preservation.

Isolate identification by 16S rDNA sequencing

Bacterial isolates were revived from -80°C storage by transfer into TSBYE and incubated at 37°C for 24 h. Cultures were diluted (1:10) in DNAse-free water (Invitrogen, Carlsbad, CA) and heated at 80°C for 30 min to create crude lysates. Alternatively, DNA extraction was accomplished using the DNeasy blood and tissue kit (Qiagen, Germantown, MD) following manufacturer's instructions. Crude lysates or DNA extractions were used as the templates for PCR to amplify the 16S rDNA gene using 27F/1492R universal primers (Lane, 1991). PCR was performed in 25 µL reactions using Platinum Hot-Start Master Mix (Invitrogen, Carlsbad, CA). PCR conditions were as follows: initial denature at 95˚C for 5 minutes, followed by 30 cycles of 95˚C for 30 seconds, 51˚C for 30 seconds, and 72˚C for 2 minutes, and a final

extension at 72˚C for 10 minutes. Successful PCR amplification was confirmed by gel electrophoresis (1% agarose gel, 10 V/cm). PCR products were cleaned with the DNA Fragment Extraction Kit (IBI Scientific, Dubuque, IA). Cleaned amplicons were submitted to OSU's Center for Genome Research and Biocomputing (CGRB, Corvallis, OR) for Sanger sequencing using both 27F and 1492R primers on an ABI 3730 Capillary Sequencer. Raw sequencing reads were processed using SeqTrace (Stucky, 2012) to create consensus sequences. Bacteria were identified at >99% similarity and >90% completeness using the 16S-based ID function of EzBioCloud (Yoon et al., 2017).

Scanning electron microscopy (SEM) of drain belt sections

Drain belt sections (n = 6; 4 middle, 2 edge) were prepared for SEM to visualize and identify the location of any attached bacteria or biofilms that survived sanitation. Belt sections were snapped and broken inside the whirlpak collection bags into approximately 1-3 cm^2 pieces. Belt pieces with visible crevices or including areas that were more difficult to access during sanitation were selected for analysis. Belt pieces were placed in SEM fixative (1% Paraformaldehyde and 2.5% Glutaraldehyde in 0.1M Sodium Cacodylate) overnight and submitted to the OSU Core Microscopy Facility (Corvallis, OR) for dehydration with ethanol and critical point dryer (EMS 850, Electron Microscopy Sciences, Hatfield, PA). Prepared belt pieces were mounted, and sputter coated with 60% gold, 40% Palladium (Cressington 108A, Ted Pella, Redding, CA) and imaged using a FEI Quanta 600F eSEM (Thermo Fisher, Waltham, MA). Two instrument settings were used for imaging: 5.00 kV with a 5.1 mm working distance or 10.00 kV with an 8.9 mm working distance.

Data analysis

Statistically significant differences in the detection of coliforms in finished product by cheesemake were determined using a Fisher's Exact Test (GraphPad, San Diego, CA) by comparing cheesemake 1 with cheesemakes 22-30 with a Bonferroni correction for multiple comparisons. Differences in coliform levels in raw milk samples by production day and time of day classification (start, middle, end) were tested

using a mixed model ANOVA followed by a post-hoc Student's t-test for pairwise differences (JMP Pro 15.1.0, SAS Institute, Inc., Cary, NC).

RESULTS AND DISCUSSION

Frequency of coliform detection in finished cheese

Coliform level in fresh Cheddar cheese over a 60-day production period are shown in Figure 2. On the majority of production days, this commercial facility produces between 29 and 31 individual cheese makes that sequentially move through the process and equipment shown in Figure 2A. Coliforms were detected in at least one cheese sample on 28 out of 60 production days (46.7%). The longest stretch of production days without a coliform-positive test was 6 days and the longest stretch of production days with coliform-positive tests was 8 days. The number of positive cheesemakes per day ranged from 1 (7 days) to 7 (2 days). Coliform levels in positive cheese samples varied from 1.0 log CFU/g (22 samples) to >3.0 log CFU/g (1 sample; 3.08 log CFU/g). Coliforms were only detected in cheese made later in the production day, specifically on or after cheese make 22 (approximately 14 hours into production). There were two production days where coliforms were detected earlier in the production day: cheesemake 16 (day 49) and cheesemakes 18 and 19 (day 50). Interestingly, cheese made on day 48 was a production day with 7 coliform-positive cheesemakes with the final 5 makes of the day being positive at high levels (1.30-2.46 log CFU/g). This was the first day in a string of 8 days with coliform-positive cheese samples with each day having at least 2 coliform-positive cheesemakes.

Trends in coliform-positive results as a function of cheesemake/production time were examined further using a subset of data from production days with ≥ 30 makes (Figure 2B; 37 production days with 1110 cheese makes). The frequency of coliform detection in finished cheese ranged 5.4% (2 of 37 cheeses) for cheesemake 23 to 37.8% (14 of 37 cheeses) for cheesemake 30. The likelihood of detecting coliforms in finished cheese was significantly higher for cheeses produced in makes 28, 29, and 30 compared to cheesemakes earlier in the production day (before cheesemake 16).

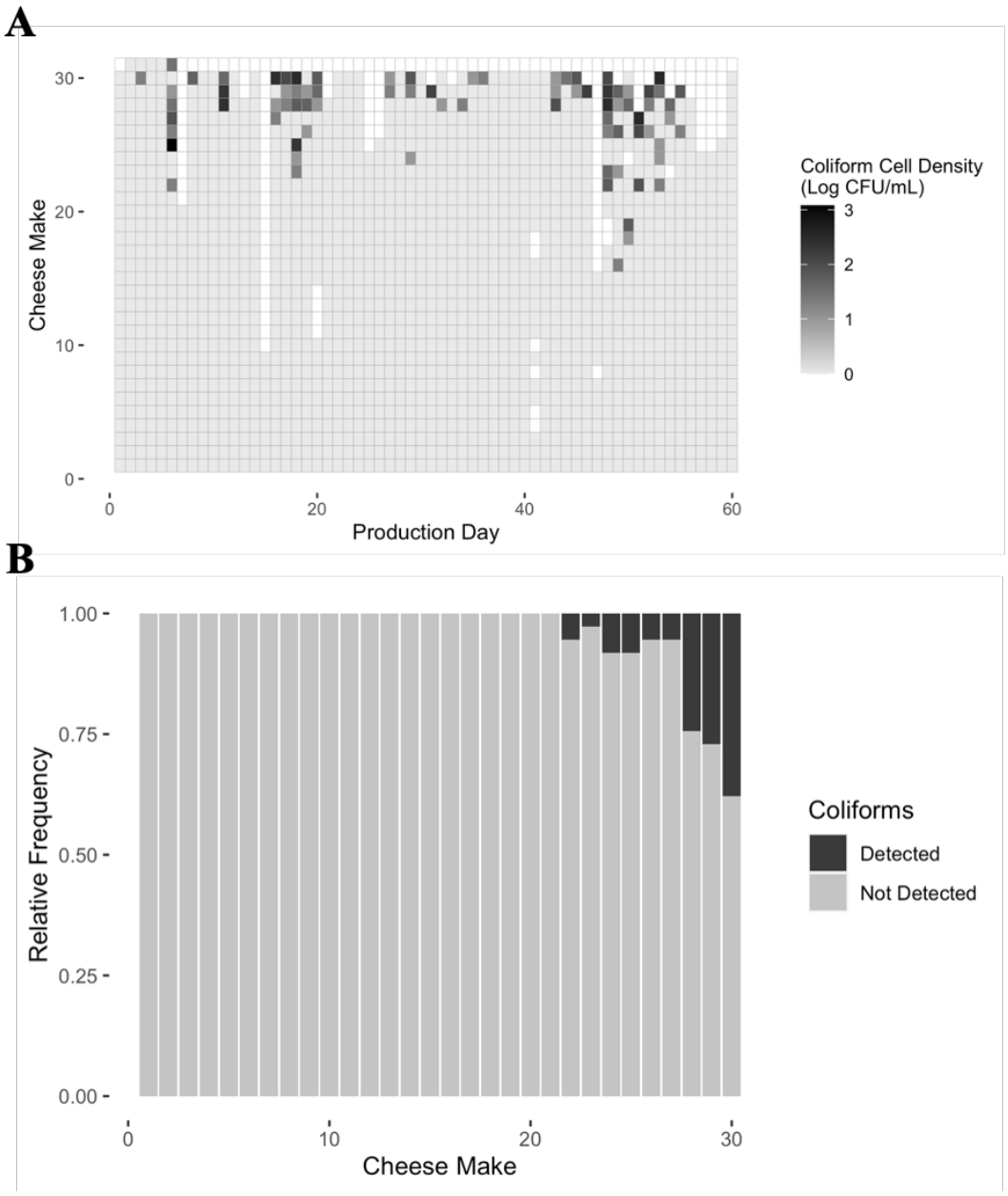

Figure 2.2. Coliform cell density and detection frequency in fresh pressed cheese based on historic production data collected by the cheese manufacturer. A) Coliform cell density by production date (60 days) and cheese make number. Grey squares represent cheese makes where coliform data was not available or when cheese was not produced. B) Coliform detection frequency by cheese make number for production days with at least 30 cheese makes (37 days). Cheese samples were analyzed by the quality assurance department of the commercial cheese manufacturer using the Coliform Petrifilm test. A sample was considered to contain coliforms if the CFU value was ≥ 10.

This high-level analysis of the historical production data suggests the growth of coliforms somewhere in the production system throughout the day which leads to detectable coliforms in finished cheeses. Another study also found an increase in coliforms during cheese production from raw milk (7.16 CFU/mL) and post pasteurization (<1 CFU/mL), to just before salting (3.63 CFU/g) on 10 separate days, indicating sources of coliform contamination within the plant (Temelli et al., 2006). This information informed in-plant sampling to investigate potential sources or harborage sites of coliforms in the facility.

Coliform levels in raw and thermally treated milk

Coliform levels in raw milk ranged from 1.87 log CFU/ml to 3.92 log CFU/ml over 6 production days, average range of 0.95 log CFU/ml between the low and high samples on individual days (Figure 3). Raw milk processed by Facility A is sourced from several hundred dairy farms and multiple silos are used for storage with a single day of processing. These factors likely contribute to variability in coliform counts. Coliform levels in raw milk were significantly higher at the beginning of the production day and lower at the end of the day (p-value 0.0061). Interestingly, coliform counts in raw milk were significantly lower before the mid-day wash when compared to after the midday wash (p-value <0.0005). The largest difference between coliform levels before and after midday was an increase of 1.4 log CFU/mL on day 2 Other studies show a wide range of coliform counts in raw milk, from 1.48 CFU/mL (Ranieri et al., 2009) to 7.16 CFU/mL (Temelli et al., 2006). During the midday wash, raw milk is held stagnant in unrefrigerated pipes prior to the pasteurizer for ~30 min as an abbreviated sanitation cycle is performed on equipment from just before the pasteurizer to immediately before the DMC (Figure 1). This time and temperature may not be sufficient for increases in coliform levels in stagnant raw milk.

Following sub-pasteurization heat treatment (67-70^{o} F, 28 sec), coliform levels in milk were reduced to <1 CFU/ml on all days at all sampling points (n = 48, data not shown). There have been limited studies on the efficacy of sub-pasteurization thermal treatments on the microbial profile of milk. Several studies have demonstrated the inability of sub-pasteurization treatments (60-74°C, 16-18 sec) to achieve a 5-log

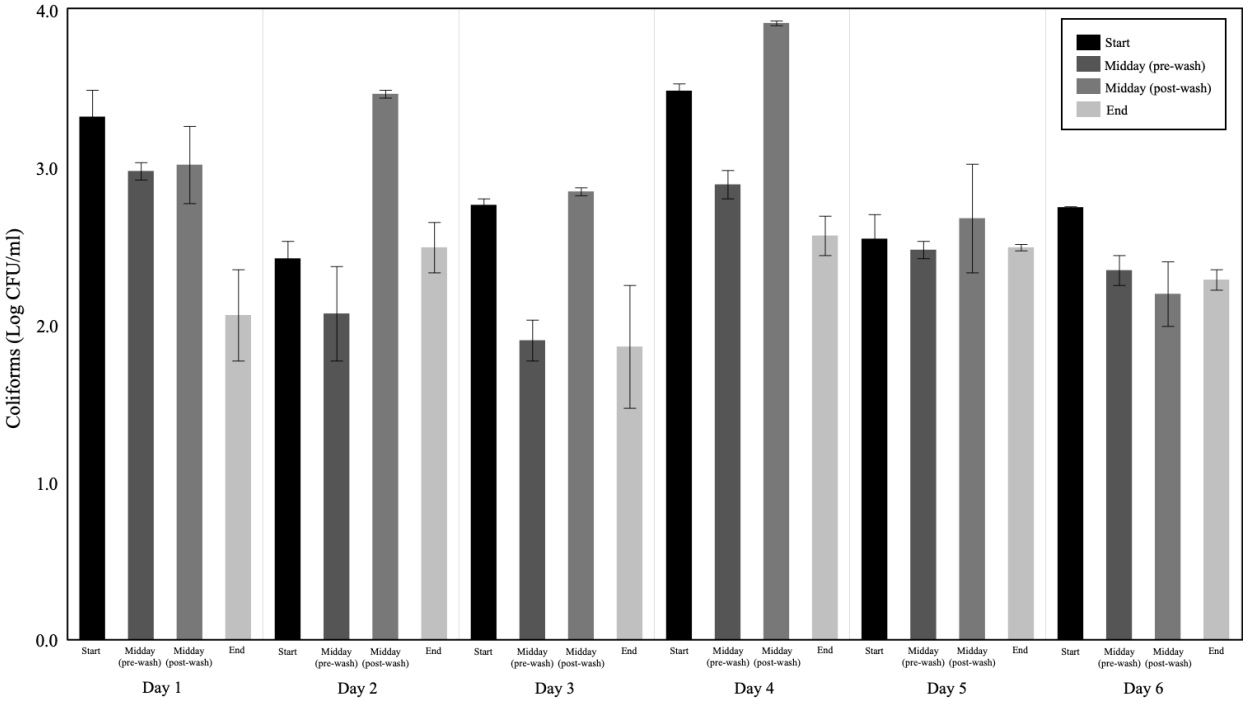

Figure 2.3. Coliform counts (log CFU/ml) in raw milk throughout the day on six production days. Results are presented as the mean ± standard error (n = 4). Days 1-3 and days 4-6 were consecutive. Coliform counts were determined counting typical colonies on MacConkey agar after incubation at 37°C for 24 hrs.

reduction of various foodborne pathogens, including *Salmonella* (D'Aoust et al., 1987), *L. monocytogenes* (Farber, JM, Sanders, GW, Malcolm, 1988), and *Campylobacter, Yersinia enterocolitica,* and *E. coli* O157:H7 (D'Aoust et al., 1988). The sub-pasteurization treatment used by the commercial cheese manufacturer occurs at a substantially lower temperature than previous studies; therefore, no significant reduction in coliform levels in milk would be predicted by this treatment. However, the commercial-scale application of this time/temperature combination consistently reduced natural coliform levels from up to 3.92 log CFU/ml to below the detection limit of the assay (<1 CFU/mL). These findings indicate that commercial-scale application of this sub-pasteurization treatment is effective at reducing coliform populations and may reduce pathogen levels in naturally contaminated milk.

Follow up sampling and analysis of heat-treated milk indicated that very low levels of coliforms (0.183 MPN/ml; 1 coliform/5.46 ml) survive sub-pasteurization heat treatment and move into later stages of processing (Table 1). Baranceli et al. (2014) also found intermittent, low levels of coliforms (1.2-1.9 log MPN/ml; 3/12 samples) in pasteurized milk used for cheese production, and (Trmčić et al., 2016) detected coliforms (1/16 samples) in finished product semi-hard cheese made from fully pasteurized milk, showing that coliforms can survive heat treatments and move on to contaminate the production process.

