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The survival and growth of *Bacillus cereus* and *Listeria monocytogenes*, during the manufacture of Ricotta Salata cheese

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Abstract

This study was conducted with the following objectives: 1) to investigate the survival and growth of *Bacillus cereus* during the manufacture of Ricotta Salata cheese; and 2) to investigate the survival and growth of *Listeria monocytogenes* during the manufacture of Ricotta Salata cheese.

The Ricotta Salata cheese was made by heating the whole milk to 95°C, and adding citric acid to coagulate the cheese curd. The cheese curd was inoculated with 7 log₁₀ CFU/g *B. cereus* broth and 8 log₁₀ CFU/g *L. monocytogenes* broth. After moulding for 12h, Ricotta Salata cheese was stored at 4°C for 1 week. During manufacture, the physico-chemical properties [pH, water activity (a_w), and Sodium chloride (NaCl) concentration] and bacterial counts were recorded.

The pH change fluctuated between 6.00 to 6.10 on the surface and 6.00 to 5.95 in the centre; the lowest a_w was approximately 0.96 on the surface and 0.97 in the centre; and the highest NaCl concentration was 3.3% on the surface and 3% in the centre.

The survival and growth of the two *B. cereus* strains (D1 and ATCC 13061) during the manufacture of Ricotta Salata cheese were similar. The *B. cereus* grew from approximately 5 log₁₀ CFU/g to a maximum of 7.7 log₁₀ CFU/g of cheese curd during moulding (20h at room temperature).

The survival and growth of the two *L. monocytogenes* strains (W1 and ATCC 35152) during the manufacture of Ricotta Salata cheese were similar. The difference between the bacteria count on the surface and in the centre was very small. *L. monocytogenes* increased from 5 to 6 log₁₀ CFU/g to a maximum of 8.6 log₁₀ CFU/g during manufacture and maintained a level of around 8 log₁₀ CFU/g in the final product.

The Ricotta Salata supported the survival and growth of *B. cereus* and *L. monocytogenes* during manufacture. It is important to improve the management of process hygiene for reducing the environmental contamination. Ideally, some lethal treatments should be

applied after the packaging of the cheese, to limit the contamination of Ricotta Salata with these two bacteria.

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1. Introduction

Ricotta Salata is a traditional salted whey protein cheese that originated in Italy. Conventionally it is made from sheep, cow, goat or buffalo milk whey by heat-coagulation of the whey proteins (Sattin et al., 2016). There is no standard procedure for Ricotta Salata manufacture. Traditionally, the whey is heated to 85°C, for approximately 30min. Floating curd is collected and transferred into plastic moulds. The curd is then pressed to drain away the water in the cheese, in order to dry salt at a controlled temperature (10 to 20°C) (Spanu et al., 2013). Nowadays, manufactures add whole or skimmed milk during the process or use whole milk instead of whey to increase yield of Ricotta Salata. In New Zealand market, Perfect Italiano Ricotta® and Bouton d'or Ricotta® both use milk instead of whey as ingredient. Due to the varied ingredients used in the manufacture of Ricotta Salata, the physical chemical analysis of Ricotta Salata varies. Spanu et al. (2012) and (2013) obtained Ricotta Salata ranges from 6.1 to 6.9 pH, 0.940 to 0.970 a_w , 50 to 60% moisture, 28 to 33% fat, and 14 to 23% protein, and the commercial product is vacuum-packed and stored at 4°C, which provides suitable conditions for the growth of some food-borne pathogens, such as *B. cereus* and *L. monocytogenes*. Fresh Ricotta is a ready-to eat product that can be stored for 1 week; while the Ricotta Salata as a salted Ricotta can be kept for up to 2 months (Nantet, 1996). Generally, there are no preservatives added to extend shelf life.

The manual manufacture of Ricotta Salata is a potential risk for contamination with foodborne pathogens, such as *B. cereus* and *L. monocytogenes*. (Lioliou et al., 2001; Pintado & Malcata, 2000; Spanu et al., 2013). However, most studies focus on the contamination of commercial products during storage time. None of the studies investigated the survival and growth of bacteria at the early stages of cheese-making.

B. cereus and *L. monocytogenes* are a concern in Ricotta Salata cheese because the physical and chemical properties provide suitable conditions for the growth of these bacteria, and there are reports world-wide of natural contamination of this cheese and outbreaks caused by consuming Ricotta Salata cheese contaminated by these two bacteria (Heiman et al., 2015; Spanu et al., 2016).

B. cereus is a spore-forming microorganism; its spores can survive at both the high processing temperatures and the low storage temperatures. Strains can grow at 4 to 8°C, more rapidly at 10°C, and spores can germinate and grow at temperatures up to 50 to 55°C (Choma et al., 2000; Dufrenne et al., 1995; Guinebretiere et al., 2001; Johnson et al., 1983; Valero et al., 2000, 2003; Van Netten et al., 1990).

L. monocytogenes is Gram-positive, non-sporeforming aerobic bacterium. In the United States, *L. monocytogenes* is probably believed to be responsible for 19% of all mortality caused by food borne illnesses (Scallan et al., 2011; Ramaswamy et al., 2007). Outbreaks of listeriosis have been related to dairy products (milk, cheese, and so on), vegetables, salads, and meat products (Te Giffel & Zwietering, 1999).

B. cereus and *L. monocytogenes* in Ricotta Salata have been studied including the occurrence and behavior and toxin production. As mentioned before there is a lack of information on the survival and growth of these two bacteria during the manufacture of Ricotta Salata, especially from the early stages of cheese-making. Therefore, the objective of this study is to evaluate the survival and growth of *B. cereus* and *L. monocytogenes* during the manufacture of Ricotta Salata.

2. Literature review

This limited review of the pertinent literature focusses on the connection between the two bacteria (*B. cereus* and *L. monocytogenes*) and Ricotta Salata cheese, which involves the cheese manufacture process, physico-chemical parameters of the cheese, shelf-life and consumption of the cheese; the contamination cases of *B. cereus* and *L. monocytogenes*; and the Hazard Analysis Critical Control Points (HACCP) of the cheese manufacture process.

2.1 Manufacture processes for Ricotta Salata cheese

There is no standard for the manufacture of Ricotta Salata, as the ingredients used vary. An example of the processes of manufacture Ricotta Salata from whey is given in Figure 1.

The whey used to produce Ricotta Salata cheese in the example is from Pecorino cheese, which is the most important sheep cheese in Italy (Mughetti et al., 2012).

Spanu et al. (2016) mentioned in their study about natural contamination of Ricotta Salata cheese during refrigerated storage, after whey heating process, there is a slow cooling of

curds, which reveal the risk of spore germination and sequential growth with toxins production.

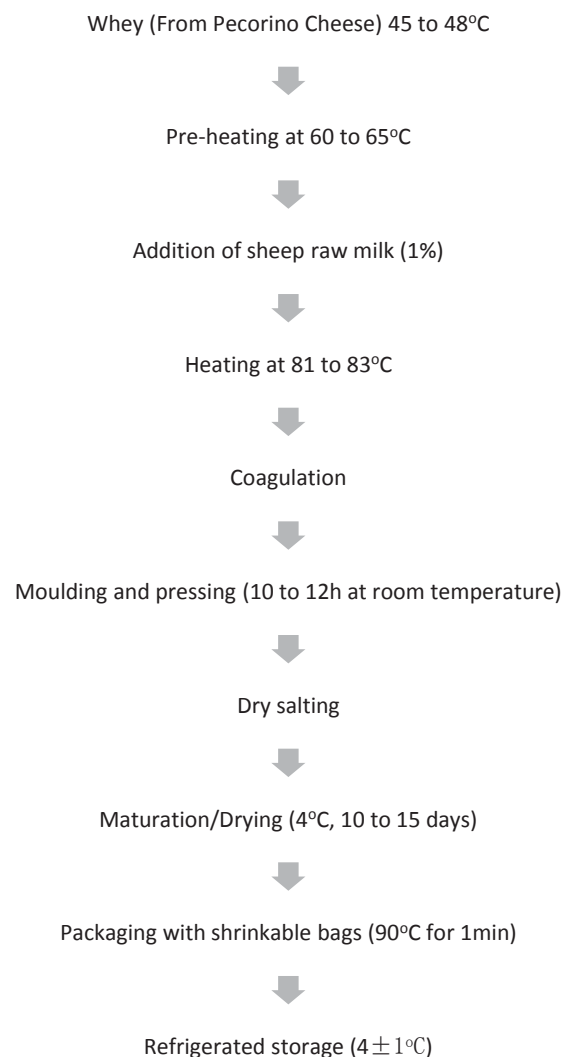


Figure 1. Process flow of traditional Ricotta Salata cheese made from whey (Casti et al., 2016).

As illustrated in Figure 1, some manufacturers add raw milk to increase the yield, which endangers the safety of this product. Although this addition is followed by a heating step, spore-forming pathogens, such as *B. cereus* still can survive and pose a threat to food safety. In New Zealand market, Ricotta type cheeses are made from pasteurised whole milk instead of whey. *B. cereus* is a spore-forming microorganism that may survive heat treatment. However, the detection of *B. cereus* in refrigerated fresh pasteurised milk is rare due to low

number of spores. The number of spores present in pasteurised milk is depend on contamination on farm during grazing (Meer et al., 1991; Te Giffel et al., 1997).

The moulding and pressing process at room temperature for 12h is potentially risky for some pathogens. On one hand, many studies confirmed that, some *B. cereus* strains can grow at 4 to 8°C, with more rapid growth at 10°C (Choma et al., 2000; Dufrenne et al., 1995; Guinebretiere et al., 2001; Valero et al., 2000, 2003; Van Netten et al., 1990). An earlier study by Andersson et al. (1995) also agreed the storage temperature is an important factor to limit *B. cereus* growth. They discovered an increase in numbers of *B. cereus*, when the storage temperature increased from 6°C to 8°C. *L. monocytogenes* can also grow at low temperatures with the growth rate at 8°C being greater than at 4°C (Coroneo et al., 2016).

Figure 1 shows a high temperature at packing (90°C for 1min) (Casti et al., 2016). Spanu et al. (2015) recorded a 5 log₁₀ reduction in *L. monocytogenes* by applying heat treatment at 90°C for 40min. *B. cereus* is a spore-forming bacterium, which is more heat-resistant than *L. monocytogenes* with the spores likely to survive the heat treatment.

Ricotta Salata cheese packaging is depending on the final application of the product. Commonly the Ricotta Salata wheels are vacuum packed in shrink bags. The cheese is often grated before being consumed grated and may also be, mixed with other cheeses or use as an ingredient in different foods. It also can be cut into wedges before packaging for straight consumption (Spanu et al., 2015). Without the use of preservatives such as bacteriocins like nisin in Ricotta-type unsafe levels of *L. monocytogenes* have been found within 1 to 2 weeks of incubation (Davies, 1997). Therefore, there is a high risk of *L. monocytogenes* threatening the safety of Ricotta Salata.

2.2 Physico-chemical parameters of Ricotta Salata cheese

2.21 Temperature

Temperature abuse is documented as the major safety risk for Ricotta Salata, since *B. cereus* survives pasteurisation and grows over a wide temperature range, similar to the temperatures used during manufacture and storage of the Ricotta Salata. Temperature abuse will exacerbate the problem with warmer storage temperatures likely to increase growth of this pathogen (Rajkowski & Mikolajcik, 1987; Doyle, 2001).

B. cereus strains can be found growing at temperatures from 5°C to 55°C (Guinebretière et al., 2008), because they contain both psychrotrophic and mesophilic strains. Psychrotrophic strains grow well at refrigeration temperatures but rarely grow at 37°C (Wijnands et al., 2006), Mesophilic strains grow well at 37°C but do not grow under 10°C.

The traditional manufacture of Ricotta Salata involves whey heating followed by slow cooling of curds, which provides a suitable condition for *B. cereus* spore germination (Spanu et al., 2016).

Comprehensive studies conducted by the United States Food and Drug Administration (FDA) and the United States Department of Agriculture along with Health and Welfare Canada have shown that *L. monocytogenes* is unable to survive milk pasteurisation. Heat treatments, such as thermisation and pasteurisation, applied to milk during Ricotta cheese making inactivate *Listeria* cells by approximately 3 to 6 log₁₀ CFU/g. The temperature range for *L. monocytogenes* growth is -1.5°C to 45 to 50°C. Therefore, after thermal treatment if *L. monocytogenes* from the environment contaminates product, it is difficult to control in the processes that follow (Donnelly & Diez-Gonzalez, 2013).

2.22 pH value

The physico-chemical characteristics of Ricotta Salata are shown in Table 1 (Spanu et al., 2016).

Table 1.

Intrinsic properties [mean±standard deviation (SD)] and composition (%±SD) evolution of Ricotta Salata cheeses (Casti et al., 2016; Spanu et al., 2015, 2016).

Parameters	Spanu et al. (2016)	Spanu et al. (2015)	Casti et al. (2016)
pH	6.54 ± 0.03	6.49 ± 0.10	6.42 ± 0.09
a _w	0.973 ± 0.005	0.978 ± 0.001	0.963 ± 0.01
% NaCl	3.42 ± 0.24	2.60 ± 0.30	4.56 ± 1.38

The minimal pH of survival and growth of *B. cereus* varies among strains and also depends on the acidulant (ICMSF, 1996). pH values of the commercial Ricotta Salata decrease during storage with an initial pH above 6.0 and after storing for 90 days, the pH drops to around 5.68. The production of diarrhoeal enterotoxins of *B. cereus* occurs between pH 5.5 to 10, with an optimum of pH 8 (Sutherland & Limond, 1993), and they are stable over a pH range of 4 to 11 and inactivated by heating to 56°C for 5min (Jenson et al., 2003). *L.*

monocytogenes can grow over a pH range from 4.3 to 9.6. Consequently, the pH range of Ricotta Salata is suitable for *B. cereus* and *L. monocytogenes* growth.

2.23 a_w value

a_w is the second most important criteria, after temperature, for microbial growth in food (Van den Berg, 1986). a_w is determined by the chemical composition of salted curd, cheese moisture and physical and structural stability of the cheese. The range of a_w is from 0 to 1, however for microbial metabolism, it ranges from above 0.6 (Pitt & Christian, 1968). The a_w of free unbound water was closed to 1.

As Ricotta Salata is a salted cheese, the a_w will decline from an initial high value of fresh salted curd compared to a lower value in the final product as the NaCl concentration increases. The a_w values of cheese are determined from a mean value obtained from samples taken at a particular point in time or throughout the shelf-life of the cheese.

Spanu et al. (2016) studied the occurrence and behaviour of *B. cereus* in naturally contaminated Ricotta Salata cheese during refrigerated storage. The results showed that, along with the storage time increase, the a_w values changed slightly, from 0.976 to 0.980. An earlier study by the same authors showed the a_w values ranged from 0.940 to 0.970 (Spanu et al., 2012; Spanu et al., 2013).

The minimum a_w for *B. cereus* growth is controversial. Scott (1957) stated the value is 0.950, Skjelkvale and Tjaberg (1974) reported the value in laboratory medium, when glycerol was used, was 0.930, and Kramer and Gilbert (1989) stated the value was 0.920. However, there is no debate that there is potential for *B. cereus* growth in Ricotta Salata.

According to Donnelly & Diez-Gonzalez (2013), the minimal range a_w for *L. monocytogenes* growing is from 0.900 to 0.970, which indicates that it can grow in Ricotta Salata.

2.24 NaCl concentration

As illustrated in Table 1, the sodium chloride content of Ricotta Salata is over 3%. Generally, when the NaCl concentration increases, the a_w decreases. Raevuori and Genigeorgis (1975) studied the effect of NaCl on growth of *B. cereus* in laboratory media and certain foods. The results showed that the a_w of brain heart infusion (BHI) broths with 0, 2.5, 5.0, 7.5, and 10% NaCl were 1.000, 0.985, 0.965, 0.955, and 0.935. Since the minimal a_w for *B. cereus* growth is 0.950, the NaCl concentration for growing *B. cereus* should be lower than 7% (Granum, 2005).

Compared with *B. cereus*, *L. monocytogenes* is more resistant to NaCl. Seeliger (1961) reported *L. monocytogenes* can grow at 10% NaCl, and survive at 16% NaCl for one year. Papageorgiou and Marth (1989) reported that *L. monocytogenes* can grow at 6% NaCl (0.96 a_w), and survive at least 132 days at 12% NaCl (0.92 a_w , 4°C) in skim milk and whey.

Commercial Ricotta Salata cheese has a_w above 0.97 and NaCl content above 3% (Table 1), therefore it is capable of supporting the survival and growth of *B. cereus* and *L. monocytogenes*.

2.3 Pathogen growth in Ricotta Salata cheese

As mentioned above, *B. cereus* and *L. monocytogenes* are able to survive and grow in Ricotta Salata cheese at refrigeration temperatures, resulting in a health risk for the consumption of this product.

2.31 *B. cereus*

B. cereus is one of the most important opportunistic food pathogens; causing foodborne gastro-enteritis in broad-type of foods, including dairy products, meats, vegetables, and rice (Carlin et al., 2000; Dufrenne et al., 1994; Guinebretiere et al., 2003; Nissen et al., 2002). In 2011, there was one food-borne outbreak caused by *B. cereus* in New Zealand. It was reported in April on the West Coast of the South Island where a fish fillet provided cooked at home was contaminated by *B. cereus* and caused two cases of illness (Cruz et al., 2013). *B. cereus* can cause two types of food poisoning: An emetic type and a diarrhoeal type (Ehling-Schulzi et al., 2004; Kramer & Gilbert, 1989). The emetic toxin is produced by the growth of the micro-organisms in food and will cause vomiting (Kramer & Gilbert, 1989); while the diarrhoeal toxin is produced by multifarious enterotoxins (Beecher & Wong, 1997; Lund & Granum, 1997) during *B. cereus* growing in the small intestine (Granum, 1994). According to Bennett and Belay (2011) until the multiplication of *B. cereus* reaches the populations larger than 5 log₁₀ CFU/g, its existence of in foods does not represent a health hazard.

Raw milk or dairy environments are the main sources of *B. cereus*, which can survive pasteurisation (Christiansson et al., 1989; Crielly et al., 1994; Davies, 1977; Griffiths & Phillips, 1990). The presence of *B. cereus* in milk is associated with the undesirable flavours, sweet curdling and bitty cream and outbreaks of food poisoning (Christiansson et al., 1989; Johnson, 1984; Overcast & Atmaram, 1974).

Ölmez and Aran (2005) designed a model for *B. cereus* growth that included variations in temperature, pH, sodium lactate and sodium chloride concentrations. They showed that under the same growing conditions, the times for emetic and diarrhoeal stains of *B. cereus* to produce toxin were different. Wong and Chen (1988) and Sooltan et al. (1987) reported similar results.

B. cereus contains both psychrotrophic and mesophilic strains. There is an increasing trend for the isolation of psychrotrophic strains of *B. cereus* from food stored at refrigerator temperatures. The generation times of these two different strains at 7°C have a wide range, from 9.4h to 75.2h in both milk and BHI. The mesophilic strains normally cause emetic food poisoning, and psychrotrophic strains are responsible for diarrhoeal type at temperatures down to 4°C (Granum & Baird-Parker, 2000).

