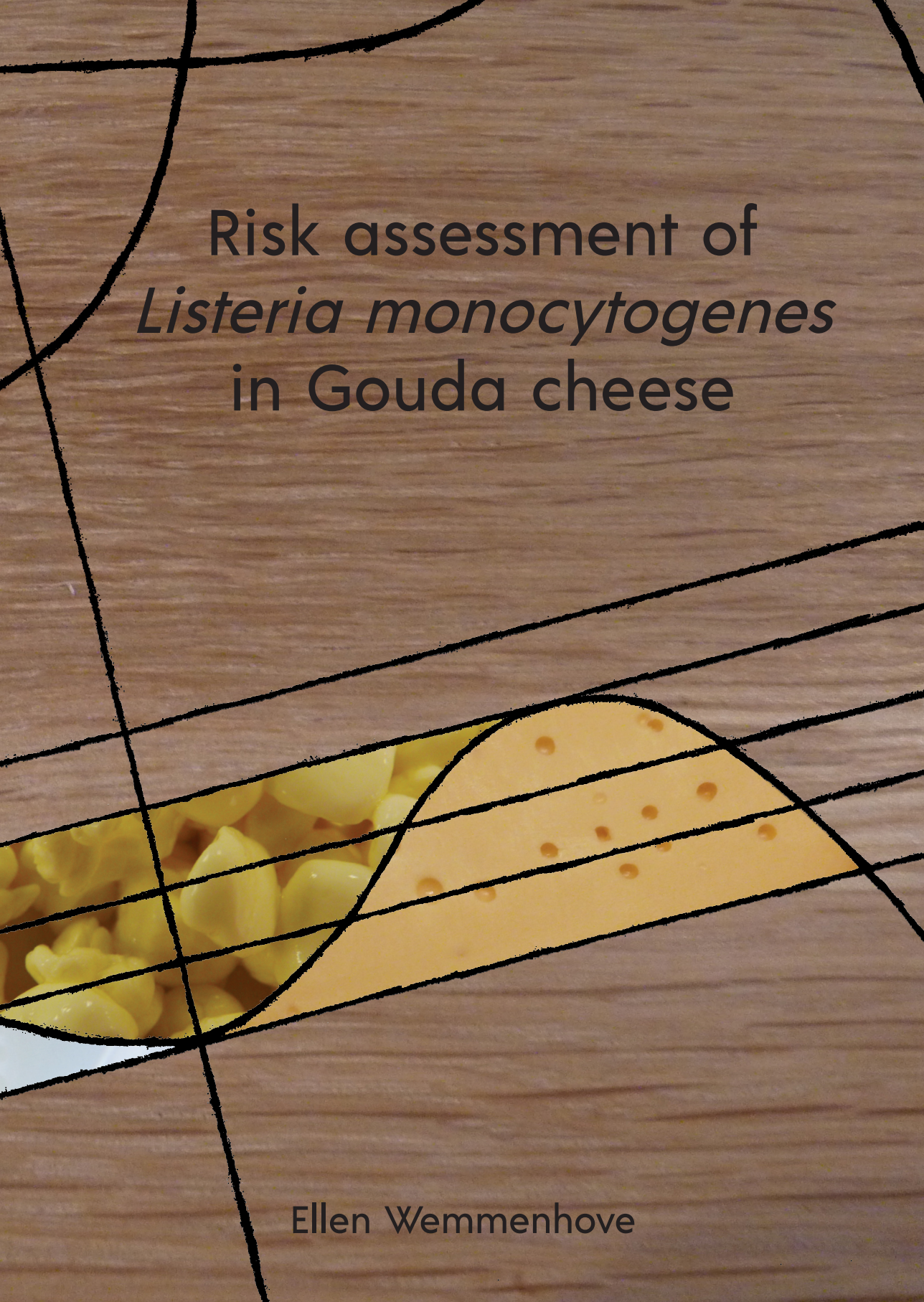
Risk assessment of *Listeria monocytogenes* in Gouda cheese



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Thesis

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Abstract

In this PhD thesis, the potential of outgrowth of *L. monocytogenes* was assessed in Dutch-type Gouda cheese. It was demonstrated that *L. monocytogenes*, which can cause listeriosis, is not able to grow in or on Dutch-type Gouda. The factors present in Gouda cheese that can lead to full inhibition of growth of *L. monocytogenes* were identified and safety criteria aiming for complete inhibition of growth of *L. monocytogenes* in Dutch-type Gouda cheese are suggested.

Dutch-type Gouda cheese is a semi-hard cheese made from bovine milk that is pasteurized when produced at an industrial scale. It is a ready-to-eat food with a pH > 5.0 and water activity a_w > 0.94. In absence of scientific evidence that this product does not support growth, Dutch-type Gouda is classified by the European legislation as a ready-to-eat food product able to support growth of *L. monocytogenes*.

In two challenge studies described in this thesis, it was demonstrated that Dutch-type Gouda cheese does not support growth of *L. monocytogenes* when inoculated in and on the product. During the cheese making process, entrapment but no growth of *L. monocytogenes* in the curd was observed. During subsequent ripening of the cheeses, no growth was observed, and upon prolonged ripening periods (>2 months) inactivation was found. In the second challenge study, a limited transfer of *L. monocytogenes* from brine to the outer layers of cheeses was observed, and during brining and ripening viable numbers of *L. monocytogenes* did not increase.

The variation in a_w inside Gouda cheese was assessed by determining the profiles of water and NaCl and the resulting a_w in nature-ripened and foil-ripened Gouda cheese during brining and ripening. An empirical model was derived for Gouda cheese in which a_w is expressed as a function of the NaCl-in-moisture content.

Dutch-type cheeses contain organic acids that are known to have potential inhibitory effects on *L. monocytogenes*. The MICs of organic acids for 6 different *L. monocytogenes* strains were established at pH values that are relevant to Dutch-type Gouda. The MICs were established for lactic acid (which is the main organic acid in Gouda), acetic acid, propionic acid, and citric acid. Variations in MICs between strains were observed.

In an overall review of the factors present in Gouda cheese that are relevant to growth inhibition of *L. monocytogenes*, undissociated lactic acid was evaluated as the primary growth-inhibiting factor that can lead to full growth inhibition in Gouda. Additionally, low a_w in the cheese rind and after prolonged ripening times can cause full growth inhibition.

This thesis lends support to categorizing Gouda as a ready-to-eat food product that does not support growth of *L. monocytogenes*. Furthermore, it is justifiable to include undissociated lactic acid (together with pH and a_w) in future food safety criteria for ready-to-eat products related to absence of growth of *L. monocytogenes*.

Chapter 1

General introduction

1.1 Food safety management

Food safety is critical to assure that food will not cause harm to the consumer when it is prepared and/or eaten according to its intended use (CAC, 2003). In the European general food law EC 178/2002 it is stated that free movement of safe and wholesome food is an essential aspect of the internal European market, and that it contributes significantly to the health and well-being of citizens, and to their social and economic interests. Consumers must be able to rely on the fact that the foods purchased and consumed will be safe and of high quality.

Food safety management systems are implemented to control foodborne hazards and thereby prevent foodborne illnesses (Meng & Doyle, 2002). Ensuring food safety is important to maintain the trust of consumers (Raspor, Smozina & Ambrožič, 2014). A food safety system comprises a combination of external (legal and governmental) and internal (food producer) requirements and procedures that are integrated in management and control systems. Risk management decisions should be taken based on technical and scientific input. The international ISO 22000 standard sets out the requirements for a food safety management system and maps out what an organization needs to do to demonstrate its ability to control food safety hazards and ensure that food is safe (ISO, 2005). Food safety management systems in the food industry include hazard analysis and critical control points (HACCP) approaches. Such approaches are suitable to analyse potential risks related to complex manufacturing processes and constitute a more cost-effective and reliable way of assuring food safety than traditional inspection and end-product testing. HACCP as a food safety management system is recommended by the Codex Alimentarius (van Schothorst, 2004). The aim of an HACCP analysis is to identify, evaluate and control hazards which are significant for food safety (CAC, 2003) wherein good manufacturing practice (GMP) and good hygiene practice (GHP) are prerequisites. Using an HACCP approach, it is possible to assess the likelihood of the occurrence of hazards in foods during the manufacturing, distribution and use of a food product, and to define measures for adequate control of hazards. Hazards may originate from different sources throughout the entire food production chain, for instance from the raw materials produced by farmers, from processing and production environments of food products, and from handling and storage at the level of retail, food service and consumer. Hazard analysis is the procedure to identify significant potential hazards and the conditions that lead to the presence thereof in food. It evaluates the likelihood of the hazard being present and the severity of an adverse health effect when it occurs, in order to determine whether the hazard is significant to threaten food safety. A critical control point is a raw material, location, practice, formulation or process where measures can be applied to prevent or minimize the likelihood of the presence of hazards at unacceptable levels. Specific aspects of GMP are essential for food safety and have to be singled out as CCP. Monitoring is

checking the conformity of the control at a CCP and consists of systematic observation, measurement, recording and evaluation. Corrective actions need to be taken when monitoring indicates loss of control (van Schothorst, 2004). Examples of corrective actions are re-pasteurization and adjustment of the processing conditions. By such corrective actions, the likelihood of exposure to biological hazards such as pathogenic bacteria, viruses and mycotoxin-producing moulds can be reduced.

1.2 *Listeria monocytogenes* causing listeriosis

Listeria monocytogenes is known as an important bacterial foodborne pathogen (Table 1.1) and is the causative agent of foodborne listeriosis. Listeriosis is an illness that may have a severe outcome with a high case-fatality rate (Swaminathan & Gerner-Smidt, 2007). It can be contracted after consumption of food that is contaminated with *L. monocytogenes*. *L. monocytogenes* is specifically a problem for the food industry, being a robust pathogen that can be present in food ingredients and in food processing facilities due to its ability to survive in highly acidic, salty and low-temperature environments and due to its ability to form biofilms. Cases of foodborne listeriosis are often related to consumption of ready-to-eat products (i.e. products that are not heat-treated before consumption), including meat, seafood and dairy products. Specific microbiological criteria for *L. monocytogenes* in RTE foods have been set for these foods in regulation (EC) No 2073/2005. Different categories of RTE foods are defined therein, based on intended use and on the ability of *L. monocytogenes* to grow in or on the RTE food. Foodborne transmission of listeriosis has only been known since 1981, after an outbreak in Canada that was associated with consumption of contaminated coleslaw. The probability that a food product leads to listeriosis depends on the virulence of the strain, (human) host susceptibility and the level of exposure (WHO/FAO, 2004). The time between ingestion of contaminated food and the onset of listeriosis symptoms is eight days on average, but can be as long as two months. A long incubation period between the time of consumption and manifestation of disease symptoms can make it extremely difficult to trace the food product that contained the pathogen and it hampers an accurate assessment of the dose that led to the disease (Buchanan, Gorris, Hayman, Jackson & Whiting, 2017).

Listeriosis may manifest as a range of symptoms. Non-invasive listeriosis can occur in any person after consumption of a high concentration of *L. monocytogenes*. This type of listeriosis usually manifests as mild gastroenteritis (Ooi & Lorber, 2005). Invasive listeriosis manifests as an infection of the central nervous system and a systemic infection, leading to the high case-fatality rate of listeriosis (Low & Donachie, 1997). In such cases, *L. monocytogenes* spreads from the intestines to the blood stream and from there to other organs. *L. monocytogenes* can cross the intestinal barrier, placental barrier and the blood-

brain barrier. It enters, survives and multiplies in both phagocytic and non-phagocytic cells (Cossart, Pizarro-Cerda & Lecuit, 2003). Listeriosis is mainly a risk for people with a weak immune system, such as pregnant women, newborns and people older than 65 years (Ramaswamy et al., 2007).

Table 1.1 Overview of microbial pathogens associated with foodborne outbreaks, and the proportion of food classes associated with outbreaks by that pathogen. The table was extracted from Greig & Ravel (2009) who created a foods and aetiology cross tabulation based on 4093 foodborne outbreaks that occurred globally between 1988 and 2007 (Greig & Ravel, 2009)

		% of food class associated with outbreak				
Microbial pathogen	**Number of outbreaks**	**Dairy**	**Seafood**	**Meat**	**Multi-ingredient**	**Other**
Bacillus cereus	74	4.1	4.1	22.9	56.8	12.3
Campylobacter spp.	191	34.6	2.6	41.8	14.1	6.8
Clostridium botulinum	108	3.7	25	26.8	0.9	43.5
Clostridium perfringens	248	0.4	2	74.6	20.2	2.8
Escherichia coli	389	9.8	0.5	52.9	11.8	24.9
Listeria monocytogenes	**53**	**41.5**	**11.3**	**39.7**	**5.7**	**1.9**
Salmonella enterica serovar Enteritidis	991	6.4	4.2	18.9	10.1	60.4
Salmonella enterica serovar Typhimurium	270	11.9	4.8	34.5	10.7	38.1
Other *Salmonella enterica*	657	6.2	2.6	36.9	13.9	40.6
Shigella spp.	83	14.5	9.6	14.4	30.1	31.3
Staphylococcus aureus	182	11	3.3	51	22	12.9

The number of reported cases of human listeriosis was 0.25-0.32 per 100,000 population in the US between 2004 and 2009 (Cartwright et al., 2013) and 0.44 per 100,000 population in the EU in 2013 (EFSA, 2015). Hospitalization rates in the case of listeriosis may be as high as 94% (Melo, Andrew & Faleiro, 2015) and fatality rates as high as 20-30% (Swaminathan & Gerner-Smidt, 2007) and 15.6% (EFSA, 2015). Leclercq, Charlier & Lecuit (2014) assessed the global disease burden of listeriosis in 2010 and estimated that 23,150 cases related to listeriosis occurred worldwide, resulting in 5,463 deaths (case-fatality rate of 23.6%) and 172,823 Disability Adjusted Life Years (DALYs) (Leclercq et al., 2014). *L. monocytogenes* was the third most costly foodborne pathogen per case in the US in 2010, due to the high hospitalization costs for listeriosis (Scharff, 2012). Listeriosis not only has negative consequences for public health, but also for the economy. When excluding medical costs, the total costs of food safety incidents in the US are around 7 billion USD per year (Hussain & Dawson, 2013). Thomas et al. (2015) estimated the total costs of an outbreak with *L. monocytogenes* in a meat processing plant at nearly 242 million Canadian dollars, while the medical costs were 2.8 million Canadian dollars.

L. monocytogenes is subdivided into 12 serotypes, of which serotype 1/2 strains (especially 1/2a and 1/2b) account for more than 50% of the isolates from foods and food environments. Serotype 4b, however, is most often related to listeriosis (Cartwright et al., 2013; Nho, Abdelhamed, Reddy, Karsj & Lawrence, 2015). The pathogen is rod-shaped, Gram-positive and facultative anaerobic. It belongs to the Firmicutes, but cannot form spores, and depending on the temperature, this bacterium can show motility by means of flagellae (Kathariou, Kanenaka, Allen, Kok & Mizomoto, 1995; Lemon, Higgins & Kolter, 2007; O'Neil & Marquis, 2006). *L. monocytogenes* is ubiquitous in the environment where it can be very persistent (Farber & Peterkin, 1991), and it is able to grow at a low pH, low temperatures, and high salinity levels (Table 1.2). Also, the bacterium can form biofilms (Borucki, Peppin, White, Loge & Call, 2003; Cossart et al. 2003; Carpentier & Cerf, 2011) and is known for its proteolytic activity (Verheul, Rombouts & Abee, 1998). The heat resistance of *L. monocytogenes* can vary per strain and food product (Aryani, Zwietering & den Besten, 2016; van Lieverloo, de Roode, Fox, Zwietering & Wells-Bennik, 2013).

Table 1.2 Temperature (τ), salt, a_w, pH limits for growth of *L. monocytogenes*, as obtained from ICMSF (1996)

Factor	Limit	
	Minimum	Maximum
τ (°C)	-0.4	~45
Salt (% NaCl in water phase) and concomitant a_w	<0.5 and >0.997	13 – 16 and 0.92 (minimum a_w growth limit)
pH (using HCl)	4.39	9.4-9.5

1.3 Detection and identification of *L. monocytogenes*

The international standard for the method for detection of *L. monocytogenes* is described in ISO 11290, with part 1: detection and part 2: enumeration (ISO, 2017a;b). Enumeration of *L. monocytogenes* in positive food samples is performed on representative samples by colony count on *L. monocytogenes* differential selective agars in conjunction with MPN enumeration using selective enrichment in Buffered *Listeria* Enrichment Broth (BLEB) with subsequent plating on *L. monocytogenes* differential selective agars (Hitchins, Jinneman & Chen, 2016). *L. monocytogenes* can be detected and identified using traditional phenotypic subtyping methods and by using molecular subtyping methods. Brain Heart Infusion (BHI) broth or BHI agar is the most commonly used non-selective medium for cultivation of *Listeria* species (Jones & D'Orazio, 2013), but chemically defined minimal media supporting growth of *L. monocytogenes* are also available (Premaratne, Lin & Johnson, 1991). There is a wide range of selective media available for *L. monocytogenes*, including Oxford medium, Palcam agar, ALOA agar and Rapid L'mono (Hitchins et al., 2016). Molecular subtyping methods can discriminate *L. monocytogenes* in a more

sensitive way than traditional phenotyping (Wiedmann, 2015). Different methods are available: banding pattern-based methods such as pulsed field gel electrophoresis (PFGE), ribotyping, repetitive extragenic palindromic sequence analysis and sequence-based subtyping. Whole-genome sequencing (WGS) reveals the complete DNA sequence of a microorganism. WGS is currently considered as a key method to identify variation between *L. monocytogenes* strains, and is increasingly used to link clinical isolates from patients with contaminated foods (Wiedmann, 2015; FDA, 2016b).

1.4 *L. monocytogenes*, a hazard for the dairy industry

The dairy industry considers *L. monocytogenes* as a microbial hazard. The pathogen may be present in raw milk and may reside in the processing environment. Multiple reported cases of foodborne listeriosis outbreaks are related to the consumption of dairy products (Table 1.1) including raw milk cheeses and cottage cheeses (Greig & Ravel, 2009; Martinez-Rios & Dalgaard, 2018). The first recognized foodborne listeriosis outbreak that was related to consumption of dairy occurred in 1983 and was associated with pasteurized milk that was contaminated post-processing (Fleming et al., 1985).