Very low levels of coliforms were detected in a whey sample from the vat (0.036 MPN/g; 1 coliform/27.8 ml). These very low levels of coliforms were detected in samples collected midway through the production day (before the midday wash) while their matched samples at the end of the day were below the detection limit (<0.03 MPN/ml; 1 coliform/33.3 ml). Coliform levels in whey and curd pumped from the vat into the DMC weir were very low at midday (<0.003 MPN/mL, 0.101 MPN/g, respectively) and near the same level at days end (0.032 MPN/mL, 0.009 MPN/g).

Taken together these results suggest that the sub-pasteurization heat treatment is consistently effective at reducing coliform levels and that production time (i.e., time since last sanitation) does not contribute to increased levels of coliforms in milk/whey after the pasteurizer nor in the vat.

Table 2.1. Prevalence and concentration of coliforms in milk, whey, and curd at different locations and times in the Cheddar cheese production system. Results represent the coliform levels during a single production day in a commercial production facility.

Sample Information		**Production Day Timing**	
Type	**Location**	**Mid-day (pre-wash)**	**End**
Milk (raw)	Silo port	1000 CFU/ml	800 CFU/ml
Milk (heat-treated)	Post pasteurizer line	0.183 MPN/ml	ND[a]
	Milk-to-Vat line	ND[a]	ND[a]
Whey	Vat (after starter addition)	0.036 MPN/ml	ND[c]
	Vat (before pumping to DMC[b])	ND[c]	ND[c]
	DMC (weir)	ND[d]	0.032 MPN/ml
	DMC (below drain belt)	0.105 MPN/ml	110,000 CFU/ml
Curd	Vat (before pumping to DMC)	ND[e]	ND[e]
	DMC (weir)	0.101 MPN/g	0.009 MPN/g
	DMC (before mill)	0.147 MPN/g	160 CFU/g

[a]ND = Coliforms not detected; Detection limit 1 coliform per 12 ml sample (-1.08 log CFU/g).
[b]DMC = Draining and matting conveyor
[c]ND = Coliforms not detected; Detection limit 1 coliform per 33.3 ml sample (-1.52 log CFU/ml).
[d]ND = Coliforms not detected; Detection limit 1 coliform per 132 ml sample (-2.12 log CFU/ml).
[e]ND = Coliforms not detected; Detection limit 10 CFU/g (1.00 log CFU/g).

Coliform growth in curd, whey, and on surfaces of the draining and matting conveyor (DMC) during the production day

As whey and curd are pumped into the DMC, coliform levels were very low (0.009-0.101 MPN/g; 1 coliform in 9.9 to 111 g of curd) midway through production and at the end of day (Table 1). These results demonstrated that fresh product entering the DMC was consistently low in coliform load. At the midday point there were low levels of coliforms in whey below the drain belt in the DMC (0.105 MPN/ml) and in curd exiting belt 2 just before the mill (0.147 MPN/g). However, by the end of the day, coliform levels increased dramatically to 5.04 log CFU/ml in whey below the drain belt and to 2.20 log CFU/g in curd at the mill. These differential levels of coliforms detected in curd at midday versus the end of the day confirm the patterns and levels identified in the historical production data discussed earlier.

Increases in coliform levels on food-contact surfaces (FCS) in the DMC during the production day were confirmed by further testing over three consecutive production days (Table 2). Coliforms were not detected on 8 out of 9 FCS samples in the DMC at the start of production. The single positive was from belt 2 with a very low level of coliforms detected (1 CFU/swab). Coliform levels increased dramatically on all three belts (drain belt, belt 1, and belt 2) in the DMC throughout the production day. On the drain belt, coliform levels went from undetectable at the beginning of production to 2.79 log CFU/swab at mid-day and had reached 5.53 log CFU/swab by the end of the production day. On the drain belt, there is a constant supply of warm whey (37°C/98°F) at a relatively high pH (6.0-6.3) making it favorable for coliform growth. Belt 1 followed a similar trend to the drain belt with coliform counts increased to 1.52 log CFU/swab at midday and to 5.46 log CFU/swab at the end of production. The curd temperature slightly increases through the DMC (99^{o}F) due to fermentation and the pH decreases to 5.5 on belt 1. On belt 2, coliforms were only intermittently detected at mid-day, but had increased to 3.47 log CFU/swab by the end of the day. Slower coliform growth on belt 2 is likely indicative of the decreased pH (5.0) and reduced moisture late in the Cheddaring process. Our sampling approach was unable to determine

Table 2.2. Coliform population levels on belt surfaces in the draining and matting conveyor (DMC) at different times throughout the processing day during Cheddar cheese production. Results are presented as the mean ± standard error (log CFU/swab) for three consecutive days of production in a commercial cheese facility.

Sample Location	**Coliform population levels (log CFU/swab)**		
	Production Day Timing		
	Start	**Mid-day**	**End**
Drain belt	ND[a]	2.79 ± 0.81	5.53 ± 0.10
Belt 1	ND	1.52 ± 0.29	5.46 ± 0.63
Belt 2	ND	(1/3)[b]	3.47 ± 0.39
Salter Belt	NA[c]	NA	0.57 ± 0.13

[a]ND = Coliforms not detected in any samples collected over three days; detection limit 1 CFU/swab.

[b]Coliforms detected (1 CFU/swab) in fractional number of samples collected (positive samples/total samples)

[c]NA = not analyzed; samples were not collected at these time points.

whether coliforms were growing on belt 2 or if they were transferred via curd or whey from the upper belts (drain belt or belt 1). Similarly, low levels of coliforms (0.57 log CFU/swab) were detected at the end of the day on the salter belt. Coliforms would be incapable of growing in the harsh conditions of the salter belt, so their detection demonstrates their transfer from one part of the production system to another as curd moves through the process.

Collectively, these data demonstrate that conditions (temperature, time, nutrients) in the DMC, particularly in the drain belt and belt 1 sections, promote significant growth of coliforms during the time course of a single production day (up to 18 hrs). As coliform levels increase in the DMC, the likelihood of detecting coliforms in fresh product increases. We believe that this is the first study to demonstrate coliform growth within the DMC. Other studies have investigated the microbial community dynamics relative to the production day. Sullivan et al., (2015) used 16S rRNA sequencing to assess the microbial shift over a production day in finished semi-hard brine-salted cheese and found higher diversity at the end of the production day, with some genera undetectable early in the day. Kable et al., (2019) used the same sequencing approach in milk processing, finding shifts in both diversity and abundance over a processing day, but also within specific milk processing equipment. Both of these studies indicate that there are bacterial genera that begin production at low and sometimes undetectable levels and, in the right conditions within specific equipment, grow throughout the day in certain parts of the production process to contaminate finished cheese later in the production day.

Evaluation of belt pieces for evidence of biofilm development

Due to the presence of low levels of detectable coliforms on one sample at the beginning of the day, there were concerns that the sanitation program may not be eliminating coliforms from the DMC. If coliforms were surviving through sanitation, then this could be an important source of coliforms that contributes to finished product contamination. To evaluate the likelihood of the DMC harboring coliforms between sanitation cycles, belt pieces were removed from DMC after a sanitation cycle and evaluated for the

presence of resident bacteria and of viable coliforms by scanning electron microscopy (SEM) and cultural methods, respectively. SEM micrographs of three locations on the underside of two unique pieces of the drain belt are shown in Figure 4. Degradation of the polypropylene belt surface is evident. Even after sanitation, bacteria remain as a few cells in crevices (Figure 4, top row), but also in high density in other belt defects (Figure 4, middle and bottom row). It is likely that as the belt degrades, sanitation programs become less effective at eliminating bacteria from surfaces within the DMC. Culturing methods confirmed that the bacteria that exist on these belt pieces after sanitation remain viable. Coliforms, specifically *Enterobacter* sp., *Escherichia fergusonii, Klebsiella pneumoniae,* and *K. variicola,* were detected on 7 out of the 32 belt sections (22%) that were analyzed by cultural methods (Table 3). *Escherichia* and *Klebsiella* were previously identified in finished cheese by the commercial producer's laboratory (personal communication), and 3 bacterial genus have been identified in other studies of finished cheese (Trmčić et al., 2016) Coliforms were detected on both side belt (1/8; 12.5%) and middle belt sections (6/24 25%) after sanitation. These results demonstrate that the sanitation program fails to eliminate bacteria, including coliforms, on a significant percentage of the drain belt pieces which have the potential to grow to high cell density on subsequent processing days.

The DMC is a complex combination of FCS and non-FCS surfaces that vary in surface material, shape, and accessibility. The approach to sanitizing the DMC is through the use of an automated clean-in-place system that delivers cycles of hot caustic, acid and detergent through fixed spray nozzles at fixed locations within the DMC. Swab analyses demonstrated the general efficacy of the sanitation program to reduce coliform load on easily accessed surfaces from relatively high levels at the end of the production shift (>5 log CFU/swab) to non-detectable (8 out of 9 swabs). However, a more thorough investigation of belt pieces demonstrated survival and harborage of coliforms, and potentially other bacteria, in cracks and crevices in a high percentage of drain belt pieces. Overall, the sanitation program is effectively reducing, but not eliminating, coliforms in the DMC. Taken with the historical pattern of multiple days in a row with detectable coliforms, it suggests that these survivors are an important source of contamination across

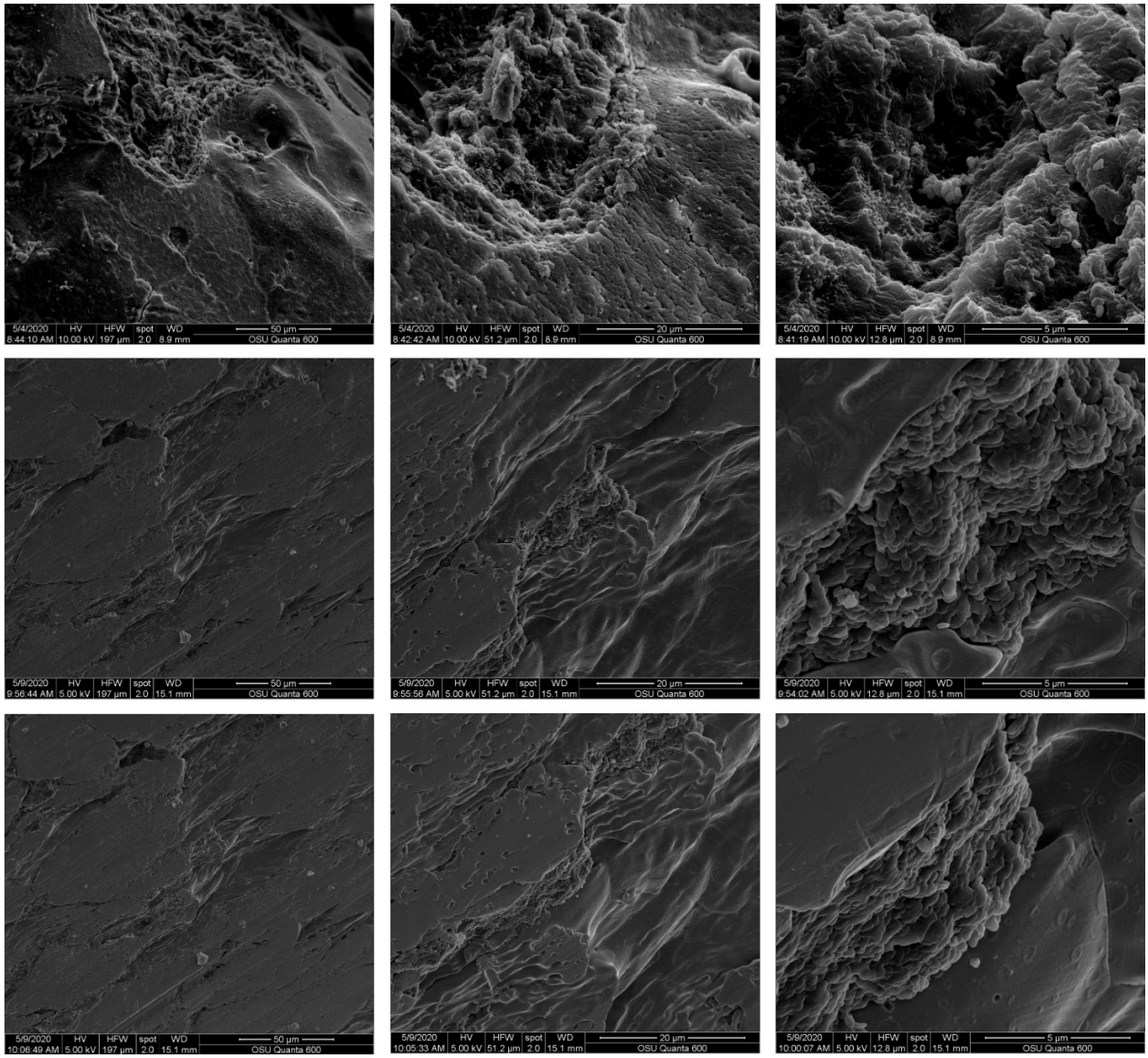

Figure 2.4. Scanning electron micrographs of underside of the drain belt conveyor after sanitation. From right to left, the magnification of a single point is increased on areas of interest with clusters of bacterial cells. Each row of images is a different area of interest. The middle and bottom row are two nearby areas of interest on the same belt piece.

Table 2.3. Prevalence and identification of coliforms from drain belt sections from the draining and matting conveyor (DMC) after sanitation and disassembly.

Location of belt section	**Prevalence of coliforms on belt sections**	**16S rRNA Identification**
Edge of belt	1/8 (12.5%)	*Escherichia fergusonii* *Klebsiella variicola*
Middle of belt	6/24 (25%)	*Enterobacter* sp. *Escherichia fergusonii* *Klebsiella pneumoniae* *Klebsiella variicola*

production days. We were able to demonstrate the cracks and crevices exist and harbor bacteria after they have been in use for more than 1 year. An important follow up would be to determine how long these belts can be used before significant defects form which could guide the replacement schedule. It would also be helpful to determine what is causing the formation of these defects and potentially modify practices (sanitation chemicals, etc) to improve the stability of these belts and extend their usable lifetime.

CONCLUSIONS

Intermittent coliform detection in finished product Cheddar cheese increased as the production day (time since sanitation) extended. Extensive in-plant sampling over multiple days of production demonstrated that significant growth of coliforms occurs during the production day in the primary draining sections (drain belt and belt 1) of the DMC. As coliform populations increase on FCS sections within the DMC, curds in contact with the belts likely accumulate significant numbers of coliforms which leads to detection in finished product. Using culturing methods coupled with SEM, two sources of coliforms were identified: low-level survivors in heat-treated milk and low-level survivors on sanitized surfaces in the DMC. Further research is necessary to determine the relative impact of these two sources to support strategic mitigation.

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CHAPTER 3 – GROWTH POTENTIAL AND BIOFILM DEVELOPMENT OF NON-STARTER BACTERIA ON SURFACES EXPOSED TO A CONTINUOUS WHEY STREAM

Title: Growth potential and biofilm development of non-starter bacteria on surfaces exposed to a continuous whey stream

Running head: Non-starter growth in whey stream

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ABSTRACT

Commercial Cheddar cheese production uses an automated, continuous production system that provides favorable conditions for specific undesirable bacterial subpopulations in certain sections of the processing system. The draining and matting conveyor (DMC) is a large, fully enclosed series of conveyor belts that separates curd and whey on the first drain belt and performs the Cheddaring process in subsequent sections. In a previous study, we demonstrated that coliforms increase in the draining section of the DMC (pH 6.0-6.3, 36°C) over a typical 18-hr production shift and can lead to detectable coliforms in finished cheese. Sampling at the commercial plant indicated two sources of coliforms: i) sub-pasteurized whey and curd entering the DMC and ii) surfaces in the DMC after sanitation; however, mitigation of these sources would require a different approach. The aim of this study was to investigate whether naturally low levels of coliforms in contaminated whey increase in the bulk liquid and attach to different materials within 18 hrs in the DMC. A laboratory-scale system was created to mimic the conditions of the initial draining section of the DMC and consisted of single pass, naturally contaminated whey (pH 6.3, 35°C) flowing through a bioreactor (1.11 L/h) containing coupons of surface types in the DMC (stainless steel and polypropylene). Whey inside the bioreactor chamber and surface coupons were enumerated for bacterial subpopulations on selective media (MacConkey, *Pseudomonas* Isolation, CHROMagar™ *Acinetobacter*, Rogosa SL and MRS agars) for planktonic and attached bacteria, respectively, at 0, 12, 15 and 18 hrs. Bacterial isolates were identified by 16S rDNA sequencing. Non-starter bacteria present in the whey at 0 h included coliforms (*Enterobacter*), *Pseudomonas*, and *Acinetobacter* (0.80, 2.55, 2.32 log CFU/mL respectively), with each increasing significantly in whey (6.18, 7.00. 5.89 log CFU/mL) and on coupons (5.20, 6.85, 5.29 log CFU/cm^2, respectively) after 18 hrs in the continuous flowing

system. SEM confirmed bacterial attachment and on both surfaces with early biofilm development on polypropylene coupons by 18 hrs. Results from this lab-scale study demonstrated that naturally low levels of coliforms entering the DMC in the whey could replicate within the conditions of the draining section of the DMC to the levels found in the commercial production environment.