2.32 *L. monocytogenes*

L. monocytogenes, an intracellular food-borne pathogen, which is potentially life-threatening for humans, is classified into two groups by the International Commission on Microbiological Specifications for Foods (ICMSF, 2002). Group I is a disease of serious hazard, but not lethal for the general population. For pregnant women and immunocompromised population, it is classified into Group IB, which is the disease of severe hazard for a restricted population, lethal or tending to substantial chronic sequel or having prolonged effects on human health.

The distribution of *L. monocytogenes* is very wide, and it can be isolated from soil, water, sewage, green plant material, decaying vegetation and numerous species of birds and mammals, including humans (Donnelly & Diez-Gonzalez, 2013). *L. monocytogenes* directly contaminates foods or food processing equipment, which results in outbreaks of food-borne disease, is called listeriosis (Allenberger et al., 2010).

Strains of *L. monocytogenes* can be divided into serotypes according to somatic and flagellar antigens. Although there are at least 13 serotypes have been identified, only serotypes 1/2a, 1/2b, and 4b are responsible for the majority of human disease (Schuchat et al., 1991; Nadon et al., 2001), furthermore, serotype 1/2a is the most frequently isolated from food.

The contamination of dairy products with *L. monocytogenes* is normally due to the dairy plant environment. Genigeorgis et al. (1991) evaluated the growth and survival of *L. monocytogenes* in 24 types of market cheeses, including soft Hispanic-type cheeses, Ricotta,

Teleme, Brie, Camembert and cottage cheese. An association was detected between the growth of *L. monocytogenes* in cheeses, pH over 5.5, and cheese manufactured without a starter culture. A recent study collected 409 environmental and food samples from 13 cheese-making plants in Sardinia (Italy) from 2011 to 2013, to study the occurrence and traceability of *L. monocytogenes* strains (Spanu et al., 2015). The results illustrated *L. monocytogenes* are widely distributed in the processing environment. Furthermore, they recovered strains from Ricotta Salata that showed a similar profile with strains isolated from the handling and storing areas. Considering Ricotta Salata supports the growth of *L. monocytogenes*, the manual handling, and the wide distribution of *L. monocytogenes* in the processing environment, there is a high risk of contaminating *L. monocytogenes* in Ricotta Salata cheese (Acciari et al., 2015).

2.4 Food poisoning cases and outbreaks associated with consumption of food contaminated with *B. cereus* and *L. monocytogenes*

2.4.1 *B. cereus*

B. cereus causes different types of disease and this differs from country to country. Although the clinical symptoms of contaminating *B. cereus* are clear, the reporting systems of different countries vary. In Europe and North America, the diarrhoeal type is the major food poison toxin, while in Japan the emetic type has been documented approximately 10 times more frequently than the diarrhoeal type, which is because the emetic type *B. cereus* is closely linked to starch, rice is a diet staple in Japan. The difficulty of collecting the data of food poisoning caused by *B. cereus* is in part due to the fact that this is not a reportable disease in any country. The recovery from this disease is quick, which enhances the difficulty in obtaining accurate data as illness is not always recorded (Kramer & Gilbert, 1989).

From 1988 to 1993, *B. cereus* caused 33% of the total cases of food poisoning in Norway; from 1985 to 1992, 47% in Iceland; 1992, 22% in Finland; 1991, 8.5% in The Netherlands; and from 1990 to 1992, 5% in Denmark. The data reported from other countries was much lower, for instance, 0.7% in England and Wales, 0.8% in Japan, 1.3% in United States and 2.2% in Canada (Granum & Lund, 1997). Although Ricotta Salata cheese is frequently contaminated with *B. cereus*, there are no documented cases of *B. cereus* causing food poisoning due to the consumption of Ricotta Salata cheese (Casti et al., 2016).

There was one outbreak caused by *B. cereus* in 2003. Five children from the same family became sick after consuming pasta salad. The pasta salad was made on Friday, and the remainders had been kept in the fridge until the next Monday evening. The salad was served to the children with dinner. There was a noticeable odour from the salad, and three of the five children, a boy aged 14 (B14), girl aged 10 (G10) and girl aged 9 (G9), ate a small quantity. The other children, a girl aged 7 (G7) and a boy aged 9 (B9) ate a larger quantity. After 6h from consuming the food, the youngest girl (G7) started vomiting. The vomiting occurred on her brothers and sisters was after she had been sent to the hospital. The clinical condition of two youngest children (G7 and G9) deteriorated rapidly. All the five children were transported to the University Hospital in Leuven. During transfer, G7 had severe pulmonary haemorrhage and she was moribund with coma, profuse bleeding, and severe muscle cramps. She died 13h after the meal. On autopsy *B. cereus* was detected in her gut content. All four other children were successfully cured (Dierick et al., 2005).

The pasta salad contained the highest *B. cereus* count (7 to 8 log₁₀ CFU/g), while in vomit the count was the lowest (2 log₁₀ CFU/g). From each positive sample, three (or four) isolates were phenotypically confirmed as *B. cereus* by use of the API 50 gallery. Further characterisation of the 22 isolates obtained consisted of repetitive sequence-based PCR (rep-PCR) (Gevers et al., 2001), pulsed-field gel electrophoresis (PFGE) of genomic DNA (Rivera & Priest, 2003), and PCR analysis indicated that it was emetic toxin (Ehling-Schulz et al., 2004).

This case study indicates the possible severity of the emetic syndrome and the importance of adequate refrigeration of prepared food. Because the emetic toxin is produced in the food and not inactivated by heat treatment (Granum & Lund, 1997) it is important to prevent growth and the production of the toxin, ceramide, during storage. In this case study, the temperature of the fridge was 14°C, and some *B. cereus* strains are psychrotrophic and have the highest emetic toxin production between 12 and 15°C (Finlay et al., 2000). In this case, conditions were favourable for the growth of *B. cereus* to > 8 log₁₀ CFU/g in 3 days with a very high toxin level.

In Sardinia 2014, from September to October there was a large case of Ricotta Salata cheese contaminated by *B. cereus*. The presence of *B. cereus* in Ricotta Salata was observed during routine microbiological testing by a local food business operator. This test was conducted as

part of their procedure based on HACCP principles. The mean level of contamination was $5.57 \pm 0.15 \log_{10}$ CFU/g in a batch. Even though there were no food safety criteria for *B. cereus* in this food product (EC Regulation No. 2073/2005), the food business operator withdrew the entire batch of Ricotta Salata (Spanu et al., 2016). However, the subsequent investigations showed that this was only part of the story as nine positive batches were detected over three months the highest level detected as $8.33 \log_{10}$ CFU/g. The actual source of the *B. cereus* in this product is unknown but the possibility of the raw milk added to the process is a possible source.

2.42 *L. monocytogenes*

The reports or outbreaks associated with *L. monocytogenes* are much greater than *B. cereus*. This may be due to the wide distribution of *L. monocytogenes* and the wide variation in growth conditions supporting *L. monocytogenes*. There are some cases related with the consumption of Ricotta Salata cheese.

From March 28th, 2012 to October 6th, 2012, the Centres for Disease Control (CDC) cooperated with public health and regulatory officials in several states and the United States Food and Drug Administration (FDA) to investigate a multistate outbreak of *L. monocytogenes* infections associated with the consumption of Ricotta Salata cheese. Those cheeses were imported from Italy, and caused 20 hospitalisations from 13 states and the District of Columbia. Among them nine were pregnant and three were new-borns. The other infected persons were aged from 30 years to 87 years, with a median age of 77 years, and 54% were female. Four deaths were reported, from Minnesota, New York, Nebraska, and California. The number of ill people identified in each location was as follows: California (3), Colorado (1), District of Columbia (1), Maryland (3), Massachusetts (1), Minnesota (1), Nebraska (1), New Jersey (3), New Mexico (1), New York (1), Ohio (1), Pennsylvania (2), Virginia (2), and Washington (1) (Acciari et al., 2015).

The cheese was made from pasteurised sheep's milk in a plant in Apulia, which processed semi-finished cheeses from five plants in Sardinia. To confirm the origin of the outbreak, 758 cheeses and 183 environmental samples were collected from the five plants. Among them 179 (23.6%) cheese samples tested positive for *L. monocytogenes*, with contamination levels from <10 CFU/g to $6 \log_{10}$ CFU/g, and 1.1% of 183 environmental samples were contaminated. There were two serotypes were identified, 1/2a (78%) and 4b (22%).

Table 2

Hazard analysis chart (Sorting from Kamel et al., 2013).

Ingredient/Processes Step	Potential Hazard introduced, controlled or enhanced at this step	Is the potential food safety hazard significant?	Justification for decision	What control measures can be applied to prevent the significant hazards?	Is this step a critical control point (CCP)?
Heating of milk	Physical: None is identified at this time Chemical: None is identified at this time Biological: Microbiological contamination	Yes	High temperature will destroy the pathogenic microorganisms present in the milk. Heating will destroy parasites	- Monitoring the temperature and time - Check the equipment is properly running - Proper personnel hygiene and handling	Yes
Adding salt (during heating)	Physical: Foreign body Chemical: None is identified at this time Biological: Pathogens	Yes Yes	Salt quality to which may contain metals - Hazards may include potential growth of undesirable microorganisms either environmental or personnel - Inhibit the growth and activity of pathogens and food poisoning microorganisms	It is better to purchase food salt from a supplier - The quantity of added salt (0.5 to 1%) should be respected - Qualified product supply should be stored at room temperature - Proper personnel hygiene and handling - The program of cleaning and disinfection of equipment should be respected	No No
Coagulation	Physical: None is identified at this time Chemical: None is identified at this time Biological: Microbiological contamination	Yes	Improper addition of citric acid will cause microbiological contamination	Citric acid should be obtained from certified suppliers and stored at room temperature Does should be respected (between 400 and 500mL)	No
Moulding and pressing	Physical: None is identified at this time Chemical: None is identified at this time				

Ingredient/Processes Step	Potential Hazard introduced, controlled or enhanced at this step	Is the potential food safety hazard significant?	Justification for decision	What control measures can be applied to prevent the significant hazards?	Is this step a critical control point (CCP)?
Salting	Biological: Potential pathogen growth	Yes	Improper cooling temperature and long exposure of ricotta to relatively high temperature could result in pathogen growth	- Proper cold drainage and cooling and the exposure of Ricotta Salata to a time below 30min reduce the potential growth of pathogens - Proper personnel hygiene and handling	Yes
	Physical: Foreign body Chemical: None is identified at this time	Yes	Salt quality to which may contain metals	It is better to purchase food salt from a supplier	No
	Biological: Pathogens	Yes	- Hazards may include potential growth of undesirable microorganisms either environmental or personnel - Inhibit the growth and activity of pathogens and food poisoning microorganisms	- The quantity of added salt (2g each time) should be respected - salted Ricotta Salata should be stored at refrigerating temperature - Qualified product supply should be stored at room temperature - Proper personnel hygiene and handling - The program of cleaning and disinfection of equipment should be respected	No
Refrigerated storage	Physical: None is identified at this time Chemical: None is identified at this time Biological: Potential pathogen growth	Yes	Storage temperature must be maintained at or less in order to ensure the microbiological safety of this product	- Weekly calibration of temperature recording device - Temperature must be maintained below - Good hygienic condition (cleaning and disinfection)	Yes

2.5 Hazard Analysis Critical Control Points (HACCP)

2.51 HACCP system

In any food manufacture process, a Hazard Analysis Critical Control Point plan is regarded as the best method to control pathogens such as those mentioned above. Zottola and Smith (1993) define Hazard Analysis Critical Control Points (HACCP) as “a systematic approach to the identification and assessment of microbial hazards and risks associated with a food process”. Once established a HACCP system identifies, the points in the process that will prevent and control the hazards and risks.

To develop a HACCP system for a cheese manufacture, there are several steps: 1) safe and wholesome products through complete management of the manufacture process; 2) the hazards and risks associated with the ingredients and products should be identified; 3) the points to control the identified hazards and risks in the process should be identified; 4) the mechanisms that will monitor the control points should be developed; 6) operating the HACCP system to test it does control the identified hazards and risks (Zottola & Smith, 1993). Initial development of HACCP system for Ricotta Salata manufacture, requires the identification of hazards followed by defining critical control points in the manufacture of products. Succinctly, a critical control point is a point in the process, that will control the identified hazard or risk (Zottola & Smith, 1993). For instance, during Ricotta Salata manufacture, the critical control points can be pasteurisation of raw milk, adding citric acid to lower pH, and cooling storage of product.

2.52 Hazard analysis

The purpose of Hazard identification is to identify potential physical, chemical and microbiological hazards that may occur during processing steps. Physical hazards are the foreign material, which come from incorrect handling or processing environment. Chemical hazards indicate the chemical contaminants added during product processing. Microbiological hazards are pathogens or harmful bacteria contaminated during manufacture, commonly are *L. monocytogenes*, *Salmonella*, *Staphylococcus aureus*. However, the identification of hazards must be relevant and verified practically due to the individual conditions of the plant. A grade will be given to each hazard by identification and evaluation each part of the plant, which is based on the severity of the hazards, the occurrence risk, and its ability to be detected. Table 2 shows the results of the analysis for

the safety hazards and CCPs in the Ricotta Salata cheese processing which is according to the manufacture process listed in Figure 1.

2.53 Critical control points determination

There are three CCPs had been determined as listed in Table 3. The first CCP is the milk heat treatment. The second CCP is the temperature and time of drainage and storage are critical to control growth of pathogens. The third CCP is the temperature during storage must be maintained at 5°C or below in order to limit the pathogens' growth in this product.

2.54 HACCP control chart

The HACCP control chart of Ricotta Salata (Table 3) includes parameters, such as significant hazards and critical limits of CCPs, monitoring contents, and corrective actions. Monitoring is the measurement or observation at a CCP level to evaluate the Ricotta Salata process operating within the critical limits. When the results of monitoring shown there is a need to prevent deviation from a CCP, corrective actions will be considered. Records are the evidence that the processes were under control; also, they are helpful for products' traceability. Last but not the least, the verification is ensuring that the whole process is safe from day to day (Kamel et al., 2013).

2.6 Microbiological hurdle technology

The microbiological hurdle technology is used to maintain the stability and safety of the food products. The preservation factors are called hurdles. Normally the hurdle methods are combined to preserve the food. The most successful hurdles are heat treatment, pH and a_w (Rahman, 2015).

2.61 Heat treatment

During Ricotta Salata cheese manufacture, the milk was heated up to 95°C, which can kill *L. monocytogenes* and the vegetative cells of *B. cereus*. Therefore, it can be regarded as a hurdle for Ricotta Salata.

2.62 pH value

pH under 4.6 can inhibit the growth of many pathogens, which is generally accepted as a safety margin (Brown & Booth, 1991). However, the pH of Ricotta Salata as mentioned before is around 6 (Casti et al., 2016; Spanu et al., 2015, 2016), which cannot limit the

growth of *B. cereus* and *L. monocytogenes*, thus it cannot be accepted as a hurdle for Ricotta Salata.

2.63 A_w value

The a_w below 0.85 can limit the growth of pathogenic bacteria, and lower than 0.6 can limit the growth of yeasts and moulds (Labuza et al., 1972). The a_w of Ricotta Salata is as high as 0.97 (Casti et al., 2016; Spanu et al., 2015, 2016), which means it cannot be accepted as a hurdle.

2.64 NaCl concentration

Salt has been used as a preservative for a long period time. Oren (2011) identified 34.6% NaCl can stop all microbial process, however the high NaCl concentration is under rate for health and sensory. Thus, NaCl is normally used with other hurdles. In Ricotta Salata cheese the NaCl concentration is around 3% (Casti et al., 2016; Spanu et al., 2015, 2016), which means it cannot be accepted as a hurdle.

2.6 Shelf life and consumption of Ricotta Salata cheese

The shelf-life of Ricotta Salata cheese is generally several months under refrigeration (Casti et al., 2016). The variability of the shelf-life is depending on a number of factors, such as storage temperature, product composition, packaging conditions, presence of preservatives and competitive microflora (Casti et al., 2016).

Table 3.

HACCP control chart for Ricotta Salata cheese (Sorting from Kamel et al., 2013).

Process step	Hazard	Preventive measure	Critical limits	Monitoring procedure	Monitoring frequency	Corrective action
Milk heat treatment	Survival of pathogens such as <i>Salmonella</i> , <i>L. monocytogenes</i> , <i>Staphylococcus aureus</i> and pathogenic <i>E. coli</i>	Temperature must achieve 90°C	Heating temperature need to reach 90°C	- Temperature measurement - The application of the rules of good manufacture practices - Calibration of the thermometer	Every heating time	- Temperature corrected
Drainage and cooling	Potential pathogen growth	Temperature is set for 30min	Temperature set below 25°C for 30min	Time temperature measurement weekly calibration of temperature recording device	Every time drainage	- Temperature should be adjusted by setting the equipment in a temperature controlled room. - Time of the drainage should be recorded and consistent.
Storage	Potential pathogen growth	Temperature must be maintained during storage	Storage temperature < 5°C during storage	- Temperature measurement - Weekly calibration of temperature recording device	Everyday	- The cause of deviation should be identified and eliminated - The CCP should be brought under control after corrective action is taken - Measures to prevent recurrence are established

Ricotta Salata cheese is a ready-to-eat product, which can be consumed alone or with other meals, such as salad, soup or other dishes. The flavour of Ricotta Salata does alter with the ingredients used, the traditional ingredient is whey, but nowadays milk and milk powder are added and contribute to the flavour. Such a variable flavour appeals to the global market for Ricotta Salata cheese. However, the storage and handling during the preparation of the cheese for consumption can be a challenge for food safety.

This study aimed to test the hypothesis that *B. cereus* and *L. monocytogenes* can survive and grow during the manufacture of Ricotta Salata cheese.

3. Materials and methods

3.1 Materials

The Farmhouse milk (Meadow Fresh Ltd, New Zealand) and non-iodine table salt (Cerebos Ltd, New Zealand) were bought from a local supermarket in Palmerston North, New Zealand. The milk was stored in a 4°C store room, and salt was stored at room temperature at around 25°C. The milk was used before the “Best-Before” date.

3.2 Microorganism

The following strains, *B. cereus* D1 and *B. cereus* ATCC13061, *L. monocytogenes* W1 and *L. monocytogenes* ATCC35152 were cultured for use in this study. *B. cereus* D1 was isolated from a dairy factory, and *L. monocytogenes* W1 was obtained from Hill Laboratories (Ltd). According to the sample confidentiality, the exact sources of the two strains *B. cereus* D1 and *L. monocytogenes* W1 were unidentified; however, they both represent isolates from the food industry. PCR identification with species specific primers identified them belongs to *B. cereus* and *L. monocytogenes*, respectively.

All cultures were obtained by inoculating a loopful of culture into 10mL of Brain Heart Infusion (BHI) broth (Oxoid Ltd, England). *B. cereus* strains were incubated at 30°C and *L. monocytogenes* strains were incubated at 37°C for 24 h.