Table 1.3 Prevalence of *L. monocytogenes* in raw milk based on presence/absence of *L. monocytogenes* detected in 25 g or ml

Total samples (n)	Amount of positive samples	Prevalence (%)	Country of origin	References
137	6	4.4	The Netherlands	Beckers, Soentoro & Delgou-van Asch (1987)
176	27	15.6	Northern Ireland	Harvey & Gilmour (1992)
589	29	4.9	Ireland	Rea, Cogan & Tobin (1992)
774	28	3.6	Spain	Gaya, Sanchez, Medina & Nuñez (1998)
295	58	19.7	Sweden	Waak, Tham & Danielsson-Tham (2002)
143	9	6.3	Belgium	De Reu, Grijspeerdt & Herman (2004)
24	1	4.2	Ireland	Fox et al. (2009)
230	0	0	Austria	Schoder et al. (2011)
297	2	0.7	New Zealand	Hill, Smythe, Lindsay & Shepherd (2012)
446	97	21.7	Iran	Jamali, Radmehr & Thong (2013)
468	11	2.4	France	Meyer-Broseta, Diot, Bastian, Rivière & Cerf (2003)
182	10	5.5	Finland	Ruusunen et al. (2013)

Cheese may get contaminated with *L. monocytogenes* via the raw milk, or after pasteurization via the manufacturing environment and through the addition of additional ingredients (Lomonaco et al., 2009). The prevalence of *L. monocytogenes* in raw milk varies

from 0 to 21.7% worldwide, as follows from the overview of studies shown in Table 1.3. Pasteurization leads to a reduction in viable numbers of *L. monocytogenes* in milk by 10.4 log cfu g^{-1} based on an average *D* value and by 2.7 log cfu g^{-1} based on a 95% upper confidence interval for the *D* value (den Besten & Zwietering, 2012). Recontamination of the cheese milk, cheese curd or pressed cheese may occur after pasteurization, for instance via contact surfaces. *L. monocytogenes* has for instance been detected on cheese shelves (Notermans, 1994) and from machines used to turn cheeses during ripening (Yde et al., 2012). Raw milk (and thus raw milk cheeses) may contain *L. monocytogenes* through contamination of the milk at the farm level. For cheese made from pasteurized milk, the cheese milk usually undergoes a heat treatment of 72 °C for 15 s to inactivate microbes, including *L. monocytogenes*. However, if such products are contaminated later in the process with the pathogen and those support the growth of *L. monocytogenes*, then consumers may be exposed to the pathogen as these are ready-to-eat products that do not undergo heating prior to consumption.

Listeriosis outbreaks with cheese are often related to the consumption of contaminated soft cheeses (Melo et al., 2015), but in one case also to hard Mimolette cheese containing mites at the surface (Yde et al., 2012). Table 1.4 presents an overview of reported listeriosis outbreaks in the EU and the USA that were linked to cheese since 1983. Several cheese companies were closed after recalls or outbreaks related to cheeses contaminated with *L. monocytogenes*. An US company was shut down after a large outbreak of listeriosis in 1985 that was related to its Mexican-style cheese (Flynn, 2011). More recently, the Food and Drug Authority (FDA) enforced the shutdown of several US companies producing Mexican-style cheese (FDA, 2012; FDA, 2013).

Table 1.4 Overview of listeriosis outbreaks in 1983-2017 that were related to consumption of cheese. Compiled from EFSA reports (Eurosurveillance), CDC reports and a general search in Web of Science and Scopus (keywords 'listeriosis' and 'cheese', or 'Listeria', 'outbreak' and 'cheese', sorted on relevance, first 300 hits)

Year of outbreak	Cheese group	Cases	Mortality (%)	Reference
1983-1987	Soft cheese made from raw milk	122	27	Bula, Bille & Glauser (1995)
1985	Mexican-style soft cheeses	142	34	Linnan et al. (1988)
1989	Camembert	2	0	Ries, Dicato, Hemmer & Arendt (1990)
1989/1990	Blue cheese	26	23	Jensen, Frederiksen & Gerner-Smidt (1994)
1995	Brie de Meaux	NR	NR	Goulet et al. (1995)
1995	Soft cheese	37	30	Vaillant, Maillot, Charley & Stainer (1998)
1997	Soft cheese	14	0	Jacquet, Brouillé, Saint-Cloment, Catimel & Rocourt (1999)
1997	Soft cheese	NR	NR	RNSP (1997)

Table 1.4 (continued)

2000	Mexican-style cheese	13	0	Cartwright et al. (2013)
2001	Soft cheese	33	0	Carrique-Mas et al. (2003)
2001	Soft cheese	120	NR	Danielsson-Tham et al. (2004)
2001	Cheese	19	0	Makino et al. (2005)
2002	Cheese made from pasteurized milk	86	0	Pagotto, Ng, Clark & Farber (2006)
2003	Soft cheese from raw milk	17	0	Gaulin, Ramsay, Ringuette & Ismail (2003)
2005	Mexican-style cheese	23	22	MacDonald et al. (2005)
2005	Queso fresco	9	NR	FIOD (2005)
2005	Tomme cheese	10	50	Bille et al. (2006)
2006	Soft cheese	189	14	Koch et al. (2010)
2006	Soft cheese	78	17	EFSA (2007)
2007	Camembert cheese	17	18	Johnsen, Lingaas, Torfoss, Strom & Nordoy (2010)
2007	Mature cheese	NR	NR	Vít et al. (2007)
2008	Cheese	92	NR	Taillefer, Boucher, Laferriere & Morin (2010)
2008	Brie cheese	91	5	ProMed (2008)
2008	Mexican-style cheese	8	0	Cartwright et al. (2013)
2009-2012	Queso fresco	30	37	Magalhaes et al. (2015)
2010	Soft cheese (Panela, Queso fresco, Requeson)	5	0	FIOD (2010)
2010	Soft cheese	28	11	FIOD (2015)
2010	Acid-curd cheese (Quargel)	14	36	Fretz et al. (2010)
2011	Fresh cheese (chives)	2	NR	FIOD (2011)
2011	Mexican-style cheese	7	29	Jackson et al. (2011)
2011	Pave de Nord (Mimolette-type cheese made from pasteurized milk)	12	33	Yde et al. (2012)
2012	Latin-style fresh cheese (pasteurized milk)	2	0	De Castro et al. (2012)
2012	Ricotta cheese	22	18	CDC (2012)
2013	Camembert / Brie	18	17	Newsdesk (2013b)
2014	Fresh cheese	1	14	FIOD (2014)
2014	Soft cheese	5	20	CDC (2014)
2015	Cheese made from unpasteurized milk	2	0	Del-Valdivia-Tapia, Pinelo-Chumbe & Carreazo (2015)
2015	Fresh cheese	3	33	FIOD (2015)
2017	Smear cheese	8	25	CDC (2017)

NR: Not reported

In the EU, 0.3% of hard, semi-soft and soft cheeses made from pasteurized milk tested positive for *L. monocytogenes* in 2013 (EFSA, 2015). In 2009, *L. monocytogenes* was detected in Dutch-type Gouda cheese exported to the USA following sampling by the FDA (FDA, 2016a), but this Gouda cheese was not linked to listeriosis, and it was unclear whether the Gouda cheese in question was made from pasteurized or unpasteurized milk, nor was information available on concentrations of *L. monocytogenes* in this cheese.

1.5 Dutch-type Gouda cheese and *L. monocytogenes*

Dutch-type cheeses are semi-hard cheeses that are made from bovine milk that is pasteurized when produced at an industrial scale. In 2017, 865 million kg of cheese was produced in The Netherlands (ZuivelNL, 2017), the majority of which being Gouda cheese. Gouda Holland cheese has the protected designation of origin (PDO) status. The first production of Gouda cheese was recorded in the 17th century (Fox & McSweeney, 2004).

A global overview of the production process of Dutch-type Gouda cheese is presented in Fig. 1.1. Upon receipt of the raw milk, the milk is thermized. After thermization, cream is removed and the skimmed milk is optionally bactofugated to reduce the concentrations of bacterial spores of *Clostridium tyrobutyricum*, thereby improving the final quality of the cheese and whey. Subsequently, the milk is standardized by adding pasteurized cream, therewith obtaining cheese milk with a protein content (~3.4%) and fat content (~3.7%) for a Dutch-type Gouda cheese with 48% w/w fat in dry matter. The standardized cheese milk is then pasteurized (min. 72 °C for 15 s) and cooled to ~31 °C. At this stage, $CaCl_2$ (~0.02%) is added to the cheese milk, followed by a starter culture consisting of ~0.5-1.0% mesophilic lactic acid bacteria (primarily of *Lactococcus lactis* subsp. *cremoris* and *Lactococcus lactis* subsp. *lactis*, and additionally *Leuconostoc* and *Lactococcus lactis* subsp. *lactis* var. *diacetylactis*), and rennet (~0.02%). Subsequently, the milk is kept for ~20 minutes at ~31 °C. The resulting curd is cut and the whey is drained from the curd. After drainage, hot water (25-30% of the initial milk volume) is added to wash the curd and to increase the temperature to ~35 °C. The latter temperature is maintained during stirring, residual heating and drainage of residual whey (~50 minutes). The curd is placed in a cheese mold and pressed. The resulting cheese is demolded after 80-90 minutes and is soaked for 2-3 days in a brine bath of pH 4.4-4.8 that contains ~18% NaCl. After brining, the cheese is coated and ripened at 12-13 °C for 4-48 weeks depending on age type, resulting in nature-ripened cheese (van den Berg, Meijer, Düsterhöft & Smit, 2004). For foil-ripened cheese, the brined cheese is packed in foil before ripening.

HACCP plans have been established for Dutch cheese factories to monitor and to prevent recontamination of the cheese with *L. monocytogenes* after pasteurization of the milk and during processing, with procedures in place that focus on prevention of occurrence of *L. monocytogenes* in the cheese product (raw and pasteurized milk, curd, cheese) and cheese processing facilities (e.g. brine baths, waste pits, floors).

Fig. 1.1 Overview of production process of Gouda cheese.

1.6 Dutch-type Gouda cheese and legislation with regard to *L. monocytogenes*

Dutch-type Gouda cheese is a ready-to-eat food product (RTE food) as it does not undergo a heat treatment prior to consumption. Good manufacturing practices (GMP), in compliance with criteria established by food authorities, are required for manufacturing and selling of food. Sampling procedures must be established to estimate contamination levels in food products and processing environments.

Measures are implemented by cheese producers to prevent contamination of cheese with *L. monocytogenes*, thereby ultimately preventing the occurrence of listeriosis cases due to the consumption of contaminated cheese.

The EU criteria for RTE foods rely on concentrations of *L. monocytogenes* (maximum of 100 cfu g^{-1} at the moment of consumption and/or absence in 25 g). An overview of the European food safety criteria in RTE foods defined in EC 2073/2005 is presented in Fig. 1.2. According to those criteria, RTE foods are divided into three categories.

Category 1.1 RTE foods intended for infants and RTE foods for special medical purposes*;

Category 1.2 RTE foods able to support growth of *L. monocytogenes*, other than those intended for infants and for special medical purposes;

Category 1.3 RTE foods unable to support growth of *L. monocytogenes*, other than those intended for infants and for special medical purposes*.

RTE foods intended for infants and for special medical purposes fall within category 1.1 and these foods have to be monitored for the absence of *L. monocytogenes* (absence in 10 samples of 25 g). Other RTE foods that are very acidic (pH ≤ 4.4), very salty and/or dry (a_w ≤ 0.92, which corresponds with ~13% (or 2.2 M) NaCl in liquid food), or acidic as well as salty (pH ≤ 5.0 and a_w ≤ 0.94, with the latter corresponding with ~10% (or 1.7 M) NaCl in liquid food) fall within category 1.3 of 'foods unable to support growth'. Other foods can also belong to category 1.3 if scientific evidence of non-growth of *L. monocytogenes* is available (Fig. 1.2). Such scientific evidence can consist of predictive mathematical modelling, durability tests and/or challenge tests.

* As described in EC 2073/2005, regular testing against the criterion applicable to category 1.1 and 1.3 foods is normally unnecessary for RTE foods that are heat-treated or processed effectively to eliminate *L. monocytogenes* and for which recontamination is not possible. This also applies to fresh, uncut and unprocessed vegetables and fruits excluding sprouted seeds, for bread, biscuits and similar products, for bottled or packed waters, soft drinks, beer, cider, wine and similar products, for sugar, honey and confectionary products, and for live bivalve molluscs. In EC 365/2010 amending EC 2073/2005, food-grade salt is added to the list of RTE foods for which regular testing against the criterion of category 1.1 and 1.3 is normally unnecessary. Although not described in EC 2073/2005 and/or EC 365/2010, this unnecessary regular testing can also apply to category 1.2 foods.

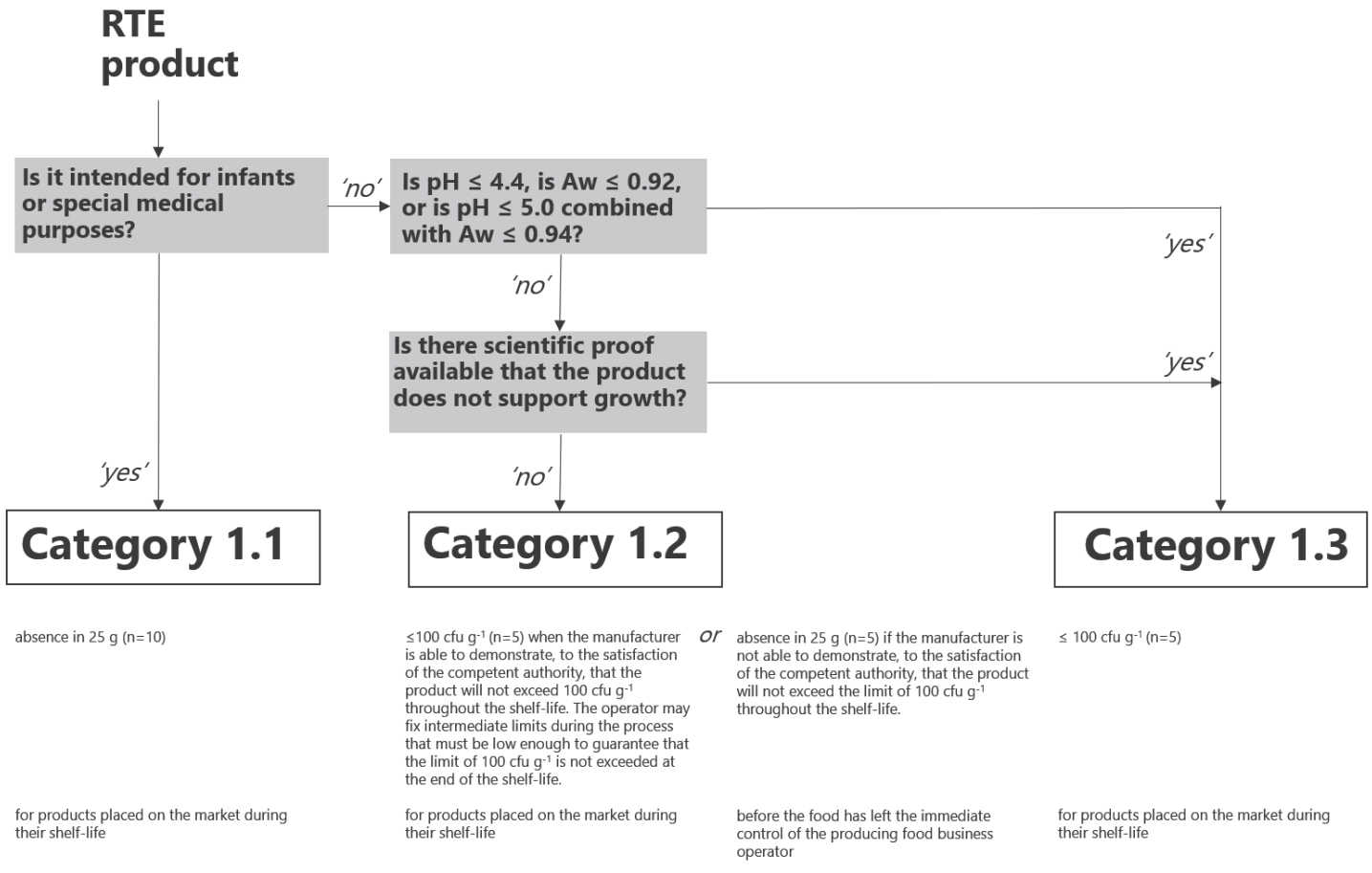

Fig. 1.2 Overview of the European food safety criteria for *L. monocytogenes* in RTE foods defined in EC 2073/2005. Below each category, the sampling plan and the stage where each criterion applies are displayed.

The fate of *L. monocytogenes* in a food product can be experimentally determined in two different ways: by durability testing or by challenge testing (Fig. 1.3). According to the most recent draft technical guidelines for such studies in RTE foods (Beaufort, Bergis & Lardeux, 2014), durability studies allow for an assessment of the shelf-life of the food regarding *L. monocytogenes* in a naturally contaminated food during its storage, according to reasonably foreseeable conditions. A challenge test assesses the growth potential or the maximum growth rate of *L. monocytogenes*, simulating as closely as possible the likely storage conditions of the product. In challenge studies, the contamination is artificial. Results generated in durability studies may be more realistic, but may be more difficult to interpret because the prevalence of the initial contamination is often low and the initial contamination levels may be unevenly distributed (Spanu et al., 2014). Challenge studies seem more suitable for Dutch-type Gouda cheese, as absence of *L. monocytogenes* is expected in a standard Gouda made from pasteurized milk.

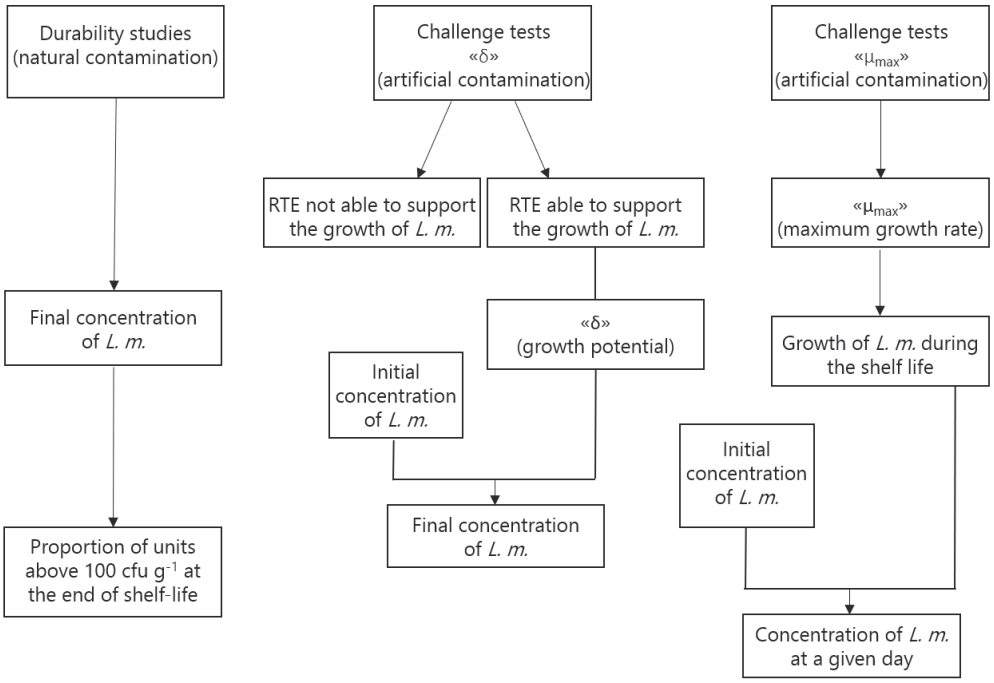

Fig. 1.3 Microbiological procedures for determining the growth of *L. monocytogenes* in RTE foods using durability and challenge tests. Obtained from the draft Technical guidance document for conducting shelf-life studies on *L. monocytogenes* in RTE foods of Beaufort et al. (2014).