Keywords: Whey, Biofilm, Bioreactor, Dairy

INTRODUCTION

Cheddar cheese is produced all over the world and one of the most widely consumed cheeses in the USA (Gosalvitr et al., 2019). The microbial ecology of cheese is dynamic and often contributes both positive and negative impacts for cheese quality. Controlling bacteria with negative quality and safety outcomes is a challenge for cheese producers, especially during large scale production. Milk used to make cheese also has a diverse microbial ecology that includes low levels of *Enterobacteriaceae*, *Pseudomonas*, *Acinetobacter*, *Bacillus*, and a wide range of other bacteria (Li et al. 2018). The majority of these bacteria are reduced to low levels (1.69-2.18 CFU/ml) during thermal treatment of milk; however, at commercial-scale these low levels may represent a significant source of contamination (Ranieri et al., 2009). In addition, subpopulations of bacteria can establish themselves in specific environmental niches in the processing environment where they can proliferate. Effective solutions to manage bacterial ecology in a production environment require recognition and prioritization of the sources of the initial contamination. Production schedules and sanitation frequency should be designed to mitigate substantial growth of bacterial populations that could lead to food safety and/or quality issues, including managing indicator organisms (i.e., coliforms). However, even more ideally, the source of these problematic bacteria would be eliminated completely.

The draining and matting conveyor is a large, fully enclosed piece of equipment that is designed to automate the Cheddaring process. Curds and whey are pumped into the top of the DMC and move down a series of three conveyors as the curd acidifies and is stretched and stacked as whey drains off. The curd will exit the DMC after approximately 90 min and will be milled, salted, and pressed before going into the aging room. As curd moves and folds through the DMC,

fermentation is rapid, causing a pH drop and resulting in whey expulsion. As product exits the DMC, the pH of the curd pH will be approximately 5.2. This reduced pH along with subsequent salt addition will inhibit growth of many bacterial subgroups after pressing and through aging (Ong et al., 2017).

A previous investigation into the intermittent detection of coliforms in Cheddar cheese at a commercial facility identified a significant growth niche in the draining and matting conveyor (DMC) (Selver et at., 2020). As whey and curd enter the DMC, the starter lactic acid bacteria are active, but have not yet acidified the curd. Therefore, the drain belt section of the DMC hosts a favorable environment (98°F/37°C, pH 6.3) with a constant flow of fresh nutrients for 18 hrs. Coliform levels in the drain belt section of the DMC increase dramatically from <1 CFU/mL to 5.04 log CFU/mL throughout the course of an 18-hr production day. The daily sanitation regime reduced levels to <1 CFU/swab; however, coliform levels would bloom again to similar levels by the end of the following day.

Further sampling in the commercial environment identified two potential sources of coliforms in the production system. Previous research (Selover et al., 2020) demonstrated that low levels (<1 CFU/mL) of coliforms were present in heat-treated milk entering the vat, but also persisted at low levels in food contact surfaces in the DMC after sanitation. To support industry decision-making to mitigate challenges associated with non-starters, it is important to determine which of these bacterial sources contribute to high cell density in the DMC within the production period. The complexity of multiple sources of non-starter bacteria in the commercial processing environment made it nearly impossible to determine their relative contribution to the ecosystem. Therefore, an alternative model system was necessary to determine whether naturally low levels

of non-starter bacterial populations could grow to high cell density within the timeframe of a production shift (18 hrs).

The objective of the study was to determine if low level bacterial contaminants in whey can increase to levels found in previous studies (Selover et al., 2020) in a continuously flowing whey system similar to a DMC, and if bacterial attachment can occur on surfaces in these conditions and in an 18 hr timeframe. This study also aimed to measure the length of time necessary to achieve this growth and attachment, and if this time frame matches a common 18 h production schedule in commercial cheese production.

MATERIALS AND METHODS

Simulated Whey Draining System

A laboratory-scale system (Figure 1) was created to mimic the conditions of the initial draining section of a draining and matting conveyor (DMC) used in commercial Cheddar cheese processing facilities. The system was designed to flow whey over representative food contact surfaces while maintaining the elevated pH (6.0) and temperature (95°F) of fresh whey entering the DMC. Bulk input whey was held in a 20 L carboy at 4°C throughout the experiment. Using a peristaltic pump (Masterflex L/S Precision Modular Pump Cole-Parmer, Vernon Hills, IL), whey was pumped (1.11 L/h) through silicone tubing (#17 Masterflex L/S platinum-cured, Cole-Parmer, Vernon Hills, IL) submerged in a water bath to temper the whey to 35°C before entering the 500 mL CBR 90 CDC Bioreactor (Biosurface Technologies, Bozeman MT). The bioreactor was housed in a 35°C incubator and whey was constantly stirred throughout the experiment. The constant input of whey into the bioreactor forced “spent” whey through the overflow outlet and

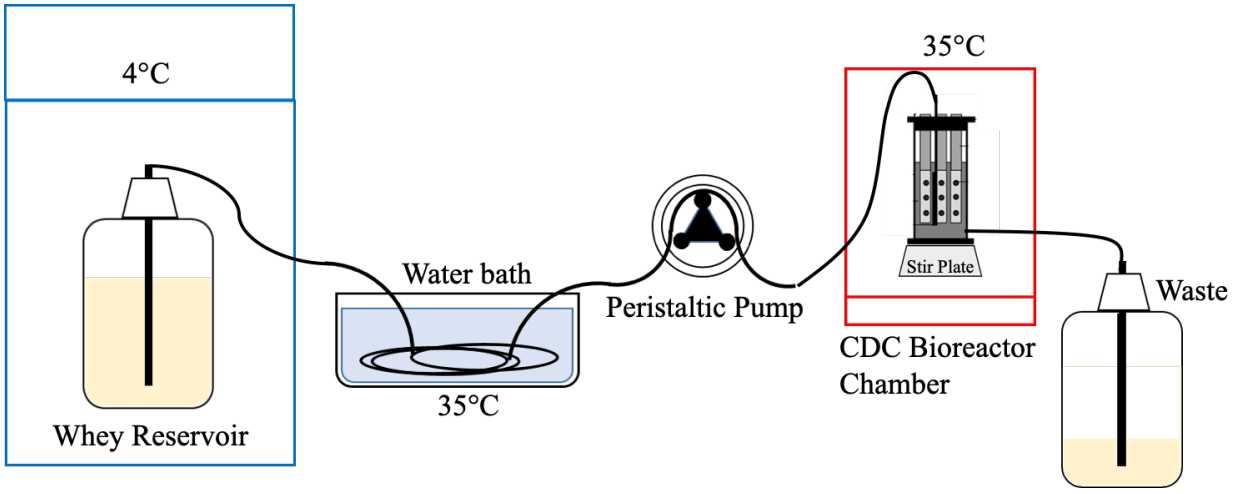

Figure 3.1. Laboratory-scale system used to mimic environmental conditions within the draining and matting conveyor (DMC) during commercial-scale Cheddar cheese production. The system was run continuously for up to 18 h. The bioreactor contained coupons made of materials representing food contact surfaces (stainless steel, polypropylene, high density polyethylene).

was collected in a second 20 L carboy. Waste whey was sterilized by autoclaving prior to disposal. Prior to each experiment, the bioreactor rods were randomly loaded with coupons (stainless steel and polypropylene; 2.53 cm^2) and the bioreactor system, including tubing, was assembled and sterilized in the autoclave (121°C, 15 min).

Whey Collection and Processing

Fresh whey was sourced from Oregon State University's Beaver Classic Creamery (Corvallis, OR) during the production of Cheddar cheese. Beaver Classic Cheddar cheese is made with the following starter culture mixture: *Lactococcus lactis* subsp. *lactis* biovar diacetylactis, *L. lactis* subsp. *lactis, L. lactis* subsp. *cremoris,* and *Streptococcus thermophilus*. After the cook/fermentation steps, whey (pH 6.35) was drained from the vat and collected into sterile 20 L carboys (Nalgene, Rochester, NY). Whey was immediately transferred to the -20°C freezer to arrest fermentation and acidification. Once the whey temperature was reduced to 4°C (~3 h), it was moved to the refrigerator (4°C) and used within 48 h. The whey carboy was transferred to the incubator (4°C) the day of the experiment, connected to the bioreactor, and ran continuously for 18 h. The entire experiment was replicated three times using whey collected from three different production days.

Microbiological Analysis - Whey

Whey samples were collected from the refrigerated carboy at the beginning of the experiment and from the bioreactor chamber after 12, 15, and 18 hrs after initiating the pump. The pH of whey samples was determined using a portable food and dairy pH meter (Apera Instruments, Columbus, OH). Microbial subpopulations in the whey were determined using standard serial

dilution (0.1% BPW, Neogen, Lansing, MI) followed by plating on De Man, Rogosa, and Sharpe (MRS) agar (Neogen), Rogosa SL agar (HiMedia Laboratories, West Chester, Pennsylvania), MacConkey agar (Neogen), *Pseudomonas* Isolation agar (PIA; Neogen), and CHROMagar™ *Acinetobacter* (DRG International, Springfield, NJ). MRS and Rogosa SL plates were incubated in a hypoxic environment (Bactrox, Sheldon Manufacturing Inc., Cornelius, OR) at 35°C for 48-72 h prior to enumeration. The remaining media were incubated at 37°C for 24-72 h prior to enumeration. Typical colonies for each agar media were counted and population levels were calculated and reported as log CFU/ml.

Microbiological Analysis - Coupons

At each time point (12, 15 and 18 hrs), coupons of each material (n = 3) were removed for bacterial enumeration and imaged via scanning electron microscopy. For bacterial enumeration, coupons were washed with phosphate buffered saline (PBS, Sigma-Aldrich, St. Louis, MO) to remove any planktonic cells and transferred to 50 ml conical tubes containing 6 g of glass beads (3 mm) and 3 ml PBS with 1% Tween 80 (Sigma-Aldrich, St. Louis, MO). Tubes containing coupons were vortexed for 5 min to facilitate removal of attached bacteria. Enumeration was accomplished by serial dilution with spread plating on growth media with incubation as described in previous section. Cell density on coupons were reported as log CFU/cm^2.

One coupon of each surface type for each time point was removed, rinsed with PBS and placed in SEM fixative (1% Paraformaldehyde and 2.5% Glutaraldehyde in 0.1M Sodium Cacodylate) and refrigerated overnight. Samples were prepared by the OSU Core Microscopy Facility (Corvallis, OR) by dehydrating with ethanol and critical point dryer (EMS 850, Electron Microscopy Sciences, Hatfield, PA). Samples were mounted and sputter coated with 60% gold,

40% Palladium (Cressington 108A, Ted Pella, Redding, CA) and SEM imaging was performed using the FEI Quanta 600F eSEM (Thermo Fisher, Waltham, MA) at the OSU Core Microscopy Facility (Corvallis, OR) using 5.00 kV with a 10.7 mm working distance.

Isolate Identification – 16S rDNA Sequencing

Typical colonies from each selective media were chosen in duplicate and isolated from whey and biofilm enumerations representing both surface types. Isolates were transferred to Tryptic Soy Broth with 3% yeast extract (TSBYE, Neogen) and incubated at 37°C for 24 h. Following incubation, cultures were mixed with glycerol (Fisher Scientific, Hampton, NH) to achieve a final concentration of 35% (v/v) and stored at -80°C.

Bacterial isolates were revived from -80°C storage by transfer into TSBYE and incubated at 37°C for 24 h. Cultures were diluted (1:10) in DNAse-free water (Invitrogen, Carlsbad, CA) and heated at 80°C for 30 min to create crude lysates. Alternatively, DNA extraction was accomplished using the DNeasy blood and tissue kit (Qiagen, Germantown, MD) following manufacturer's instructions. Crude lysates or DNA extractions were used as the templates for PCR to amplify the 16S rDNA gene using 27F/1492R universal primers (Lane, 1991). PCR was performed in 25 μL reactions using Platinum Hot-Start Master Mix (Invitrogen, Carlsbad, CA). PCR conditions were as follows: initial denature at 95˚C for 5 minutes, followed by 30 cycles of 95˚C for 30 seconds, 51˚C for 30 seconds, and 72˚C for 2 minutes, and a final extension at 72˚C for 10 minutes. Successful PCR amplification was confirmed by gel electrophoresis (1% agarose gel, 10 V/cm). PCR products were cleaned with the DNA Fragment Extraction Kit (IBI Scientific, Dubuque, IA). Cleaned amplicons were submitted to OSU's Center for Genome Research and Biocomputing (CGRB, Corvallis, OR) for Sanger sequencing using both 27F and

1492R primers on an ABI 3730 Capillary Sequencer. Raw sequencing reads were processed using SeqTrace (Stucky, 2012) to create consensus sequences. Bacteria were identified at the genus (95% similarity) or species (99% similarity) level using the 16S-based ID function of EzBioCloud (Yoon et al., 2017).

RESULTS

Microbiological profile changes in continuously flowing whey over 18 hours.

Bacterial subpopulation cell densities in continuously flowing whey in the bioreactor over an 18-hour period are shown in Figure 1. Lactic acid bacteria, presumably starters (*L. lactis* and *S. thermophilus*), were enumerated on MRS agar and maintained a near constant cell density (7.18-7.85 Log CFU/ml) throughout the 18-hr period. *Pseudomonas* and *Acinetobacter* subpopulations were present in the fresh whey at populations of 2.55 and 2.32 log CFU/g, respectively. Both of these subpopulations increased significantly throughout the 18 hr flow to achieve cell densities of 7.00 and 5.89 log CFU/ml, respectively. As enumerated on MCA, low levels of coliforms (0.80 log CFU/ml) were present in the fresh whey and increased to 6.18 log CFU/ml within 18 hrs. Very low levels of non-starter lactobacilli (0.42 log CFU/ml) were detected in fresh whey using Rogosa SL agar. Interestingly, the lactobacilli subpopulation remained stable throughout the 18 hr flowing conditions. Throughout the course of the 18-hr flow, chamber whey pH was maintained at pH >5.9 which demonstrated the turnover of whey in the bioreactor was sufficient to prevent acidification. Maintenance of the pH was critical for this system to be considered comparable to conditions of the draining section of a DMC. These results demonstrate that naturally low levels of several subpopulations of non-starter bacteria in whey can significantly increase in continuously flowing whey under conditions representative of the DMC.