3.3 Experimental design

Ricotta Salata cheese samples were first produced following by the instructions in the Artisan Cheese Kit (Mad Millie Ltd, New Zealand) as shown in Figure 2. The pH, a_w and NaCl concentration were tested on Days 3, 5, 7, 14 (aging week 1), and Day 21 (aging week2). The results were compared with the published information and modifications were made to produce a product that met the expected criteria for Ricotta Salata cheese.

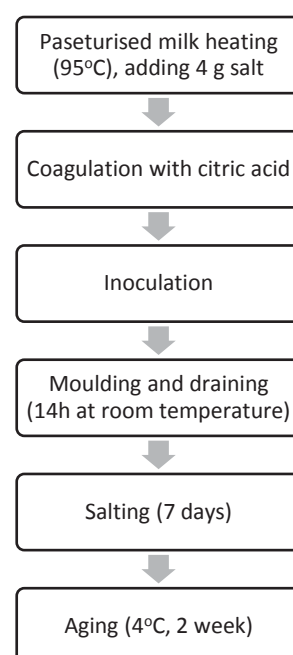


Figure 2. Process flow of the manufacture of Ricotta Salata cheese made from whey.

After modification, the Ricotta Salata cheese samples were analysed at different times during the manufacture processes. Sampling times were: When bacteria culture inoculated into Ricotta Salata cheese curd, defined as time zero (T0), and time 20 (T20), time 92 (T92), time 140 (T140), and time 308 (T308) were 20, 92, 140 and 308h after the inoculation of bacteria cultures. Among them, T92, T140, and T308 were sampled at both surface and centre of the cheese, which is because salting process was started after sampled at T20.

The Ricotta Salata samples were analysed for the determination of the microbiological profile and physicochemical properties (pH, a_w and NaCl concentration).

3.4 Preparation of Ricotta Salata cheese

The study was conducted on Ricotta Salata cheese, made using a modified process based on the Artisan Cheese Kit (Mad Millie Ltd, New Zealand). The major steps of the process flow for the Ricotta Salata cheese examined in the present study are shown in Figure 2. The modifications involved shortening the salting and aging steps to 5 days and 1 week respectively.

For every experiment, two identical Ricotta Salata cheeses were made at the same time. The mean $\pm$ SD result for the two cheeses was regarded as one trial. In total seven trails of Ricotta Salata cheese inoculated in *B. cereus* and seven trails of Ricotta Salata cheese inoculated in *L. monocytogenes* were made.

3.41 Pasteurised milk heating

4g non-iodine salt (Cerebos Ltd, New Zealand) was dissolved into 2L pasteurised whole milk within 'Best-Before' date. The milk was heated to 95°C, took around 15min.

3.42 Coagulation

The citric acid solution was made by dissolving 3.6g citric acid powder (Miles Laboratories Inc, United States) into 60mL water. The citric acid solution was added into the milk, when it achieved 95°C. The mixture was stirred for 1min to mix. The mixture was left for 1h to coagulate and cool down to less than 35°C.

3.43 Artificial inoculation

The curd was removed and pressed with a colander in Artisan Cheese Kit (Mad Millie Ltd, New Zealand) to make it as dry as no visible whey draining out in 1min. 1mL bacteria culture (approximately 7 log₁₀ CFU/mL for *B. cereus* broth and 8 log₁₀ CFU/mL for *L. monocytogenes* broth) was added into the curd, then stirred for 10min to mix it completely. In the Ricotta Salata cheese the inoculation levels were around 4 log₁₀ CFU/g of *B. cereus* and 5 log₁₀ CFU/g of *L. monocytogenes*. These levels of

inoculation were used to detect the death or growth during the manufacture of Ricotta Salata. No uninoculated control was used as the level of inoculation used was greater than aim like natural contamination and would not interact with the experiment.

3.44 Moulding and draining

The inoculated Ricotta cheese curd was scooped into a Ricotta Basket Artisan Cheese Kit (Mad Millie Ltd, New Zealand). 1.1g cheese was sampled immediately, recorded as T0. Then the Ricotta cheese was drained for 20h. A T20 sample was taken just before salting.

3.45 Salting

The Ricotta cheese surface was rubbed with 2g salt, after draining for 20h, and it was salted every day for 5 days. The Ricotta Salata cheese was stored at 4°C during this time. 1.1g sample was taken at 92h and 140h from the surface and centre of cheese, recorded as T92 and T140.

3.46 Aging

After 2 days salting, the Ricotta Salata cheese was kept in the Ricotta basket in a plastic box in Artisan Cheese Kit (Mad Millie Ltd, New Zealand) at 4°C, for 1 week. T308 was sampled at 308h both from the surface and centre of the cheese.

3.5 Incubation of Ricotta Salata cheese

Sampling time was set as after inoculation (T0), 20 h after inoculation before salting (T20), salted for 3 days (T92), salted for 5 days (T140), and aging for 1 week (T308). T92, T140, and T308 were sampled both on the surface (1cm depth) and centre (7cm depth) of the cheese. A metallic stick was used to take samples, each time before sampling, the stick will be sterilised by 70% ethanol and a gas burner. Each sample was taken from at least 2 parts of the cheese at the same depth.

1.1g of sample was transferred into 9.9mL of buffered peptone water (Microbiology GranuCult™), and suspended blending in a Stomacher bag (Stomacher, AES laboratories, Ltd). Then 0.1mL of the suspension was added into 0.9mL peptone water, and ten-fold serial dilutions of the suspensions were prepared.

The Mannitol Egg Yolk Polymyxin (MYP) agar (Fort Richard Laboratories Ltd, New Zealand) is a *B. cereus* selective agar, which was used for the enumeration of *B. cereus*. 0.2mL of each dilution of cheese sample was spread on the surface of the agar and incubated at 30°C for 24h. The number of colonies was counted and expressed as CFU/g of sample. The plates were also examined for typical *B. cereus* colonies, which were pink in colour with zone of representing lecithinase activity (Mossel et al., 1967). Typical *B. cereus* colonies were examined by Gram staining which showed Gram-positive rod-shaped containing spores which did not stain (Harrigan & McCance, 1976).

L. monocytogenes selective agar [Oxford agar (Fort Richard Laboratories Ltd, New Zealand)] was used for the enumeration of *L. monocytogenes*. 0.2mL of each dilution of cheese was spread on the surface of the agar and incubated at 37°C for 24h. The number of colonies was counted and expressed as CFU/g of sample. The plates were also examined for typical *L. monocytogenes* colonies, which were 1mm in diameter, black in colour, and surrounding with blackened precipitate resulting from esculin hydrolysis (Van Netten et al., 1989). Typical *L. monocytogenes* colonies were examined by Gram staining which showed Gram-positive rod-shaped bacterium.

3.6 Physico-chemical analysis of Ricotta Salata cheese

pH, a_w and NaCl concentration were determined at each sampling time.

The pH values of cheese samples were measured using Seven Compact pH Meter (Mettler Toledo, China) at around 25°C. The meter electrode was calibrated with pH 4 and pH 7 buffer solution first. And the meter electrode was washed by distilled water before each sampling. Then the electrode was pat dried with a soft tissue. After that the electrode was placed inside a container of the sample. When the reading stabilised, the result was recorded. Three measurements were taken for each sample, and mean and SD were recorded.

The a_w values were determined using Dew Point Water Activity Meter (Aqua Lab, New Zealand). No more than half cup of sample was place in the special cup. There were lids for waiting samples to prevent water loss. Three measurements were taken for each sample, and mean and SD were recorded.

The NaCl concentration was measured by determining the chloride ion concentration by titration (Volhard's Method). This method uses a back titration with potassium thiocyanate to determine the concentration of chloride ions in a solution. 3g of sample was dissolved into 50mL 0.1mol/L silver nitrate solution (LabServ Ltd, New Zealand), and then 20mL of concentrated nitric acid was added, 100mL of distilled water and a few boiling chips, before boiling the solution in a fume hood. As the solution boiled 5mL of 5% potassium permanganate solution, made from potassium permanganate power (Science Supply Store, Australia) was added. The solution was kept boiling until the purple colour disappeared, and then another 5mL of potassium permanganate added. This process continued until 30mL of potassium permanganate solution had been added and the cheese particles were completely digested. 1mL of saturated ferric ammonium sulphate solution, made from ammonium ferrous sulfate-12-hydrate (Riedel-deHaën™) was added into 100mL of the cheese extract solution as an indicator. The unreacted silver ions were titrated with 0.1mol/L potassium thiocyanate solution (Normadose™). The end point is the first appearance of a dark red colour due to the ferric thiocyanate complex. For each sample three titrations were determined. The concentration of sodium chloride in the cheese was reported as grams of NaCl per 100g cheese (%NaCl), mean and SD were recorded. Tests were done on samples taken from the surface (1cm from surface), middle (3cm from surface) and centre (6cm from surface) of the cheese.

3.7 Statistical analysis

Differences in mean and SD mesophilic bacteria counts ($\log_{10}$ CFU/g), intrinsic properties (mean $\pm$ SD) and composition (% $\pm$ SD) between sampling times were compared using T-test. All statistical analyses were performed with Minitab 17 software.

4. Results

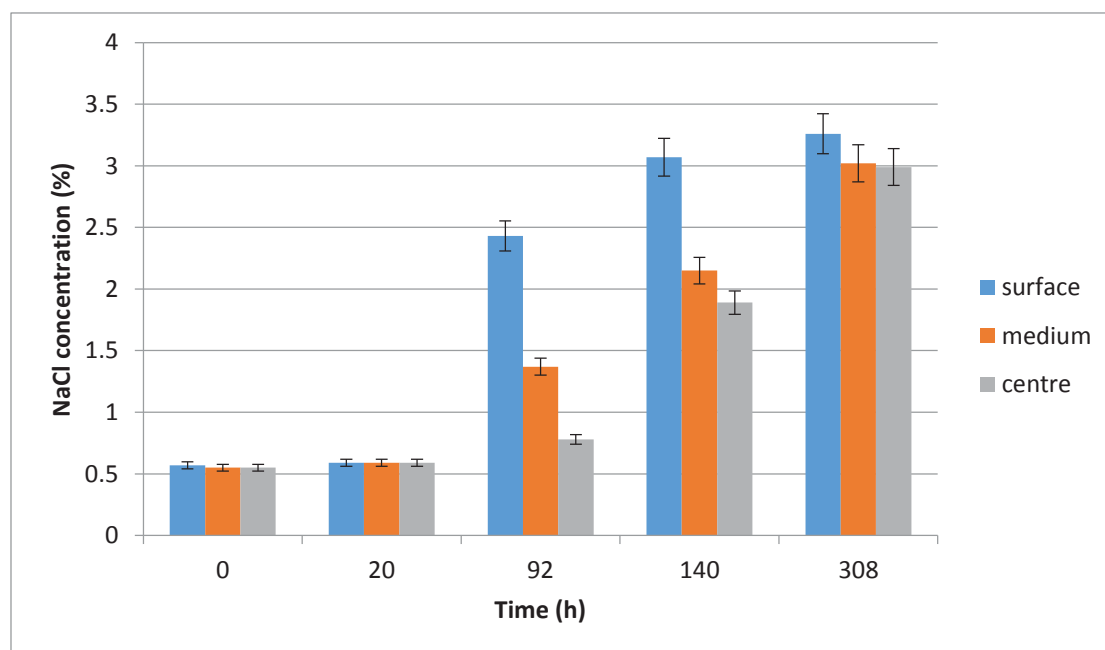
4.1 Selected physicochemical properties of Ricotta Salata cheese

The intrinsic properties and composition of Ricotta Salata have been reported (Table 1). Spanu et al. (2016) reported the intrinsic properties (mean $\pm$ SD) of a Ricotta Salata cheese were 6.49 $\pm$ 0.10 for pH, 0.978 $\pm$ 0.001 for a_w , and NaCl (% $\pm$ SD) 2.60 $\pm$ 0.30.

Spanu et al. (2015) reported the intrinsic properties (mean $\pm$ SD) were 6.42 $\pm$ 0.09 for pH and 0.963 $\pm$ 0.01 for a_w , and NaCl (% $\pm$ SD) 4.56 $\pm$ 1.38. Casti et al. (2016) 6.54 $\pm$ 0.03 for pH, 0.973 $\pm$ 0.05 for a_w , and NaCl (% $\pm$ SD) 3.42 $\pm$ 0.24. The mean values of these reported studies are 6.48 for pH, 0.971 for a_w , and 3.53% for NaCl concentration, which was used as a standard to manufacture Ricotta Salata samples used in this study.

The results of pH, a_w , and NaCl concentration tested during manufacture of Ricotta Salata cheese in this study are shown in Table 4. The pH of all samples was lower than reported pH values. Day 5 samples had a_w and NaCl concentrations closest to the reported values. After salting for 7 days, and aging for 14 days, the a_w decreased, and the NaCl concentration increased. Therefore, in order to obtain a Ricotta Salata cheese, with properties that met the contained the properties reported for this type of cheese, the Ricotta Salata cheese was slated for 5 days and aged for 1 week.

4.2 NaCl concentration of Ricotta Salata cheese



where: Surface=1cm from surface; Medium=3cm from surface; Centre=6cm from surface. At 0 h, 4 g of salt was added into the milk; at 20 h, 92 h and 140 h, 2 g of salt was added on the surface of the Ricotta Salata cheese.

Figure 3. The NaCl concentration of Ricotta Salata.

Figure 3 shows the NaCl concentrations of Ricotta Salata during manufacture. At the beginning of the process the NaCl concentrations were low, and there was no visible

difference between the surface, medium and centre. From T20 to T92 there was an obvious increase, and the gradient from the surface to the centre was significant ($p < 0.05$). Then after T92 the gradients became less. NaCl concentration, it was increased fourfold from T20 to T92, and stabilised T92 to T308. The increase of NaCl concentration of the area midway between the surface and centre of the cheese (medium) increased steadily from T20 to T308. In the centre, the NaCl concentration was slightly increased from T20 to T92, and then significant increased from T92 to T308 ($p < 0.05$). The final NaCl concentration ($\% \pm \text{SD}$) of the surface was 3.26 ± 0.13 , the medium was 3.02 ± 0.07 and the centre was 2.99 ± 0.10 , which is similar to the reported NaCl concentrations as 3.42 ± 0.24 and 2.60 ± 0.30 (Casti et al., 2016; Spanu et al., 2016).

4.3 Inoculation of bacteria

Approximately $7 \log_{10}$ CFU/mL *B. cereus* broth inoculated into milk d after heating (when the temperature lower than 30°C), and at the beginning of modelling (T0) approximately $5 \log_{10}$ CFU/g *B. cereus* remained in the cheese.

Approximately $8 \log_{10}$ CFU/mL *L. monocytogenes* broth inoculated into milk after heating (when the temperature lower than 30°C), and at the beginning of modelling (T0) the approximately 5 to $6 \log_{10}$ CFU/g *L. monocytogenes* remained in the cheese.

4.4 pH change during the manufacture of Ricotta Salata inoculated with different strains

The pH change of surface during the manufacture of Ricotta Slata cheese, which was inoculated with *B. cereus* D1 is shown in Figure 4. From T0 to T308, the pH of the seven samples all increased to a different extent. Sample 4 increased the most from 5.96 ± 0.04 to 6.23 ± 0.01 (mean $\pm$ SD), and the least increase was Sample 7, from 6.06 ± 0.03 to 6.07 ± 0.01 .

Table 4.

pH, a_w , and NaCl concentration of Ricotta Salata during manufacture (mean $\pm$ SD).

Sampling time	pH	a_w		NaCl (%)		
		Surface	Centre	Surface	Centre	
Day 3	6.08±0.02	5.96±0.05	0.9763±0.0013	0.9852±0.009	2.54±0.20	0.98±0.06
Day 5	6.06±0.03	5.99±0.11	0.9684±0.0016	0.9729±0.0015	3.32±0.05	3.09±0.11
Day7	6.04±0.10	5.91±0.19	0.9588±0.0032	0.9602±0.0021	4.55±0.15	4.23±0.21
Day 14	6.10±0.24	5.94±0.04	0.9544±0.0017	0.9565±0.0022	4.60±0.16	4.49±0.07
Day 21	5.99±0.11	5.95±0.07	0.9490±0.0012	0.9539±0.0005	4.68±0.06	4.49±0.14

where: Day3, Day 5, Day 7= salting for 3 days, 5 days, and 7 days. Day 14 and Day 21= aging for 1 week and 2 weeks, respectively.

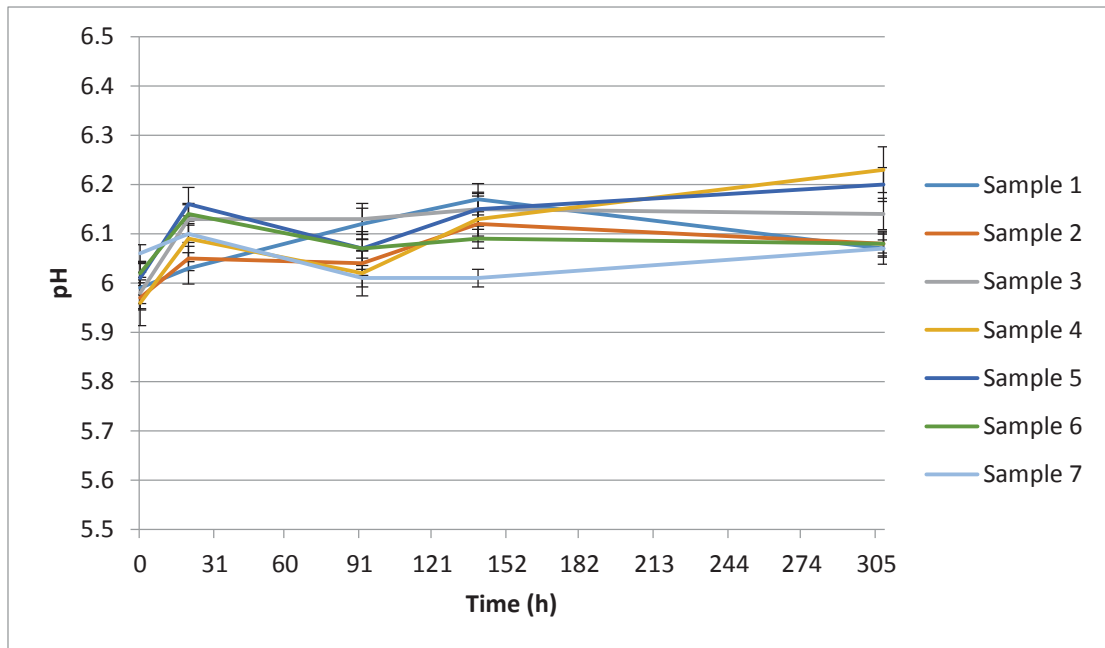


Figure 4. The pH change during the manufacture of Ricotta Salata inoculated with *B. cereus* D1 isolated from food factory at 4°C (surface) (mean±SD).