Currently, Dutch-type Gouda cheese can be categorized based on EC 2073/2005 as a category 1.2 food. When the manufacturer has not demonstrated to the satisfaction of the competent authority that the product will not exceed 100 cfu g^{-1} throughout the shelf-life, *L. monocytogenes* must be absent in 25 g (n = 5) before the food has left the immediate control of the food business operator, who has produced it. Gouda is a food with pH > 5.0

and a_w > 0.94 and merely based on pH and a_w criteria, growth of *L. monocytogenes* may not be excluded. There is evidence that Dutch-type Gouda cheese does not support growth of *L. monocytogenes* and the product has never been linked with listeriosis. Therefore, the Dutch dairy industry would like to investigate if Dutch-type Gouda cheese made from pasteurized milk could classify for the sampling plan of <100 cfu g^{-1} (n=5) for products placed on the market during their shelf-life. There are two cases in which *L. monocytogenes* has been isolated from US-type Gouda cheese, leading to recalls (Newsdesk, 2013a; 2014), but it was unclear if those recalls involved cheeses made from raw or pasteurized milk.

The published challenge study by Northolt et al. (1988) for Dutch-type Gouda cheese showed no growth, only survival of *L. monocytogenes* during six weeks of ripening. More insight in the most important production and product parameters that determine growth/no growth of *L. monocytogenes* in Dutch-type Gouda cheeses can be obtained by studying the fate of *L. monocytogenes* in the cheese. The impact of the factors determining this fate can be studied using a predictive modelling approach.

1.7 Predictive modelling

Predictive modelling is a technique with which the behavior as a response to environmental conditions can be systematically captured and converted into predictions using mathematics. It relies on the concept that responses are reproducible.

Predictive models for microorganisms in foods are constructed to describe their fate in those foods. The fate of bacteria in food can either be growth, survival or inactivation. Models can either be probabilistic, modelling the likelihood of growth or inactivation, or they can be kinetic, modelling the growth or inactivation rate and concentration of microorganisms (Ross & McMeekin, 1994). There are three types of predictive models describing bacterial behavior: primary, secondary and tertiary models (Whiting & Buchanan, 1993).

Primary models describe the change in microbial populations (bacterial growth of death) as a function of time. Gibson, Bratchell & Roberts (1987) established the Gompertz equation to describe the bacterial growth curve. Zwietering, Jongenburger, Rombouts & van 't Riet (1990) adapted this equation to the modified Gompertz equation, which is currently the most widely used primary model to describe bacterial growth.

Secondary models describe the effect of environmental factors on growth or starvation parameters, by describing the response of primary parameters to intrinsic and extrinsic parameters. Such intrinsic or extrinsic parameters often include pH, water activity (a_w) and

temperature (T), but can also include other factors such as CO_2, organic acids and nitrite (Baranyi & Roberts, 1994; Farber, Cai & Ross, 1996; Mejlholm & Dalgaard, 2009). Several types of secondary models are available to describe the growth of bacteria. Polynomial models have been developed as a technique for empirical curve fitting (Gibson, Bratchell & Roberts, 1988; Buchanan & Phillips, 1990). Ratkowsky, Lowry, McMeekin, Stokes & Chandler (1983) developed a square-root model to model the inhibiting effect of temperature on growth of bacteria at temperatures lower than the optimum growth temperature. This square-root model was extended with other growth-inhibiting factors such as a_w and pH (Presser, Ratkowsky & Ross, 1997; Presser, Ross & Ratkowsky, 1998). Zwietering, Wijtzes, de Wit & van 't Riet (1992) established the Gamma model following a multiplicative approach to evaluate the effect of single factors in various combinations by multiplication of the effects. The Gamma model assumes that there are no interactions between the growth-inhibiting parameters; therewith it models the growth-inhibiting effects of individual factors. The model allows inclusion of several datasets for each hurdle. These datasets can be less extensive in comparison to datasets required for polynomial models. The Gamma hypothesis is defined as follows:

$$\mu_{max}(x,y) = \mu_{opt} \cdot \gamma(x) \cdot \gamma(y) \quad \text{(Eq. 1.1)},$$

stating that the maximum specific growth rate of a bacterium in a food product μ_{max} is dependent on the growth rate of that bacterium at optimal conditions μ_{opt} and the growth-inhibiting effects of the intrinsic and extrinsic factors that are present $\gamma(x)$ and $\gamma(y)$. Equations representing the effects of the intrinsic and extrinsic factors can be described by the cardinal model from Rosso, Lobry, Bajard & Flandrois (1995).

Le Marc et al. (2002) used the multiplicative approach with τ, pH and organic acids as growth-inhibiting factors, but expanded the Gamma model with a synergy factor $\zeta(x,y)$ to describe interaction between factors at the growth limits:

$$\mu_{max}(x,y) = \mu_{opt} \cdot \gamma(x) \cdot \gamma(y) \cdot \zeta(x,y) \quad \text{(Eq. 1.2)}.$$

Augustin & Carlier (2000a; 2000b) also proposed a secondary model to describe the global effect of growth conditions on μ_{max}. They used a multiplicative approach as well, and assumed that T, pH, a_w, inhibitory substances c_i and qualitative factors k_{jl} have independent effects on μ_{max}:

$$\mu_{max} = \mu_{opt} \cdot \tau(T) \cdot \rho(pH) \cdot \alpha(a_w) \cdot \prod_{i=1}^{n} \gamma(c_i) \cdot \prod_{j=1}^{p} k_{jl} \quad \text{(Eq. 1.3)},$$

with $\prod_{i=1}^{n} \gamma(c_i)$ as the sum of inhibitory substances c_i and $\prod_{j=1}^{p} k_{jl}$ as the sum of qualitative factors k_{jl}.

The minimal growth parameters for the factors determining μ_{max} are described by Rosso et al. (1995) and others according to Augustin et al. (2000a).

Tertiary models are based on software programs that provide an interface between the underlying mathematics and the user, allowing model inputs to be entered and estimates to be observed through simplified graphical outputs (Pérez-Rodríguez & Valero, 2013). Examples of tertiary models are Combase Predictor (http:/modelling.combase.cc) and a predictive model for *L. monocytogenes* in Blue-type cheeses (Rosshaug, Detmer, Ingmer & Larsen, 2012).

At the start of the work described in this thesis, no predictive models had been applied to study the fate of *L. monocytogenes* in Gouda cheese. In this thesis, the Gamma model without interaction between factors has been applied to Dutch-type Gouda cheese to model growth/no growth of *L. monocytogenes*, as this model can incorporate the effects of multiple growth-inhibiting factors whilst inclusion of several datasets is allowed.

1.8 Aim and outline of the thesis

The aims of this study were to establish if *L. monocytogenes* can grow in Dutch-type Gouda cheese and to provide insight in the most important factors for inhibition of growth and the variation of these factors inside the cheese and throughout the ripening. An additional aim of this study was to provide data that support decisions with respect to food safety limits for *L. monocytogenes* in Dutch-type Gouda cheese. Establishing food safety limits requires interactions between academia/research institutes (research), policy makers (categorization and monitoring) and industry (quality management and production). Academia have an advisory role on the food safety limits, policy makers use the limits for categorization and legislation where safety supervisors monitor the conformity to the limits, and industry complies with the set limits to assure food safety throughout production (Fig. 1.4).

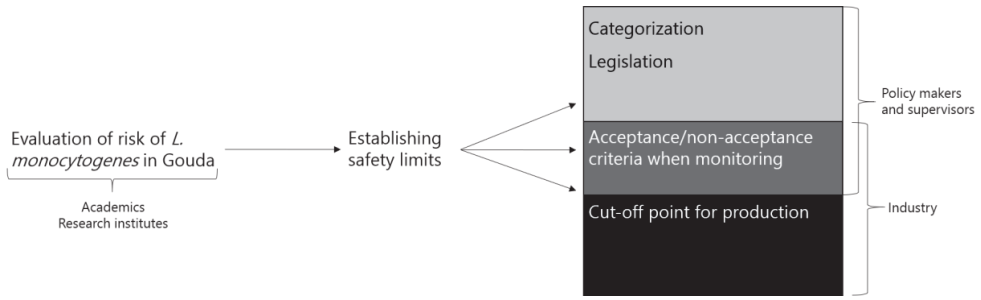

Fig. 1.4 Establishment of food safety limits is an interaction between academia/research institutes, policy makers and supervisors, and the industry.

The different research topics addressed in this thesis are as follows. The fate of *L. monocytogenes* in and on Dutch-type Gouda cheese is described in **Chapter 2** and **Chapter 3**, respectively. To improve the estimation of the effect of a_w on growth inhibition of *L. monocytogenes*, the NaCl and water content together with the a_w were established during brining and ripening of Gouda cheese for both foil-ripened and nature-ripened cheese (**Chapter 4**). In **Chapter 5**, the sensitivity of *L. monocytogenes* to organic acids prevalent in Dutch-type cheeses was assessed. In **Chapter 6,** the individual factors contributing to inhibition of growth of *L. monocytogenes* in Dutch-type Gouda were evaluated. The actual concentrations of the factors as present in Dutch-type Gouda were determined, as well as their corresponding capacity to inhibit growth of *L. monocytogenes*. **Chapter 7** is the general discussion, including a validation of growth/no growth of *L. monocytogenes* in RTE cheese when growth/no growth is predicted based on undissociated lactic acid, pH, a_w and T as growth-inhibiting factors, using an existing secondary growth model. In addition, growth/no growth of *L. monocytogenes* is predicted in the last chapter in other types of soft, semi-hard and hard cheeses. The main results are summarized and discussed, and implications for the dairy industry with regard to *L. monocytogenes* and Dutch-type Gouda cheese are presented.

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Chapter 2

Fate of *Listeria monocytogenes* in Gouda microcheese: No growth, and substantial inactivation after extended ripening times

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ABSTRACT

This challenge study demonstrates that *Listeria monocytogenes* does not grow in Gouda cheese: during the first 8 weeks of ripening no growth was observed and between 8 and 52 weeks viable numbers declined significantly in a well-established Gouda microcheese system. Cheese milk was artificially contaminated just prior to addition of the starter culture. Three individual *L. monocytogenes* strains were used, including strains originating from cheese, a cheese plant environment and a reference strain. During curd formation, viable numbers of *L. monocytogenes* increased by 0.5 log cfu g^{-1}, resulting from entrapment in the curd. No growth was observed during the first 8 weeks of ripening. A significant decline in the viable numbers of *L. monocytogenes* was observed in Gouda cheese that was ripened for longer than 8 weeks. Two factors that could possibly control the fate of *L. monocytogenes* in Gouda cheese were lactic acid and water activity.

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2.1 Introduction

The food-borne pathogen *Listeria monocytogenes* is the causative agent of listeriosis, a disease that can manifest as meningitis, encephalitis, sepsis, intrauterine infections and gastroenteritis (Vazquez-Boland et al., 2001). Although listeriosis infections are rare, the case-fatality rate is very high (20 – 30%), especially for the elderly, the unborn child, infants, and immune-compromised individuals (Doorduyn et al., 2006). *L. monocytogenes* may be found in a broad range of foods from animal or plant origin, and the microorganism can grow at pH values ranging from pH 4.4 to 9.0, at NaCl concentrations up to 12%, and temperatures from –0.4 to 44 °C (Lou & Yousef, 2000). Foodborne outbreaks of *L. monocytogenes* have been associated with many different categories of food products, including seafood, vegetables, meat products and dairy products (EFSA, 2011), like soft and acid-curd cheeses made from pasteurized milk (Gaulin, Ramsay, & Bekal, 2012; Koch et al., 2010).

In December 2007, microbiological criteria for *L. monocytogenes* in ready-to-eat (RTE) foods were implemented in the European Union through regulation 1441/2007. For RTE foods that do not support growth of *L. monocytogenes*, a maximum level of 100 cfu g^{-1} is allowed. Criteria for products that do not support growth of *L. monocytogenes* as given in the EU regulation 1441/2007 are: pH ≤ 4.4; a_w ≤ 0.92; or pH ≤ 5.0 and a_w ≤ 0.94. A product does not support growth of *L. monocytogenes* when it fulfills at least one of the previous criteria. Other conditions that may not allow for growth of *L. monocytogenes* in a product can be established using a modeling approach and/or challenge tests. For products that can support growth, EU regulation 1441/2007 requires that *L. monocytogenes* should be absent in 5 samples of 25 g after production and that the products should not exceed 100 cfu g^{-1} at the moment of consumption. Codex Alimentarius (CAC, 2007) similarly specifies absence of the pathogen in 5 samples of 25 g.

When *L. monocytogenes* was identified as a food-borne pathogen in the 1980s, Northolt et al. (1988) demonstrated the absence of growth of *L. monocytogenes* in 4.5 kg Gouda cheeses during a 6 week ripening period. Over the last 3 decades, no listeriosis incidents have been reported related to the consumption of semi-hard Gouda cheeses made from pasteurized milk, this supports the hypothesis that this organism does not grow in Gouda cheese. Nevertheless, as Gouda cheese is a RTE product, due diligence is required with regard to potential contamination of cheeses with *L. monocytogenes* during manufacturing and potential growth of the organism during ripening.

Gouda cheese is a semi-hard cheese that is made from pasteurized milk in which curd formation is induced by rennet and a mesophilic starter culture. The pH of young Gouda cheese is 5.3 (van den Berg, Meijer, Düsterhöft & Smit, 2004), and the a_w is 0.95 (Guinee &

Fox, 2004). Merely based on this pH in combination with the a_w, *L. monocytogenes* may be expected to grow during extended ripening periods, as has been found in soft cheese models with a moisture content higher than 50% and a pH higher than 5.9 (Baranyi et al., 2012). These models, however, did not take into account other growth-inhibiting factors, such as the effect of undissociated organic acids. Consequently, there is a need to extend predictive models for *L. monocytogenes* in semi-hard cheeses by obtaining additional quantitative data on the fate of this organism in Gouda cheese to determine the most important factors for growth/no growth of this pathogen in cheese.

The work presented here constitutes a challenge study of *L. monocytogenes* in a well-established Gouda microcheese® model, that has been developed at NIZO food research (Bachmann, Kruijswijk, Molenaar, Kleerebezem, & van Hylckama Vlieg, 2009). Different *L. monocytogenes* strains were artificially inoculated into cheese milk, and following starter-induced curd formation, microcheeses were ripened for a period of up to 12 months. The viable counts of *L. monocytogenes* were determined at the different stages of cheese making and ripening. In addition, to allow for a direct extrapolation to large scale cheeses, intrinsic parameters of cheese (a_w, moisture content, pH and organic acid concentrations) were determined for the microcheeses and in parallel for industrially produced non-inoculated 12 kg Gouda cheeses.

2.2 Materials and methods

2.2.1 Cheese milk preparation

Raw bovine bulk tank milk was standardized (3.5% fat, 3.4% protein and 4.5% lactose) and subjected to a single round of bactofugation and a heat treatment of 73 °C for 14 s in a continuous-flow pasteurizer with a capacity of 10,000 L h^{-1} (Alfa Laval, Lund, Sweden). The milk was then frozen using liquid nitrogen and stored at -40 °C until further used.

2.2.2 Bacterial strains and cultures used

Bos starter culture (CSK food enrichment, Ede, The Netherlands) was selected for the acidification of cheese milk to produce Gouda microcheeses. Bos is a mesophilic starter culture containing a mixture of mainly lactococci and *Leuconostoc* spp. that is commonly used for Gouda cheese production in The Netherlands. For pre-culturing, an aliquot of a frozen concentrated Bos culture was inoculated into sterilized (115 ºC for 10 min),

reconstituted skimmed milk powder (NIZO food research, Ede, The Netherlands) and incubated for 20h at 20 °C. Subsequently, this precultured Bos starter culture was added to the cheese milk at an inoculum level of 1%. This yielded $3.3 \cdot 10^6$ cfu mL^{-1} lactococci and $4.6 \cdot 10^5$ cfu mL^{-1} *Leuconostoc* species in the cheese milk; the viable numbers of *Leuconostoc* and lactococci were determined by plating on Lactobacillus agar (De Man, Rogosa and Sharpe agar; MRS agar, Merck, Darmstadt, Germany) and plating on M17 agar (Merck) to which 0.5% lactose was added after sterilization, respectively. Acidification during curd formation using the Bos starter culture was monitored by pH measurements of the cheese milk, the first curd, the second curd, and the whey.

Three strains of *L. monocytogenes* were selected for use in the challenge study: a cheese isolate (strain 2F serotype 1/2a), an environmental isolate from a cheese factory (strain 6E, serotype 1/2a), and a reference strain *L. monocytogenes* Scott A (milk outbreak isolate, serotype 4b) (Table 2.1). These strains were included in a previous study in which the phenotypic properties of 138 *L. monocytogenes* strains were determined (van der Veen, Moezelaar, Abee, & Wells-Bennik, 2008). Strain 6E and 2F were selected because these strains showed the highest resistance to lactic acid while strain Scott A had the lowest resistance to lactic acid (van der Veen et al. 2008; Wemmenhove, van Valenberg, Zwietering, van Hooijdonk, & Wells-Bennik, 2016). Growth of strains Scott A, 2F and 6E was assessed in the Gouda microcheese system, using four different NaCl concentrations (0.9%, 3.1%, 3.8% and 4.1% NaCl on a dry matter basis) for a ripening period of up to one year.

Table 2.1 *L. monocytogenes* strains used in this study, their serotype, resistance to lactic acid (van der Veen et al. 2008) and origin

Strain	Serotype	Minimal concentration (mM) of undissociated lactic acid needed to inhibit growth	Origin
Scott A	4b	2.5	Milk outbreak (Massachusetts, US, 1983)
2F	1/2a	4.0	Cheese (Dijon, France, 1990)
6E	1/2a	7.5	Cheese equipment of an industrial cheese-making plant (Dijon, France, 1990)

2.2.3 Production of Gouda-type cheeses with different NaCl concentrations and artificial *Listeria* contamination during curd formation

To produce Gouda microcheeses, the frozen milk was thawed and warmed to 30.5 °C. The following were added per liter of cheese milk: 200 µL rennet enzyme (Kalase®, 150 IMCU,

CSK Food Enrichment), 400 µL $CaCl_2$ (35% w/v, Akzo Nobel, Frankfurt, Germany), 0.5 g Delvocid (DSM food specialties, Delft, The Netherlands). Individual *L. monocytogenes* strains were added to separate batches to a final level of approximately 10^7 cfu mL^{-1}; the exact levels were determined by plating on PALCAM-selective agar (VWR, Amsterdam, The Netherlands). A pre-cultured Bos starter culture was then added to the artificially contaminated milk at an inoculum level of 1%.