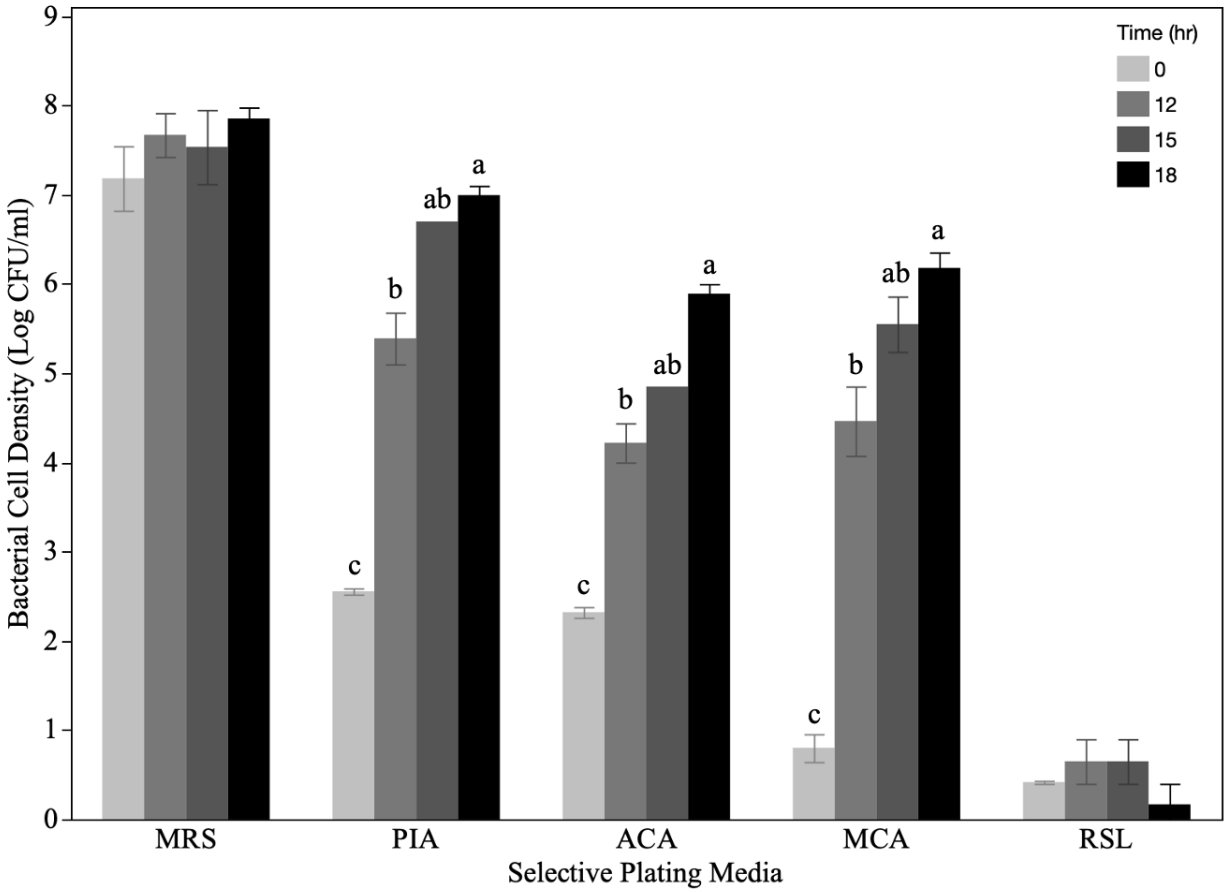

Figure 3.2. Changes in natural microbial subpopulations in continuously flowing Cheddar cheese whey through a bioreactor for up to 18 hours at 37°C . Selective media: MRS – de Man, Rogosa, and Sharpe Agar, PIA – *Pseudomonas* Isolation Agar, ACA – *Acinetobacter* CHROMAgar, MCA – MacConkey Agar, RSL - Rogosa SL Agar. Bars represent the mean and error bars indicate the standard error (n = 2 for PIA and ACA; n = 3 for all other media). Means with the same letter are not significantly different (p-value > 0.05) within selective media type (Tukey's HSD).

Microbiological profile changes on food contact surfaces exposed to continuously flowing whey for 18 hours.

Bacterial subpopulations associated with stainless-steel and polypropylene coupons during exposure to continuously flowing whey are shown in Table 1. Within 12 hrs, significant bacterial attachment of LAB (6.67-6.69 log CFU/cm^2), *Pseudomonas* (5.91-6.27 log CFU/cm^2), *Acinetobacter* (3.43-3.81 log CFU/cm^2), and coliforms (3.81-4.16 log CFU/cm^2) had occurred on both polypropylene and stainless-steel surfaces. Subpopulations of *Pseudomonas, Acinetobacter,* and coliforms increased significantly from 12 to 15 hrs, but did not significantly increase from 15 to 18 hrs. Representative isolates from PIA, ACA, and MCA were confirmed to be members of the targeted subpopulations which included *Pseudomonas aeruginosa, Acinetobacter baumanii,* and *Enterobacter horaechei*, respectively. The LAB subpopulation on coupons increased over time; however, this increase was only statistically significant on stainless-steel surfaces between 12 hrs (6.67 log CFU/cm^2) and 18 hrs (7.47 log CFU/cm^2). Only very low levels of lactobacilli (0.65-0.80 log CFU/cm^2) were detected on coupons and did not increase throughout the 18-hr flow. Cell density of all subpopulations were similar on stainless steel and polypropylene surface coupons at all time points.

Bacterial attachment to polypropylene and stainless-steel coupons was confirmed using SEM (representative micrographs shown in Figure 3). After 18 hrs, the pattern and distribution of attached bacteria differed between stainless-steel and polypropylene. Bacterial attachment on stainless-steel coupons was evident; however, attachment was not confluent, instead small clusters of cells with one or two morphological types were observed. Micrographs of stainless-steel coupons showed morphologies characteristic of starter bacteria (*L. lactis* and *S.*

Table 3.1. Attachment and growth of natural microbial subpopulations on stainless-steel and polypropylene coupons in continuously flowing Cheddar cheese whey in a bioreactor for up to 18 hrs at 37°C. Values are represented as the mean (log CFU/coupon) ± standard deviation. Rows with different uppercase subscript letters indicate a significant difference in the subpopulation at different timepoints (p-value < 0.05).

Media[a]	**Surface**[b]	**12 hrs**	**15 hrs**	**18 hrs**	**Isolated species**
MRS	PP	6.69 ± 0.51	7.03 ± 0.32	7.27 ± 0.46	ND[c]
	SS	6.67 ± 0.37^{A}	7.26 ± 0.41^{AB}	7.47 ± 0.31^{B}	
PIA	PP	6.27 ± 0.25^{A}	7.22 ± 0.23^{B}	6.87 ± 0.44^{AB}	*Pseudomonas aeruginosa*
	SS	5.91 ± 0.47	6.27 ± 0.00	6.85 ± 0.60	
ACA	PP	3.81 ± 0.76^{A}	5.25 ± 0.17^{B}	5.29 ± 0.37^{B}	*Acinetobacter baumanii*
	SS	3.43 ± 0.76^{A}	4.90 ± 0.43^{B}	5.31 ± 0.20^{B}	
MCA	PP	4.16 ± 0.61^{A}	5.11 ± 0.27^{B}	5.20 ± 0.39^{B}	*Enterobacter horaechei*
	SS	3.81 ± 0.95^{A}	5.15 ± 0.25^{B}	5.37 ± 0.39^{B}	
RSL	PP	ND	ND	1.25 (1/6)	ND
	SS	0.68 (1/6)	ND	ND	

[a]Selective growth media: MRS – deMan, Rogosa, and Sharpe; PIA – *Pseudomonas* Isolation Agar, ACA – *Acinetobacter* CHROMagar, MCA – MacConkey Agar, RSL – Rogosa SL Agar.
[b]Surface typ: PP – polypropylene, SS – stainless-steel
[c]Not determined. Isolates from these media were presumed to be intentionally added starter cultures used in Cheddar cheese production.

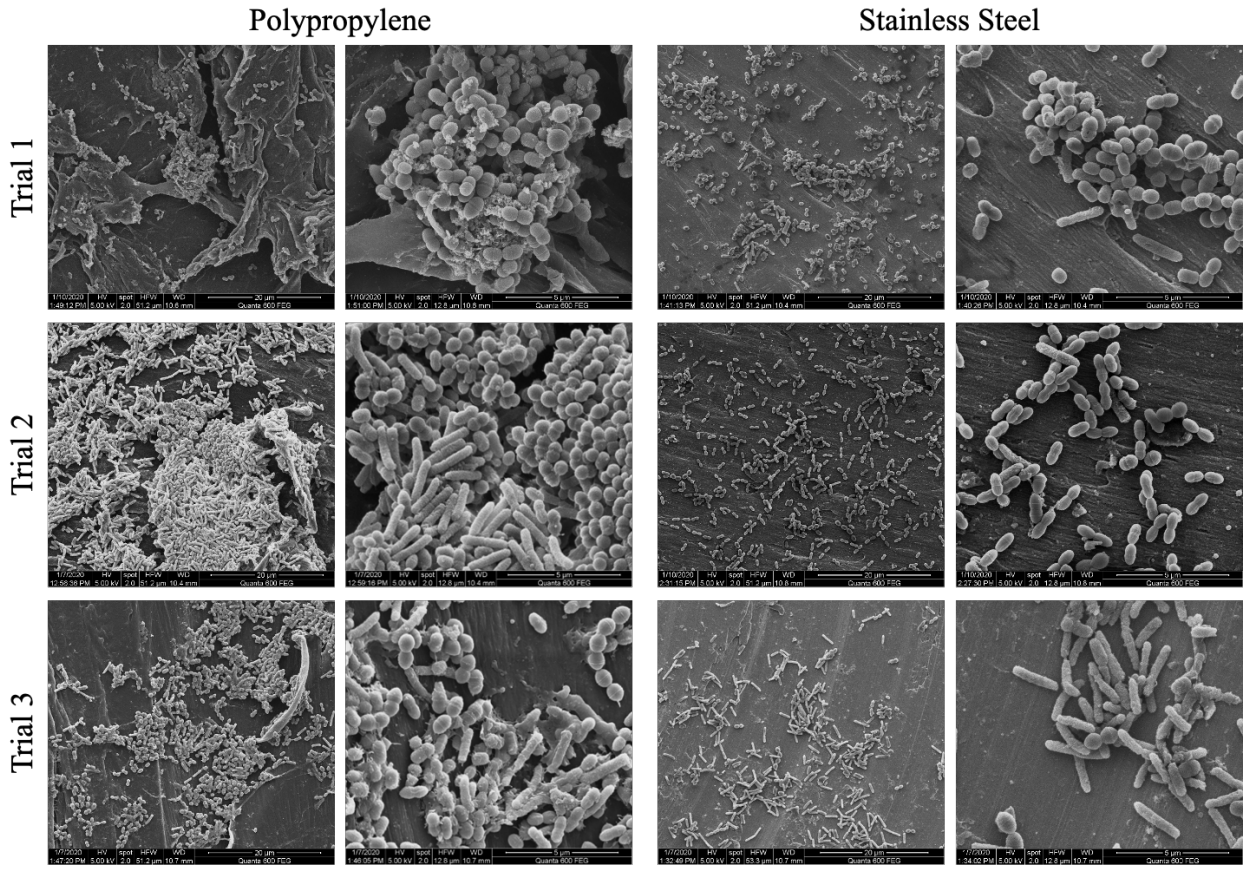

Figure 3.3. Scanning electron micrographs of bacterial attachment on polypropylene and stainless-steel coupons in continuously flowing Cheddar cheese whey after 18 hrs at 37°C.

thermophilus); however, bacilli were also observed, particularly in the micrograph from trial 3. Most of the cells on a stainless-steel surface within a field are of one morphology; however, each field contains several cells of a second morphological type. This suggests a random attachment from the whey to the stainless-steel surface after 18 hrs of whey flow.

Bacterial attachment on polypropylene is more pronounced with substantially larger clusters of closely associated cells. The micrograph of the polypropylene coupon from trial 1 shows a complex surface defect along with a large cluster of diplococci. The coupon from trial 2 shows nearly confluent growth on the polypropylene coupon surface, while also showing an interface of the three-dimensional growth of two bacterial populations: bacilli and diplococci. This arrangement suggests independent replication-in-place of these two subpopulations as opposed to random attachment from the whey to the coupon surface. The micrograph from trial 3 displays a field of cells of multiple cellular morphologies on the plane of the coupon and have not yet developed any three-dimensional structure.

DISCUSSION

The overall goal of this study was to determine if conditions within the draining section of the DMC would support significant growth of various bacterial subpopulations, including coliforms, in bulk whey and on surfaces. Our experimental system recreated the conditions inside the DMC, including a warm temperature, a constant flow of whey at a consistent pH (~6) that contained an active LAB starter that was naturally complicated by low levels of multiple bacterial subpopulations. This is the first reported attempt to create a laboratory-based system that mimics the conditions of one particular section of the DMC. The successful control and management of

the conditions of the DMC and the use of fresh Cheddar whey allowed us to characterize the development of specific subpopulations throughout a production shift.

Naturally occurring levels of non-starter bacteria can grow to high cell density in a continuously flowing whey system within the timeframe (18 hr) of a commercial cheese production shift. Analysis of fresh Cheddar whey revealed low, but quantifiable levels of various bacterial subpopulations, including *Pseudomonas, Acinetobacter,* and coliforms. Similar contaminant bacteria were found in another study by (Choi et al., 2020) investigating the microbial diversity of the OSU creamery. Several non-starter bacteria were found in this study, including *Pseudomonas* and *Escherichia coli,* detected before starter culture was added to pasteurized milk. Although these bacteria were not detected later in the cheese making process (after acidification), their presence is consistent with our results. The whey for this study was also collected from the OSU creamery, indicating the possibility of other subpopulations present in whey that we not enumerated in this study.

Pseudomonas, Acinetobacter, and coliforms, but not lactobacilli, were capable of reaching relatively high cell density (5.89-7.00 log CFU/ml) in bulk whey after 18 hrs. These subpopulations increased from <3 log CFU/ml within a dynamic, flowing system where the pH, temperature, and nutrient stream remained consistently hospitable for growth. Haugen et al., (2006) measured the growth rate of *Pseudomonas* and *Serratia* in skim milk and observed an 5-6 log CFU/mL increase after 18 h with <3 log CFU/mL starting cell density. The increase in our study occurred in spite of the relatively high turnover of bulk fluid in the bioreactor (pump rate 1.115 L/hr with a maximum volume of 500 ml within the bioreactor chamber). Increased

residence time of whey in the bioreactor, or within the DMC, would likely enhance the growth rate of these subpopulations.

This study demonstrated that bacteria attach and replicate on surface materials used in dairy processing environments within the timeframe of a single production day (18 hrs). LAB, *Pseudomonas, Acinetobacter,* and coliforms each demonstrated ability to attach to both polypropylene and stainless-steel surfaces under the time, temperature, pH, and nutrient conditions that exist in the draining section of the DMC. Selover et al. (2020) detected cell densities of 5.04 log CFU/mL in whey during in-plant sampling for coliforms in the draining section of a DMC after 18 h of production after pre-production coliform counts of <1 CFU/mL. These time frames and growth rates show that the lab-scale bioreactor in this study can be a useful model for the draining section of a DMC. This 18-hr time-frame was also important in Kable et al., (2019), who found *Pseudomonas* and *Acinetobacter* in milk processing equipment only exceeded 3.51 log CFU/mL when time between sanitation events was greater than 19 hrs. Previous studies of biofilm development indicate low abundant species can also facilitate biofilm development (Liu et al., 2017) which is important because whey can have a diverse microbial community with members at low cell densities (Randazzo et al., 2002).

Bacterial attachment and localized replication differed by surface type. SEM analysis revealed that distribution and organization of cell clusters differed by surface type. Polypropylene surfaces were more densely colonized and demonstrated local bacteria replication, particularly near defects or cracks. By comparison, bacterial attachment to stainless-steel was sparse and colonization was immature at the same time point (18 hrs). Other studies

comparing bacterial attachment on stainless steel and plastics found mixed results. Oulahal et al., (2008) observed *Listeria innocua* attached better to stainless steel than polypropylene in milk, whereas with *Staphylococcus aureus* the opposite was observed which shows the microbial ecology is also a factor in the rate of attachment.

Once a FCS hosts a significant population of bacteria, it can serve as a source of contamination for subsequently processed product. Flint et al., (2001) demonstrated this using a similar bioreactor apparatus with milk as the fluid matrix. Pasteurized milk passing through the bioreactor was contaminated by contact with stainless-steel coupons hosting a mature *Bacillus* biofilm, showing the potential for biofilms to contaminate passing bulk liquids.