For the first 20h, the pH of all seven samples increased. For this point, there was some fluctuation in pH with most showing a decrease. The change was only about 0.1 of a pH unit.

At T308, the pH of seven samples was from 6.00 ± 0.03 to 6.23 ± 0.01 , which is close, but slightly lower than the values reported by Spanu et al. (2016) (6.23 to 6.67). In other reported studies, the pH values of Ricotta Salata cheese were 6.42 ± 0.09 and 6.54 ± 0.05 (Casti et al., 2016; Spanu et al., 2015).

The pH values of the centre of the Ricotta Salata inoculated with *B. cereus* D1 were more similar for all 7 samples compared with the values of the surface (Figure 5). The major trend in pH change was the increase from T0 to T20, followed by a decrease from T20 to T92, followed by a slight increase from T92 to T140. The pH stabilised to between 5.92 ± 0.01 and 5.99 ± 0.01 at the end of the trial. Interestingly, the decrease in pH for Samples 1 and 2 between T140 and T308 followed the trend in the surface of Ricotta Salata.

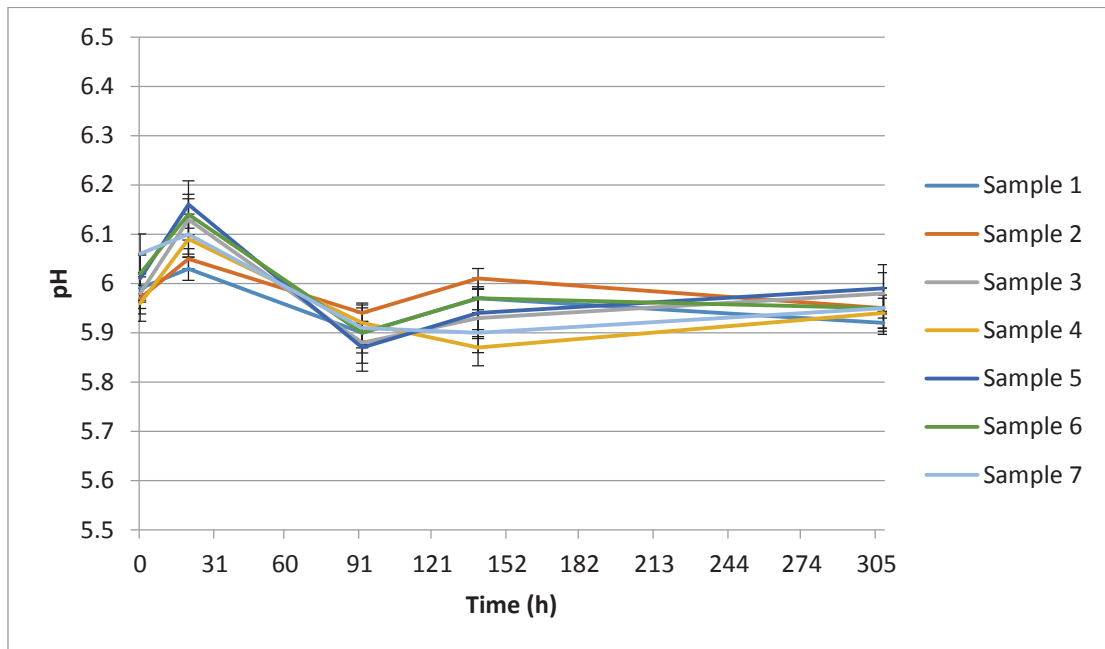


Figure 5. The pH change during the manufacture of Ricotta Salata inoculated with *B. cereus* D1 isolated from food factory at 4°C (centre) (mean±SD).

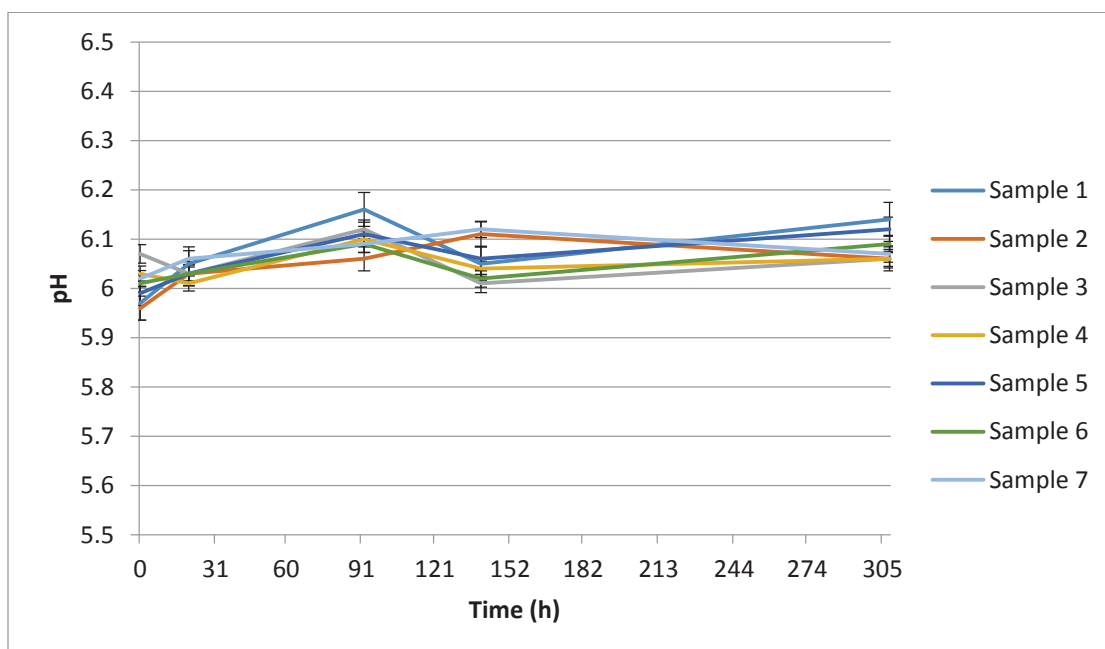


Figure 6. The pH change during the manufacture of Ricotta Salata inoculated with *B. cereus* (ATCC13061) at 4°C (surface) (mean±SD).

The surface pH change during the manufacture of Ricotta Salata inoculated with *B. cereus* (ATCC13061) is shown in Figure 6. The pH values of most samples (except Sample 3) increased at the start of the process. Most of the samples increased from

T0 to T92, and then decreased from T92 to T140 followed by an increase. The pH of most samples was between 6.05 and 6.10 at the end of the trial. Although Sample 1 and 5 produced a slightly higher pH than the others at the end of the trail, the difference between each sample was no more than 1.

The pH change of the centre of Ricotta Salata inoculated with *B. cereus* (ATCC35152) during manufacture is shown in Figure 7. The pH of all but two samples (4 and 6) decreased in the first 20 h, and then decreased for all samples from T0 to T92. From this point the pH appeared to transition from a decrease to an increase from T140 to T308. During this transition zone T92 to T140 samples 2 and 7 increased while the other samples decreased slightly. Then from T140 to T308 Sample 7 had a slightly decrease, while other samples all increased.

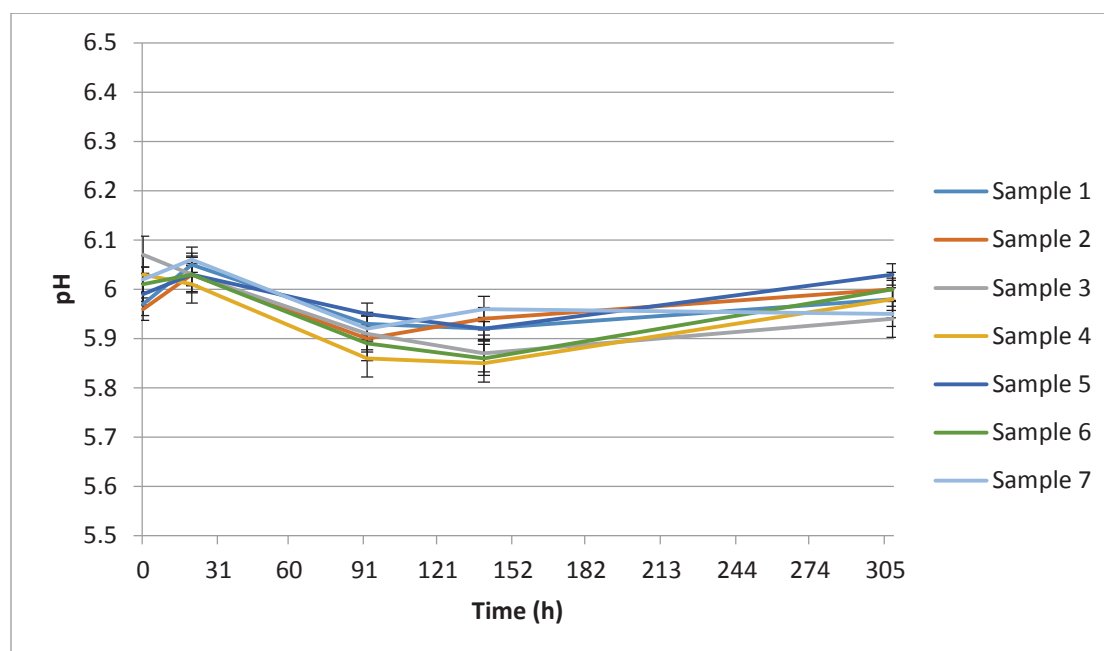


Figure 7. The pH change during the manufacture of Ricotta Salata inoculated with *B. cereus* (ATCC13061) at 4°C (centre) (mean±SD).

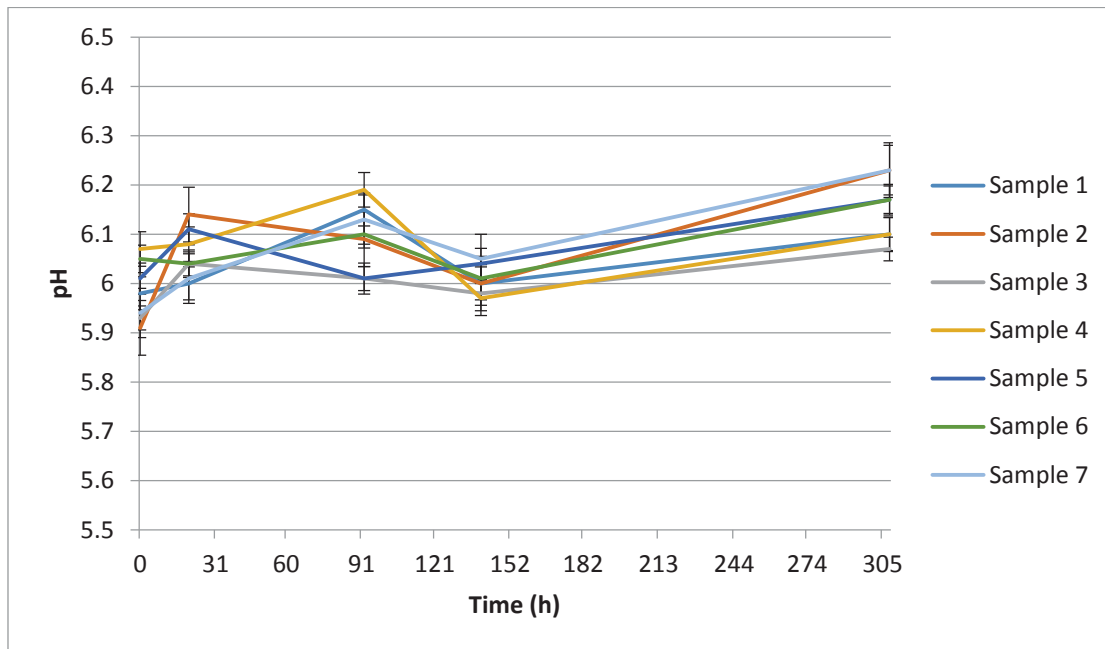


Figure 8. The pH change during the manufacture of Ricotta Salata inoculated with *L. monocytogenes* W1 at 4°C (surface) (mean±SD).

The pH change for the surface of Ricotta Salata cheese inoculated with *L. monocytogenes* W1 is shown in Figure 8. The pH change fluctuated from T0 to T140, and then all of the seven samples showed an obvious increase. The final pH values were between 6.07 and 6.23.

The centre pH change during the manufacture of Ricotta Salata inoculated with *L. monocytogenes* W1 is shown in Figure 9. Sample 6 decreased slightly from T0 to T20. All of the other samples showed an increase over that period. This was followed by a decrease in pH for all samples, reaching the same level as beginning of the trial. Then, the pH values started to disperse, with Sample 7 showing an obvious increase while the other samples decreased from T92 to T140. From T140 to T308, the pH all of the seven samples all significant increased, which is as same as the trend of the surface pH ($p<0.05$).

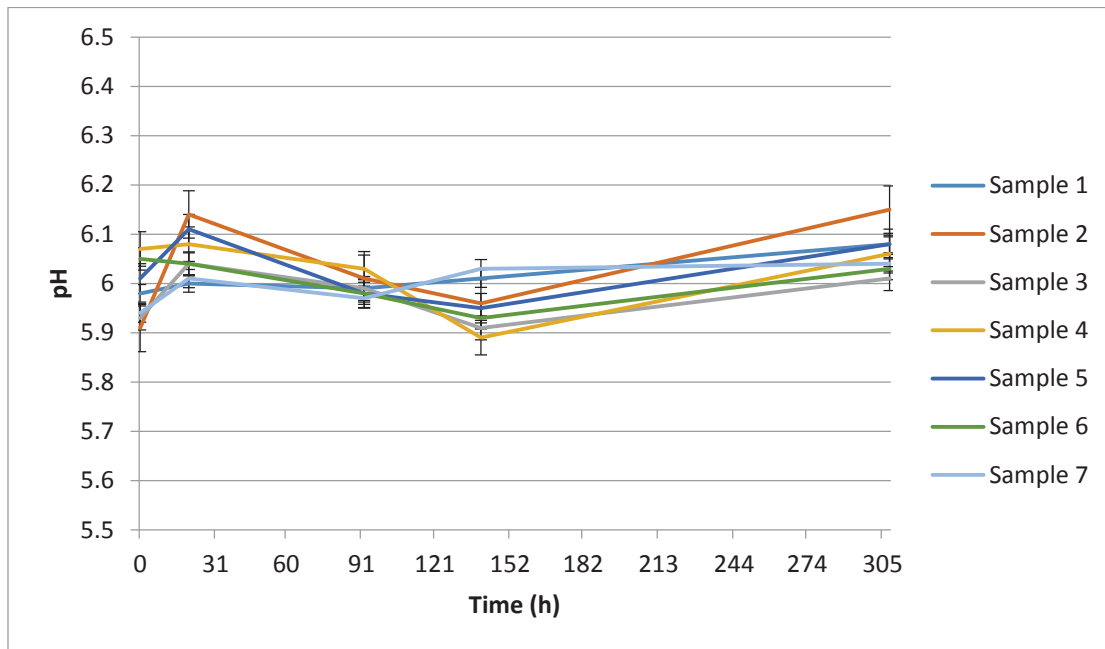


Figure 9. The pH change during the manufacture of Ricotta Salata inoculated with *L. monocytogenes* W1 at 4°C (centre) (mean±SD).

The trend in pH during the manufacture of Ricotta Salata inoculated with *L. monocytogenes* (ATCC 35152) in both the surface and centre were similar to *L. monocytogenes* W1. The figures are shown in Appendix (Figure A1 & A2).

4.5 a_w change during the manufacture of Ricotta Salata inoculated with different strains

The surface a_w change during the manufacture of Ricotta Salata inoculated with *B. cereus* D1 is shown in Figure 10. All samples showed a similar trend with a decrease in a_w . From T0 to T20 the decrease was slow, however due to the salting process, there was a more rapid decrease from T20 to T92, then from T92 to T140 the a_w values decreased slightly. Afterwards, all of the a_w turned to be stable. The final a_w values of the surface of Ricotta Salata were between 0.9598 ± 0.0005 and 0.9694 ± 0.0007 , which are close to the reported value 0.963 ± 0.01 (Spanu et al., 2015).

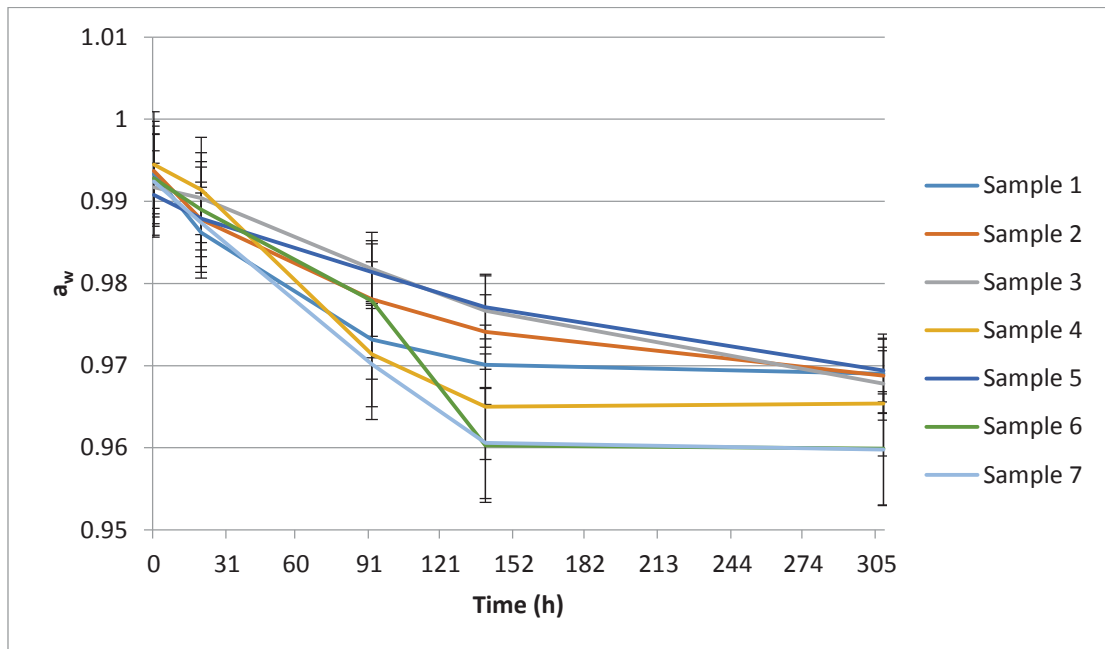


Figure 10. The a_w change during the manufacture of Ricotta Salata inoculated with *B. cereus* D1 isolated from food factory at 4°C (surface) (mean±SD).