Subsequently, 1.7 mL aliquots of the inoculated cheese milk were put in a deep-well microplate (Greiner, Alphen a/d Rijn, The Netherlands) and incubated at 30.5 °C. After approximately 60 min the curd was cut, using a custom-made stirring device, as described before (Bachmann et al., 2009). The next steps consisted of stirring, settling and resting, taking an overall time of approximately 25 min. Then deep-well microplates were centrifuged (1000 x g for 5 min) to slightly compact the curds and facilitate whey removal. Following centrifugation, 620 µL of whey was removed and 550 µL of sterile washing water of 55 ºC was added to mimic the curd washing step in the large-scale process and simultaneously increase the temperature of the microcheeses to 35.5 °C. The microplates were incubated at 35.5 ºC for 40 min with stirring and an additional 20 min without stirring. Subsequently, the plates were subjected to centrifugation (4000 x g for 60 min), after which the second whey was removed using pipettes, and by turning the plates upside down on a sterile tissue paper for 10 min. More detailed information on the sample size, pH, time and temperature of each of the phases is presented in Table 2.2. The pH and moisture content were measured 4 h after the addition of the starter to check the acidification capacity of the Bos starter culture and to assess curd formation. After 24 h the pH was determined again.

To determine the effect of NaCl in cheese on *L. monocytogenes*, Gouda cheeses with different concentrations of NaCl were produced and stored under controlled conditions throughout the ripening. To adjust the NaCl concentrations in the microcheeses, 17 µL aliquots of sterile NaCl solution (different concentrations in the range of 80, 200, 280 and 350 g L^{-1}) were added to each microcheese, and microcheese plates were then centrifuged (1000 x g for 5 min). This resulted in NaCl concentrations of 0.9, 3.1, 3.8 and 4.1% on a dry matter basis, as determined using method NEN 3762 (van 't Westende, 1977). After 24 h, the target moisture content of the microcheese was 42–45%, and plates were then sealed in 0.8 atmospheres of 100% nitrogen, followed by storage at 12 °C for ripening periods of 2, 4, 6, 8, 16, 28 and 52 weeks. Microcheeses with different NaCl concentrations were present in different wells of one microcheese plate. For each ripening period a different microplate was opened and analysed. Two independent experiments were performed per condition and per strain, and moisture and pH analyses were performed in duplicate.

Table 2.2 Viable counts of *L. monocytogenes* strains Scott A, 2F and 6E during the successive stages of curd formation[a]

Code	Sample	Sample size (g)	pH	Time (min) after starter addition	Tempe-rature (°C)	*L. monocytogenes* viable counts (cfu g^{-1})[a]		
						Scott A	2F	6E
A	Milk	1.7	6.7	0	30.5	$6.5 \cdot 10^7$ $\pm 1.1 \cdot 10^6$	$4.8 \cdot 10^7$ $\pm 5.5 \cdot 10^5$	$2.3 \cdot 10^7$ $\pm 4.0 \cdot 10^5$
B	Renneted milk	1.7	6.6	20	30.5	$2.9 \cdot 10^7$ $\pm 1.0 \cdot 10^6$	$1.2 \cdot 10^7$ $\pm 4.9 \cdot 10^5$	$3.6 \cdot 10^6$ $\pm 7.2 \cdot 10^4$
C_1	First curd	1.08	6.4	60	30.5	$8.7 \cdot 10^7$ $\pm 1.0 \cdot 10^4$	$4.7 \cdot 10^7$ $\pm 2.0 \cdot 10^5$	$3.9 \cdot 10^7$ $\pm 2.3 \cdot 10^5$
C_2	First whey	0.62	6.6	60	30.5	$2.6 \cdot 10^6$ $\pm 4.6 \cdot 10^4$	$4.7 \cdot 10^5$ $\pm 8.3 \cdot 10^3$	$4.3 \cdot 10^5$ $\pm 2.5 \cdot 10^3$
D	First curd with 0.55 ml H_2O	1.63	6.4	60	35.5	$5.8 \cdot 10^7$ $\pm 1.0 \cdot 10^4$	$3.1 \cdot 10^7$ $\pm 2.0 \cdot 10^5$	$2.6 \cdot 10^7$ $\pm 2.3 \cdot 10^5$
E	First curd before whey separation	1.63	6.2	130	35.5	$5.7 \cdot 10^7$ $\pm 3.5 \cdot 10^5$	$3.2 \cdot 10^7$ $\pm 2.5 \cdot 10^4$	$4.7 \cdot 10^7$ $\pm 3.9 \cdot 10^5$
F_1	Second curd	0.17	5.7	200	35.5	$1.3 \cdot 10^8$ $\pm 3.4 \cdot 10^7$	$1.4 \cdot 10^8$ $\pm 3.2 \cdot 10^7$	$1.5 \cdot 10^8$ $\pm 1.1 \cdot 10^7$
F_2	Second whey	1.46	6.4	200	35.5	$1.3 \cdot 10^4$ $\pm 1.5 \cdot 10^3$	$1.6 \cdot 10^5$ $\pm 1.5 \cdot 10^3$	$2.3 \cdot 10^4$ $\pm 3.2 \cdot 10^3$

[a] The sample size, pH, time (after starter addition) and temperature of each stage of curd formation are specified. Results are expressed as average ± stdev of triplicate determinations. Codes A–F: see Fig. 2.1A.

2.2.4 Microbial analyses

The viable numbers of *L. monocytogenes* Scott A, 2F, and 6E were determined in triplicate in milk, in first and second whey (60 min and 200 min after addition of starter to the milk, respectively), and in the first (60 and 130 min after addition of starter) and second curd. For the analysis of curd, whey and cheese, samples from five wells of the 96 wells plate were collected and pooled. Before making ten-fold serial dilutions, the cheese and curd samples were homogenized using a stomacher and dissolved by adding 9 mL of 2% trisodium citrate to 1 g cheese or curd. Ten-fold serial dilutions were made in peptone physiological NaCl and samples were plated on PALCAM-selective agar (VWR). The viable numbers of *L. monocytogenes* during ripening were determined in duplicate in the microcheeses after 2, 4, 6, 8, 16, 28 and 52 weeks. The numbers enumerated throughout the 12 month ripening period at different time points were compared with initial levels of *L. monocytogenes* in microcheeses directly after curd formation to correct for small differences in inoculation levels of different strains for the different experiments.

The logarithmic increase of *L. monocytogenes* throughout ripening was calculated as follows:

$$Log\ increase = \log C_t - \log C_0 \quad \text{(Eq. 2.1)},$$

where C_t = viable numbers of *L. monocytogenes* (cfu g^{-1}) determined in Gouda microcheeses after t weeks of ripening and $C_0 = C_{second\ curd}$ = initial viable numbers (cfu g^{-1}) determined in Gouda microcheeses directly after curd formation.

2.2.5 Chemical parameters during ripening of Gouda microcheeses

Organic acids, pH, moisture content, and water activity were determined in the Gouda microcheeses after 2, 4, 6, 8, 16, 28 and 52 weeks of ripening. The acidification by the starter bacteria in the Gouda microcheeses was determined by measuring the pH immediately after curd formation. The pH was measured with a 3 mm-electrode (BioTrode, Metrohm, Herisau, Switzerland) directly in the microcheeses. Organic acid concentrations (lactic, acetic and propionic acid) present in the microcheeses were determined after 2, 4, 6, 8, 16, 28 and 52 weeks of ripening using analytical reverse phase high pressure liquid chromatography (RP-HPLC) as described by Visser, Slangen and Rollema (1986). The moisture content was determined according to NEN 3755 (van 't Westende, 1998). Water activity was measured using the dewpoint method (Decagon Aqualab, Pullman, WA, USA) at 2, 4, 8, 28 and 52 weeks. Factory-scale (12 kg) Gouda cheeses aged 2 weeks, 2 months and 6 months were collected from 5 different Gouda cheese factories and for these cheeses the pH, organic acid concentrations and moisture content were determined.

2.3 Results

Gouda microcheeses were artificially contaminated by introducing three different strains of *L. monocytogenes* in the cheese milk before starter addition, to establish the fate of the pathogen throughout curd formation and during ripening of the cheeses for a period of up to 1 year. This situation mimics the hypothetical presence of *L. monocytogenes* in cheese milk after pasteurization, in a worst case scenario as a result of post-processing contamination or due to incidents relating to failure in the pasteurization process.

2.3.1 Curd formation of Gouda microcheeses: concentration and limited growth of added *L. monocytogenes*

To establish the fate of *L. monocytogenes* during curd formation after artificial contamination of the cheese milk, the viable counts of *L. monocytogenes* were determined in the cheese milk, renneted milk, in the first curd, first whey, in the first curd with added washing water, in the first curd just before separation of the second whey, and in the second curd and whey. These data are presented in Fig. 2.1 The pH decreased from 6.7 in the cheese milk to 6.4 in the renneted milk just before separation of the first curd and first whey (Table 2.2). The concentration of *L. monocytogenes* strains Scott A, 2F and 6E decreased by 0.6 log cfu g^{-1} during this part of the process (Table 2.2, Fig. 2.1). Upon separation of the first curd and first whey, 97–99% of the *L. monocytogenes* cells were recovered from the curd of the Gouda microcheese. At the same time, the weight was reduced from 1.7 g to 1.08 g due to whey removal, and concentrations of *L. monocytogenes* Scott A, 2F and 6E showed an increase of 0.7 log cfu g^{-1}, respectively. At the time of second curd formation, a decrease in viable numbers was observed (Table 2.2, Fig. 2.1A) for all three *L. monocytogenes* strains. At this time, the temperature was 35.5 °C, and the pH was 6.2 before separation of the second whey and was 5.7 after separation. The overall increase in viable numbers of *L. monocytogenes* Scott A, 2F and 6E, was 0.3, 0.5 and 0.8 log cfu g^{-1}. There was a 10-fold decrease in volume from cheese milk to second curd, and 99.9% of the *L. monocytogenes* cells were trapped in the cheese matrix (Table 2.2, Fig. 2.1A). This implies that the increase of *L. monocytogenes* during the initial stages of fermentation and curd formation is primarily due to concentration and not to growth. In cheese milk without starter culture that was kept at 30 °C for 200 min, the observed increase of the different *L. monocytogenes* strains due to growth was significantly higher, i.e. 1.6 log cfu g^{-1}, 1.0 cfu g^{-1} and 0.67 cfu g^{-1} for Scott A, 2F and 6E, respectively (Fig. 2.1B).

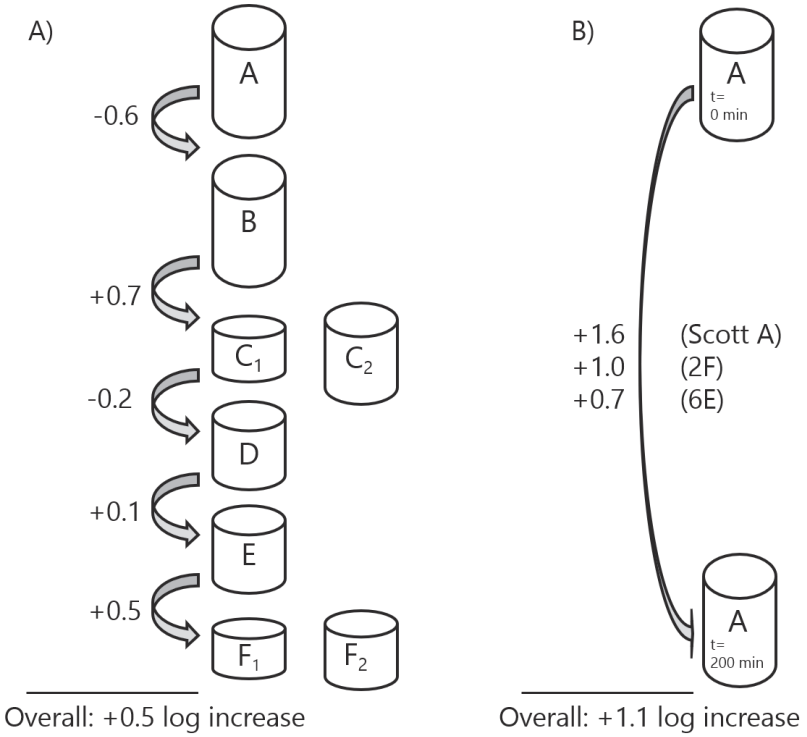

Fig. 2.1 The curd formation process. Changes in viable counts (cfu g^{-1}) of *L. monocytogenes* strains Scott A, 2F and 6E during curd formation of Gouda microcheeses (one experiment, in triplicate per strain per time point) are presented. Cheese milk was artificially contaminated with either *L. monocytogenes* strain Scott A, 2F or 6E, and the concentrations were subsequently determined in the cheese milk (sample A), renneted milk (sample B), first curd (sample C_1), first whey (sample C_2), first curd with added washing water (sample D), first curd before whey separation (sample E), second curd (sample F_1) and second whey (sample F_2). Changes in viable counts (cfu g^{-1}) during curd formation (Fig. 2.1A) were compared with changes in viable counts of *L. monocytogenes* in milk kept at 30 °C for 200 min (Fig. 2.1B).

2.3.2 No growth of *L. monocytogenes* during the first 8 weeks of ripening of Gouda microcheeses

The viable counts of *L. monocytogenes* during the ripening of the Gouda microcheeses with different NaCl concentrations are presented in Fig. 2.2. Using the definition of growth and inactivation of Koutsoumanis and Sofos (2005) and Skandamis et al. (2007), an increase of more than 0.5 log cfu g^{-1} was considered as growth, and a decrease in numbers of more than 1.0 log cfu g^{-1} is defined as inactivation. For all three *L. monocytogenes* strains (Scott A, 2F and 6E) an increase in viable numbers of more than 0.5 log was not observed under any of the conditions tested (different strains and NaCl concentrations throughout ripening). Inactivation (i.e. decrease of ≥ 1.0 log cfu g^{-1}) was not observed in the first 8 weeks. Young Gouda cheese is typically consumed within 4–6 weeks after production.

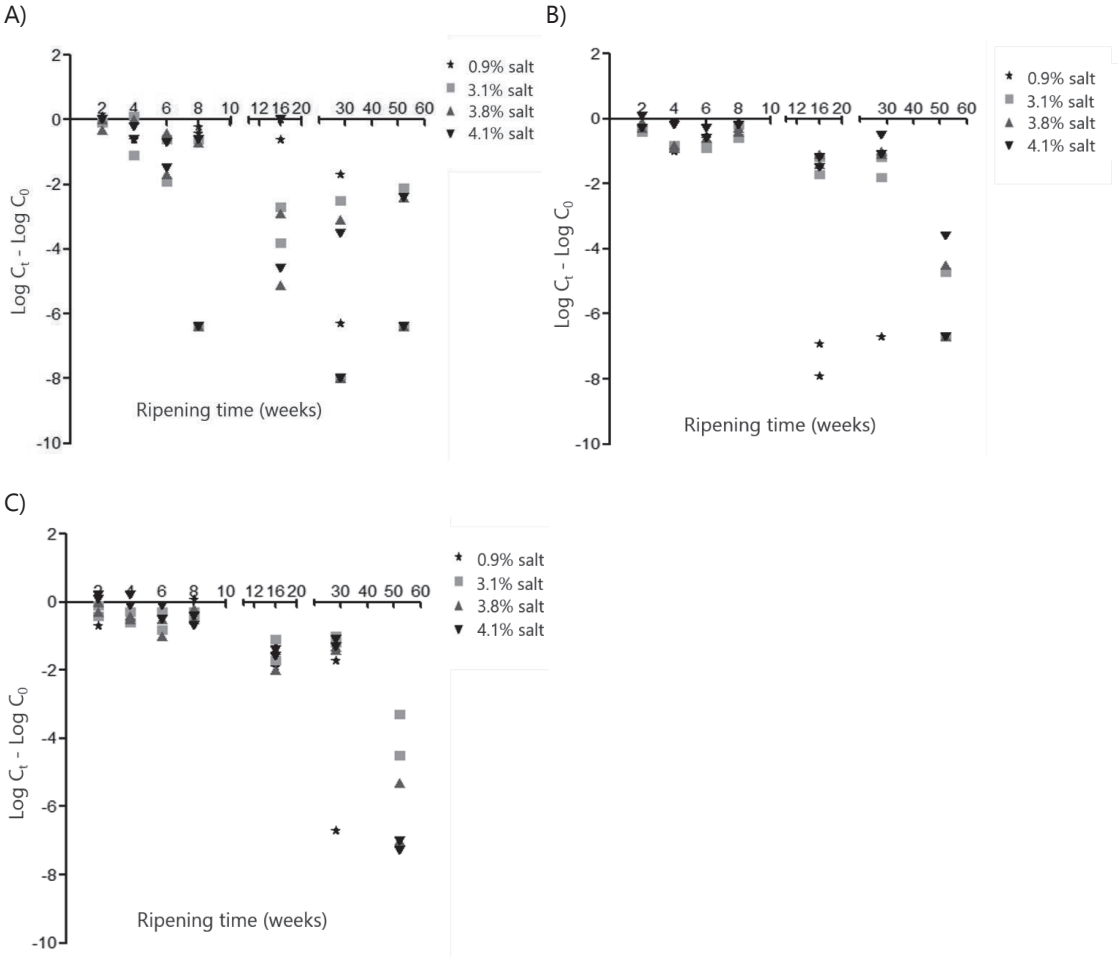

Fig. 2.2 Inactivation of *L. monocytogenes* strain Scott A (Fig. 2.2A), strain 2F (Fig. 2.2B) and strain 6E (Fig. 2.2C) during early (2–8 weeks) and prolonged (16–52 weeks) ripening of Gouda microcheeses. Initial concentrations (C_0 in log cfu g^{-1}) in the cheese curd (t = 0 weeks) were subtracted from viable numbers (C_t in log cfu g^{-1}) at a given time point. Values are displayed as individual determinations (n = 2 per strain and per time point and per salt concentration). Salt concentrations were: ★, 0.9%; ■, 3.1%; ▲, 3.8%, ▼, 4.1%. Negative log values represent lower viable counts at the given time point than initial levels.