CONCLUSIONS

This study is important in identifying the ability of naturally low levels of diverse bacterial subpopulations in whey to significantly grow and attach to surfaces under conditions representative of the draining section of the DMC. This lab scale model system highlights the importance of managing the production and sanitation schedule, and is consistent with other in-plant research showing that significant microbial growth occurs on whey after 12 hrs of continuous production.

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CHAPTER 4 - CONCLUSION

There are sections within the production environment of commercial Cheddar cheese production that provide a suitable combination of pH, temperature, moisture and nutrients to support significant growth of coliforms and other non-starter bacteria during the production day. The study demonstrated that conditions within the DMC, particularly the drain belt and belt 1, encourage growth of coliforms to levels that lead to intermittent contamination of cheese produced during the latter half of the production day. End of day sanitation inside the DMC is effective in reducing coliform levels on accessible surfaces but is ineffective in eliminating bacteria harbored in cracks and defects in food contact surfaces. The source of these coliforms within the production environment include low levels of survivors in heat-treated milk as well as low levels of survivors within cracks and defects in sanitized belts of the DMC. This study demonstrated in a lab-scale model that bacteria, including coliforms, can increase to detectable and potentially problematic levels in the continuous whey stream and on food contact surfaces in the draining section of the DMC.

This study clearly demonstrates that coliforms increase to high levels in the draining section of the DMC and that this is likely the primary section of cheese production that leads to detection of coliforms in finished product. There are two main approaches to mitigate this problem: modify processes to mitigate or prevent growth or eliminate the source(s). Historical production data and time of day sampling demonstrated that coliform levels increase to a problematic level around cheesemake 22 (16-17 hrs of production). A sanitation break of the DMC prior to cheesemake 22 could mitigate the problem. Alternatively, an approach to eliminate the sources could allow for longer production runs without a sanitation break. To reduce low levels of coliforms coming into

the DMC with product, the thermal treatment of the milk either during “heat shock” or in the vat could be increased. To eliminate low levels of survivors on cracked belts, more frequent monitoring of belt quality or more frequent belt replacement would be valid approaches.

Cheese producers experiencing intermittent coliform detection in finished product should evaluate the timing within the production day to determine patterns. If this is the case, processors may consider adjusting their sanitation program or frequency of the problematic production zone. Controlling coliform growth in production equipment is important to reduce potential contamination in finished product cheese. This study provides information on potential locations of bacterial contamination “hot spots” like the DMC, and can assist in the diagnosis of coliform contamination problems during Cheddar cheese production

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APPENDIX A – SUPPLEMENTAL SANGER SEQUENCING DATA FOR CHAPTER 2

Bacterial isolates from post-CIP belt pieces from the Draining and Matting Conveyor with typical morphology on MacConkey Agar.

T391 – Side belt after CIP, 2/24/20

NTGNAGTCGAGCGGTAGCACAGAGAGCTTGCTCTCGGGTGACGAGCGGCGGACGGGTGAGTAATGTCTGGGAAACTGCCT
GATGGAGGGGGATAACTACTGGAAACGGTAGCTAATACCGCANAANGTCGCAAGACCAAAGTGGGGGACCTTCGGGCCTC
ATGCCATCAGATGTGCCCAGATGGGATTAGCTAGTAGGTGGGGNANNGGCTCACCTAGGCGANNNCNGATCCCNTAGCTG
GTCTGAGAGGATGACCAGCCACACTGGAACTGAGACANCGGTCCAGACTCCTACGGNGAGNGCAGCAGTGGGGAATATTG
CACNAATGGGCGCAAGCCTGATGCAGCCATNGCCGCGTGTGTGAAGAAGGCCTTCGGGTTGTAAAGCACTTTCAGCGGGG
AGGAAGGCGNTNAGGTTAATAACCTTGTCGATTGACGTTACCCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCG
CGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTCTGTCAAGTCGGATGTGA
AATCCCCGGGCTCAACCTGGGAACTGCATTCGAAACTGGCAGGCTAGAGTCTTGTAGAGGNGGGGTAGAATTCCAGGTGT
AGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACAAAGACTGACGCTCAGGTGCGA
AAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGATTTGGAGGTTGTGCCCTTGAG
GCGTGGCTTCCGGAGCTAACGCGTTAAATCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGG
GGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTGGTCTTGACATCCACAGAAC
TTTCCAGAGATGGATTGGTGCCTTCGGGAACTGTGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTTGTGAAATGTT
GGGTTAAGTCCCGCAACGAGCGCAACCCTTATCCTTTGTTGCCAGCGGTNNGGCCGGGAACTCAAAGGAGACTGCCAGTG
ATAAACTGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGACNNNNCAGGGCTACACACGTGCTACAATGG
CATATACAAAGAGNAAGCGACCTCGCGAGAGCAAGCGGACCTCATAAAGTATGTCGTAGTCCGGATTGGAGTCTGCAACT
CGACTCCATGAAGTCGGAATCGCTAGTAATCGTAGATCAGAATGCTACGGTGAATACGTTCCCGGGCCTTGTACACACCG
CCCGTCACACCATGGGAGTGGGTTGNAAAAGAAGTAGGTAGCNN

Hit taxon name	Hit strain name	Accession	Similarity	Variation Ratio	Completeness (%)
Klebsiella variicola subsp. variicola	DSM 15968(T)	CP010523	99.63	5/1368	100.0
Klebsiella pneumoniae subsp. ozaenae	ATCC 11296(T)	Y17654	99.63	5/1365	99.3
Klebsiella quasipneumoniae subsp. quasipneumoniae	01A030(T)	HG933296	99.63	5/1364	100.0
Klebsiella pneumoniae subsp. pneumoniae	DSM 30104(T)	AJJI01000018	99.56	6/1368	100.0
Klebsiella quasivariicola	KPN1705(T)	CP022823	99.56	6/1368	100.0

T392 – Side belt after CIP, 2/24/20

GTCGNNCGGTNACANNNNNNNNGCTTGNNNNNNTNCTGACGAGTGGCGGACGGGTGAGTAATGTCTGGGAAACTGCCTGA
TGGAGGGGGATAACTACTGGAAACGGTAGCTAATACCGCANAANGTCGCAAGACCAAAGAGGGGGACCTTCGGGCCTCTT
GCCATCGGATGTGCCCAGATGGGATTAGCTNGNNNGTGGGGTAACGGCTCACCTAGGCGACNGATCCCTAGCTGGTCTGA
GNNAGNGANTGACCNNAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCAC
AATGGGCGCAAGCCTGATGCANGCCATGCCGCGTGTATGAAGAAGGCCTTCGGGTTGTAAAGTACTTTCAGCGGGGAGGA
AGGGAGTAAAGTTAATACCTTTGCTCATTGACGTTACCCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGT
AATACGGAGGGTGCAAGCGTTAATCGGAANTTACTGGGCGTAAAGCGCACGCAGGCGGTTTGTTAAGTCAGATGTGAAAT
CCCCGGGCTCAACCTGGGAACTGCATCTGATACTGGCAAGCTTGAGTCTCGTAGAGGGGGGTAGAATTCCAGGTGTAGCG
GTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACGAAGACTGACGCTCAGGTGCGAAAGC
GTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGACTTGGAGGTTGTGCCCTTGAGGCGT
GGCTTCCGGAGCTAACGCGTTAAGTCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGC
CCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTGGTCTTGACATCCACGGAAGTTTT
CAGAGATGAGAATGTGCCTTCGGGAACCGTGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTTGTGAAATGTTGGGT
TAAGTCCCGCAACGAGCGCAACCCTTATCCTTTGTTGCCAGCGGTCCGGCCGGGAACTCAAAGGAGACTGCCAGTGATAA
ACTGGAGGAAGGTGGGGATGACNGTCAAGTCATCATGNNNNGCCCTTACGACCAGGGCTACACACGNTGCTACAATGGCG
CATACAAAGAGAAGCGACCTCGCGAGAGCAAGCGGACCTCATAAAGTGCGTCGTAGTCCGGATTGGAGTCTGCAACTCGA
CTCCATGAAGTCGGAATCGCTAGTAATCGTGGATCAGAATGCCACGGTGAATACGTTCCCGGGCCTTGTACACACCGCCC
GTCACACCATGGGAGTGGGTTGCAAAAGAAGNN

Hit taxon name	Hit strain name	Accession	Similarity	Variation Ratio	Completeness (%)
Escherichia fergusonii	ATCC 35469(T)	CU928158	99.85	2/1347	100.0
Shigella flexneri	ATCC 29903(T)	X96963	99.78	3/1347	100.0
Shigella sonnei	CECT 4887(T)	FR870445	99.70	4/1437	100.0
Shigella boydii	GTC 779(T)	AB273731	99.55	6/1347	100.0

T394 – Middle belt after CIP, 2/24/20

NTTNCTGACGAGTGGCGGACGGGTGAGTAATGTCTGGGAAACTGCCTGATGGAGGGGGATAACTACTGGAAACGGTAGCT
AATACCGCANAANGNCGCAAGACCAAAGAGGGGGACCTTCGGGCCTCTTGCCATCGGATGTGCCCAGATGGGATTAGCTN
GNNNGTGGGGTAACGGCTCACCTAGGCGANNNCGATCCCTAGCTGGTCTGAGAGGATGACCAGCCACACTGGAACTGAGA
CACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATNATTGCACNAATGGGCGCAAGCCTGATGCAGCCATGCCGCGT
GTATGAAGAAGGCCTTCGGGTTGTAAAGTACTTTCAGCGGGGAGGAAGGGAGTAAAGTTAATACCTTTGCTCATTGACGT
TACCCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACT
GGGCGTAAAGCGCACGCAGGCGGTTTGTTAAGTCAGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATCTGATACTG
GCAAGCTTGAGTCTCGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGG
CGAAGGCGGCCCCCTGGACGAAGACTGACGCTCAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTC
CACGCCGTAAACGATGTCGACTTGGAGGTTGTGCCCTTGAGGCGTGGCTTCCGGAGCTAACGCGTTAAGTCGACCGCCTG
GGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAT
GCAACGCGAAGAACCTTACCTGGTCTTGACATCCACGGAAGTTTTCAGAGATGAGNATGTGCCTTCGGGAACCGTGAGAC
AGGTGCTGCATGGCTGTCGTCAGCTCGTGTTGTGAAATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATCCTTTGT
TGCCAGCGGTCCGGCCGGGAACTCAAAGGAGACTGCCAGTGATAAACTGGAGGAAGGTGGGGATGACGTCAAGTCATCAT
GGCCCTTNNACGACCAGGGCTACACACGTGCTACAATGGCGCATACAAAGAGAAGCGACCTCGCGAGAGCAAGCGGACCT
CATAAANTGCGTCGTAGTCCGGATTGGAGTCTGCAACTCGACTCCATGAAGTCGGAATCGCTAGTAATCGTGGATCAGAA
TGCCACGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAGTGGGTTGCAAAAGAAGTAGNNTAG
CNTANCCNTCNGGAGGGN

Hit taxon name	Hit strain name	Accession	Similarity	Variation Ratio	Completeness (%)
Escherichia fergusonii	ATCC 35469(T)	CU928158	100.00	0/1353	100.0
Shigella flexneri	ATCC 29903(T)	X96963	99.93	1/1353	100.0
Shigella sonnei	CECT 4887(T)	FR870445	99.85	2/1353	100.0
Shigella boydii	GTC 779(T)	AB273731	99.70	4/1353	100.0

T395 – Middle belt after CIP, 2/24/20

NNTGCTGACGAGTGGCGGACGGGTGAGTAATGTCTGGGAAACTGCCTGATGGAGGGGGATAACTACTGGAAACGGTAGCT
AATACCGCANAANGNCGCAAGACCAAAGAGGGGGACCTTCGGGCCTCTTGCCATCGGATGTGCCCAGATGGGATTAGCTN
GTNNGTGGGGTAACGGCTCACCTAGGCGACGATCCCTAGCTGGTCTGAGAGGATNGACCAGCCACNACTGGANACTGAGA
CACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGT
ATGAAGAAGGCCTTCGGGTTGTAAAGTACTTTCAGCGGGGAGGAAGGGAGTAAAGTTAATACCTTTGCTCATTGACGTTA
CCCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGG
GCGTAAAGCGCACGCAGGCGGTTTGTTAAGTCAGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATCTGATACTGGC
AAGCTTGAGTCTCGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCG
AAGGCGGNCCCCTGGACGAAGACTGACGCTCAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCA
CGCCGTAAACGATGTCGACTTGGAGGTTGTGCCCTTGAGGCGTGGCTTCCGGAGCTAACGCGTTAAGTCGACCGCCTGGG
GAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGC
AACGCGAAGAACCTTACCTGGTCTTGACATCCACGGAAGTTTTCAGAGATGAGAATGTGCCTTCGGGAACCGTGAGACAG
GTGCTGCATGGCTGTCGTCAGCTCGTGTTGTGAAATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATCCTTTGTTG
CCAGCGGTCCGGCCGGGAACTCAAAGGAGACTGCCAGTGATAAACTGGAGGAAGGTGGGGATGACGTCAAGTCATCATGG
CCCTTACGACCAGGGCTACACACGNTGCTACANATGGCGCATACAAAGAGAAGCGACCTCGCGAGAGCAAGCGGACCTCA
TAAANTGCGTCGTAGTCCGGATTGGAGTCTGCAACTCGACTCCATGAAGTCGGAATCGCTAGTAATCGTGGATCAGAATG
CCACGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAGTGGGTTGCAAAAGAAGTAGNNTAGCN
TANCCTTCGNGAGGGNG

Hit taxon name	Hit strain name	Accession	Similarity	Variation Ratio	Completeness (%)

Escherichia fergusonii	ATCC 35469(T)	CU928158	100.00	0/1356	100.0
Shigella flexneri	ATCC 29903(T)	X96963	99.93	1/1356	100.0
Shigella sonnei	CECT 4887(T)	FR870445	99.85	2/1356	100.0
Shigella boydii	GTC 779(T)	AB273731	99.70	4/1356	100.0

T396 – Middle belt after CIP, 2/24/20

NNTGCAGTCGAGCGGTAGCACNGAGAGCTTGCTCTCGGGTGACGAGCGGCGGACGGGTGAGTAATGTCTGGGAAACTGCC
TGATGGAGGGGGATAACTACTGGAAACGGTAGCTAATACCGCANAANGTCGCAAGACCAAAGTGGGGGACCTTCGGGCCT
CATGCCATCAGATGTGCCCAGATGGGATTAGCTAGTAGGTGGGGTAANGGCTCACCTAGGCGACGATCCCTAGCTGGTCT
GAGAGGATGACCAGNCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCNAGCAGTGGGGAATATTGCACAA
NTNGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTGTGAAGAAGGCCTTCGGGTTGTAAAGCACTTTCAGCGGGGAGGAA
GGCNNNNNGGTTAATAACCTTGTCGATTGACGTTACCCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTA
ATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTCTGTCAAGTCGGATGTGAAATCC
CCGGGCTCAACCTGGGAACTGCATTCGAAACTGGCAGGCTAGAGTCTTGTAGAGGGGGGTAGAANTTCCAGGTGTAGCGG
TGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACAAAGACTGACGCTCAGGTGCGAAAGCG
TGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTNAACGATGTCGATTTGGAGGTTGTGCCCTTGAGGCGTG
GCTTCCGGAGCTAACGCGTTAAATCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCC
CGCACNAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTGGTCTTGACATCCACAGAACTTTCC
AGAGATGGATTGGTGCCTTCGGGAACTGTGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTTGTGAAATGTTGGGTT
AAGTCCCGCAACGAGCGCAACCCTTATCCTTTGTTGCCAGCGGNNNGGCCGGGAACTCAAAGGAGACTGCCAGTGATAAA
CTGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGACCAGGGCTACACACGTGCTACNAATGGCATATACA
AAGAGAAGCGACCTCGCGAGAGCAAGCGGACCTCATAAAGTATGTCGTAGTCCGGATTGGAGTCTGCAACTCGACTCCAT
GAAGTCGGAATCGCTAGTAATCGTAGATCAGAATGCTACGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACA
CCATGGGAGTGGGTTGCAAAAGAAGTAGGTAGCNTANCCTTCNGGAGGGN