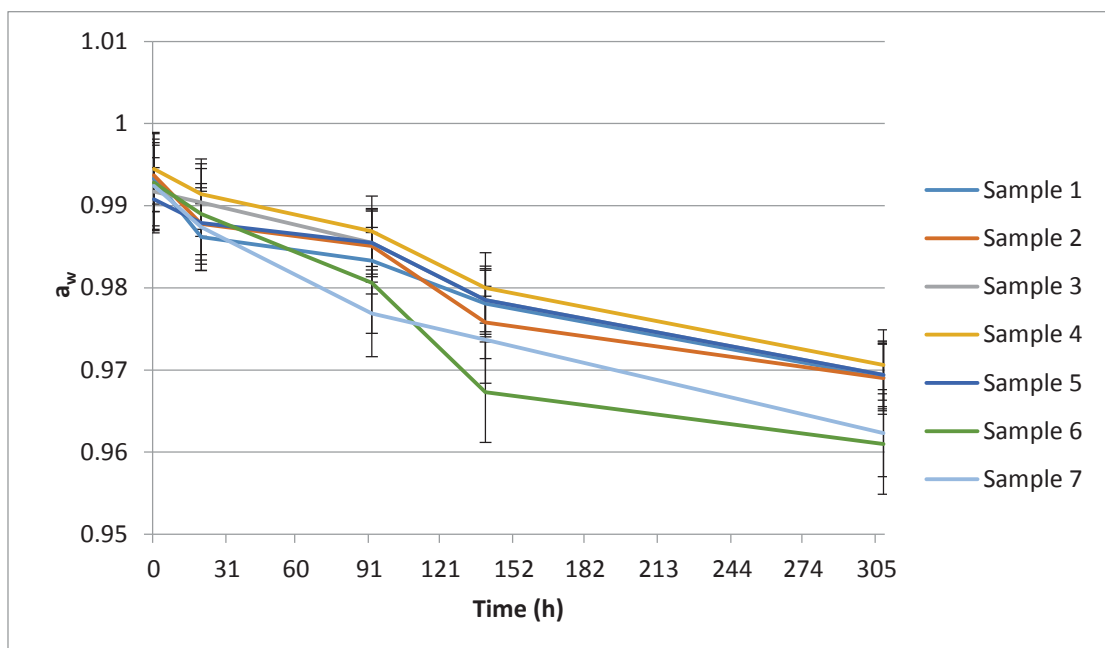


Figure 11. The a_w change during the manufacture of Ricotta Salata inoculated with *B. cereus* D1 isolated from food factory at 4°C (centre) (mean±SD).

The centre a_w change during the manufacture of Ricotta Salata inoculated with *B. cereus* D1 is shown in Figure 11. The results for the centre of Ricotta Salata were similar to the surface (Figure 8). a_w values of the seven samples started between

0.9908±0.0010 and 0.9945±0.0009, then decreased to 0.9610±0.0002 to 0.9706±0.0006. At T308, five out of the seven samples were between 0.9690±0.0007 and 0.9706±0.0006. Samples 6 and 7 reached a lower a_w values than the others, which is similar to the a_w values of the surface. The a_w change for Ricotta Salata inoculated with *B. cereus* (ATCC13061) was similar to that recorded for the cheese inoculated with *B. cereus* D1 for both the surface and the centre. The detailed figures are shown in the Appendix (Figures A3 & A4).

The similar trend for *B. cereus* (ATCC 13061) growth and survival both on the surface and centre of Ricotta Salata was observed. The detailed figures are shown in the Appendix (Figures A5 & A6).

Figure 12 shows the a_w change during the manufacture of Ricotta Salata inoculated with *L. monocytogenes* W1 (surface). The results for all seven samples were similar. The decrease from T0 to T20 was slow. Then from T20 to T140, there was a strong continued significant decrease ($p<0.05$). This was followed by a stabilisation in the values. A similar trend was shown for the centre samples, with details shown in the Appendix (Figure A7).

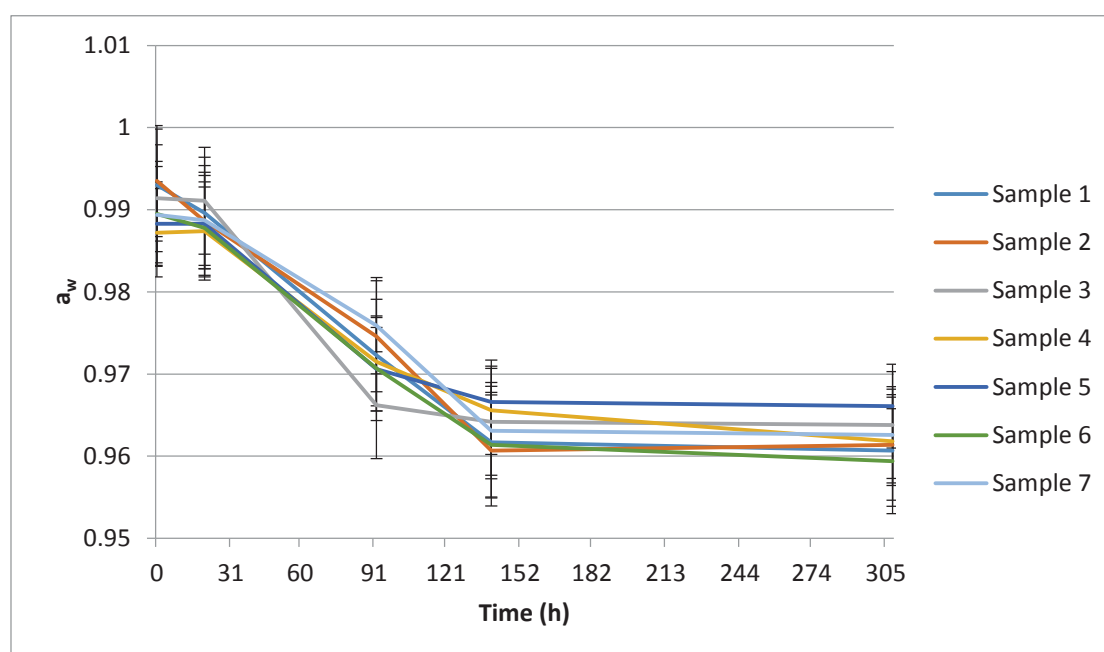


Figure 12. The a_w change during the manufacture of Ricotta Salata inoculated with *L. monocytogenes* W1 at 4°C (surface) (mean±SD).

The similar trends for a_w change both the surface and centre were observed on the Ricotta Salata inoculated with *L. monocytogenes* (ATCC 35152) compared with the cheese inoculated with *L. monocytogenes* W1. The detailed figures are shown in the Appendix (Figure A8 and A9).

4.6 Bacteria count change during the manufacture of Ricotta Salata inoculated with different strains

The bacteria count in the surface during the manufacture of Ricotta Salata inoculated with *B. cereus* D1 is shown in Figure 13 with all samples producing similar results. There was a significant increase of almost 3 $\log_{10}$ CFU/g from T0 to T20 ($p < 0.05$). Then the bacteria count decreased by about 0.5 $\log_{10}$ CFU/g from T20 to T92, and continued to decrease then all of the samples continued decreased by approximately 1 $\log_{10}$ CFU/g from T92 to T140. This was followed by an increase of approximately 0.5 $\log_{10}$ CFU/g from T140 to T308.

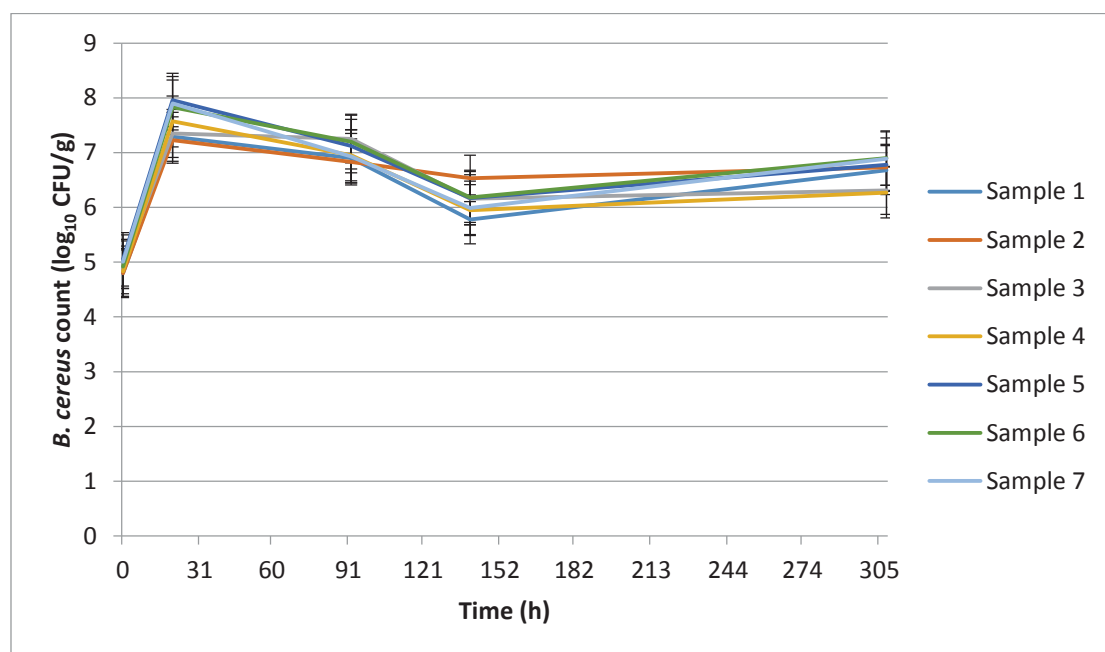


Figure 13. *B. cereus* count in the surface samples taken during the manufacture of Ricotta Salata inoculated with *B. cereus* D1 over a 308h period (mean $\pm$ SD).

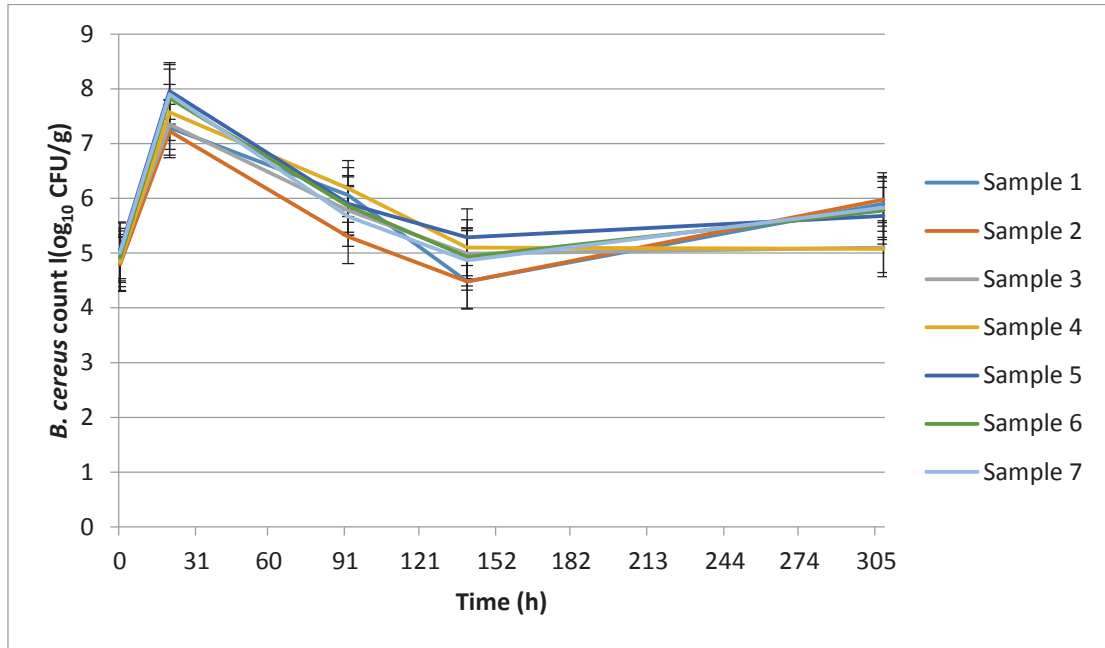


Figure 14. *B. cereus* count in the centre samples taken during the manufacture of Ricotta Salata inoculated with *B. cereus* D1 over a 308h period (mean $\pm$ SD).

The change in the bacteria count in the centre of sampled Ricotta Salata inoculated with *B. cereus* D1 is shown in Figure 14. From T0 to T20, bacteria count significant increased by about 3 log₁₀ CFU/g ($p < 0.05$), the difference between the seven samples was nearly 1 log₁₀ CFU/g. This was followed by a significant decreased of approximately 2 log₁₀ CFU/g in from T20 to T92 ($p < 0.05$), which was followed by a 1 log₁₀ CFU/g reduction from T92 to T140. Then most of the seven samples showed a slight increase, and some were stable.

The trends of *L. monocytogenes* growth in the surface during manufacture of Ricotta Salata cheese surface are shown in Figure 15. From T0 to T20 the bacteria count significant increased ($p < 0.05$), followed by a slight increase from T20 to T140. Then the bacteria count decreased slightly. The bacteria count of *L. monocytogenes* of the centre cheese observed a similar trend, with the details in the Appendix (Figure A10).

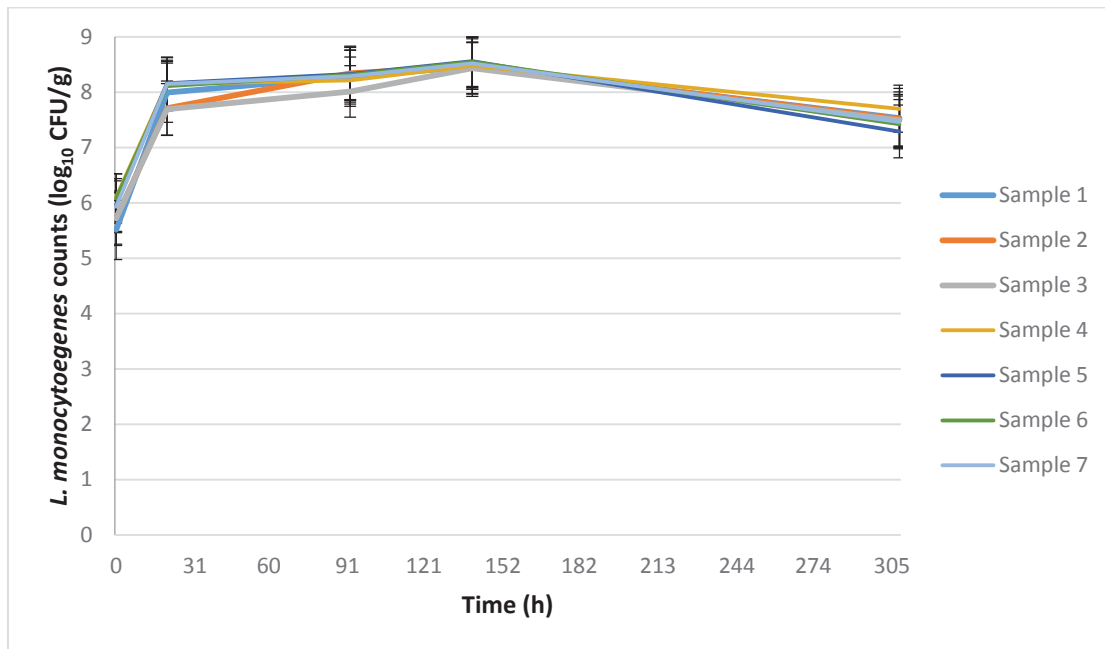


Figure 15. *L. monocytogenes* count in the surface samples taken during the manufacture of Ricotta Salata inoculated with *L. monocytogenes* W1 over a 308h period (mean±SD).

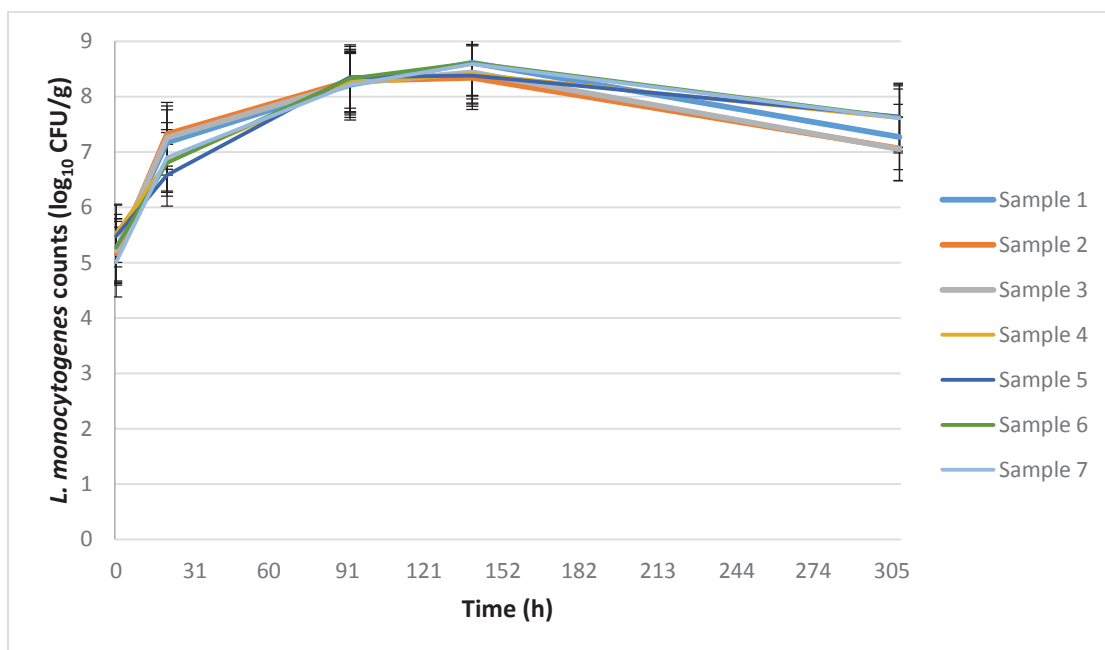


Figure 16. *L. monocytogenes* count in the surface samples taken during the manufacture of Ricotta Salata inoculated with *L. monocytogenes* (ATCC 35152) over a 308h period (mean±SD).

The bacteria count of *L. monocytogenes* (ATCC35152) on the surface of Ricotta Salata is shown in Figure 16. There was a steady increase occurred from T0 to T140, then the growth stopped and bacteria count start to decrease slightly. The *L. monocytogenes* (ATCC35152) in the centre showed similar results to the surface, with the details shown in Appendix (Figure A11).