2.3.3 Reduction of *L. monocytogenes* during extended ripening of Gouda microcheeses.

The viable numbers of *L. monocytogenes* significantly decreased in Gouda microcheeses during ripening for an extended period of time (16, 28 and 52 weeks). The viable numbers of strains 2F and 6E in microcheeses were at least 1.0 log units lower after ripening for 16 weeks (except for one experiment with strain 2F in microcheeses ripened for 28 weeks). In comparison with the counts in the second curd; viable counts of strain Scott A were reduced by more than 1.0 log after 28 weeks and longer (Fig. 2.2).

The reduction in viable numbers of *L. monocytogenes* strain Scott A in microcheeses in which the NaCl content was 0.9% on dry matter basis was lower than in microcheeses with 3.1, 3.6 and 4.1% on dry matter basis. The inactivation of strains 2F and 6E was more profound in microcheeses with 0.9% NaCl on dry matter basis than in cheeses with 3.1, 3.6 and 4.1% NaCl on dry matter basis. Overall, the survival and inactivation of *L. monocytogenes* strains was not affected by the NaCl concentrations set in different microcheeses. At extended ripening times, variability in the decrease of viable counts between independent duplicates was observed; this varied from 0 log cfu g^{-1} to 4 log cfu g^{-1}.

2.3.4 Chemical parameters during ripening of Gouda microcheeses.

The pH attained in the curd of the microcheeses (24 h after inoculation of the starter culture) was 5.3. During ripening, the pH of the microcheeses increased from pH 5.3 after 1 day, to pH 5.5 after 7 months and pH 6.1 after 1 year (Fig. 2.3A). The organic acids that were detected in the microcheeses were lactic acid and acetic acid, that were present at concentrations of 13.9 g kg^{-1} and 1.1 g kg^{-1}, respectively. The concentrations did not significantly change over time (Fig. 2.4A). As a reference, the pH and organic acid concentrations in the 12 kg commercial Gouda cheeses were also determined; the pH was between 5.3 and 5.5 in Gouda cheeses after 2 weeks of ripening and was not significantly higher after 6 months (Fig. 2.3B). The organic acids that were detected were also lactic acid and acetic acid at concentrations of 13.7 g kg^{-1} and 0.9 g kg^{-1}, respectively. No significant changes in the organic acid concentrations were observed over time (Fig. 2.4B). Citric acid and propionic acid were not detected in Gouda microcheeses or commercial 12 kg cheeses after ripening times of 2 weeks or longer.

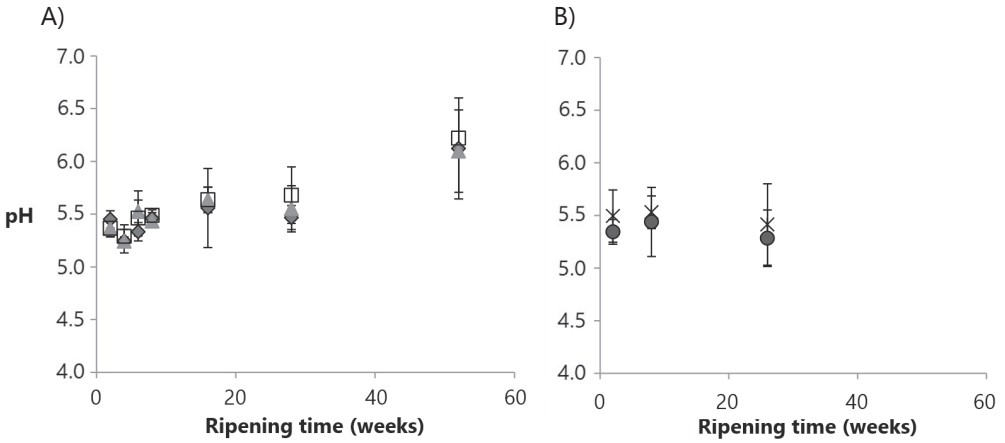

Fig. 2.3 pH (average±stdev with n = 2 per strain and per time point and per salt concentration) of Gouda microcheeses, with microcheeses challenged with *L. monocytogenes* strain Scott A depicted as ◆), 2F (depicted as ▲) and 6E (depicted as □) (Fig. 2.3A), versus 12 kg factory scale cheeses (average±stdev with n = 10 per time point) with (x) depicting the crust and (●) depicting the core of factory-scale Gouda cheese during ripening (Fig. 2.3B).

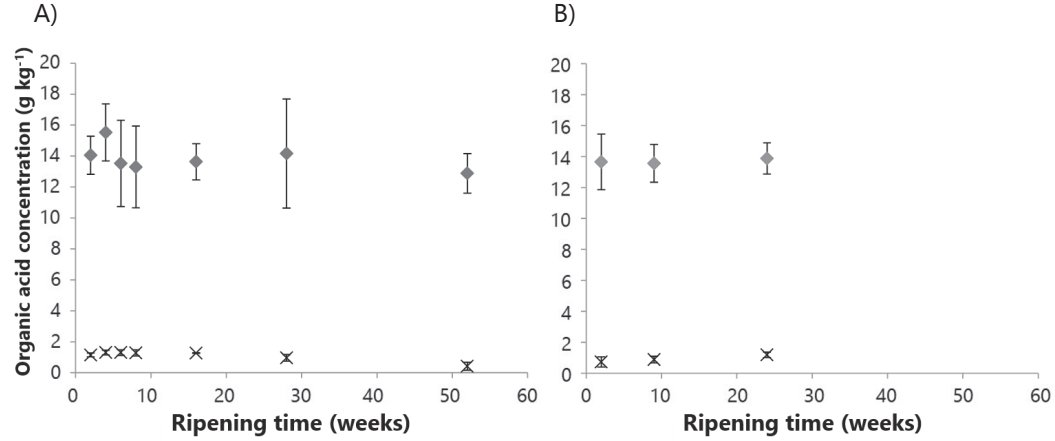

Fig. 2.4 Total concentrations (average±stdev based on independent measurements, n = 5) of lactic (◆) and acetic (x) acid (g kg^{-1}) of Gouda microcheeses (Fig. 2.4A) and of 12 kg Gouda cheeses (Fig. 2.4B), analysed during ripening.

The microcheeses had a moisture content of 45% directly after curd formation, 42% after 8 weeks of ripening (young Gouda cheese) and 39% after 1 year of ripening (Fig. 2.5). The reduction of the moisture content in time corresponds with a decline in water activity (a_w = 0.98 after 8 weeks; 0.92 after 7 months, and 0.84 after 1 year). These water activities are comparable with those of large Gouda cheeses, in which the a_w is 0.92 in the crust and 0.94 in the core after 7 months (data not shown). The NaCl concentration (concentrations varied

between 0.9 and 4.1% dry matter) and the pH did not have an effect on the moisture content within the range tested (results not shown).

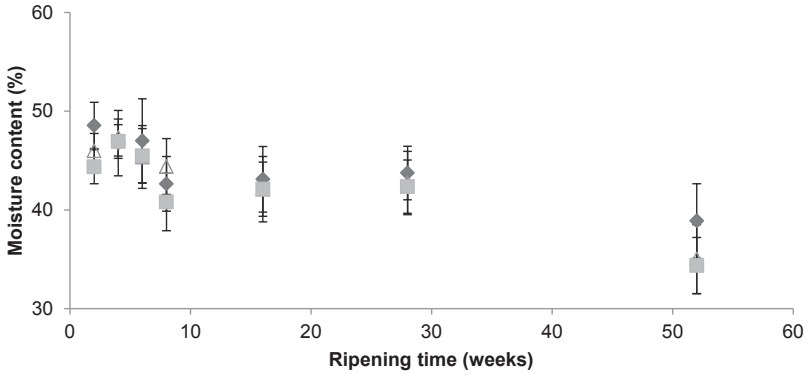

Fig. 2.5 Moisture content of Gouda microcheeses (average±stdev based on independent measurements, n=9 per time point) during ripening of the microcheeses that were inoculated with *L. monocytogenes* strains Scott A (◆), 2F (Δ) and 6E (■).

2.4 Discussion

In this challenge study, no growth of *L. monocytogenes* was observed in Gouda microcheeses throughout the Gouda cheese making process and a 52 week ripening period; in fact, the numbers declined during ripening. The cheese milk was artificially contaminated with 3 different *L. monocytogenes* strains. Because this took place at the very start of the cheese making process, it mimicked post pasteurization contamination of the milk or failure of the pasteurization process of contaminated raw milk.

The viable numbers of this pathogen were determined in the curd and the whey, as well as in the cheese during a ripening period of 52 weeks. During the first stages of cheese production, *L. monocytogenes* was concentrated in the curd upon whey separation: for the three different strains, 97–99% of *L. monocytogenes* cells were retrieved from the curd. Such enclosures in the curd are in line with findings of Yousef & Marth (1988), showing that 97.6% of *L. monocytogenes* cells were trapped in the curd during manufacture of Colby cheese. For other bacteria, notably the lactic acid bacteria in starter cultures, it is also known that only a small fraction of the bacteria ends up in the whey (Kalab, 1979). Growth of *L. monocytogenes* during formation of the first curd is another factor contributing to the increase in viable counts in the first hour after starter inoculation and artificial contamination of the cheese milk.

Acidification at that stage (pH 6.4) is still limited in the first curd and the lactic acid concentrations are not high enough to fully inhibit growth of *L. monocytogenes*, based on

previously established lactic acid concentrations that are minimally needed to fully inhibit growth of *L. monocytogenes* (Coroller et al., 2005; Houtsma, de Wit, & Rombouts, 1996; Wemmenhove, van Valenberg, Zwietering, van Hooijdonk, & Wells-Bennik, 2016). The increase in viable counts of *L. monocytogenes* strains Scott A, 2F and 6E during curd formation was +0.3 to +0.8 log cfu g^{-1} cheese. Considering that a 10-fold concentration of *L. monocytogenes* resulted from enclosure in the curd, the actual concentration of *L. monocytogenes* in the cheese milk until the separation of whey and curd decreased by -0.2 to -0.7 log cfu g^{-1} cheese. The increase in pasteurized cheese milk that was left at 30 °C was +0.7 to +1.6 log cfu g^{-1} milk, to final concentration of 8.5 log cfu g^{-1}. In a recent study, Schvartzman et al. (2011) studied the fate of *L. monocytogenes* during curd formation in a model system in which pasteurized milk was acidified with F-DVS CHN-19 starter culture that contained *Lactococcus* subsp. *cremoris*, *lactis* and *diacetylactis*, and *Leuconostoc mesenteroides*. These authors reported a 2.0 log increase of *L. monocytogenes,* starting with cheese milk and ending up with cheese curd after incubation at 30 °C for 5 h. The inoculation level of the starter culture was 0.02%, resulting in 0.17 g lactic acid per kg cheese, which is lower than the 13.9 g lactic acid per kg Gouda cheese obtained in our study using an inoculation level of 1%. The acidification level of the cheeses in the study by Schvartzman et al. (2011) was relatively low, *i.e.* a pH decrease from 6.7 to 6.3 after 4.4 h while in this study, a pH decrease from 6.7 to 5.7 was observed in Gouda cheeses. Clearly, it is important to take the effect of the starter culture and the degree of acidification into account when assessing the growth potential of *L. monocytogenes*.

No growth of *L. monocytogenes* was observed during the first 8 weeks of ripening of Gouda. These findings correspond with a previous Gouda challenge study that was performed by Northolt et al. in 1988, showing no growth of *L. monocytogenes* in 4.5 kg cheeses during the first six weeks of ripening. Lactic acid is the most prominent organic acid in Gouda cheese (Fig. 2.4). The potential of this acid in its undissociated form to inhibit the growth of *L. monocytogenes* has been described in various papers (Ahamad & Marth, 1989; Coroller et al., 2005; Vasseur, Baverel, Hébraud, & Labadie, 1999; Young & Foegeding, 1993). Van der Veen et al. (2008) also demonstrated that 2% sodium lactate at pH ≤ 5.2 led to full inhibition of growth of 138 different *L. monocytogenes* strains. Once formed, the concentration of lactic acid did not change during ripening of Gouda (Fig. 2.4A). Another factor that may contribute to growth inhibition of *L. monocytogenes* in Gouda cheese is nutrient limitation or absence of fermentable sugars (Degeest, Vaningelgem, & de Vuyst, 2001; Loubiere, Cocaign-Bousquet, Matos, Goma, & Lindley, 1997). Viable counts of all *L. monocytogenes* strains clearly decreased in all Gouda microcheeses after ripening periods of 7 months and longer, with a drop in viable numbers of at least 1 log unit and up to 7 log units. Gradual inactivation after prolonged ripening has also been observed in previous experiments with *L. monocytogenes* in Swiss, Cheddar, Colby and Feta cheese

(Buazzi, Johnson, & Marth, 1992; Genigeorgis, Carniciu, Dutulescu, & Farver, 1991; Ryser & Marth, 1987; Shrestha, Grieder, McMahon, & Nummer, 2011).

After a ripening time of ≥ 8 weeks, the pH of the microcheeses increased from 5.3 to 5.5 and even to pH 6.1 after 1 year of ripening. Such a pH increase in the microcheeses can be explained by proteolysis and by yeast and mould growth, although the latter was not observed in the analyzed cheeses. Between 8 and 52 weeks of ripening, the moisture content of the microcheeses declined, resulting in a lowered water activity. This coincided with inactivation of *L. monocytogenes* in cheese.

The NaCl contents (tested range 0.9–4.1% dry matter) corresponded very well with the NaCl concentrations of large-scale Gouda cheeses of 2.9–3.6% dry matter (van den Berg et al., 2004). In this range, NaCl did not affect the fate of *L. monocytogenes* during ripening. The concentrations of NaCl to which *L. monocytogenes* strains were exposed in cheese were substantially lower than the NaCl concentrations that constitute the growth limits of strains Scott A, 2F and 6E in culture medium; these were 11.7% w/v NaCl (van der Veen et al., 2008). Furthermore, the NaCl content does not influence the water activity to a large extent within the range of 0.9–4.1% NaCl in dry matter (results not shown). Based on these results, the probability of growth of *L. monocytogenes* is not expected to be higher in Gouda microcheeses than in large-scale cheeses in the range of 0.9–4.1% salt dry matter. This outcome is confirmed in a recent study on *L. monocytogenes* in low-salt Cheddar cheese (Shrestha et al. 2011).

The Gouda microcheese model has previously been validated as a model for the Gouda cheese process to systematically screen cheese starter cultures for their flavour profiles over time (Bachmann et al., 2009). This study substantiated the use of the Gouda microcheese as a model system for larger scale 12 kg Gouda cheeses, as the main intrinsic parameters, namely, pH, organic acid concentrations, NaCl concentrations, a_w and moisture content were very comparable during ripening. The microcheese system is also a suitable model system for Gouda cheese challenge tests with pathogens or spoilage organisms in the presence of a starter culture, as temperature, pH, organic acid and water activity profiles in microcheeses were similar to those of factory-scale cheeses. Generated data from the microcheese system can be used for validation of quantitative microbiological risk models on semi-hard cheese made from starter-induced curd formation.

This study has shown that 3 individual *L. monocytogenes* strains with origins from cheese, dairy plant environment and a reference strain do not grow in Gouda cheese and that their numbers decline during extended ripening periods. The main factors that contributed to the inhibition of *L. monocytogenes* are likely to be lactic acid and the decreasing water activity during ripening.

2.5 Conclusions

L. monocytogenes does not grow in Gouda cheese during the first 8 weeks of ripening. Viable numbers of *L. monocytogenes* in Gouda cheese decrease in the ripening period between 8 and 52 weeks. A 0.5 log cfu g^{-1} increase of *L. monocytogenes* during curd formation results from entrapment in the curd and is not due to growth.

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Chapter 3

The fate of *Listeria monocytogenes* in brine and on Gouda cheese following artificial contamination during brining

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ABSTRACT

The fate of 3 different *Listeria monocytogenes* strains (Scott A, 2F and 6E) was studied independently in brine and on factory-scale Gouda cheeses that had been submerged in brine that was artificially contaminated with these individual strains. Viable numbers of *L. monocytogenes* in the brine decreased during brining (0, 1, 2.9 and 8.9 d). *L. monocytogenes* was enumerated on the surface of Gouda cheese directly after brining and over 26 weeks of ripening at 12.5 °C. Transfer of *L. monocytogenes* from brine to cheese during brining was limited. *L. monocytogenes* was detected in the outer layer of Gouda cheese but not in the center directly after brining or during ripening. Throughout the ripening period, the viable numbers of *L. monocytogenes* declined significantly. This study adds to the understanding of the fate of *L. monocytogenes* in brine and on Gouda cheese, and demonstrates that growth of *L. monocytogenes* on Gouda cheese is not supported following contamination during brining.

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3.1 Introduction

Listeria monocytogenes is a food-borne pathogen that is the causative agent of listeriosis. Infection with *L. monocytogenes* can result in meningitis, encephalitis, sepsis and gastroenteritis, with high fatality rates (20–25%), especially in unborn children, infants, the elderly and immune-compromised people (Vázquez-Boland et al., 2001). The bacterium has the ability to grow at refrigeration temperatures and form biofilms, and has a high tolerance to salt and acid (Gandhi & Chikindas, 2007). Food-borne infections of *L. monocytogenes* have been associated with ready-to-eat foods like seafood, vegetables and meat products, as well as dairy products (EFSA/ECDC, 2011; FDA, 2003). Strict guidelines for *L. monocytogenes* have been set for ready-to-eat foods (EFSA/ECDC, 2011; FDA, 2003), a category of foods that includes cheese.

Gouda cheese is a dairy product with an average pH of 5.3, an average water activity of 0.95 and a shelf-life longer than 5 days (Guinee & Fox, 2004; Fox, McSweeney, Cogan, & Guinee, 2004b; WHO, 2004). For products with an average pH higher than 5.0 in combination with an average water activity above 0.94 and a shelf-life more than 5 days, the EU law requires the absence of *L. monocytogenes* after production in 5 samples of 25 g, and concentrations may not exceed 100 cfu g^{-1} in 5 samples during shelf-life (European Commission, 2007). This specification applies unless producers can demonstrate that growth of *L. monocytogenes* is not supported in the food; in that case, concentrations may not exceed 100 cfu g^{-1} in 5 samples during shelf-life.

Using the growth and inactivation definition of Koutsoumanis and Sofos (2005) and Skandamis et al. (2007), an increase of more than 0.5 log cfu g^{-1} is considered to indicate growth, and a decrease in numbers of more than 1.0 log cfu g^{-1} as inactivation.

In published challenge studies, the fate of *L. monocytogenes* in cheese has been assessed in semi-hard cheese made from pasteurised milk after artificial contamination of the milk with *L. monocytogenes* (Northolt et al., 1988; Wemmenhove, Stampelou, van Hooijdonk, Zwietering, & Wells-Bennik, 2013). Growth of *L. monocytogenes* in semi-hard cheeses made of pasteurised milk such as Gouda has not been observed so far, nor have these cheeses been associated with listeriosis. As *L. monocytogenes* is highly resistant to acid and salt, contamination of cheese with this pathogen could potentially also occur during brining. The fate of *Listeria* on cheese when contamination occurs *via* this route has so far not been described in literature.