Hit taxon name	Hit strain name	Accession	Similarity	Variation Ratio	Completeness (%)
Klebsiella variicola subsp. variicola	DSM 15968(T)	CP010523	99.71	4/1383	100.0
Klebsiella pneumoniae subsp. ozaenae	ATCC 11296(T)	Y17654	99.71	4/1380	99.3
Klebsiella quasipneumoniae subsp. quasipneumoniae	01A030(T)	HG933296	99.71	4/1379	100.0
Klebsiella pneumoniae subsp. pneumoniae	DSM 30104(T)	AJJI01000018	99.64	5/1383	100.0
Klebsiella quasivariicola	KPN1705(T)	CP022823	99.64	5/1383	100.0

T397 – Middle belt after CIP, 2/24/20

NNTGCAGTCGAGCGGTAGCNCAGAGAGCTTGCTCTCGGGTGACGAGCGGCGGACGGGTGAGTAATGTCTGGGAAACTGCC
TGATGGAGGGGGATAACTACTGGAAACGGTAGCTAATACCGCANAANGTCGCAAGACCAAAGTGGGGGACCTTCGGGCCT
CATGCCATCAGATGTGCCCAGATGGGATTAGCTAGTAGGTGGGGTANNGGCTCACCTAGGCGACGATNCCTAGCTGGTCT
GAGAGGATGACCAGCCACACTGGANACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCNACAA
TGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTGTGAAGAAGGCCTTCGGGTTGTAAAGCACTTTCAGCGGGGAGGAAGG
CGNTNAGGTTAATAACCTTGTCGATTGACGTTACCCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAAT
ACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTCTGTCAAGTCGGATGTGAAATCCCC
GGGCTCAANCTGGGAACTGCATTCGAAACTGGCAGGCTAGAGTCTTGTAGAGGGGGGTAGAANTTCCAGGTGTAGCGGTG
AAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACAAAGACTGACGCTCAGGTGCGAAAGCGTG
GGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTNAACGATGTCGATTTGGAGGTTGTGCCCTTGAGGCGTGGC
TTCCGGAGCTAACGCGTTAAATCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCCCG
CACNAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTGGTCTTGACATCCACAGAACTTTCCAG
AGATGGATTGGTGCCTTCGGGAACTGTGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTTGTGAAATGTTGGGTTAA
GTCCCGCAACGAGCGCAACCCTTATCCTTTGTTGCCAGCGNTCNGGCCGGGAACTCAAAGGAGACTGCNNNNNCAGTGAT
AAACTGGAGGAAGGTGGGGATGACGTNCAAGTCATCATGGCCCTTACGACCAGGGCTACACACGTGCTACAATGGCATAT
ACAAAGAGAAGCGACCTCGCGAGAGCAAGCGGACCTCATAAAGTATGTCGTAGTCCGGATTGGAGTCTGCAACTCGACTC
CATGAAGTCGGAATCGCTAGTAATCGTAGATCAGAATGCTACGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTC
ACACCATGGGAGTGGGTTGCAAAANAAGTAGGTAGCNNA

Hit taxon name	Hit strain name	Accession	Similarity	Variation Ratio	Completeness (%)
Klebsiella pneumoniae subsp. ozaenae	ATCC 11296(T)	Y17654	99.71	4/1369	99.3
Klebsiella quasipneumoniae subsp. quasipneumoniae	01A030(T)	HG933296	99.71	4/1367	100.0
Klebsiella variicola subsp. variicola	DSM 15968(T)	CP010523	99.64	5/1372	100.0
Klebsiella pneumoniae subsp. pneumoniae	DSM 30104(T)	AJJI01000018	99.56	6/1372	100.0
Klebsiella quasivariicola	KPN1705(T)	CP022823	99.56	6/1372	100.0

T398 – Middle belt after CIP, 2/24/20

NNGTCGAGCGGTAGCACAGAGAGCTTGCTCTCGGGTGACGAGCGGCGGACGGGTGAGTAATGTCTGGGAAACTGCCTGAT
GGAGGGGGATAACTACTGGAAACGGTAGCTAATACCGCANAANGTCGCAAGACCAAAGTGGGGGACCTTCGGGCCTCATG
CCATCAGATGTGCCCAGATGGGATTAGCTAGTAGGTGGGGNANNANGGCTCACNCTAGGCGACGATCCCTAGCTGGTCTG
AGAGGATGACCAGCCACACTGGAACTGAGACACGGTCCAGACTNNCCTACGGGAGGCAGCAGTGGGGAANTATTGCACAA
TGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTGTGAAGAAGGCCTTCGGGTTGTAAAGCACTTTCAGCGGGGAGGAAGG
NGNTNAGGTTAATAACCTTGTCGATTGACGTTACCCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAAT
ACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTCTGTCAAGTCGGATGTGAAATCCCC
GGGCTCAANCTGGGAACTGCATTCGAAACTGGCAGGCTAGAGTCTTGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGA
AATGCGTAGAGATCTGGAGGAATACCGGTGGCGAANGCGGNCCCCTGGACAAAGACTGACGCTCAGGTGCGAAAGCGTGG
GGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGATTTGGAGGTTGTGCCCNTGAGGCGTGGCT
TCNGGAGCTAACGCGTTAAATCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCCCGC
ACNAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTGGTCTTGACATCCACAGAACTTTCCAGA
GATGGATTGGTGCCTTCGGGAACTGTGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTTGTGAAATGTTGGGTTAAG
TCCCGCAACGAGCGCAACCCTTATCCTTTGTTGCCAGCGGTNNGNCCGGGAACTCAAAGGAGACTGCCAGTGATAAACTG
GAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGACCAGGGCTACACACGTGCTACAATGGCATATACAAAGA
GAAGCGACCTNNCGCGNAGANGCAAGCGGACCTCATAAAGTATGTCGTAGTCCGGATTGGAGTCTGCAACTCGACTCCAT
GAAGTCGGAATCGCTAGTAATCGTAGATCAGAATGCTACGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACA
CCATGGGAGTGGGTTGCAAAAGAAGTAGGTAGCNTANCCTTCNGGAGGGNGNT

Hit taxon name	Hit strain name	Accession	Similarity	Variation Ratio	Completeness (%)
Klebsiella variicola subsp. variicola	DSM 15968(T)	CP010523	99.85	2/1377	100.0
Klebsiella pneumoniae subsp. ozaenae	ATCC 11296(T)	Y17654	99.85	2/1374	99.3
Klebsiella quasipneumoniae subsp. quasipneumoniae	01A030(T)	HG933296	99.85	2/1373	100.0
Klebsiella pneumoniae subsp. pneumoniae	DSM 30104(T)	AJJI01000018	99.78	3/1377	100.0
Klebsiella quasivariicola	KPN1705(T)	CP022823	99.78	3/1377	100.0

T399 – Middle belt after CIP, 2/24/20

TGNNGTCGAGCGGTAGCNCAGANAGCTTGCTCTCGGGTGACGAGCGGCGGACGGGTGAGTAATGTCTGGGAAACTGCCTG
ATGGAGGGGGATAACTACTGGAAACGGTAGCTAATACCGCANAANGTCGCAAGACCAAAGTGGGGGACCTTCGGGCCTCA
TGCCATCAGATGTGCCCAGATGGGATTAGCTAGTAGGTGGGGNAANGGCTNCACCTNAGGCGACGATCCCTAGCTGGTCT
GAGAGGATGACCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATG
GGCGCAAGCCTGATGCAGCCATGCCGCGTGTGTGAAGAAGGCCTTCGGGTTGTAAAGCACTTTCAGCGGGGAGGAAGGCG
NNNAGGTTAATAACCTTGTCGATTGACGTTACCCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATAC
GGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTCTGTCAAGTCGGATGTGAAATCCCCGG
GCTCAACCTGGGAACTGCATTCGAAACTGGCAGGCTAGAGTCTTGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAA
TGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACAAAGACTGACGCTCAGGTGCGAAAGCGTGGGG

AGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGATTTGGAGGTTGTGCCCTTGAGGCGTGGCTTC
CGGAGCTAACGCGTTAAATCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCCCGCAC
AAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTGGTCTTGACATCCACAGAACTTTCCAGAGA
TGGATTGGTGCCTTCGGGAACTGTGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTTGTGAAATGTTGGGTTAAGTC
CCGCAACGAGCGCAACCCTTATCCTTTGTTGCCAGCGGTCNGGCCGGGAACTCAAAGGAGACTGCCAGTGATAAACTGGA
GGAAGGTGGGGATGACGTCAAGTCATCATNNNGGCCCTTACGACCAGGGCTACACACGTGCTACAATGGCATATACAAAG
AGAAGCGACCTCGCGAGAGCAAGCGGACCTCATAAAGTATGTCGTAGTCCGGATTGGAGTCTGCAACTCGACTCCATGAA
GTCGGAATCGCTAGTAATCGTAGATCAGAATGCTACGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCA
TGGGAGTGGGTTGCAAAAGAAGTAGGTAGCNTANCCTTCGGGAGGGNGNT

Hit taxon name	Hit strain name	Accession	Similarity	Variation Ratio	Completeness (%)
Klebsiella pneumoniae subsp. ozaenae	ATCC 11296(T)	Y17654	99.64	5/1384	99.3
Klebsiella quasipneumoniae subsp. quasipneumoniae	01A030(T)	HG933296	99.64	5/1382	100.0
Klebsiella variicola subsp. variicola	DSM 15968(T)	CP010523	99.57	6/1387	100.0
Klebsiella pneumoniae subsp. pneumoniae	DSM 30104(T)	AJJI01000018	99.50	7/1387	100.0
Klebsiella quasivariicola	KPN1705(T)	CP022823	99.50	7/1387	100.0

T402 – Middle belt after CIP, 2/24/20

NNTGCAGTCGAGCGGTAGCNCAGAGAGCTTGCTCTCGGGTGANGAGCGGCGGANGGGTGAGTAATGTCTGGGAAACTGCCTGAT
GGAGGGGGATAACTACTGGAAACGGTAGCTAATACCGCANAANGTCGCAAGACCAAAGTGGGGGACCTTCGGGCCTCATGCCAT
CAGATGTGCCCAGATGGGATTAGCTAGTAGGTGGGGNAANGGCTCACCTAGGCGACGATCNNCCTAGCTNGGTNCTGAGAGGAT
NNGACCAGCCACACTGGAACTNGAGACANCGGTCCAGACTCCTACGNGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGNCA
AGCCTGATGCAGCCATGCCGCGTGNTGTGAAGAAGGCCTTCGGGTTGTAAAGCACTTTCAGCGGGGAGGAAGGNGNTNAGGTTA
ATAACCTTGTCGATTGACGTTACCCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGC
GTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTCTGTCAAGTCGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGC
ATTCGAAACTGGCAGGCTAGAGTCTTGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATA
CCGGTGGCGAAGGCGGCCCCCTGGACAAAGACTGACGCTCAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTA
GTCCACGCCGTNAACGATGTCGATTTGGAGGTTGTGCCCTTGAGGCGTGGCTTCCGGAGCTAACGCGTTAAATCGACCGCCTGGG
GAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACG
CGAAGAACCTTACCTGGTCTTGACATCCACAGAACTTTCCAGAGATGGATTGGTGCCTTCGGGAACTGTGAGACAGGTGCTGCAT
GGCTGTCGTCAGCTCGTGTTGTGAAATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATCCTTTGTTGCCAGCGGNTCNGGCC
GGGAACTCAAAGGAGACTGCCAGTGATAAACTGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGACCAGGGCTA
CACACGTGCTANCAATGGCATATACAAAGAGAAGCGACCTCGCGAGAGCAAGCGGACCTCATAAAGTATGTCGTAGTCCGGATT
GGAGTCTGCAACTCGACTCCATGAAGTCGGAATCGCTAGTAATCGTAGATCAGAATGCTACGGTGAATACGTTCCCGGGCCTTGT
ACACACCGCCCGTCACACCATGGGAGTGGGTTGCAAAAGAAGTAGGTAGCNTANCCTTCNGGAGGGNGNT

Hit taxon name	Hit strain name	Accession	Similarity	Variation Ratio	Completeness (%)
Klebsiella variicola subsp. variicola	DSM 15968(T)	CP010523	99.71	4/1385	100.0
Klebsiella pneumoniae subsp. ozaenae	ATCC 11296(T)	Y17654	99.71	4/1383	99.3
Klebsiella quasipneumoniae subsp. quasipneumoniae	01A030(T)	HG933296	99.71	4/1381	100.0
Klebsiella pneumoniae subsp. pneumoniae	DSM 30104(T)	AJJI01000018	99.64	5/1385	100.0
Klebsiella quasivariicola	KPN1705(T)	CP022823	99.64	5/1385	100.0

T403 – Middle belt after CIP, 2/24/20

TGNNGTCGAGCGGNAGCNCNGAGAGCTTGCTCTCGGGTGACGAGCGGCGGACGGGTGAGTAATGTCTGGGAAACTGCCTG

ATGGAGGGGGATAACTACTGGAAACGGTAGCTAATACCGCANAANGTCGCAAGACCAAAGTGGGGGACCTTCGGGCCTCA
TGCCATCAGATGTGCCCAGATGGGATTAGCTAGTAGGTGGGGTANNGGCTCACCTAGGCGACGATCCCTAGCTGGTCTGA
GAGGNNATGANNCCAGCCACACTGGAACTGAGACACGGTCCAGACTCNCTACNGGGAGGCAGCAGTGGGGAATATTGCAC
AATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTGTGAAGAAGGCCTTCGGGTTGTAAAGCACTTTCAGCGGGGAGGAA
GGCGNTNAGGTTAATAACCTTGTCGATTGACGTTACCCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTA
ATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTCTGTCAAGTCGGATGTGAAATCC
CCGGGCTCAACCTGGGAACTGCATTCGAAACTGGCAGGCTAGAGTCTTGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGT
GAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGNCCCCTGGACAAAGACTGACGCTCAGGTGCGAAAGCGT
GGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGATTTGGAGGTTGTGCCCTTGAGGCGTGG
CTTCCGGAGCTAACGCGTTAAATCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCCC
GCACNAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTGGTCTTGACATCCACAGAACTTTCCA
GAGATGGATTGGTGCCTTCGGGAACTGTGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTTGTGAAATGTTGGGTTA
AGTCCCGCAACGAGCGCAACCCTTATCCTTTGNTTGCCAGCGGTCNGGCCGGGAACTCAAAGGAGACTGCCAGTGATAAA
CTGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTANCNNNNGACCAGGGCTACACACGTGCTACAATGGCATA
TACAAAGAGAAGCGACCTCGCGAGAGCAAGCGGACCTCATAAAGTATGTCGTAGTCCGGATTGGAGTCTGCAACTCGACT
CCATGAAGTCGGAATCGCTAGTAATCGTAGATCAGAATGCTACGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGT
CACACCATGGGAGTGGGTTGCAAAAGAAGTAGGTAGCNNANCCTNCGGGAGGNNG

Hit taxon name	Hit strain name	Accession	Similarity	Variation Ratio	Completeness (%)
Klebsiella pneumoniae subsp. ozaenae	ATCC 11296(T)	Y17654	99.64	5/1373	99.3
Klebsiella quasipneumoniae subsp. quasipneumoniae	01A030(T)	HG933296	99.64	5/1371	100.0
Klebsiella variicola subsp. variicola	DSM 15968(T)	CP010523	99.56	6/1376	100.0
Klebsiella pneumoniae subsp. pneumoniae	DSM 30104(T)	AJJI01000018	99.49	7/1376	100.0
Klebsiella quasivariicola	KPN1705(T)	CP022823	99.49	7/1376	100.0