4.7 *B. cereus*

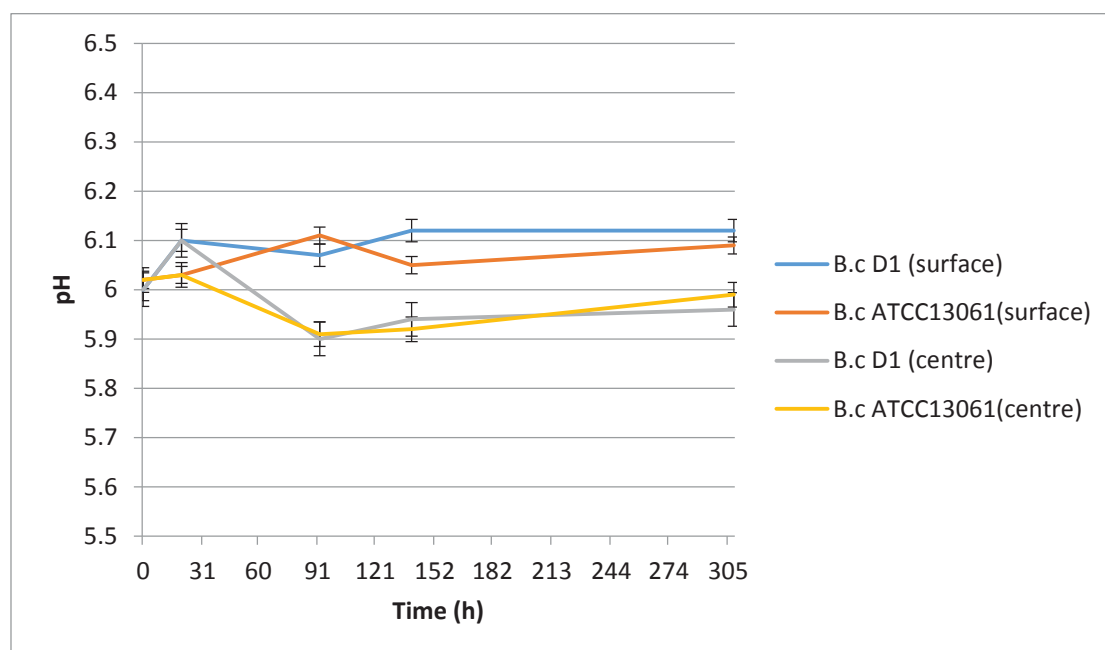


Figure 17. The pH change during the manufacture of Ricotta Salata inoculated with *B. cereus* D1 and ATCC 13061 (mean±SD).

The pH change during the manufacture of Ricotta Salata inoculated with *B. cereus* D1 and ATCC 13061 is shown in Figure 17. The pH increase in the cheese inoculated with *B. cereus* D1 was larger than the increase in cheese inoculated with of *B. cereus* ATCC35152 from T0 to T20. The pH of surface cheese increased to around 6.1 from T20 to T92, and stabilised. The pH of centre of Ricotta Salata decreased to about 5.9, and slightly increased from T92 to T308. There is no significant difference between the two strains ($p>0.05$), but a significant difference between the surface and the centre ($p<0.05$).

The change in a_w during the manufacture of Ricotta Salata inoculated with *B. cereus* D1 and ATCC 13061 is shown in Figure 18. The initial a_w of the cheeses were close.

From T92, the difference between surface and centre became obvious for a while, with the a_w of both cheeses becoming similar again towards the end of the trail. There is no significant difference between the two strains ($p>0.05$). The surface and the centre of both cheeses showed similar results, with no significant differences ($p>0.05$).

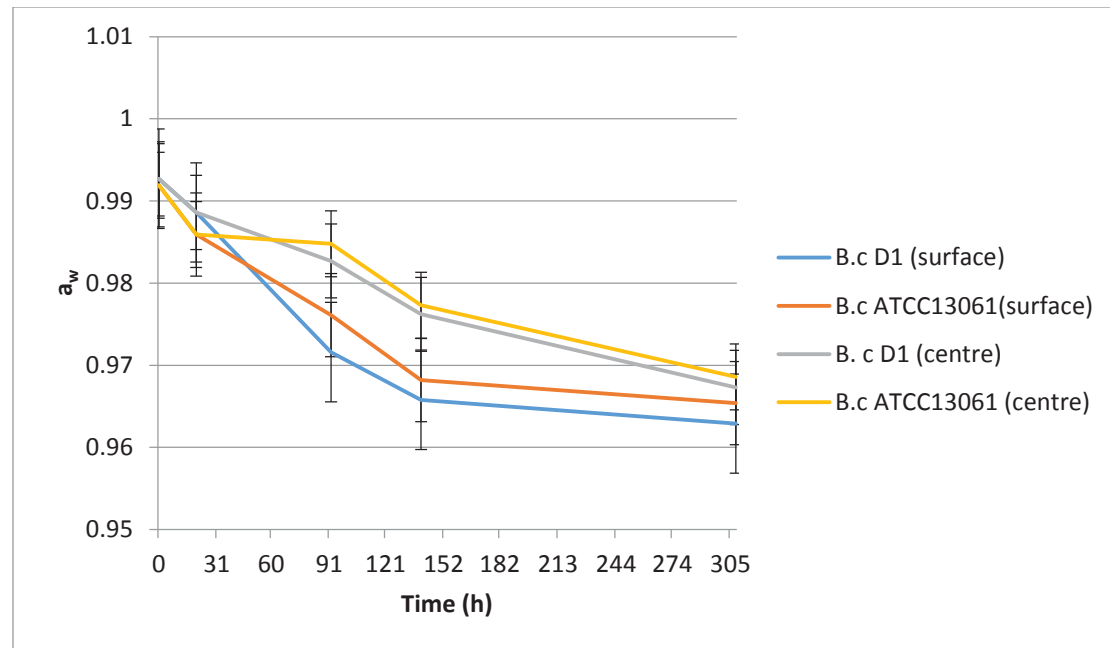


Figure 18. The a_w change during the manufacture of Ricotta Salata inoculated with *B. cereus* D1 and ATCC 13061 (mean $\pm$ SD).

The bacteria count during the manufacture of Ricotta Salata inoculated with *B. cereus* D1 and ATCC 13061 is shown in Figure 19. The trends in the counts these two strains both on the surface and centre were similar, with no significant neither between the two strains or the surface and the centre ($p>0.05$). There was a significant increase from T0 to T20 ($p<0.05$), then the bacteria count decreased, with the decrease in the centre larger than the surface. The decrease stopped at T140, and then the bacteria count increased slightly. The difference between ring and centre got larger from T20 to T140, and turned to be constant from T140 to T308.

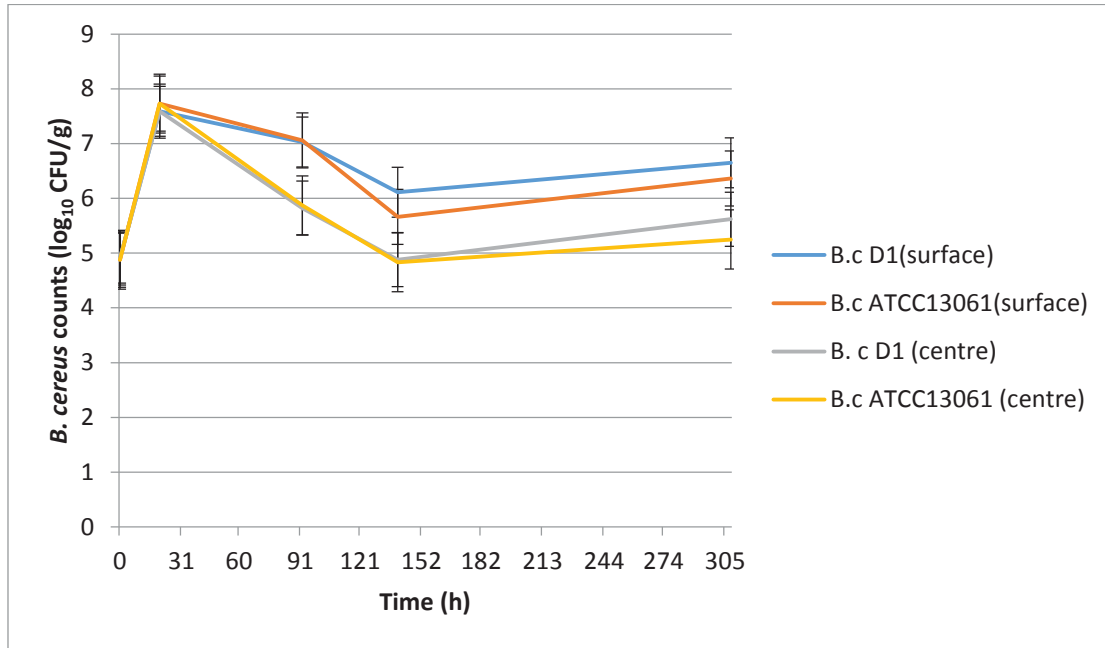


Figure 19. *B. cereus* counts taken during the manufacture of Ricotta Salata inoculated with *B. cereus* D1 and ATCC 13061 over a 308h period (mean±SD).

4.8 *L. monocytogenes*

The pH change during the manufacture of Ricotta Salata inoculated with *L. monocytogenes* W1 and ATCC 35152 is showing in Figure 20. The trends in cheese inoculated with each strain were similar, with no significant difference ($p>0.05$). There was an initial increase of pH from T0 to T20, and then the surface pH continued to increase. The centre pH decreased between T20 and T92. From T92 to T140, the pH of both the surface and the centre decreased. This was followed by a significant increase in pH ($p<0.05$). There is no significant difference between the surface and the centre ($p>0.05$).

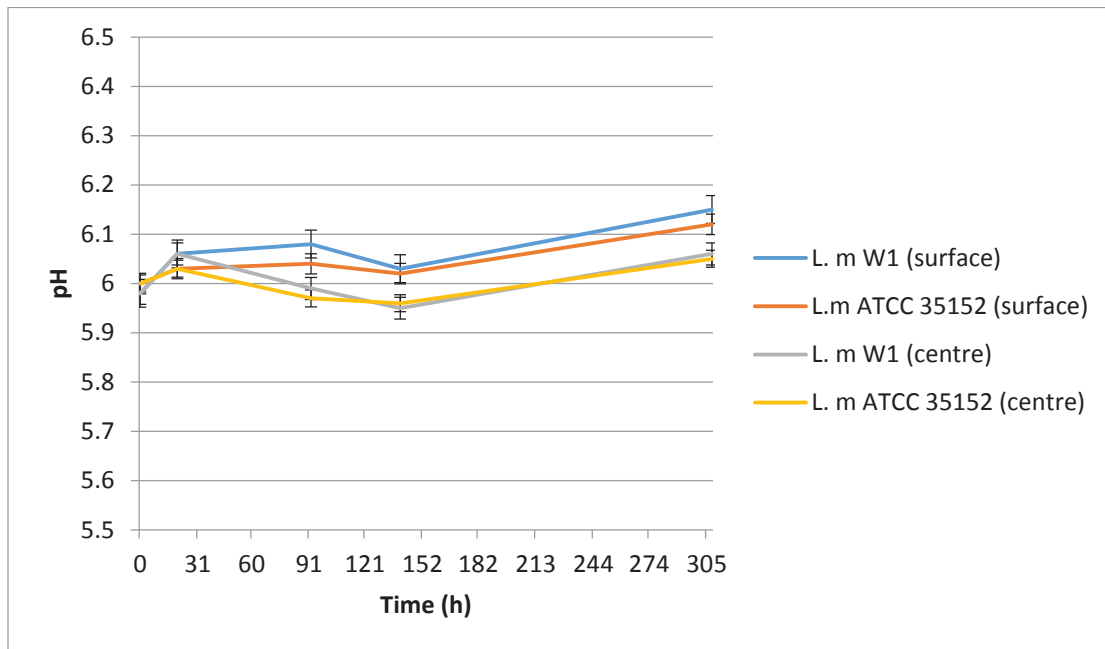


Figure 20. The pH change during the manufacture of Ricotta Salata inoculated with *L. monocytogenes* W1 and ATCC 35152 (mean $\pm$ SD).

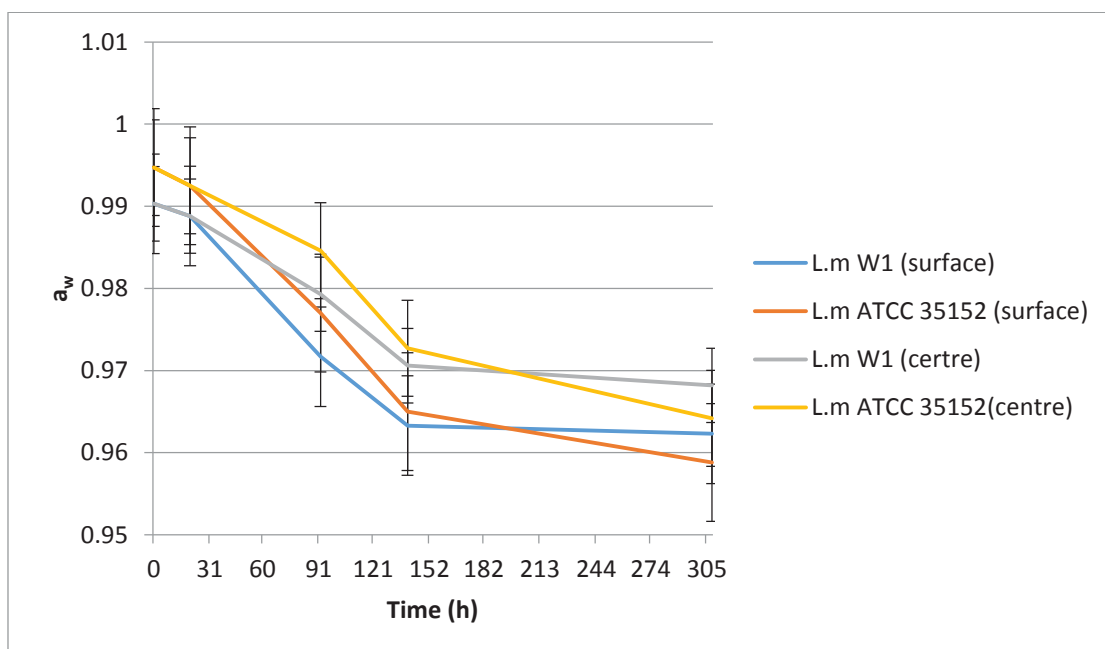


Figure 21. The a_w change during the manufacture of Ricotta Salata inoculated with *L. monocytogenes* W1 and ATCC 35152 (mean $\pm$ SD).

The a_w change during the manufacture of Ricotta Salata inoculated with *L. monocytogenes* W1 and ATCC 35152 is shown in Figure 21. The a_w decreased from T0 to T308, with a significant decrease from T0 to T140 followed by a smaller

decrease after T140 ($p>0.05$). A difference between surface and centre was occurred from T20, and the difference was maintained from T20 to T308, ($p>0.05$). There was no significant difference in results for cheese inoculated with either strain ($p>0.05$).

The bacteria count during the manufacture of Ricotta Salata inoculated with *L. monocytogenes* W1 and ATCC 35152 is shown in Figure 22. From T0 to T308 there was no obvious difference between the two strains, in neither the surface nor the centre samples ($p>0.05$). The bacteria count initially significant increased from 5 to 6 $\log_{10}$ CFU/g to nearly 8 $\log_{10}$ CFU/g then stabilised at around this level ($p<0.05$).

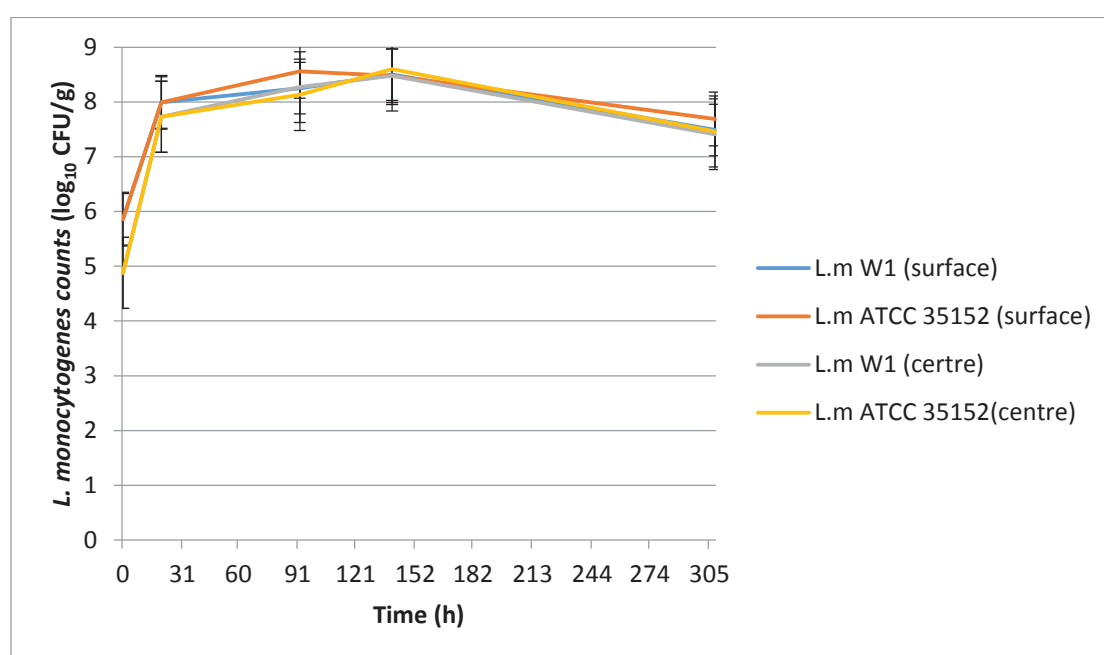


Figure 22. *L. monocytogenes* counts taken during the manufacture of Ricotta Salata inoculated with *L. monocytogenes* W1 and ATCC 35152 over a 308h period (mean $\pm$ SD).

5.0 Discussion

The survival and growth of the two *B. cereus* strains (D1 and ATCC 13061) during the manufacture of Ricotta Salata cheese were similar. The *B. cereus* grew from approximately 5 $\log_{10}$ CFU/g to a maximum of 7.7 $\log_{10}$ CFU/g of cheese curd during moulding (20h under room temperature). A similar amount of growth of *B. cereus* was also found during the manufacture of Gouda cheese, with an increase of 2 $\log_{10}$

CFU/g (Rukure & Bester, 2001). This initial growth of *B. cereus* was expected because the combination of production technology and intrinsic characteristics.

The production technology factors influencing *B. cereus* growth include, as the heating process and the absence of starter culture. The heating process (whey heating or pasteurisation of milk) inactivates the competitor micro-organisms in milk, allowing more opportunity for *B. cereus* growth, as this bacterium is a poor competitor in unpasteurised dairy products (Andersson et al., 1995). The absence of starter in the Ricotta Salata cheese encourages *B. cereus* growth as the pH does not reduce as it does with starter bacteria. However, the lactic acid bacteria (LAB) used in starter cultures does not affect *B. cereus* growth, when they reach a high number (Rukure & Bester, 2001; Wong & Chen, 1988). In order to enhance the ripening of cheese, the LAB should reach the numbers as high as $9 \log_{10}$ CFU/g of cheese (Kosikowski, 1979). Therefore, the presence of starter culture in the early stages of cheese manufacture would not affect *B. cereus*, but it does decrease rapidly with continued fermentation (Rukure & Bester, 2001; Wong & Chen, 1988).