The aim of the current study was to assess the fate of *L. monocytogenes* on cheese following artificial contamination of the cheese *via* brine. Firstly, the fate of three different *L. monocytogenes* strains in brine was established using brine conditions typical for the

Dutch-type cheese industry. Subsequently, Gouda cheeses were submerged in brine that was artificially contaminated with *L. monocytogenes* and the level of transfer of *L. monocytogenes* to the cheese was assessed. The fate of the pathogen on the cheese was also determined throughout a ripening period of 6 months, at different depths in the cheese from the surface to the center.

3.2 Materials and methods

3.2.1 Bacterial strains

L. monocytogenes strain Scott A (serotype 4b, milk outbreak isolate), strain 2F (1/2a, cheese isolate; see also Table S1 and S3 in van der Veen, Moezelaar, Abee, & Wells-Bennik, 2008) and strain 6E (1/2a, isolate from wall in cheese factory, see also Table S1 and S3 in the supplemental material of van der Veen et al., 2008) were selected from the NIZO culture collection. *L. monocytogenes* strains were individually inoculated into Brain Heart Infusion Broth (BHIB) (Merck, Darmstadt, Germany) of pH 7.0 and incubated at 30 °C for 18 h. Bacterial cells from the individual cultures were subsequently concentrated by centrifugation at 4800 x g for 15 min.

3.2.2 Exposure to brine and enumeration of *L. monocytogenes* from the brine

For adaptation to brine conditions, the cells of *L. monocytogenes* cultures that were individually incubated overnight were sedimented by centrifugation at 4800 x g for 15 min and the cell pellets were resuspended in a mixture of 1 part BHIB and 1 part industrial brine (consisting of 18% NaCl, 0.04% w/v lactic acid, with pH adjusted to 4.4 with HCl) from a Dutch Gouda cheese production plant and left for 3 days at 12 °C. Subsequently, the cells were sedimented again by centrifugation and the pellets were dispersed in brine. The industrial brine that was obtained from the factory, and transported to NIZO for inoculation with *L. monocytogenes,* was monitored regularly in the factory as part of routine analyses (e.g., 75 samples in 2011, with experiments being performed in May 2011). In all 75 samples (including 4 samples in May 2011), *L. monocytogenes* was shown to be absent. In the routine analysis, the brine was analysed as follows: 10 mL brine was enriched in 100 mL of Half Fraser broth (incubation at 30 °C for 24 h) followed by secondary enrichment in Fraser broth (incubation at 37 °C for 48 h) according to ISO 11290, and then a loopful (1 µL) was spread plated on Rapid'Lmono agar plates (BioRad, Marne la Coquette,

France) and on Oxoid Chromogenic *Listeria* Agar agar plates (Oxoid, Landsmeer, The Netherlands). Confirmation of suspected colonies, normally showing as typical blue colonies on Rapid L'mono (Biorad) and blue colonies surrounded by semi-transparent white halos on OCLA (Oxoid), was routinely performed using the Bax® Lmono system PCR Assay (Dupont, Dordrecht, The Netherlands) to detect *L. monocytogenes*. This brine was separated into three brine baths of 200 L and these baths were inoculated with cells of either strain Scott A, 2F or 6E that were diluted and resuspended in the brine (and adapted to brine, see above), to a final concentration of approximately $7 \cdot 10^5$ cfu mL^{-1} (5.9 log cfu mL^{-1}). Each brine solution was stirred continuously (80 rpm) at 12ºC to mimic the brining procedure at industrial level. Viable numbers of *L. monocytogenes* were determined in brine over 8.9 days by taking triplicate samples of 1 mL from each brine solution at t=0 and after 1.0, 2.9 and 8.9 d, and determining viable numbers by plating appropriate dilutions in triplicate on PALCAM-*Listeria* selective agar (VWR, Amsterdam, The Netherlands), followed by enumeration after incubation at 30ºC for 48 h.

3.2.3 Exposure of Gouda cheeses to *L. monocytogenes* from the brine

Eighteen wheel-type Gouda cheeses of 4.5 kg from a commercial production lot were supplied by an industrial producer of Gouda cheese from pasteurised milk. For cheeses in the batch, absence of *L. monocytogenes* in 5 samples of 25 g of cheese was demonstrated by the producer, according to ISO 11290, and the cheese production lot was released for human consumption. The cheeses were obtained immediately after pressing and separated into three groups of six cheeses. Each group was put into a different brine solution that had been artificially contaminated with one of the three strains of *L. monocytogenes*, 3 h after pressing of the cheeses. In each group, two cheeses were brined for 0.33 d, two for 2.1 d and two for 8.9 d (i.e., duplicate samples) to obtain low-salt, normal-salt and high-salt cheeses. Immediately after brining and after ageing at 12.5ºC and sampling, cheeses were coated with commercially available coating solution (CSK food enrichment, Ede, The Netherlands) containing 0.02% natamycin to prevent mould growth during ripening, according to standard cheese-making protocols.

3.2.4 Cheese sampling and enumeration of *L. monocytogenes* from the cheeses

Immediately after brining (t=0) and after 2, 4, 7, 12, 19 and 26 wk of ripening, cheese wheels were placed on the flat side and cheese samples, consisting of blocks of

approximately 1 x 1 x 1 cm (length x width x height), were taken approximately 5 cm from the center of the cheese (cheese diameter of 22 cm) using a sterile cheese scoop. Subsequently, each cheese sample was analysed at different distances from the crust: 0–1 cm (duplicate samples), 2–3 cm (duplicate samples) and at 4–5 cm (center). Considering these determinations to be independent measurements, the viable counts were analysed in four replicates for the 0–1 cm sample (crust) as well as for the 2–3 cm sample. For enumeration of *L. monocytogenes*, cheese samples of 1 g were obtained from the different sections, and diluted 10-fold using 9 mL of 2% sodium citrate of 40 °C. Each cheese diluent was divided into two parts of 5 g and put in large sterile petri dishes (VWR, Amsterdam, The Netherlands) with a diameter of 145 mm. Subsequently, 50 mL of PALCAM-selective agar was added to each petri dish to obtain two pour plates with PALCAM-selective agar and the cheese suspension. Viable numbers were determined after 48 h incubation at 30ºC by pour-plating using PALCAM-selective agar.

3.2.5 Validation of plating method

The method of pour-plating, using PALCAM *Listeria*-selective agar, of brine and cheese samples containing *L. monocytogenes* was validated by comparing the results of this enumeration method with results of the plating method described in ISO 11290. Appropriate dilutions were pour-plated using PALCAM-selective agar, and spread-plated onto PALCAM agar, Rapid L'mono agar (Biorad, Marne la Coquette, France) and OCLA agar (Oxoid, Landsmeer, The Netherlands). For this evaluation, an overnight culture of *L. monocytogenes* grown in BHIB was used; furthermore, cells of *L. monocytogenes* were used that were pre-exposed to a 50/50 volume ratio of brine and BHIB for 3 days at 12 °C. All three individual cultures of the *L. monocytogenes* strains that were incubated overnight in BHIB were diluted approximately one thousand fold in BHIB to ~5.6 log cfu mL^{-1} prior to plating. The viable counts of the diluted cultures were subsequently determined by pour-plating using PALCAM-selective agar and by spread plating onto PALCAM agar, Rapid L'mono agar or OCLA agar. In addition, the viable counts were determined using these plating methods and media for cultures of all three *L. monocytogenes* strains that were pre-exposed for 3 days to a mixture of brine and BHIB using a 50/50 volume ratio, having a concentration of ~ 5.6 log cfu mL^{-1} prior to plating. After incubation of the plates at 37 °C for 48 h, the viable counts were recorded and 5 suspected colonies were obtained from each plate. Each of those colonies was enriched in 100 mL Half Fraser broth, subsequently enriched in Fraser broth, and then a loopful (1 µL) was spread plated on Rapid'Lmono and OCLA agar. Blue colonies on Rapid'Lmono and blue colonies surrounded by semi-transparent white halos on OCLA plates were suspected to be *L. monocytogenes*, and these were further confirmed as *L. monocytogenes* according to ISO 11290 using the

Bax® Lmono PCR Assay (Dupont, Dordrecht, The Netherlands). The logarithmic average and 95% standard deviation were taken to minimise the influence of outliers in viable counts, assuming that the measurement error is normally distributed after log transformation.

3.2.6 Determination of the salt content, moisture content, water activity and pH of cheese

The salt content of cheese was determined according to NEN 3762 (NEN, 1977), and the moisture content of cheese was determined using method NEN 3755 (NEN, 1998). The pH was measured by inserting a 3 mm electrode (BioTrode, Metrohm, Herisau, Switzerland) directly into the cheese. The dew point method (Decagon Aqualab, Pullman, WA, USA) was used to determine the water activity of the cheese.

3.3 Results and discussion

Viable counts of *L. monocytogenes* that were determined using PALCAM pour plates did not significantly differ ($p>0.05$) from counts on spread plates using either PALCAM-selective agar, Rapid L'mono agar or OCLA agar. This was the case for cells of *L. monocytogenes* that were cultured overnight and then plated. This implies that the recovery of *L. monocytogenes* did not differ for the different plating media. For the cells of *L. monocytogenes* that were pre-exposed to brine and BHIB at a 50/50 ratio, no significant differences ($p>0.05$) were observed between the different media for strain 2F, but for strains Scott A and 6E, the viable counts were slightly higher ($p=0.02$) in PALCAM-pour plates than on spread plates of PALCAM, Rapid L'mono and OCLA (Table 3.1). As the recovery from the different media was very comparable for cells that were pre-exposed to brine and BHIB at a 50/50 ratio, it was concluded that pre-exposure to brine did not affect the sensitivity of *L. monocytogenes* to different plating media. Furthermore, counts of *L. monocytogenes* in and on cheese were determined with pour-plating.

Brining for 0.33, 2.1 and 8.9 d resulted in low-salt, normal-salt and high-salt cheeses, respectively, with salt in dry matter contents (mean ± 95% standard deviation in %) varying between the surface and the center of the cheese from 1.4%±0.05 to 2.4%±0.01 in cheeses brined for 0.33 d (with 1.9% on average), salt in dry matter contents varying from 3.0%±0.23 to 5.0%±0.04 in cheeses brined for 2.1 d (with 4.0% on average) and salt in dry matter contents varying from 5.1%±0.49 to 8.6%±0.13 in cheeses brined for 8.9 d (6.9% on average).

No typical colonies were found in the brine before inoculation with *L. monocytogenes*. After inoculation, inactivation during brining was observed for the three strains tested. The extent of inactivation of *L. monocytogenes* in brine significantly differed between the tested strains; strain Scott A (serotype 4b) showed a decrease of 2.8 log cfu g^{-1}, corresponding to a decimal reduction of 3.2 days, while strains 2F and 6E (serotype 1/2a) each only showed a 0.7 log decrease, corresponding to a decimal reduction of 14.4 and 14.9 days, respectively (Fig. 3.1). The serotype 1/2a strains had previously been shown to be relatively acid-resistant (van der Veen et al., 2008). *L. monocytogenes* strains with serotype 1/2a are more often isolated from cheese (or cheese equipment) than serotype 4b (Farber & Peterkin, 1991). A previous study reported that serotype 4b isolates generally show a higher resistance to NaCl at 30 °C, but that serotype 1/2 is more resistant to NaCl at 7 °C (van der Veen et al., 2008). Natural selection and/or adaptation to a high-salt and acidic environment could be an explanation for the higher resistance of strains with serotype 1/2a to high salt and acid conditions. Lack of proliferation but rather inactivation of *L. monocytogenes* in brine was likely the result of the high salt and acidic conditions. The highest concentrations of NaCl at which growth of *L. monocytogenes* have been observed are 10–12% (Lou & Yousef, 2000; Swaminathan, Cabanes, Zhang, & Cossart, 2007). The absence of growth, but survival and inactivation of *L. monocytogenes* in brine is consistent with previous studies (Durmaz, Aygun, & Ardic, 2009; Larson, Johnson, & Nelson, 1999). In a recent study from Schirmer, Heir, Lindstedt, Møretrø & Langsrud (2014), *L. monocytogenes* was inactivated in brine of pH 4.5– 6.0 that contained 14.1–26.9% NaCl. This inactivation was faster in brine of low pH (4.5) and at a low NaCl concentration (15%) than at higher pH values and NaCl concentrations. This observation suggests that lowering the NaCl concentration of brine from 18% to 15% whilst maintaining a low pH (4.5) would not cause a risk to food safety.

In this study, the sample sizes were 1 g, to allow for sampling of each cheese at seven time points (0, 2, 4, 7, 12, 19 and 26 weeks) in duplicate. Variation in the observed inactivation of *L. monocytogenes* strains in cheese could be partly due to the sample size. The probability of detection was increased in this work by artificially contaminating the brine with a high concentration of *L. monocytogenes* and by stirring the brine solutions during cheese salting to obtain an equal distribution of *Listeria* on the cheese surface. In practice, concentrations of *L. monocytogenes* in brine that would be as high as the concentrations studied in this work would be very unlikely, even when the brine is strongly contaminated.

Table 3.1 Viable counts of *L. monocytogenes* cultures (overnight and brine-treated)[a] obtained with the pour plating method using PALCAM-selective agar and with the spread plating methods for enumeration of *L. monocytogenes* in cheese, as used in ISO 11290

L. monocytogenes culture	Overnight culture (log cfu mL^{-1})				Brine-treated culture (log cfu mL^{-1})			
	PALCAM pour plate	PALCAM spread plate	Rapid L'mono-spread plate	OCLA-spread plate	PALCAM pour plate	PALCAM spread plate	Rapid L'mono spread plate	OCLA-spread plate
Scott A	5.7±0.11	6.0±0.25	5.7±0.08	5.7±0.08	6.0±0.09	5.4±0.07	5.4±0.17	5.5±0.01
2F	5.7±0.02	5.6±0.01	5.7±0.12	5.6±0.15	5.8±0.47	5.5±0.31	5.6±0.03	5.5±0.01
6E	5.7±0.01	5.7±0.09	5.7±0.06	5.7±0.09	5.6±0.05	5.3±0.05	5.4±0.01	5.5±0.03

Viable counts (log cfu mL^{-1}, ±95% standard error of the mean (n = 3)) of a prediluted overnight culture of *L. monocytogenes* in BHIB and of a culture that was pre-exposed to a mixture of brine (consisting of 18% NaCl, 0.04% w/v lactic acid, with pH 4.4 adjusted with HCl) and BHIB using a 50/50 volume ratio for 3 days at 12 °C prior to plating.

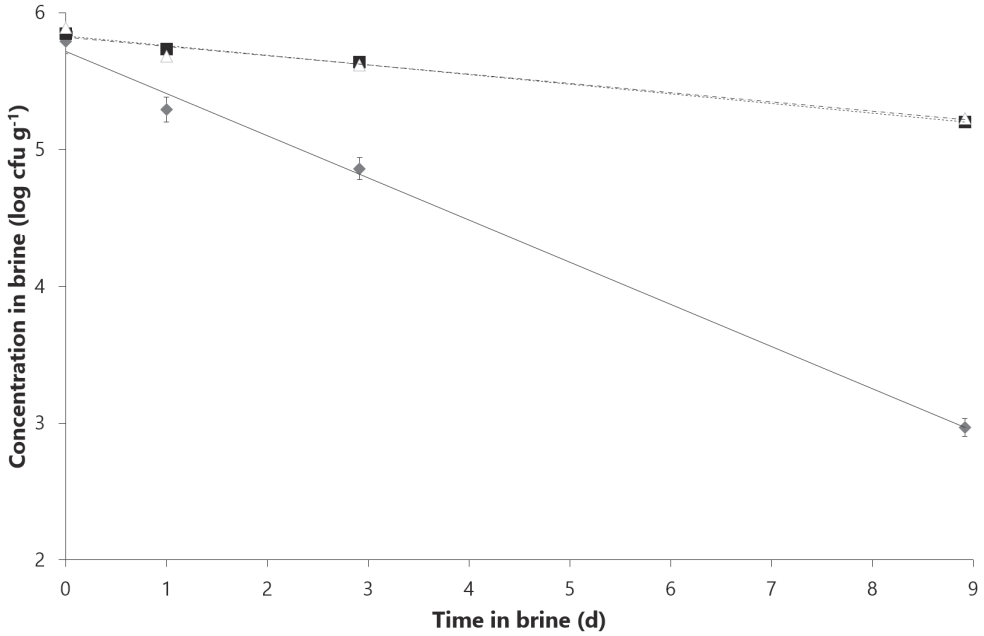

Fig. 3.1 Levels of *L. monocytogenes* strains Scott A (◆), 2F (■) and 6E (△) after artificial contamination of brine with individual strains. Viable numbers (logarithmic mean ± 95% standard error of the mean; n = 3) are expressed in log cfu g^{-1}, based on a density of brine of 1.12 g mL^{-1}. The calculated decimal reduction times and corresponding r^2 values are 3.2 days and 0.995 for Scott A, 14.4 days and 0.995 for 2F, and 14.9 days and 0.954 for 6E, respectively (in certain cases, the standard deviation was so small that the error bar is not visible).

The transfer of *L. monocytogenes* from brine to cheese was assessed by comparing the concentrations of the bacteria on the crust of Gouda cheese with the initial concentration in the brine. A weight loss of 2% occurred during brining, as 5% w/v of moisture was expelled from the cheese and 3% w/v salt migrated into the cheese during brining, which is typical for Gouda cheese (Fox, McSweeney, Cogan, & Guinee, 2004b). For all three *L. monocytogenes* strains, the viable counts in cfu per g in the outer layer of the cheeses (0–1 cm) immediately after removal from the brining solutions at t = 0 weeks were 100 times lower than the concentrations present in brine immediately after immersion of the cheeses into the brine solutions.