T404 – Middle belt after CIP, 2/24/20

NTGNAGTCGANCGGNNNCNNNNANNNNNNNNNNNNNNNNNNNNNNNNNNNNGNNNNNNNNNNNNNNNNNNNNNNNNNNN
NNNNGNNNNNNNNNNNNNNNNNNNNNNNNTNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
NNNNNNNNNNNNNNNNNNNNNNNNNNNNTNNNNNNNNNGNNNNNNNNNNNNNNNNNNNNNNNNNNTNNNTNGNTNGNNNN
NNNNNNNNACCANNCNNNNNGNNNNNNNNNNNNNCNGNANNNNANNNNNNNNNGNNNNNNNNNNNNNNNNGNNNNNNNN
NNNGNNNNCNNGNCNNATGCANNCNTNNNNGTGNNNGNNNNGNNNNNNNNNNGNNTNNNAANNNNNTTNNNANNGNNNGN
NNNGCNNNNNAGNNANNNNNNNNTNCNGATNGANCNTNCCNCANNNANCANNNGNNNGCTANNTNCNTGCCNGCNNNNNN
NNTNNATACNGAGGGTGNANNNNNNNNTNNNANNNACTGGGNNTNAAGCGCACGCNGGNNGTNNGTCAAGNNGNANNNGA
AATCNCCNGNCNTNNNNNGNNNNACTGCNTTCNNANCTGGCNANGNTAGAGTCTNNTNGAGGGGGNTNNNNNNCCNGNGT
GTAGCGNNTGNNNTGCNTANAGNNNTGNANGAATANCGGTGNCGAAGGCGGCCCCCNGGACAANGACTGNCNCTCAGGTG
CGAANGCGTGGGGAGCAANCAGGATTAGATACCNTGGTAGNCCACNCCNTAAACGATGTCGATTTNNNGAGGTNGTGCCC
NTGAGGCGTGNGCTTCCGGAGCTAACGCGTTNANTCGNACCGCCTGGGGAGTACNGGCCGCAAGNTTAAAACTCAAATGA
ATTGACGGGGGCCCGCACNNAGCGGTNGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCNTGGTCTTGAC
ATCCACAGAACTTTCCAGAGATNGGATTGGTGNCCTTCGGGAACTGTGAGACAGGTGCTGCATGGCTNNGTCGTCAGCTC
GTGTTGTGAAATGTTGGGTTAAGTCCCGCNAACGAGCNGCAACCCTTATCCTTTGTTGCCAGCGGNNNGNCCGGGAACTC
AAAGGAGACTGCCAGTGATAAACTGGNNAGGAAGGTGNGGGATGACGTCAAGTCATCATGGCCCTTACNGACCAGGGCNT
ACACACGTGCTANCAATGGCATATACAAAGAGAAGCGACNCTCGCGAGAGCAAGCGGACCTCATAAAGTATGTCGTAGTC
CGGATTGGAGTCTGCAACTCGACTCCATGAAGTCGGAATCGCTAGTAATCGTAGATCAGAATGCTACGGTGAATACGTTC
CCGGGCCTTGTACACACCGCCCGTCACACCATGGGAGTGGGTTGCAAAAGAAGTAGGTAGCNTANNCTNCNGGAGG

Hit taxon name	Hit strain name	Accession	Similarity	Variation Ratio	Completeness (%)
Klebsiella pneumoniae subsp. ozaenae	ATCC 11296(T)	Y17654	99.02	9/915	99.3
Klebsiella pneumoniae subsp. pneumoniae	DSM 30104(T)	AJJI01000018	98.58	13/918	100.0
Klebsiella quasivariicola	KPN1705(T)	CP022823	98.58	13/918	100.0
Klebsiella variicola subsp. variicola	DSM 15968(T)	CP010523	98,58	13/918	100.0

Klebsiella pneumoniae subsp. ozaenae	ATCC 11296(T)	Y17654	98.36	15/914	99.3

T405 – Middle belt after CIP, 2/24/20

NTGNAGTCGAGCGGTAGCACNGAGAGCTTGCTCTCGGGTGACGAGCGGCGGACGGGTGAGTAATGTCTGGGAAACTGCCT
GATGGAGGGGGATAACTACTGGAAACGGTAGCTAATACCGCANAANGTCGCAAGACCAAAGTGGGGGACCTTCGGGCCTC
ATGCCATCAGATGTGCCCAGATGGGATTAGCTAGTAGGTGGGGNAANNGCTCACCTAGGCGACGATCCCTAGCTGGTCTG
AGNNAGGATNGACCAGNCCACNACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCAC
AATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTGTGAAGAAGGCCTTCGGGTTGTAAAGCACTTTCAGCGGGGAGGAA
GGCGNTNAGGTTAATAACCTTGTCGATTGACGTTACCCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTA
ATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTCTGTCAAGTCGGATGTGAAATCC
CCGGGCTCAACCTGGGAACTGCATTCGAAACTGGCAGGCTAGAGTCTTGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGT
GAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACAAAGACTGACGCTCAGGTGCGAAAGCGT
GGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGATTTGGAGGTTGTGCCCTTGAGGCGTGG
CTTCCGGAGCTAACGCGTTAAATCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCCC
GCACNAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTGGTCTTGACATCCACAGAACTTTCCA
GAGATGGATTGGTGCCTTCGGGAACTGTGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTTGTGAAATGTTGGGTNT
AAGTCCCGCAACGAGCGCAACCCTTATCCTTTGTTGCNCAGCNNGGTNNNNNNCNGCCGGGAACTNCAAAGGAGACTGCC
AGTGATAAACTGGAGGAAGGTGGGGATGACGTCANAGTCATCATGGCCCTTACGACCAGGGCTACACACGTGCTACAATG
GCATATACAAAGAGAAGCGACCTCGCGAGAGCAAGCGGACCTCATAAAGTATGTCGTAGTCCGGATTGGAGTCTGCAACT
CGACTCCATGAAGTCGGAATCGCTAGTAATCGTAGATCAGAATGCTACGGTGAATACGTTCCCGGGCCTTGTACACACCG
CCCGTCACACCATGGGAGTGGGTTGCAAAAGAAGTAGGTAGCNTAN

Hit taxon name	Hit strain name	Accession	Similarity	Variation Ratio	Completeness (%)
Klebsiella pneumoniae subsp. ozaenae	ATCC 11296(T)	Y17654	99.49	7/1362	99.3
Klebsiella quasipneumoniae subsp. quasipneumoniae	01A030(T)	HG933296	99.49	7/1361	100.0
Klebsiella variicola subsp. variicola	DSM 15968(T)	CP010523	99.41	8/1365	100.0
Klebsiella pneumoniae subsp. pneumoniae	DSM 30104(T)	AJJI01000018	99.34	9/1365	100.0
Klebsiella quasivariicola	KPN1705(T)	CP022823	99.34	9/1365	100.0

T406 – Middle belt after CIP, 2/24/20

NTGNAGTCGAGCGGTANCACAGAGAGCTTGCTCTCGGGTGACGAGCGGCGGACGGGTGAGTAATGTCTGGGAAACTGCCT
GATGGAGGGGGATAACTACTGGAAACGGTAGCTAATACCGCATAACGTCGCANGACCAAAGTGGGGGACCTTCGGGCCTC
ATGCCATCAGATGTGCCCAGATGGGATTAGCTAGTAGGTGGGGTAACGGCTCACCTAGGCGACGATCCCTAGCTGGTCTG
AGAGGATGACCAGCCACACTGGAACTGAGACACGNGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATG
GGCGCAAGCCTGATGCAGCCATGCCGCGTGTGTGAAGAAGGCCTTCGGGTTGTAAAGCACTTTCAGCGGGGAGGAAGGNG
NTNAGGTTAATAACCTTGTCGATTGACGTTACCCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATAC
GGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTCTGTCAAGTCGGATGTGAAATCCCCGG
GCTCNACCTGGNAACTGCATTCGAAACTGGCAGGCTAGAGTCTTGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAA
TGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGNCCCCTGGACAAAGACTGACGCTCAGGTGCGAAAGCGTGGGG
AGCAAACAGGATTAGATACCNTGGTAGTCCACGCCGTAAACGATGTCGATTTGGAGGTTGTGCCCTTGAGGCGTGGCTTT
CNGGAGCTAACGCGTTAAATCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCCCGCA
CNAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTGGTCTTGACATCCACAGAACTTTCCAGAG
ATGGATTGGTGCCTTCGGGAACTGTGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTTGTGAAATGTTGGGTTAAGT
CCCGCAACGAGCGCAACCCTTATCCTTTGNTTGCCAGCNGGTNNNNTAGGCCGGGAACTCAAAGGAGACTGCNCAGTGAN
TAAACTGGANNGGAAGGTGGGGATGNACGTCAAGTCATCATGGCCCTTACGACCAGGGCTACACACGTGCTACAATGGCA
TATACAAAGAGAAGCGACCTCGCGAGAGCAAGCGGACCTCATAAAGTATGTCGTAGTCCGGATTGGAGTCTGCAACTCGA
CTCCATGAAGTCGGAATCGCTAGTAATCGTAGATCAGAATGCTACGGTGAATACGTTCCCGGGCCTTGTACACACCGCCC
GTCACACCATGGGAGTGGGTTGCAAAAGAAGTAGGTAGCNTAN

Hit taxon name	Hit strain name	Accession	Similarity	Variation Ratio	Completeness (%)
Klebsiella variicola subsp. variicola	DSM 15968(T)	CP010523	99.78	3/1373	100.0
Klebsiella quasivariicola	KPN1705(T)	CP022823	99.71	4/1373	100.0
Klebsiella quasipneumoniae subsp. quasipneumoniae	01A030(T)	HG933296	99.71	4/1368	100.0
Klebsiella pneumoniae subsp. pneumoniae	DSM 30104(T)	AJJI01000018	99.64	5/1373	100.0
Klebsiella pneumoniae subsp. ozaenae	ATCC 11296(T)	Y17654	99.64	5/1370	99.3

APPENDIX B – SUPPLEMENTAL SANGER SEQUENCING DATA FOR CHAPTER 3

Bacterial isolates from polypropylene and stainless-steel coupons isolated on various selective media.

B110 – Stainless Steel coupon, 12/10/2019

CNGGNAGCAGCTTGCTGCNNNNNTGACGAGTGGCGGANGGGTGAGTAATGTCTGGGAAACTGCCTGATGGAGGGGGATAACTAC
TGGAAACGGTAGCTAATACCGCANAACGTCGCAAGACCAAAGAGGGGGACCTTCGGGCCTCTTGCCATCGGATGTGCCCAGATG
GGATTAGCTAGTAGGTGGGGTAACGGCTCACCTAGGCNACGATCCCTAGCTGGTCTGAGAGGATGACCAGCCACACTGGAACTGA
GACACGGTCCAGACTCCTACGGGAGGCAGCAGTNGGGGAATATTGCACAATGGGCNGCAAGCCTGATGCAGCCATGCCGCGTGT
ATGAAGAAGGCCTTCGGGTTGTAAAGTACTTTCAGCGGGGAGGAANGNGNNNAGGTTAATAACCTNNNNNNATTGACGTTACCC
GCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAA
GCGCACGCANGCGGTCTGTCAAGTCGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATTCGAAACTGGCAGGCTAGAGTCT
TGTAGAGGGGGGTAGAATTCCAGGNGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAANNCGGNCCCCTGGA
CAAAGACTGACGCTCNNNNGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTNANCGATGTCGACT
TGGAGGTTGTGNCCNTGAGGCGTGGCTTCCGGAGCTAACGCGTTAAGTCGACCGCCTGGGGGAGTACGGCCGCAAGGTTAAAACT
CAANTGAATTGANGGGGGCCCGCACNAGCGGTGGNAGCATGNGGTTTAATTCGATGCAACGCGANGAACNTTACCTACTCTTGAC
ATCCAGAGAACTTTCCAGAGATGGATTGGTGCCTTCGGGAACTCTGAGACAGGTGCTGCATGGCNGTCGTCAGCTCGNGTTGNNG
AAATGTTGGGTTAAGTCCCGCAACGANGCGCAACCCTTATCCTTTGTTGCCAGCGGTCCGNCCGGGAACTCAAAGNGAGACTGCC
AGTGATAAACTGGAGGAAGGTGGGGANGACGTCAAGTCATCATGGCCCTTACGAGTAGGGCTACACACNTGCTNACAANNTGGC
GCATACAAAGAGAAGCGACCTCGCGAGAGCAAGCGGACCTCATAAANNGCGTNGTAGTCCGGATTGGAGTCTGCAACTNGACTC
CATGAAGTNGGAATCGNTNGTAATCGTGGATCAGAATGCCACGGTGAATACGTTCCCGGNCCTTGTACACACCGCCCGTCACACC
ANGGGAGNGGGTTGCAAAAGAAGNAN

Hit taxon name	Hit strain name	Accession	Similarity	Variation Ratio	Completeness (%)
Enterobacter hormaechei subsp. oharae	DSM 16687(T)	CP017180	99.92	1/1307	100.0
Enterobacter hormaechei subsp. steigerwaltii	DSM 16691(T)	CP17179	99.54	6/1307	100.0
Enterobacter hormaechei subsp. *xiangfangensis*	LMG 27195(T)	FYBF01000083	99.46	7/1307	100.0
Enterobacter quasihormaechei	WCHEs12000e(T)	MK567958	99.46	7/1307	100.0
Enterobacter sichuanensis	WCHECI1597(T)	POVL01000141	99.46	7/1306	100.0

B111 – Polypropylene coupon, 12/10/2019

GNANGGTAACNGGNNGCANCNNNNNNNNNNNNNNNNNNNNNNNCNNNNNNNNNNNNNNNNNNNNGGANNNNNNNTGATGGN
NNNNNNNNNCTACTGGAAACGGTAGCTAATACCGCANAACGTCGCANGNCCAAAGAGGGGGNNNNTCGGGCCTCTTGCCA
TCGGANGTGCCCAGATGGGATTAGCTAGTAGGTGGGGTAACGGCTCACCTAGGCGACGATCCCTAGCTGGTNCTGAGAGN
GANTGACCAGCCACACTGGANNNNACTGAGNACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAAT
GGGCGCAAGCCTGATGCAGCCATGCCGCGTGTATGAAGAAGGCCTTCGGGTTGTAAAGTACTTTCAGCGGGGAGGAANGN
GNTGAGGTTAATAACCTNNNNCNATTGACGTTACCCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAAT
ACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTCTGTCAAGTCGGATGTGAAATCCCC
GGGCTCAANCTGGGAACTGCATTCGAAACTGGCAGGCTAGAGTCTTGTAGAGGGGGGTAGAANTTCCAGGTGTAGCGGTG
AAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACAAAGACTGACGCTCAGGTGCGAAAGCGTG
GGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTNANCGATGTCGACTTGGAGGTTGTGCCCTTGAGGCGTGGC
TTCCGGAGCTAACGCGTTAAGTCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCCCG
CACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTACTCTTGACATCCAGAGAACTTTCCAG
AGATGGATTGGTGCCTTCGGGAACTCTGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTTGTGAAATGTTGGGTTAA
GTCCCGCAACGAGCGCAACCCTTATCCTTTGTTGCCAGCGGTCNGGCCGGGAACTCAAAGGAGACTGCCAGTGATAAACT
GGAGGAAGNGNTGGGGATGACGTCAAGTCATCATNGGCCCTTACGAGTAGGGCTACACACGTGCTACAATGNGCGCATAC
AAAGAGNAAGCGACCTCGCGAGAGCAAGCGGACCTCATAAANTGCGTCGTAGTCCGGATTGGAGTCTGCAACTCGACTCC
ATGAAGTCGGAATCGCTAGTAATCGTGGATCAGAATGCCACGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCA

CACCATGGGAGTGGGTTGCAAAAGAAGTAGGTAGNNTA

Hit taxon name	Hit strain name	Accession	Similarity	Variation Ratio	Completeness (%)
Enterobacter hormaechei subsp. oharae	DSM 16687(T)	CP017180	99.84	2/1287	100.0
Enterobacter hormaechei subsp. hormaechei	ATCC 49162(T)	AFHR01000079	99.38	8/1287	100.0
Enterobacter hormaechei subsp. *steigerwaltii*	DSM 16691(T)	CP017179	99.38	8/1287	100.0
Enterobacter xiangfangensis	LMG 27195(T)	FYBF01000083	99.38	8/1287	100.0
Enterobacter quasihormaechei	WCHEs120003(T)	MK567858	99.38	8/1287	100.0