The intrinsic properties of Ricotta Salata were favourable for the growth of *B. cereus*. The pH change fluctuated between around 6.00 to 6.10 on the surface and 6.00 to 5.95 in the centre; the lowest a_w was approximately 0.96 on the surface and 0.97 in the centre; and the highest NaCl concentration was 3.3% on the surface and 3% in the centre. According to the published studies, *B. cereus* cannot grow, when the pH is lower than 5.0, a_w lower than 0.95 or NaCl concentration higher than 7% (Chorin et al., 1997; Granum, 2005; Raevuori & Genigeorgis, 1975).

The effect of temperature on the growth of *B. cereus* varies depending on the strain. Few *B. cereus* strains are able to grow below 6°C (Van Netten & Kramer, 1990; Rangasamy et al., 1993; Larsen & Jørgensen, 1997). Te Giffel et al. (1997) isolated 106 strains from pasteurised milk, finding 53% of the strains were able to grow at 7°C, and 87% of the strains capable to grow at 37°C with, 40% able to grow both at 7 and 37°C. Although spores can survival during pasteurisation, the detection of *B. cereus* spores in fresh pasteurised milk is rare (Larsen & Jørgensen, 1997, 1999; Te Giffel et al., 1997).

Although theoretically the conditions of the manufacture of Ricotta Salata cheese should not limit the *B. cereus* growth, during the salting process, the *B. cereus* decreased to approximately 1 log₁₀ CFU/g both on the surface and in the centre of the cheese. According to Spanu et al. (2016), the growth and survival of *B. cereus* is also influenced by a series of complex interactions including temperature, pH, a_w, NaCl concentration, nutrients and presence of competitive microbiota, which could explain the decrease of *B. cereus*. However, a model system based on pH, a_w, and temperature of *B. cereus* did not agree with that (Lanciotti et al., 2001). Although the combination of the physico-chemical conditions has effect on the growth rate of *B. cereus* (Benedict et al., 1993; Quntavalla & Parolari, 1993), when those conditions exceed the minimal values required for growth, the effect of the combination of conditions (pH, a_w and temperature) on the growth of *B. cereus* is weak. However, in the present study, when the physico-chemical conditions are above the minimum values for growth of *B. cereus*, the interactions to some extent limited the growth and survival of *B. cereus*. The difference between the results could be explained by the difference in strains. Spanu et al. (2016) studied the *B. cereus* ISO 7932, and Lancitotti et al. (2001) isolated the *B. cereus* from spinach. In the present study, one of the strains of *B. cereus* was isolated from a dairy factory and another one was reference strain, *B. cereus* ATCC13061.

The decrease in the numbers of *B. cereus* stopped, and at the end of aging process and increased slightly on the surface to approximately 6.5 log₁₀ CFU/g and 5.3 log₁₀ CFU/g in the centre. This slightly increase may be due to the death of vegetative cells and survival of spores during salting. The survived spores would then be able to germinate during aging, explaining the increase in bacteria count. This has been reported by Spanu et al. (2016), where the spores of *B. cereus* germinated after manufacture and largely present as vegetative cells at the beginning of the Ricotta Salata storage.

The bacteria count of *B. cereus* in the present study is higher than 5 log₁₀ CFU/g, which is the number published by Overcast and Atmaram in 1974 that could cause illness. A high level of natural contamination of *B. cereus* in Ricotta Salata was once

reported by Spanu et al. (2016), $8.33 \log_{10}$ CFU/g and nine positive batches over three months. However, the follow-up HACCP investigation indicated that there was no *B. cereus* contamination on any production batch during the rest of the year. Therefore, they suggested this incident was a sporadic event, which could be associated with the season in Sardinia (Spanu et al., 2016). Generally, the detection of *B. cereus* contamination in Ricotta Salata is rare, and the contamination level is low, with the maximum level reported is ca. $3 \log_{10}$ CFU/g (Cosentino et al., 1997; Fadda et al., 2012; Spanu et al., 2012).

The survival and growth of the two *L. monocytogenes* strains (W1 and ATCC 35152) during the manufacture of Ricotta Salata cheese were similar. The difference between the bacteria count on the surface and in the centre was very small. The bacteria count of the *L. monocytogenes* increased from 5 to $6 \log_{10}$ CFU/g to a maximum of $8.6 \log_{10}$ CFU/g during manufacture and maintained a level of around $8 \log_{10}$ CFU/g in the final product. This increase was supported by the study of Genigeorgis et al. (1991), where *L. monocytogenes* was found increased in Ricotta cheese (pH 5.9 to 6.1), from 1.53 to $4.18 \log_{10}$ CFU/g.

The influence of heating and the absence of starter culture on the growth of *L. monocytogenes* were similar the effect on *B. cereus* growth. Heating is able to kill the non-sporeforming bacteria and inactivates the bacteria capable of forming spores, but not to the spores themselves. Once *L. monocytogenes* contaminates the cheese, there is no limitation for its growth. As there is no starter culture used in the manufacture of this cheese, the inhibitory properties of starter bacteria that assist in the control of pathogens in many cheeses, are not present in Ricotta Salata LAB in the starter cultures inhibit the growth of pathogens such as *L. monocytogenes* (Morgan et al., 2001; Valero et al., 2014).

The intrinsic properties of Ricotta Salata do not limit the growth of *L. monocytogenes*. According to published studies, *L. monocytogenes* cannot grow, when the pH is lower than 4.39, a_w lower than 0.92 or NaCl concentration higher than 10% (Petrán & Zotolla, 1989; Seeliger, 1961). However, in Ricotta Salata all of the intrinsic properties both for the surface and the centre, are above the limit (6.00 to 6.10 on

the surface and 6.00 to 5.95 in the centre for pH; 0.96 on the surface and 0.97 in the centre for the lowest a_w ; 3.3% on the surface and 3% in the centre for the highest NaCl concentration). The combination of intrinsic properties in Ricotta Salata cheese is suitable for *L. monocytogenes* survival and growth. *L. monocytogenes* is a robust microorganism as it can grow at 6% NaCl, a_w 0.96, and survive at least 132 days at a NaCl concentration was 12%, and a_w was 0.92, (4°C) in skim milk and whey (Papageorgiou & Marth, 1989). Refrigeration helps stabilise slow the growth of *L. monocytogenes* in Ricotta Salata (Coroneo et al., 2016).

In the present study, the initial growth of *L. monocytogenes* was due to the temperature during the moulding stage at around 25°C. The ComBase modelling system confirms the growth of *L. monocytogenes* at 25°C in conditions similar to those in Ricotta Salata (ComBase, 2017). In general, most bacteria grow better at warmer temperatures and slower at refrigeration temperatures and although *L. monocytogenes* is a psychrotrophic bacterium, its growth will be much slower at 5 to 10°C compared with 30°C (Cole et al., 1990).

Since *L. monocytogenes* is commonly detected in the food environment, the common contamination areas in cheese-making plants have been the subject of several studies (Ibba et al., 2013; Pilo et al., 2007). Ibba et al. (2013) investigated the persistent environmental contamination of *L. monocytogenes* in two sheep milk cheese factories, which all produced Ricotta Salata cheese. The contamination was detected both on the food contact surfaces and non-food contact surfaces. The contamination of *L. monocytogenes* in Ricotta Salata products was mainly influenced by the hygiene of the plants (environments, operators, and production phases). According to Ibba et al.'s study (2013), the washing, drying and packaging rooms had the higher risk of contaminating *L. monocytogenes* than other areas in the two plants. One possible reason is the difficulty in cleaning some of the environmental surfaces. In the investigation of Ibba et al. (2013) they also found contamination of *L. monocytogenes* in the Pecorino Romano production line had higher numbers of *L. monocytogenes* than the Ricotta Salata production line in the same manufacture facility. The whey from the Pecorino Romano production line was a source of

contamination for the Ricotta Salata cheese. However, according to Pilo et al.'s study (2007), whey production is not as high a risk for *L. monocytogenes* contamination as environment of the manufacture plant. Therefore, the high level of contamination of the Romano production line in Ibba et al.'s study (2013) is most likely due to environmental contamination in the manufacture plant. Since the traditional Ricotta Salata manufactures use whey as the ingredient, rather than milk, it could be argued that the more processes involved, the greater the risk of contamination.

Furthermore, in cheese-making plants, there are some share areas, which are used to produce more than one product. Such areas include washing, drying, and packaging areas, which have been reported as common areas for the detection of *L. monocytogenes* in cheese-making plants (Endrikat, 2010). There is a potential risk of cross-contamination among different production lines. In Heiman et al.'s study (2016) about the listeriosis outbreak caused by imported cheese in the United States in 2012, they detected the cutting and repackaging processes were the cause of cross-contamination of *L. monocytogenes* among Ricotta Salata, blue cheese, and farmstead cheeses. The present study indicated that the inner conditions of Ricotta Salata were able to support the growth and survival of *L. monocytogenes*, to the same extent as the surface. Although the reports of *L. monocytogenes* contamination in the interior of Ricotta Salata cheese are rare, the cross-contamination caused by cutting and repackaging can occur. The inner cheese could be contaminated through cutters, cutting boards, knives, and utensils after cutting wheels of cheese (Heiman et al., 2015). Therefore, those areas could be regarded as the monitoring points in the setting up the HACCP system for *L. monocytogenes*.

Several researchers have investigated the presence of *L. monocytogenes* in Ricotta Salata and cheese-making plants (Acciari et al., 2015; Spanu et al., 2012 and 2015). Although the initial level of *L. monocytogenes* contamination is low, there is a potential threat to human health during manufacture and storage of the cheese (Acciari et al., 2015; Coroneo et al., 2016). After heating the manufacture of Ricotta Salata, there are no further critical control points to eliminate *L. monocytogenes*. Once the cheese gets contaminated, the bacteria will survive and grow during the

manufacture. The detection of *B. cereus* in Ricotta Salata is less common than *L. monocytogenes*; however, the present study indicated that Ricotta Salata supports the growth and survival of *B. cereus*. Therefore, in order to reduce the risks associated these two bacteria, it is important to improve the management of process hygiene for controlling environmental contamination. However, it may not be enough to ensure no risk of contamination. Ideally, some lethal treatments should be applied after the packaging of the cheese, to limit the contamination of Ricotta Salata with these two bacteria (Arriari et al., 2016; Valero et al., 2014). In one of the Ricotta Salata factory in Italia, the heat treatment has been applied during packaging, as 90°C for 1 min (Casti et al., 2016). Moreover, Spanu et al. (2015) detected the effect of post-lethality thermal treatment on Ricotta Salata, they indicated that 80°C 40 min and 90°C 40 min can effectively reduce 5 log₁₀ CFU/g *L. monocytogenes* and there was no significant difference of sensory properties observed after the heat treatments.

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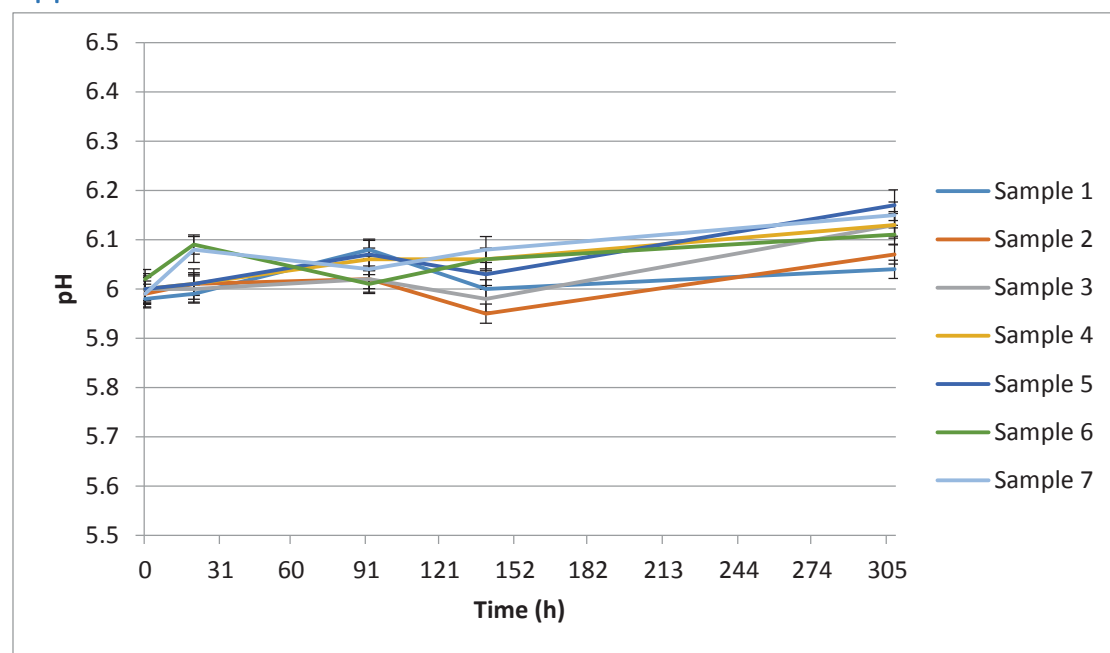
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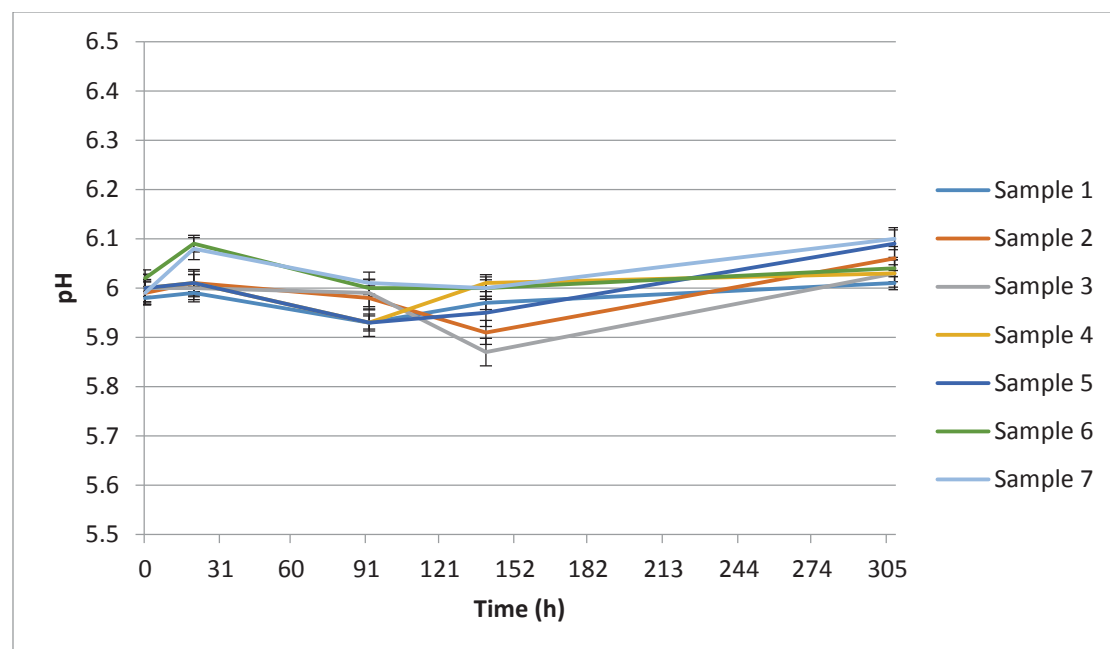
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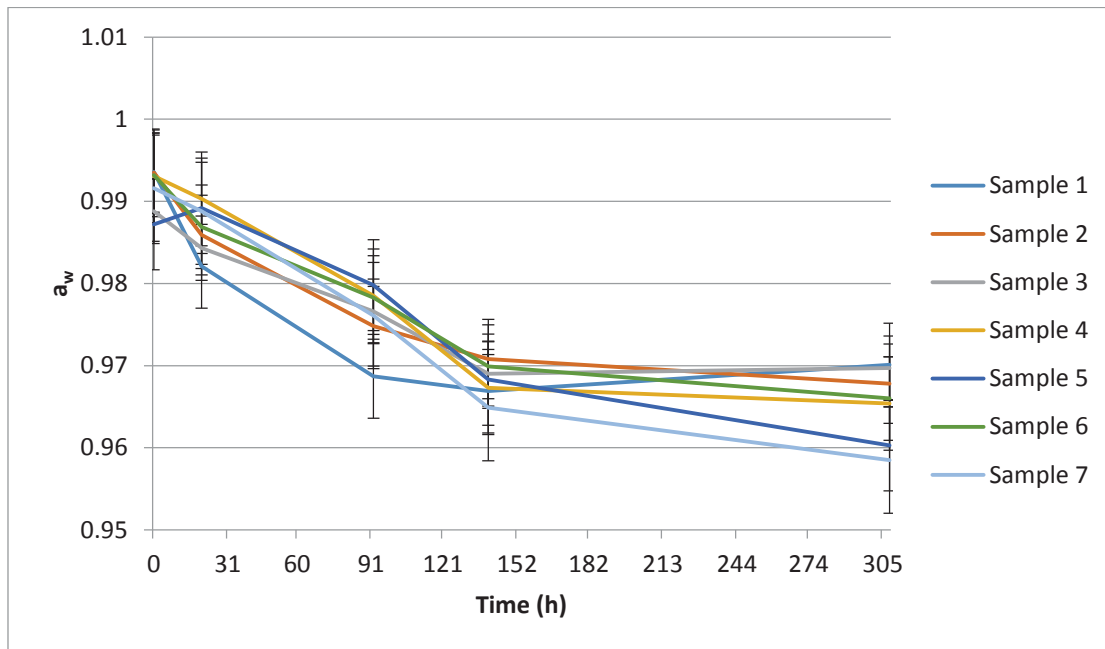
Appendices



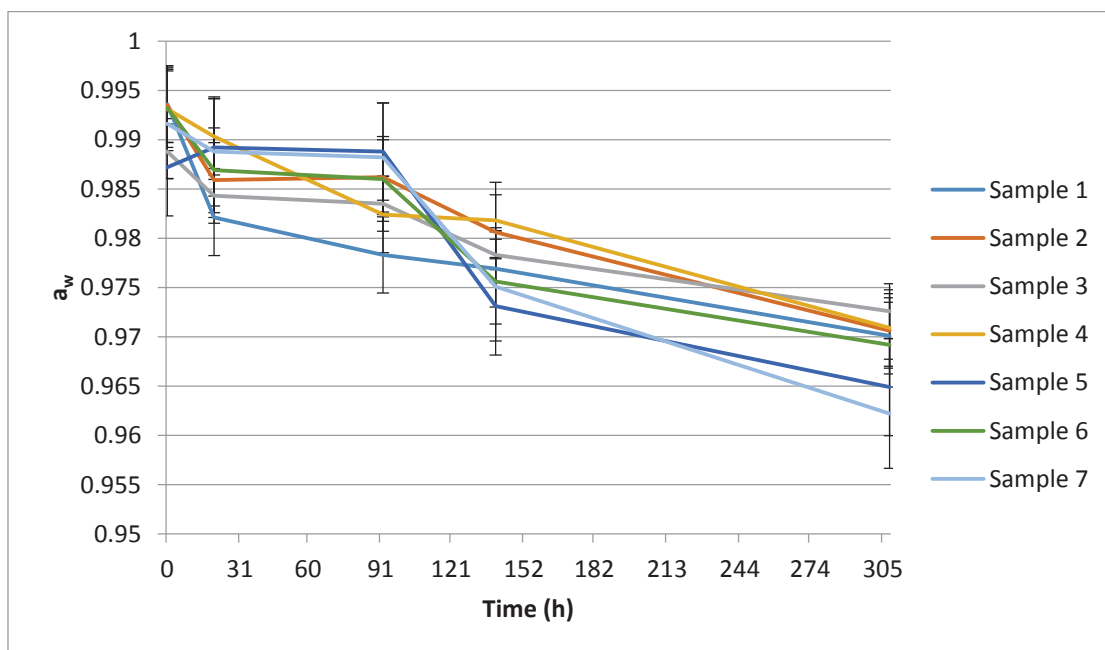
Appendix Figure A1. The pH change during the manufacture of Ricotta Salata inoculated with *L. monocytogenes* (ATCC 35152) at 4°C (surface) (mean±SD).