The levels of *L. monocytogenes* strain Scott A (mean ± 95% standard deviation in log cfu g^{-1}) were 5.8±0.09 log cfu g^{-1} in the brine and 3.4±0.5, 2.2±0.2 or 2.4±0.5 log cfu g^{-1} in the outer layer of cheeses that were brined for 0.33, 2.1 or 8.9 days, respectively; the levels of strain 2F were 5.8±0.07 log cfu g^{-1} in the brine and immediately after brining these levels were 3.1±0.5, 2.5±0.3 or 2.9±0.7 log cfu g^{-1} in the outer layer of cheeses that were brined for 0.33, 2.1 and 8.9 days, respectively; and the levels of strain 6E were 5.9±0.05 log cfu g^{-1} in the brine and immediately after brining these levels were 3.0±0.8, 2.7±0.8 or 3.8±0.7 log cfu g^{-1} in the outer layer of cheeses that were brined for 0.33, 2.1 and 8.9 days,

respectively (Fig. 3.2–3.4). *L. monocytogenes* was not found at an average concentration above 1.0 log cfu g^{-1} in the deeper layers throughout the whole study, i.e., from the time immediately after brining up to 26 weeks of ripening. Therefore, the standard deviation was only displayed for the 0–1 cm cheese samples. Although *L. monocytogenes* can be motile through its ability to express flagella at temperatures below 37 °C, this capacity is greatly reduced at lower temperatures (Farber & Peterkin, 1991; Peel, Donachie, & Shaw, 1988). In this study, no evidence was obtained that *L. monocytogenes* can spread throughout Gouda cheese during ripening. The pH did not significantly ($p>0.05$) differ between cheeses that were brined for 0.33, 2.1 or 8.9 days. The pH of the cheeses increased from 5.05±0.10 directly after brining to 5.40±0.03 after 19 weeks, and decreased to 5.25±0.03 after 26 weeks (Fig. 3.5). The pH was lower in the center than in the outer layers until 19 weeks, but after 26 weeks the pH values were equal in the different parts of the cheese. Although the acid and salt conditions are much more favourable for *L. monocytogenes* in cheese than in brine, no growth and only inactivation (≥ 1.0 log cfu g^{-1} decrease in viable counts) was observed for all three strains of *L. monocytogenes* in the crust of all cheeses tested. Compared with the initial count on the crust, a significant decrease ($p<0.05$) of at least 2.0 log cfu g^{-1} was observed after 19 weeks in all cheeses for all three strains of *L. monocytogenes*.

Inactivation of *L. monocytogenes* in Gouda cheese during ripening occurred faster in this brine study than in previous studies in which *L. monocytogenes* was dispersed throughout the cheese following artificial contamination of cheese milk. In those studies, *L. monocytogenes* survived throughout the study period of 13 and 6 weeks (Bachmann & Spahr, 1995; Northolt et al., 1988) and was inactivated after 16–28 weeks (Wemmenhove et al., 2013). The faster inactivation of *L. monocytogenes* in cheese during ripening in the latter study was likely due to the decreasing water activity in cheese over time and the presence of undissociated lactic acid. The water activity in Gouda cheese decreases during ripening as a result of salt diffusion and evaporation of water. In coated cheeses, the water activity can drop to values near or below the minimal water activity limit of 0.92 that is required for growth of *L. monocytogenes* (ICMSF, 1996), resulting in inactivation. The water activity in the outer 0–1 cm layer immediately after brining was 0.95, 0.93 and 0.90 for the cheeses that were subjected to the three different brining times, due to salt diffusion from the brine into the cheese and moisture loss from the cheese to the brine, while the water activity in the center of the cheese was 0.99. The low water activity in the outer layer of the cheese in this study probably contributed to faster inactivation of *L. monocytogenes* compared with inactivation observed in previous challenge studies of Gouda with *L. monocytogenes* during curd formation (Wemmenhove et al., 2013). In line with this, the fastest inactivation of *L. monocytogenes* was observed in the cheese with the highest salt content (Fig. 3.2C, 3.3C, 3.4C), as the water activity in this cheese was as low as 0.90.

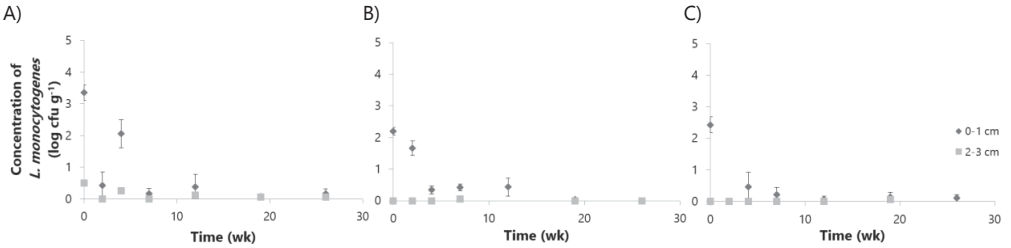

Fig. 3.2 Levels of *L. monocytogenes* Scott A in Gouda cheese containing (A) 1.9% NaCl; (B) 4.0% NaCl; and (C) 6.9% NaCl on dry matter basis after ripening for 0, 2, 4, 7, 12, 19 and 26 weeks at 12.5 °C. The logarithmic mean ± 95% standard error of the mean (n = 4) are expressed in log cfu g^{-1}. Diamonds indicate 0–1 cm (crust sample) and squares 2–3 cm (layer between 2 and 3 cm from the crust). In samples from the center of the cheese (4–5 cm samples), *L. monocytogenes* was not detected.

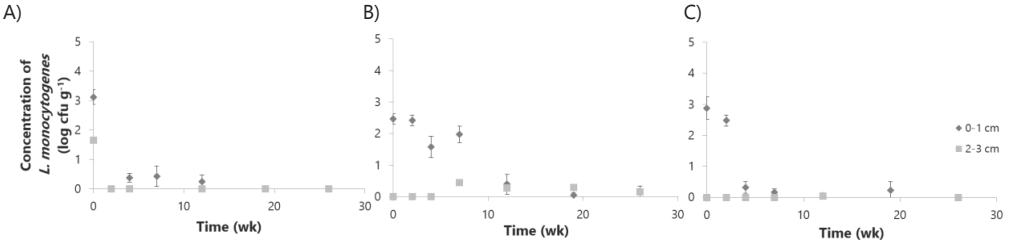

Fig. 3.3 Levels of *L. monocytogenes* 2F in Gouda cheese containing (A) 1.9% NaCl; (B) 4.0% NaCl; and (C) 6.9% NaCl on dry matter basis after ripening for 0, 2, 4, 7, 12, 19 and 26 weeks at 12.5 °C. The logarithmic mean ± 95% standard error of the mean (n = 4) are expressed in log cfu g^{-1}. Diamonds indicate 0–1 cm (crust sample) and squares 2–3 cm (layer between 2 and 3 cm from the crust). In samples from the center of the cheese (4–5 cm samples), *L. monocytogenes* was not detected.

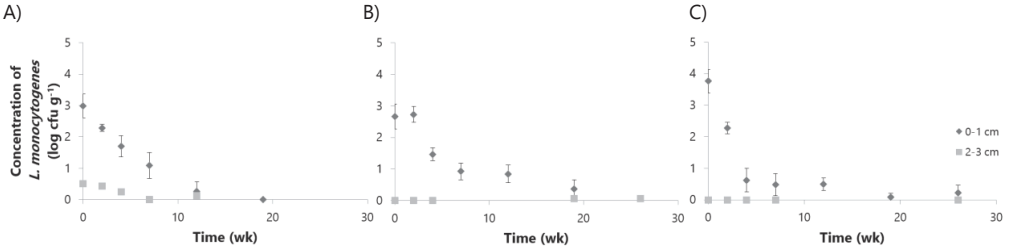

Fig. 3.4 Levels of *L. monocytogenes* 6E in Gouda cheese containing (A) 1.9% NaCl; (B) 4.0% NaCl; and (C) 6.9% NaCl on dry matter basis after ripening for 0, 2, 4, 7, 12, 19 and 26 weeks at 12.5 °C. The logarithmic mean ± 95% standard error of the mean (n = 4) are expressed in log cfu g^{-1}. Diamonds indicate 0–1 cm (crust sample) and squares 2–3 cm (layer between 2 and 3 cm from the crust). In samples from the center of the cheese (4-5 cm samples), *L. monocytogenes* was not detected.

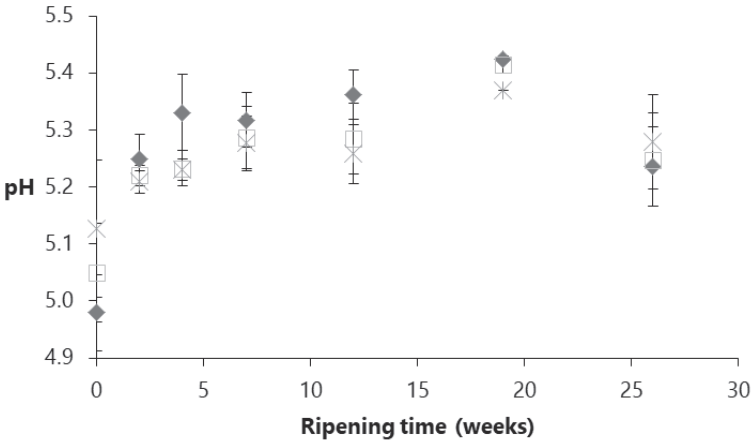

Fig. 3.5 The pH of Gouda cheese during ripening for 0, 2, 4, 7, 12, 19 and 26 weeks at 12.5 °C. The pH values represent mean ± 95% standard deviation (duplicate measurement) of cheese samples containing 1.9%, 4.0% and 6.9% NaCl on average on a dry matter basis, giving n = 6 per time point and location in the cheese, as no significant differences in pH were observed in cheeses of different salt concentrations. Diamonds indicate the pH in the 0–1 cm (crust) sample, squares in the 2–3 cm sample (layer between 2 and 3 cm from the crust) and crosses indicate the pH in the 4–5 cm samples (centre).

3.4 Conclusion

Based on the findings in this research, namely that (i) a 0.1–2.8 log decrease of *L. monocytogenes* occurred during brining; (ii) concentrations on the cheese surface were 100 times lower than in brine; and (iii) a significant decrease in *L. monocytogenes* occurred in cheese after ripening times of 2–12 weeks, it was concluded that the probability that a brine contamination will result in detectable levels (per 25 g) of *L. monocytogenes* in Gouda cheese during shelf-life is negligible. Moreover, *L. monocytogenes* that contaminated the surface of the Gouda cheese during brining declined in numbers during ripening.

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Chapter 4

How NaCl and water content determine water activity during ripening of Gouda cheese, and the predicted effect on inhibition of *Listeria monocytogenes*

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ABSTRACT

This study describes the diffusion of NaCl and water in Gouda cheese during brining and ripening. Furthermore, we established water activity as a function of the NaCl-in-moisture content in Gouda cheese during ripening. We determined NaCl content, water content, and water activity in block-type Gouda cheeses that were brined for 3.8 d and foil-ripened for a period of 26 wk, and in wheel-type Gouda cheeses that were brined for 0.33, 2.1, or 8.9 d and subsequently nature-ripened for a period of 26 wk. The calculated diffusion coefficients of NaCl during brining were $3.6 \cdot 10^{-10}$ $m^2 s^{-1}$ in the block-type Gouda cheeses and $3.5 \cdot 10^{-10}$ $m^2 s^{-1}$ in the wheel-type Gouda cheeses. Immediately after brining, gradients of NaCl and water were observed throughout both types of cheese. During ripening, these gradients disappeared, except for the water gradient in nature-ripened cheeses. An empirical model was derived for Gouda cheese, in which the water activity is expressed as a function of the NaCl-in-moisture content, as established for different brining times, locations and ripening times. Moreover, the effect of a reduced water activity on inhibition of growth of *L. monocytogenes* in Gouda cheese was calculated. In addition to the presence of lactate and a pH of 5.2-5.3, the reduced water activity as seen in Gouda cheese can substantially contribute to inhibition of microbial growth, and even to inactivation when cheeses are brined and ripened for extended times and subjected to nature-ripening.

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4.1 Introduction

Gouda cheese is a ready-to-eat (RTE) product that is made from pasteurized cow milk. Many factors contribute to the microbial safety and quality of Gouda cheese. This includes pasteurization of raw milk (72 °C for 15 s), hygienic processing, the use of acidifying starter cultures during curd formation, and salting or brining after pressing of the curd. The intrinsic properties of cheese, such as the presence of organic acids and a low pH, but also reduced water activity due to the presence of NaCl, are important factors to control the outgrowth of undesirable microorganisms (Koutsoumanis & Sofos, 2005). Gouda cheese has a typical pH value of 5.2 to 5.3, due to the activity of the starter lactic acid bacteria (including *Lactococcus lactis* and *Leuconostocs mesenteroides*) that convert lactose to lactic acid. Salt is introduced by immersing Gouda cheeses in brine (that typically has a pH of 4.4 and contains 18-19% wt/vol sodium chloride) for 2 to 4 d at 12 to 13 °C. The brining time is chosen based on the desired final sodium chloride (NaCl) content and on the geometry and mass of the cheese. The average water activity of Gouda is 0.95, but this value depends on the exact position within the cheese (e.g, close to the rind or the core), and it changes during brining and ripening due to salt and water migration (Guinee & Fox, 2004). Ripening conditions of Gouda cheese can vary. Nature-ripened Gouda cheeses (usually wheel-type cheeses) are turned and coated regularly during ripening and are stored on wooden shelves at 12 to 13 °C for a period of 4 wk up to more than 1 yr. Foil-ripened Gouda cheeses (block-type cheeses) are wrapped in plastic and ripened for 4 to 8 wk at ~5 °C, and these cheeses are not coated and turned.

An important microbiological hazard for dairy foods such as cheese is the bacterium *Listeria monocytogenes* (Greig & Ravel, 2009), which is the causative agent of listeriosis (Vazquez-Boland et al., 2001), a disease that can have a case-fatality rate as high as 20 to 30% (Swaminathan & Gerner-Smidt, 2007). The organism can grow at low temperatures and is highly tolerant to salt and acid (Lou & Yousef, 2000). Microbiological criteria have been set in EU regulation EC 2073/2005 (European Commission, 2005) for *L. monocytogenes* in RTE foods such as Gouda cheese. Based on these criteria, Gouda belongs to the category of RTE foods not intended for infants and medical purposes that can potentially support growth of *L. monocytogenes*, as the shelf life is >5 d, the average pH >5.0 and the average water activity >0.94.

Since the recognition of *L. monocytogenes* as the causative agent in the 1980s (Schlech et al., 1983), listeriosis has mainly been associated with consumption of Mexican-style cheeses (Silk et al., 2012), with soft cheeses made from pasteurized milk (Koch et al., 2010; Gaulin, Ramsay, & Bekal, 2012; Gould, Mungai, & Behravesh, 2014) and Mimolette cheese (Yde et al., 2012), but not with consumption of Dutch-type cheeses such as Gouda and Maasdam or with Emmental. The latter cheeses do not support growth of

L. monocytogenes in challenge studies with the pathogen (Northolt et al., 1988; Buazzi, Johnson, & Marth, 1992; Bachmann & Spahr, 1995; Wemmenhove et al., 2013, 2014). Undissociated lactic acid present in Gouda cheese plays an important role in inhibition of growth of this pathogen: we recently demonstrated that the highest concentration required to inhibit all growth of 6 different *L. monocytogenes* strains is 9.0 mM, and that this is around the same level as the average concentration of undissociated lactic acid that is typically present in Gouda cheese, that is 9.2 mM at pH 5.3 based on 13.9 g of lactic acid kg^{-1} cheese (Wemmenhove et al., 2016).

In addition to the effect of undissociated lactic acid, the major salt in Gouda cheese, NaCl, may further contribute to inhibition of growth of *L. monocytogenes* due to its effect on water activity of the cheese. At low water activities, less water is freely available for bacteria, which can lead to inhibition of growth. Water activity (a_w) is a physicochemical parameter equivalent to the vapor pressure of water in a system (p) divided by the vapor pressure of pure water (p_0):

$$a_w = \frac{p}{p_0} \quad \text{(Eq. 4.1).}$$

The effect of water activity on inhibition of growth of food-borne pathogens has been modeled together with other factors in secondary models based on the Gamma concept (Zwietering, Wijtzes, Rombouts, & van 't Riet, 1993) that is widely used. According to this model, the inhibiting effect of water activity is expressed in the gamma factor for growth (γ_{a_w}), which can be calculated as follows if $a_w > a_{w_{min}}$:

$$\gamma_{a_w} = \frac{a_w - a_{w_{min}}}{1 - a_{w_{min}}} \quad \text{(Eq. 4.2),}$$

in which a_w is the water activity that is actually present in the cheese, and in which $a_{w_{min}}$ is the minimal water activity at which growth of a bacterium can occur; $a_{w_{min}}$ is 0.92 for *L. monocytogenes* according to ICMSF (1996). If the water activity is >0.92, it can still contribute to inhibition of growth of *L. monocytogenes* and growth can be fully inhibited when other factors are also present (e.g. pH, organic acids), according to the Gamma concept (Zwietering et al., 1993).

As indicated above, NaCl is added to Gouda cheese during brining and is the major salt that affects the water activity of this product. It migrates into the cheese during brining and ripening, and water migrates from the cheese to the brine. Resnik & Chirife (1988) predicted the theoretical water activity based on measurements in saturated NaCl solutions at a temperature ranging from 15 to 50 °C:

$$a_w = e^{\left(\frac{\varphi \cdot v_i \cdot m_i}{-55.51}\right)} \quad \text{(Eq. 4.3),}$$

in which φ is the ionic strength and v_i is the number of particles into which each solute of molality m_i dissociates.

Several empirical models are available for other types of cheese in which the water activity depends on the solutes (e.g. NaCl) and water content of the cheese. In those models the NaCl content is expressed in NaCl-in-moisture (%, wt/wt). An empirical model has been established for fresh cheeses with a water-in-cheese content ≥ 40% by Marcos, Alcalá, León, Fernández-Salguero, & Esteban (1981) and Marcos Millán, Esteban, Alcalá, & Fernández-Salguero (1983):

$$a_w = 1.00 - 0.00565 \cdot [NaCl]_{H_2O} \quad \text{(Eq. 4.4),}$$

in which $[NaCl]_{H2O}$ as NaCl-in-moisture (%, wt/wt).

In addition, Saurel, Pajonk, & Andrieu (2004) proposed a model for Emmental cheese based on measurements of water activity and NaCl-in-moisture content during brining (a_w varying from 0.80 to 0.99 and $[NaCl]_{H_2O}$ varying from an NaCl-in-moisture of 0 to 30%, wt/wt):

$$a_w = 0.997 - 0.00604 \cdot [NaCl]_{H_2O} \quad \text{(Eq. 4.5).}$$

So far, the effect of the NaCl content and the water content on the water activity has not been investigated for Dutch-type cheeses. In the current study, we assessed the water activity of Gouda cheese based on migration of NaCl and water that occurs in the cheese during brining and ripening. An empirical model is presented and compared with the previous empirical models developed for other types of cheeses. In addition, we predict the impact of water activity on inhibition of growth of *L. monocytogenes*. The results presented in this study are based on cheeses that were brined for 3.8 d and then foil-ripened, and on cheeses that were brined for different times and subsequently nature-ripened. The goals of this study were (1) to establish the diffusion of NaCl and water in Gouda cheese during brining and ripening, (2) to establish the water activity as a function of the NaCl content and the water content, and (3) to predict the effect of water activity on growth inhibition of microbial pathogens such as *L. monocytogenes* in Gouda cheese.