B112 – Stainless Steel coupon, 12/10/2019

NNTGCAGTCGAGCGGNNGNAGGGAGCTTGCTCCTGGNNTCNGCGGCGGANGGGTGAGTAATGCCTAGGAATCTGCCTGGTAGTG
GGGGATAACGTCCGGAAACGGGCGCTAATACCGCATACGTCCTGAGGGAGAAAGTGGGGGATCTTCGGACCTCACGCTATCAGA
TGAGCCTAGGTCGGATTAGCTAGTTGGTGGGGTAAAGGCCTACCAAGGCGACGATCCGTAACTGGTCTGAGAGGATGATCAGTCA
CACTGGAACTGNAGACACGGTCCAGACTCNCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGAAAGCCTGATCCANG
CCATGCCGCNGTGTGTGAAGAAGGTCTTCGGATTGTAAAGCACTTTAAGTTGGGAGGAAGGGCAGTAAGTTNAATACCTTGCTGT
TTTGACGTTACCAACAGAATAAGCACCGNGCTAACTTCGTGCCAGCAGCCGCGGTAATACGAANGGTGCAAGCGTTAATCGGAAT
TACTGGGCGTAAAGCGCGCGTAGGTGGTTCAGCAAGTTGGATGTGAAATCCCCGGGCTCNACCTGGGAACTGCATCCAAAACTAC
TGAGCTAGAGTACGGTAGAGGGTGGNTGGAATTTCCTGTGTAGCGGTGAAATGCGTAGATATAGGAAGGAACACCAGTGGCGAA
GGCGACCACCTGGACTGATACTGACACTGAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACNCCNGGTAGTCCACGCNG
TAAACGATGTCGACTAGCNGTTGGGATCCTTGAGATCTTAGTGGCGCAGCTAACGCGATAAGTCGACNGCCTGGGGAGTACGGCC
GCAAGGTTAAAACTCAAATGAATTGANGGGGGCCC
GCACNAGCGGNGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCTGGCCTNGACATGCTGAGAACTTTCCAGAGAT
GGATTGGTGCCTTCGGGAACTCAGACACAGNTGCTGCATGGCNGTCGTCAGCTCGTGTCGTGAGATGTTNGGTTAAGTCCCGTAA
CGAGCGCAACCCTTGTCCTTAGTTANNNCCAGCACCTCGGGTGGGCACTCNNTAAGGAGACTGCCGGTGANCAAACCGGAGGAA
GGTGGGGATGACGTCAAGTCATCATGGCCCTTACGGCCAGGGCTACACACGTGCTACAATGGTCGGTACAAAGGGTTGCCAAGCC
GNGAGGNGGAGCTAATCCCATAAAACCGATNGTAGTCCGGATCGCAGTNTGCAACTNGANTGNGTGAAGTNGGAATCGNTNGTA
ATCGTGAANCAGAATGNCACGGTGAATACGTTCCCGGGCCTTGTACACACCNCCCGTCACACCANGGGAGTGGGNNNCTNCANA
AGNANNTAGTCTANCCGCNA

Hit taxon name	Hit strain name	Accession	Similarity	Variation Ratio	Completeness (%)
Pseudomonas aeruginosa	JCM 5962(T)	BAMA01000316	99.78	3/1347	100.0
CP000744 s	PA7	CP000744	99.78	3/1347	100.0
Pseudomonas otitidis	MCC10330(T)	AY953147	98.37	22/1347	100.0
CP043311 s	PE08	CP043311	97.77	30/1347	100.0
QJRX s	MB-090714	QJRX01000015	97.55	33/1347	100.0

B113 – Polypropylene coupon, 12/10/2019

NTGNAGTCGAGCGGNNGNNGGGAGCTTGCTCCTGGNNTCNGCGGCGGACGGGTGAGTAATGCCTAGGAATCTGCCTGGTAGTGG
GGGATAACGTCCGGAAACGGGCGCTAATACCGCATACGTCCTGAGGGAGAAAGTGGGGGATCTTCGGACCTCACGCTATCAGAT
GAGCCTAGGTCGGATTAGCTAGTTGGTGGGGTAAAGGCCTACCAAGGCGACGATCCGTAACTGGTNCTGAGAGGATGATCAGTCA
CANCTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGAAAGCCTGANTCCAGN
CCATGCCGCGTGTGTGAAGAAGGNTNCTTNCGGATTGTAAAGCNACTTTAAGTTGGGAGGANANGGGCAGTAAGTTAATACCTTG
CTGTTTTGACGTTACCAACAGAATAAGCACCGGCTAACTTCGTGCCAGCAGCCGCGGTAATACGAANGGTGCAAGCGTTAATCGG
AATTACTGGGCGTAAAGCGCGCGTAGGTGGTTCAGCAAGTTGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATCCNAAAC
TACTGAGCTAGAGTACGGTAGAGGGTGGGTGGAATTTNCNGTGTAGCGGTGAAATGCGTAGATATAGGAAGGAACNCCAGTGGC
GAANGCGACCACCTGGACTGATACTGACACTGAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACNCCTGGTAGTNCNAC
GCCGTAAACGATGTCGACTAGCCGTNGGGATCCTTGAGATCTTAGTGGCGCAGCTANCGCGATAAGTCGACCGCCTGGGGGAGTA

CGGCCGCANGGTTAAAACTCAAATGAATTGANGGGGGCCCGCACNAGCGGNGGAGCATGTGGTTTAATTNGAAGCAACGCGAAN
NNCNTNNCCTGNCCTNGACATGCTGAGAACTTTCCAGAGATGGATNGGTNCNTTCGGGAACTCAGACACAGGTGCTGCATGGCTG
TCGTCAGCTNNNNCNGNGTCGTGNAGATGTTGGGTTAAGNTCCNNNCNGTAACNGAGCNGCAACCCTTNGTCCTTAGTTACCAGC
ACCTNGGGTGGNCACTCTAAGGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGANGANGTCANGTCATCATGGCCCTTACGG
CCAGGGCTACACACGTGCTACAATGGTCGGTACAAAGGGTTGCCAAGCCGCGAGGTGGAGCTAATCCCATAAAACCGATNGTAG
TCCGGATCGCAGTCTGCAACTNGANTGCGNGAAGTNGGAATCGCTNGTAATCGTGAATCAGAATGTCACGGTGAATACGTNCCCG
GGCCTTGTACACACCGCCCGTCACACCANGGGAGTGGGTTNCTCCANAAGTANNTANTCTANNCGCAA

Hit taxon name	Hit strain name	Accession	Similarity	Variation Ratio	Completeness (%)
Pseudomonas aeruginosa	JCM 5962(T)	BAMA01000316	99.24	10/1321	100.0
CP000744_s	PA7	CP000744	99.24	10/1321	100.0
Pseudomonas otitidis	MCC10330(T)	AY953147	97.88	28/1321	100.0
AP013068_s	NBRC 106553	AP013068	97.27	36/1321	100.0
CP043311_s	PE08	CP043311	97.27	36/1321	100.0

B114 – Stainless Steel coupon, 12/10/2019

NTGNAGTCGAGCGGGGGNNGGNAGCTTGCTNCNNNACCTAGCGGCGGANGGGTGAGTAATGCTTAGGAATCTGCCTATTAGTGG
GGGACAACATCTCGAAAGGGATGCTAATACCGCATACGTCCTACGGGAGAAAGCAGGGGATCTTCGGACCTTGCGCTAATAGAT
GAGCCTAAGTCGGATTAGCTAGTTGGTGGGGTAAAGGCCTACCAAGGCGACGATCTGTAGCGGGTCTGAGAGGATGATCCGCCAC
ACTGGGACTGAGACACGGCCCAGACTCCTNNACGGGAGGCAGCAGTGGGGAATANNNNNTTGGACAATGGGGGGANACCCTGAT
CCAGCCATGCCGCGTGTGTGAAGAAGGCCTTATGGTTGTAAAGCACTTTAAGCGAGGAGGAGGCTANCTTTAGTTAATACCTAGA
GATAGTGGACGTTACTCGCAGAATAAGCACCGGCTAACTCTGTGCCAGCAGCCGCGGTAATACAGAGGGTGCGANGCGTTAATCG
GATTTACTGGGCGTAAAGCGTGCGTANGCGGCTTATTAAGTCGGATGTGAAATCCCCGAGCTTAACTTGGGAATTGCATTCGATA
CTGGTGAGCTAGAGTATGGGAGAGGATGNGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGATNG
CGAANGCAGCCATCTGGCCTAATACTGACGCTGAGGTACGAAAGCATGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCATG
CCGTAAACGATGTCTACTAGCCGTTGGGNGCCTTTGAGGCTTTAGTGGCGCAGCTAACGCGATAAGTAGACCGCNNGGGGAGTAC
GGTCGCAAGACTAAAACTCAAATGAATTGACGGGG
GCCCGCACNAGCGGNGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTGGCCTTGACATACTAGAAACTTTCCAG
AGATGGATTGGTGCCTTCGGGAATCTANGATACAGGTGCTGCATGGCTGNTNCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGT
NCCCGCAACGAGCGCAACCCTTTTCCTTACTTGCCAGCATTTCGGATGGGAACTTTAAGGATACTGCCAGTGACAAACTGGAGGA
AGGCGGGGANGANGTCAAGTCATCATGGCCCTTACGGCCAGGGCTACACACNTGCTACAATGGTCGGTACAAAGGGTTGCTACA
CAGCGATGTGATGCTAATCTCAAAAAGCCGATCGTAGTCCGGATTGGAGTCTGCAACTNGACTCCATGAAGTCGGAATCGNTNGT
AATCGCGGATCAGAATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGANTTTGNTGCACCANA
AGTANCTANCCTAACNNCAAAGANNGCG

Hit taxon name	Hit strain name	Accession	Similarity	Variation Ratio	Completeness (%)
Acinetobacter baumannii	ATCC 19606(T)	ACQB01000091	99.85	2/1368	100.0
Acinetobacter seifertii	NIPH 973(T)	KB851199	98.24	24/1366	100.0
Acinetobacter nosocomialis	NIPH 2119(T)	APOP01000014	98.10	26/1366	100.0
Acinetobacter junii	CIP 64.5(T)	APPX01000010	98.02	27/1367	100.0
Acinetobacter halotolerans	R160(T)	KT032155	97.88	29/1367	99.2

B115 – Polypropylene coupon, 12/10/2019

NNGTCGAGCGGGGGNNGGNAGCTTGCTACCGGACCTAGCGGCGGANGGGTGAGTAATGCTTAGGAATCTGCCTATTAGTGGGGGG
ACAACATCTCGAAAGGGATGCTAATACCGCATACGTCCTACGGGAGAAAGCAGGGGATCTTCGGACCTTGCGCTAATAGATGAGC
CTAAGTCGGATTAGCTAGTTGGTGGGGTAAAGGCCTACCAAGGCGACGATCTGTAGCGGGTCTGAGAGGATGATCCGCCACACTG
GGACTGAGACACGGCCNCAGACTCCTNACGGGAGGCAGCAGTGGGGAATATTGGANCAATGGGGGGAACCCTGATCCAGCCATG
CCGCGTGTGTGAAGAAGGCCTTATGGTTGTAAAGCACTTTAAGCGAGGAGGAGGCTACTTTAGTTAATACCTAGAGATAGTGGAC
GTTACTCGCAGAATAAGCACCGGCTAACTCTGTGCCAGCAGCCGCGGTAATACAGAGGGTGCGAGCGTTAATCGGATTTACTGGG
CGTAAAGCGTGCGTAGGCGGCTTATTAAGTCGGATGTGAAATCCCCGAGCTTAACTTGGGAATTGCATTCGATACTGGTGAGCTA
GAGTATGGGAGAGGATGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGATGGCGAAGGCAGCCA
TCTGGCCTAATACTGACGCTGAGGTACGAAAGCATGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCATGCCGTAAACGATGT
CTACTAGCCGTTNGGNGCCTTTGAGGCTTTAGTGGCGCAGCTAACGCGATAAGTAGACCGCCTGGGGAGTACGGTCGCAAGACTA
AAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTGGCCT

TGACATACTAGAAACTTTCCAGAGATGGATTGGTGCCTTCGGGAATCTAGATACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCG
TGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTTTCCTTACTTGCCAGCATTTCGGATGGGAACTTTAAGGATACTGCCA
GTGACAAACTGGAGGAAGGCGGGGACGACGTCAAGTCATCATGGCCCTTACNGGCCAGGGCTACACACNNGTGCTNACAATGGT
CGGTACAAAGGGTTGCTACACAGCGATGTGATGCTAATCTCAAAAAGCCGATCGTAGTCCGGATTGGAGTCTGCAACTCGACTCC
ATGAAGTCGGAATCGCTAGTAATCGCGGATCAGAATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCA
TGGGAGTTTGTTGCACCAGAAGTAGCTAGCCTAACNNCAAAGA

Hit taxon name	Hit strain name	Accession	Similarity	Variation Ratio	Completeness (%)
Acinetobacter baumannii	ATCC 19606(T)	ACQB01000091	99.93	1/1381	100.0
Acinetobacter seifertii	NIPH 973(T)	KB851199	98.04	27/1381	100.0
Acinetobacter nosocomialis	NIPH 2119(T)	APOP01000014	97.97	28/1381	100.0
Acinetobacter halotolerans	R160(T)	KT032155	97.83	30/1381	99.2
Acinetobacter junii	CIP 64.5(T)	APPX01000010	97.76	31/1381	100.0

C109 – Bioreactor Whey, 10/17/2019

NTGNAGTCGAGCGGGGGNNGGNAGCTTGCTNNNGGACCTAGCGGCGGANGGGTGAGTAATGCTTAGGAATCTGCCTATTAGTGG
GGGACAACATCTCGAAAGGGATGCTAATACCGCATACGTCCTACGGGAGAAAGCAGGGGATCTTCGGACCTTGCGCTAATAGAT
GAGCCTAAGTCGGATTAGCTAGTTGGTGGGGTAAAGGCCTACCAAGGCGACGATCTGTAGCGGGTCTGAGAGGATGATCCGCCAC
ACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGGGGAACCCTGATCCAGCCAT
GCCGCGTGTGTGAAGAAGGCCTTATGNGTTGTAAAGCACTTTAAGCGAGGAGNGAGGCTACTTTANNGTTAATACCTANGAGATA
NGTGGACGTTACTCGCAGAATANAGCANCCGGCTAACTCTGTGCCAGCAGCCGCGGTAANTACAGAGGGTGCGAGCGTTAATNC
GGATTTACTGGGCNGTAAAGCGTGCGTAGGCGGCTTATTAAGTCGGATNGTGAAATCCCCGAGCTNAACTTGGGAATTGCATTCG
ATACTGGTGAGCTAGAGTATGGGAGAGGATGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGAT
GGCGAAGGCAGCCANTCTGGCCTAATACTGACGCTNAGGTACGAAAGCATGGGGAGCAAACAGGATTAGATACCCTGGTAGTNN
ATGCCGTAAACGATGTCNACTAGCNNTTGGGGCCTTTGAGGCNTTNGTGGCNGCANCTANCGCGATAAGTAGACCGNCNGGGGA
GTACNGTCGCAAGANTAAANNTCAAATGAATTGACGGGGGCCCGCACNAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCG
AAGAACCTTACCTNGNNNNTGACNTNCTANAAACTTTCCANAGATNGATTGGTGCCTTCNGGNNNNTNGATACNGNTGCTGCATG
GCNGTCGTCAGCTCNNGTNNTGANATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTNTCNTTNNTTGCCAGCANTTTNNGAN
NNGAANNTTNNNGATACTNNCA

Hit taxon name	Hit strain name	Accession	Similarity	Variation Ratio	Completeness (%)
Acinetobacter baumannii	ATCC 19606(T)	ACQB01000091	99.61	4/1035	100.0
OVCN s	KCRI-348C	OVCN01000041	97.97	21/1035	100.0
Acinetobacter venetianus	RAG-1(T)	AKIQ01000085	97.97	21/1032	100.0
JFYL s	Ver3	JFYL01000147	97.87	22/1032	100.0
Acinetobacter seifertii	NIPH 973(T)	KB851199	98.04	23/1035	100.0