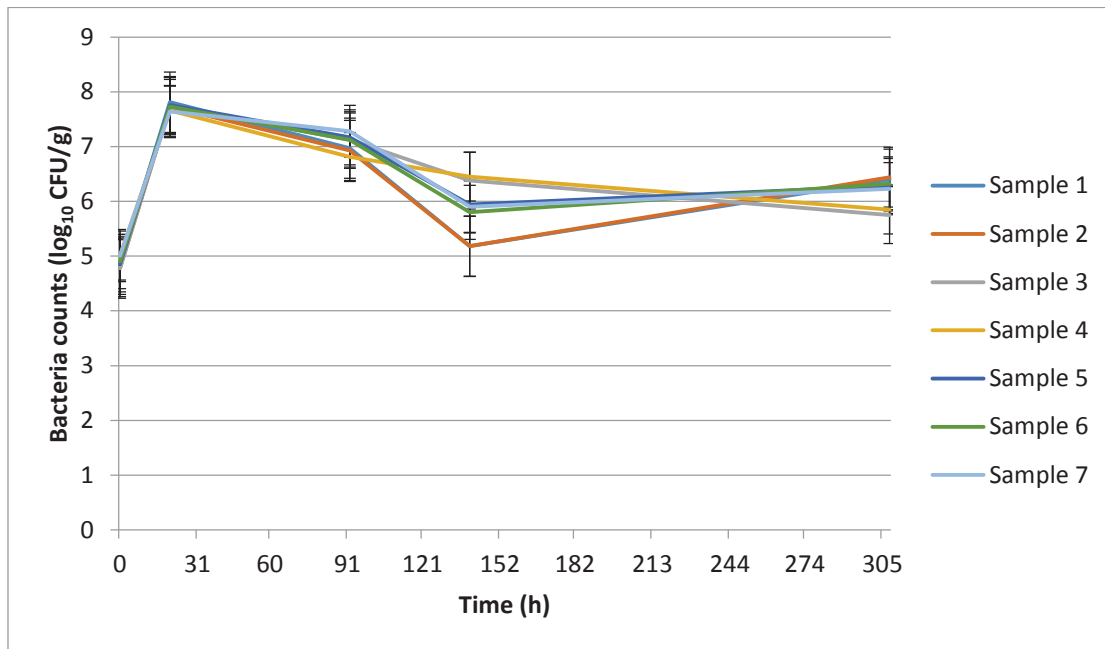
Appendix Figure A2. The pH change during the manufacture of Ricotta Salata inoculated with *L. monocytogenes* (ATCC 35152) at 4°C (centre) (mean±SD).



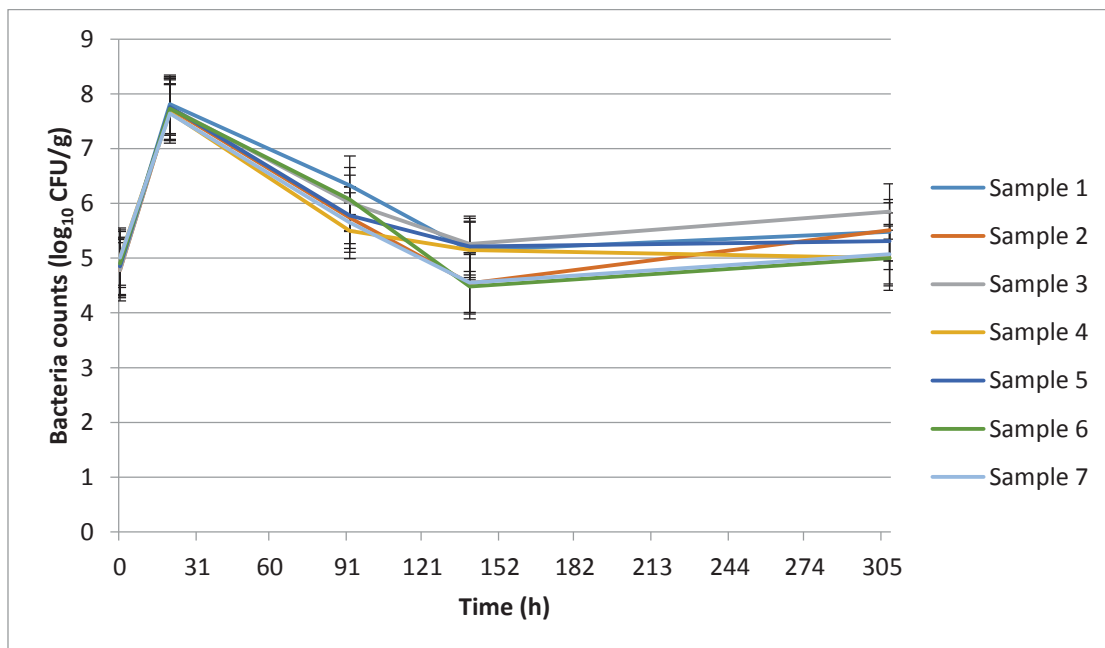
Appendix Figure A3. The a_w change during the manufacture of Ricotta Salata inoculated with *B. cereus* (ATCC13061) at 4°C (surface) (mean±SD).



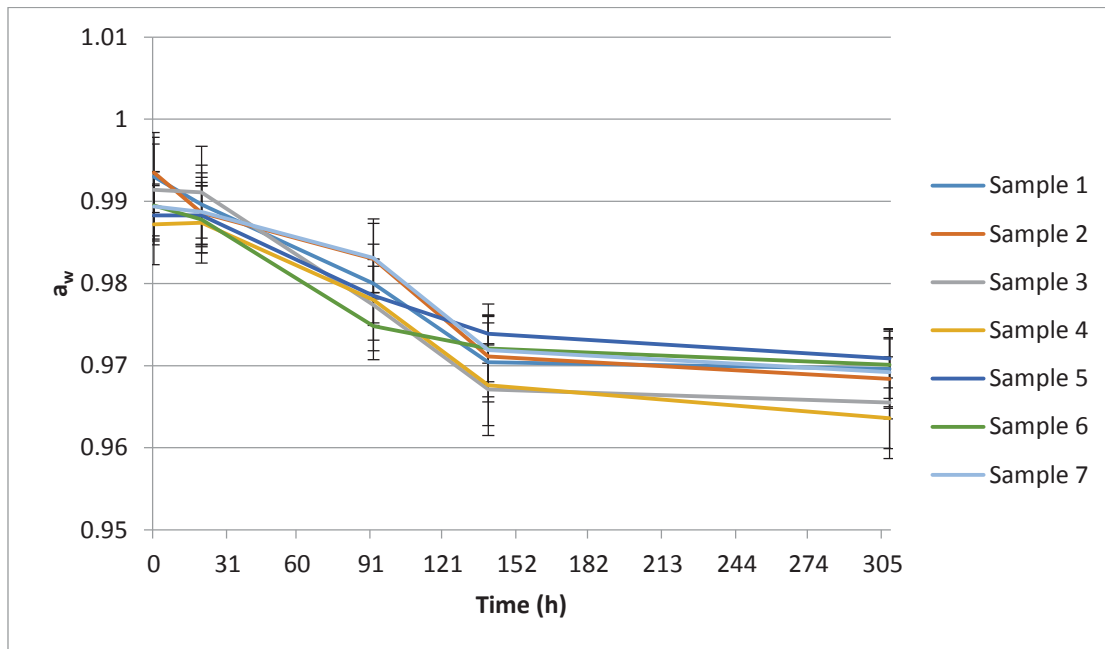
Appendix Figure A4. The a_w change during the manufacture of Ricotta Salata inoculated with *B. cereus* (ATCC13061) at 4°C (centre) (mean±SD).



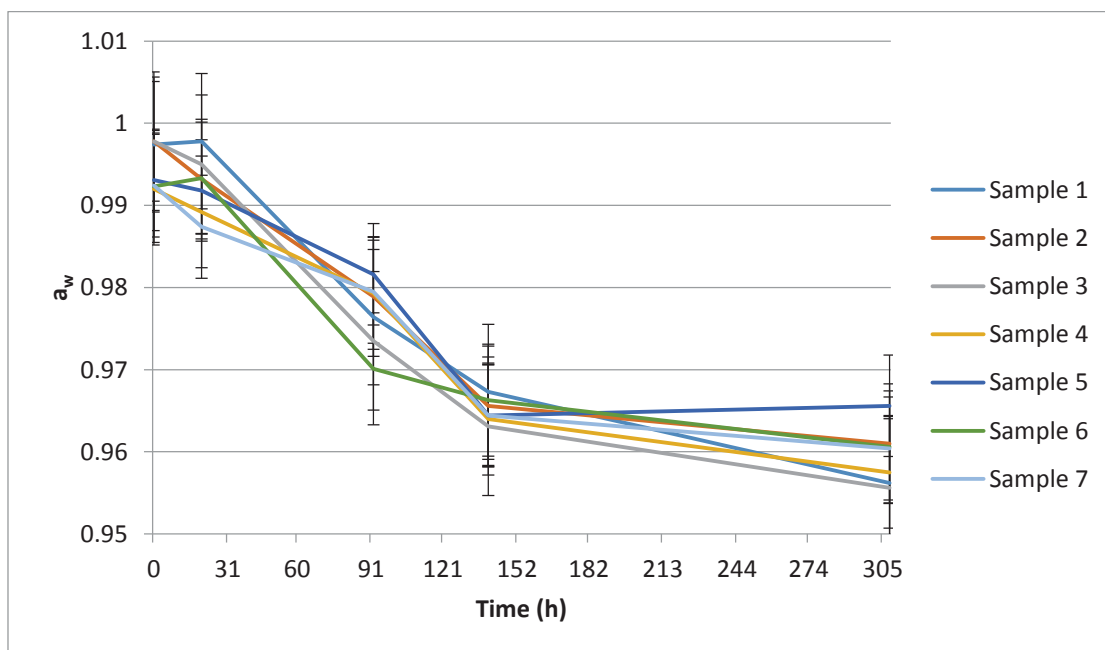
Appendix Figure A5. The bacteria count change during the manufacture of Ricotta Salata inoculated with *B. cereus* (ATCC13061) at 4°C (surface) (mean±SD).



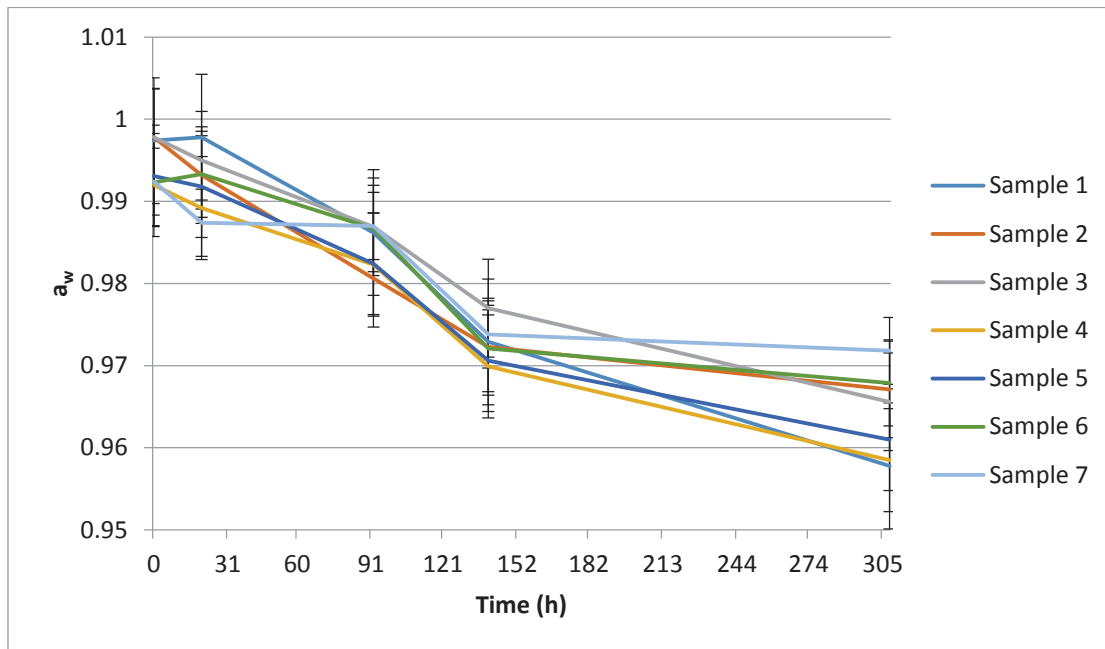
Appendix Figure A6. The bacteria count change during the manufacture of Ricotta Salata inoculated with *B. cereus* (ATCC13061) at 4°C (centre) (mean±SD).



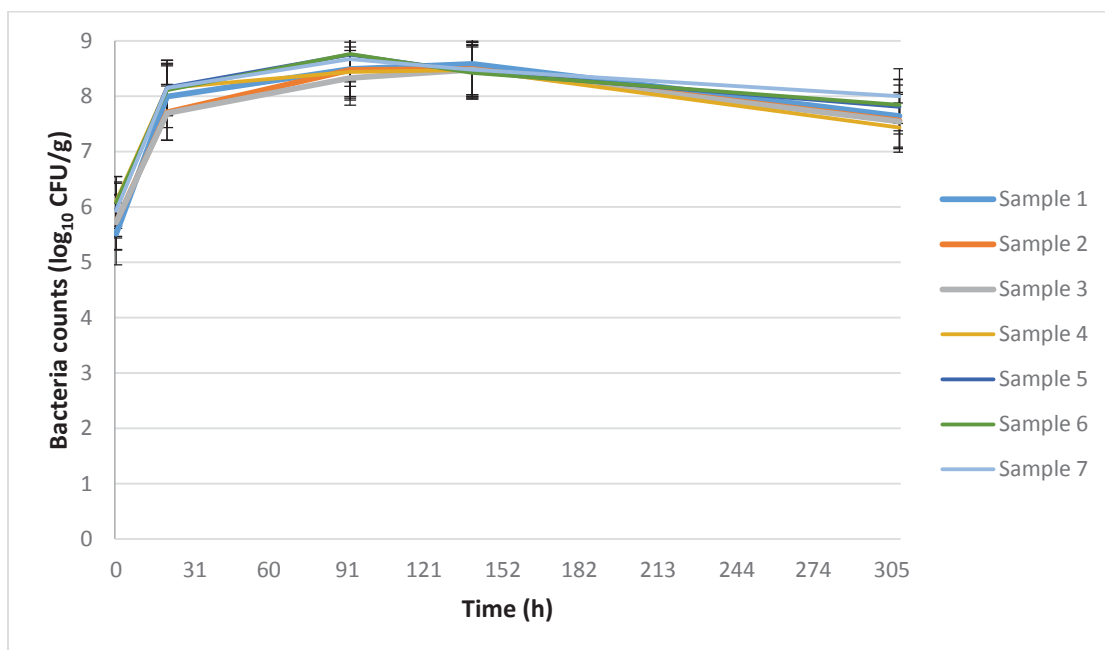
Appendix Figure A7. The a_w change during the manufacture of Ricotta Salata inoculated with *L. monocytogenes* W1 at 4°C (centre) (mean±SD).



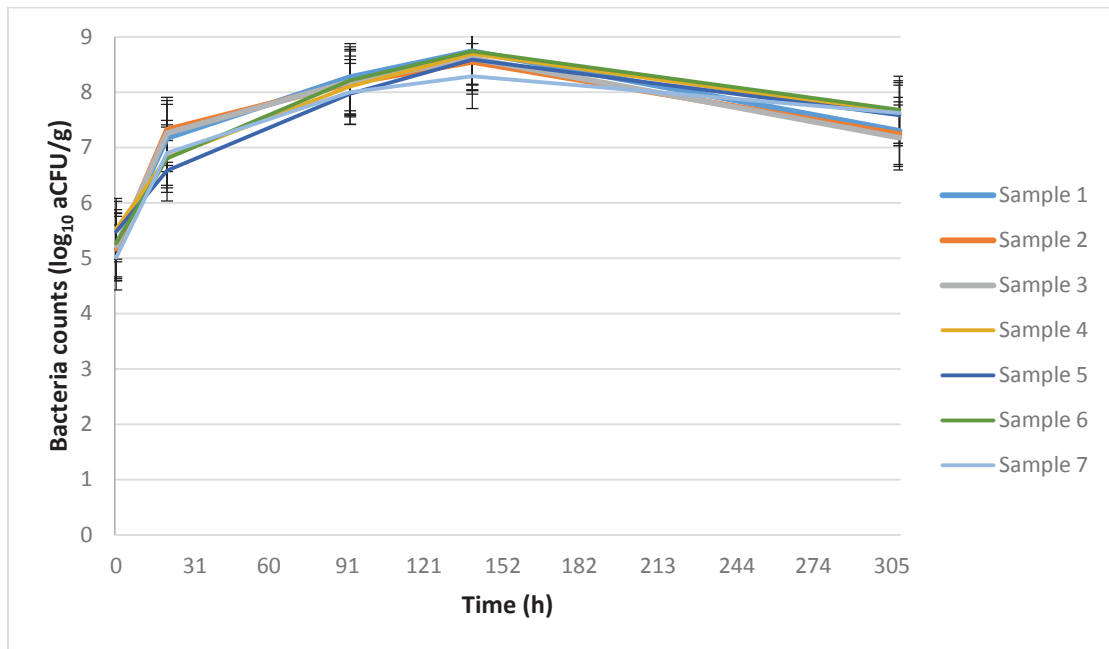
Appendix Figure A8. The a_w change during the manufacture of Ricotta Salata inoculated with *L. monocytogenes* (ATCC 35152) at 4°C (surface) (mean±SD).



Appendix Figure A9. The a_w change during the manufacture of Ricotta Salata inoculated with *L. monocytogenes* (ATCC 35152) at 4°C (centre) (mean±SD).



Appendix Figure A10. The bacteria count change during the manufacture of Ricotta Salata inoculated with *L. monocytogenes* W1 at 4°C (centre) (mean±SD).



Appendix Figure A11. The bacteria count change during the manufacture of Ricotta Salata inoculated with *L. monocytogenes* (ATCC 35152) at 4°C (centre) (mean±SD).