4.2 Materials and methods

4.2.1 Manufacturing of Gouda cheese, brining and ripening procedure

Two block-type Gouda cheeses (weight 15.7 kg, 0.30 x 0.50 x 0.11 m, 48% fat in DM) from one production lot, and 6 wheel-type Gouda cheeses (weight 4.5 kg, height 0.097 m, diameter 0.245 m, 48% fat in DM) from one production lot were supplied by an industrial producer of pasteurized-milk Gouda cheeses in The Netherlands. Pressed fresh cheeses were transported in molds (protected with aseptic plastic bags) from the production location to NIZO food research and within 3 h after pressing (as is common practice in Dutch-type Gouda cheese production), all cheeses were immersed in brine (consisting of 18.4% wt/vol NaCl, with pH adjusted to 4.4 with HCl) that was slowly but continuously agitated. The block-type cheeses were brined for 3.8 d. Brining was performed at 12 °C. Of the 6 wheel-type cheeses, 2 cheeses (i.e. duplicate samples) were brined for 0.33 d, 2 cheeses for 2.1 d, and 2 cheeses for 8.9 d, resulting in cheeses defined as low-salt, normal-salt and high-salt cheeses, respectively. The 2 cheeses brined for 2.1 d and the 2 block-type cheeses that were brined for 3.8 d had the same NaCl-in-cheese content after 4 wk of ripening (both normal-salt cheeses).

After brining, the cheeses were removed in pairs and of each cheese, samples were taken independently before ripening started (for details see Cheese sampling section). Subsequently, the block-type cheeses were vacuumed using thermoforming PA/PE foil with a thickness of 100 µm (Hevel, Zaandam, The Netherlands) and ripened at NIZO food research at 5 °C and 62.5% humidity, as normally applied in Dutch cheese-making factories for this type of cheese. The wheel-type cheeses were coated with commercially available coating solution (CSK food enrichment, Ede, The Netherlands) containing 0.02% natamycin as commonly applied for nature-ripened Gouda cheese to prevent growth of mold during ripening. The wheel-type cheeses were ripened at 12.5 °C and 85% relative humidity at NIZO Food Research.

4.2.2 Cheese sampling

Two different cheeses were analyzed per brining condition and each of these cheeses was sampled immediately after coating of the cheeses (t = 0) and after 2, 4, 7, 12, 19 and 26 wk of ripening. Cheese samples (slices of 25-30 g) were taken at approximately 0.005, 0.025 and 0.045 m from the rind of the cheese. From each slice of sampled cheese, the

coating was removed and the slice was dissected to obtain 2 samples 0-0.01 m from the rind (cheese rind or the 0.005-m sample), 2 samples 0.02-0.03 m from the rind (0.025-m sample) and 1 sample 0.04-0.05 m from the rind (cheese core or the 0.045-m sample), yielding 5 samples per cheese. Each cheese sample was grated and mixed to obtain a homogeneous sample. After grating, the samples were immediately stored in 180-mL plastic sample containers with screw caps (VWR, Amsterdam, The Netherlands) at room temperature and analyzed within 2 h after storage to prevent water evaporation during sample preparation.

4.2.3 Cheese analysis

Each cheese sample of 25-30 g was grated, and the NaCl-in-cheese content, water-in-cheese content and the water activity were analyzed. The NaCl-in-cheese content was determined on the basis of the chloride content by a potentiometric titration of the chloride ions with silver nitrate according to NEN 3762 (NEN, 1977). The NaCl-in-moisture (%, wt/wt) was calculated from the NaCl-in-cheese content by multiplying the NaCl-in-cheese content (%, wt/wt) by $\frac{100\%}{water-in-cheese\ content,\%\ (wt/wt)}$. The water-in-cheese content (%, wt/wt) was analyzed by determining the mass of the grated cheese sample before and after heating at 160 °C in an oven (Kern MLS, Balingen, Germany) for 30 min according to NEN 3755 (NEN, 1998). The water activity was determined with an Aqualab Series 3 water activity measuring device (Decagon, Washington, USA). All analyses were performed in duplicate, and the average value of each determination was reported.

4.2.4 Diffusion coefficient of NaCl during brining in Gouda cheese

The diffusion of NaCl-in-moisture (%, wt/wt) was studied during brining of block-type and wheel-type Gouda cheeses. The displacement of NaCl in the vertical direction was assumed to be unaffected by the lateral displacements for both the block-type cheeses and the wheel-type cheeses, as the width was much greater than the height for both cheeses. Assuming no influence of lateral displacement, the diffusion coefficient, D^*, was established according to the error function (erf) for surface convection based on the diffusion law of Fick, as described by Geurts (1972):

$$\frac{C' - c}{C' - C_0} = \mathrm{erf}\left(\frac{x}{2\sqrt{(D^* t)}}\right) \quad \text{(Eq. 4.6),}$$

in which C' = concentration of NaCl at the rind in NaCl-in-moisture (%, wt/wt), c = concentration of NaCl-in-moisture (%, wt/wt) in the cheese sample, C_0 = concentration of NaCl-in-moisture (%, wt/wt) in the cheese before brining, x = distance (m) from the rind in the direction to which NaCl has diffused, t = brining time (s) and D^* = diffusion coefficient of NaCl-in-moisture (in m^2s^{-1}).

The diffusion coefficient D^* of NaCl during brining of Gouda cheese was calculated for each sample according to Equation [4.6]. In the initial unbrined cheeses, chloride ions were already present at a level of 0.39 g per 100 g cheese moisture (C_0 = 0.39 %, wt/wt) due to addition of calcium chloride ($CaCl_2$) to the cheese milk. The bulk concentration of NaCl in brine (C') and the average diffusion coefficient (D^*) were estimated using the Solver function in Excel (based on the minimal residual sum of squares between the measured and fitted values for c determined per cheese sample) from Microsoft Office (Microsoft Corp., Redmond, WA).

4.2.5 Deriving an empirical model for the water activity of Gouda cheese

An empirical model for the water activity as a function of the NaCl-in-moisture content of Gouda cheese was derived using linear regression (based on the highest R^2 value and an intercept of 0.995, representing the water activity of Gouda before brining). This model was based on analysis of 70 samples from the block-type cheeses (7 ripening times x 2 cheeses x 5 samples per cheese) and 210 samples from the wheel-type cheeses (7 ripening times x 2 cheeses x 3 brining times x 5 samples per cheese). The NaCl content was expressed as NaCl-in-moisture (%, wt/wt) to incorporate the changes in the water-in-cheese content of the cheese throughout ripening.

4.2.6 Calculating the effect of water activity on growth inhibition of *L. monocytogenes*

The effect of water activity on inhibition of growth of *L. monocytogenes* was calculated using the previously established gamma factor for water activity γ_{a_w} as described in Equation [4.2]; γ_{a_w} was calculated at each location at which the NaCl content and water content were established in the cheese (0.005, 0.025 or 0.045 m from the rind) and plotted against time for all types of cheeses (block-type cheeses that were brined for 3.8 d and

wheel-type cheeses that were brined for 0.33, 2.1, or 8.9 d). The lower the value of γ_{a_w}, the greater the effect of water activity on growth inhibition of *L. monocytogenes* in that cheese at that time. When, at a given time, the γ_{a_w} was equal to or lower than zero, water activity was predicted to fully inhibit growth of *L. monocytogenes*.

4.3 Results and discussion

4.3.1 Diffusion coefficient of NaCl in Gouda

In this study, the diffusion of NaCl was determined in Dutch-type Gouda cheeses which were subjected to different brining times (Fig. 4.1). The D^* value of NaCl was determined during brining of Gouda cheeses produced in a Dutch cheese-making factory (The Netherlands) with, on average, a pH of 5.1 and water content of 47% before brining. The average D^* value determined in this study was $3.6 \cdot 10^{-10}$ m^2s^{-1} in the 15.7 kg block-type Gouda cheeses (Fig. 4.1A) and $3.5 \cdot 10^{-10}$ m^2s^{-1} in the 4.5 kg wheel-type Gouda cheeses (Fig. 4.1B). The diffusion coefficients for NaCl as determined in the block-type and the wheel-type Gouda cheeses are in the range of those reported for Emmental, Cheddar, Feta and Camembert cheeses; namely, 1.0 to $5.3 \cdot 10^{-10}$ m^2s^{-1} (Floury, Jeanson, Ally, & Lortal, 2010). The diffusion coefficient of NaCl in Gouda cheese was higher than that reported by Geurts (1972), who calculated a D^* value of 1.9 to $2.2 \cdot 10^{-10}$ m^2s^{-1} in Gouda cheese. The higher coefficient found in Gouda in this study than in Geurts (1972) might be explained by differences in processing conditions. Geurts (1972) did not add $CaCl_2$ to the cheeses, but left the freshly pressed cheeses for 2.5 d before brining, to allow for further acidification and rind formation. The matrix and rind of the Gouda cheese of Geurts (1972) may have been more rigid and less permeable, which could explain the lower diffusion of NaCl in those cheeses. The higher D^* value found in the current study compared with Geurts (1972) may also be explained by the higher water-in-cheese content of cheeses before brining in this study than in Geurts (1972). When the water-in-cheese content is increased, the protein-in-cheese fraction will be decreased, leading to a lower rigidity of the cheese and a higher diffusion of NaCl. In the study of Geurts (1972), the water-in-cheese content before brining was not reported, but the water-in-cheese content after 8.1 d of brining varied from 31% in the rind to 43% in the core. The water-in-cheese contents observed in this study were higher (38% in the rind and 46% in the core after 8.9 d of brining). The initial water-in-cheese content of the cheeses of Geurts (1972) therefore appears to be ~4% lower, likely explaining the reported lower D^* values.

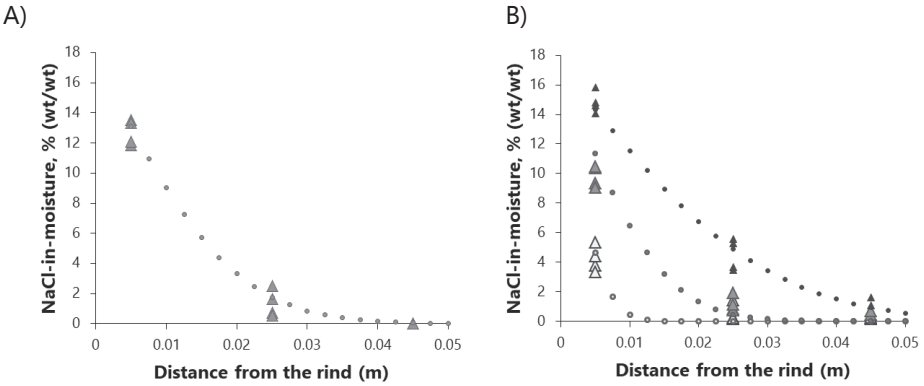

Fig. 4.1 NaCl-in-moisture (%, wt/wt) of Gouda cheeses immediately after brining. Samples were taken at distances of 0.005, 0.025 and 0.045 m from the rind of (A) 2 block-type cheeses of 15.7 kg that were brined for 3.8 d at 12 °C, and at 0.005, 0.025 and 0.045 m from the rind of (B) 6 wheel-type cheeses of 4.5 kg that were brined for 0.33 d (2 cheeses, white triangles), for 2.1 d (2 cheeses, grey triangles), and for 8.9 d (2 cheeses, black triangles) at 12 °C. Contents of NaCl for each distance are presented individually. The fitted NaCl contents (plotted as dotted lines) are based on concentration of NaCl-in-moisture (%, wt/wt) in the cheese before brining (C_0) = 0.39 % (wt/wt), diffusion coefficient (D^*) = $3.6 \cdot 10^{-10}$ $m^2 s^{-1}$, and concentration of NaCl at the rind (C') = 17.7 % (wt/wt) for the block-type cheeses and D^* = $3.5 \cdot 10^{-10}$ $m^2 s^{-1}$ and C' = 17.2 % (wt/wt) for the wheel-type cheeses, as calculated using Equation [4.6], with the NaCl concentrations in the cheeses as predicted before and after brining, brining times and distances from the rind as input parameters.

4.3.2 Profiles of NaCl and water during ripening

The gradient in the NaCl-in-moisture (%, wt/wt) disappeared in all cheeses during ripening (Fig. 4.2A and 4.2B). Immediately after brining, the NaCl-in-moisture content was ~9 times higher in the rind than in the core for the wheel-type cheeses that were brined for 0.33, 2.1 or 8.9 d. Not surprisingly, the highest concentration of NaCl-in-moisture content was measured in the rind of the cheeses with the longest brining time (8.9 d; Fig. 4.2B). Immediately after brining, the NaCl-in-moisture content was 5.1 % (wt/wt), on average, in the block-type Gouda cheeses that were brined for 3.8 d (Fig. 4.2A). In wheel-type nature-ripened Gouda cheeses that were brined for 0.33, 2.1 and 8.9 d, the average NaCl-in-moisture contents were 2.0, 4.2 and 7.2 % (wt/wt), respectively. The average NaCl-in-moisture content of the wheel-type nature-ripened cheeses increased to 4.8, 9.6 and 19.0 % (wt/wt) after ripening for 26 wk (Fig. 4.2B). NaCl migrated from the outer to the inner layers of the cheese during ripening. According to Guinee & Fox (2004), it can take 9 wk for the NaCl gradient to disappear within large Gouda cheese blocks. In the 15.7-kg block-type cheeses that were wrapped in foil, the NaCl gradient disappeared between 4 and 7 wk of ripening, resulting in an average NaCl-in-moisture content throughout the cheese of 5.3 % (wt/wt; Fig. 4.2A). After a similar time the NaCl-in-moisture gradient also disappeared in the wheel-type cheeses (Fig. 4.2B).

The average water-in-cheese content in the block-type cheeses that underwent foil-ripening was constant (Fig. 4.3A) but decreased in the wheel-type cheeses that were nature-ripened (Fig. 4.3B). The gradient in water-in-cheese content between rind and core had already disappeared in the block-type cheeses after 2 wk of ripening (Fig. 4.3A), but persisted in the nature-ripened cheeses (due to evaporation, Fig. 4.3B). The average water-in-cheese content was lower in the cheese that was brined for 8.9 d than in the cheese that was brined for 0.33 d, with a difference of 3.5% directly after brining and 2.2% after 26 wk of ripening. Overall, an increase in the content of NaCl-in-moisture was observed in the wheel-type cheeses during ripening (Fig. 4.2B) due to an inwards migration of NaCl and an outwards migration of water.

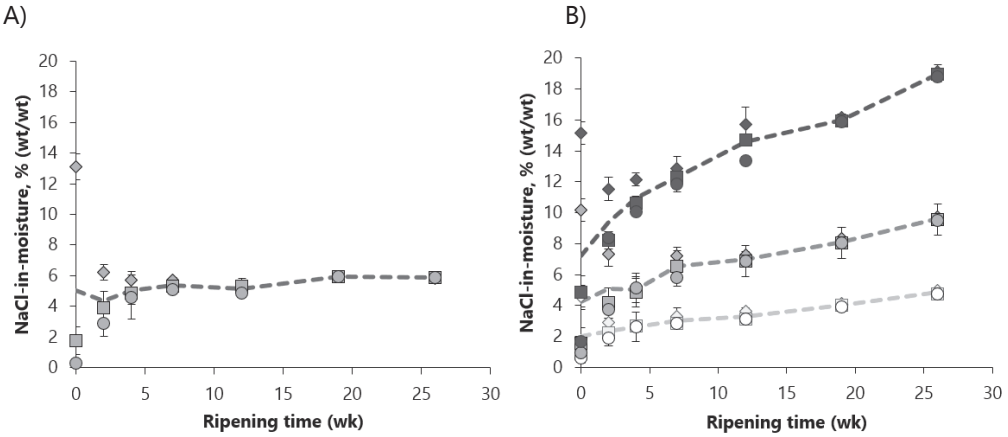

Fig. 4.2 Sodium chloride (NaCl)-in-moisture (%, wt/wt) of Gouda cheese at different time points during ripening, showing the NaCl content (mean ± SD) in samples taken at different distances from the rind at each time point: 0.005 m from the rind (diamonds, n = 4), 0.025 m from the rind (squares, n = 4), and 0.045 m from the rind (circles, n = 2). In addition, the average NaCl-in-moisture (%, wt/wt) is plotted (dashed lines) for each brining time in (A) block-type foil-ripened cheese of 15.7 kg brined for 3.8 d; and (B) wheel-type nature-ripened cheese of 4.5 kg brined for 0.33 d (open white symbols), 2.1 d (grey symbols), or 8.9 d (black symbols).

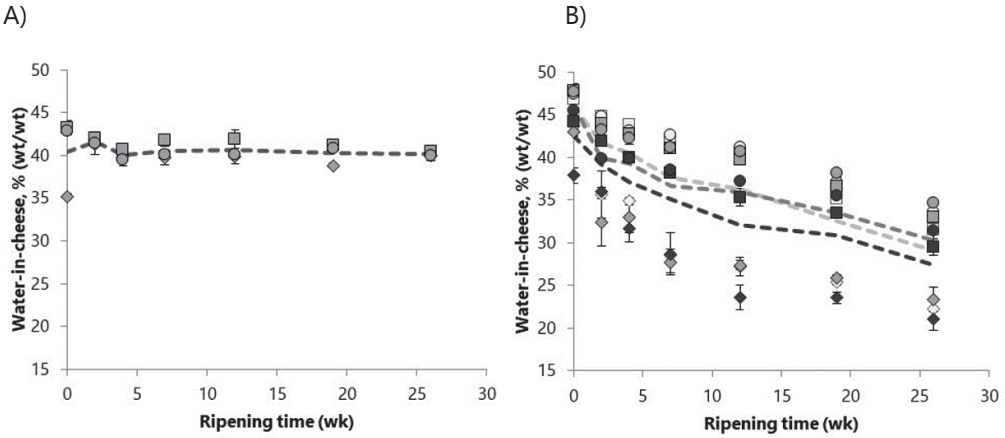

Fig. 4.3 Water-in-cheese (%, wt/wt) of Gouda cheese at different time points during ripening, showing the water-in-cheese content (mean ± SD) in samples taken at different distances from the rind at each time point: 0.005 m from the rind (diamonds; n = 4), 0.025 m from the rind (squares; n = 4), and 0.045 m from the rind (circles; n = 2). In addition, the mean water-in-cheese content (%, wt/wt) is plotted (dashed lines) for each brining time in (A) block-type foil-ripened cheese of 15.7 kg brined for 3.8 d; and (B) wheel-type nature-ripened cheese of 4.5 kg brined for 0.33 d (open white symbols), 2.1 d (grey symbols), or 8.9 d (black symbols).

4.3.3 Water activity during ripening

Fig. 4.4 displays the water activity during ripening of Gouda cheese. In the block-type cheeses that were brined for 3.8 d and subsequently foil-ripened, the water activity in the rind immediately after brining was as low as 0.91, but it rapidly increased to a value of 0.96 during ripening of the cheese (Fig. 4.4A). Subsequently, the water activity in the foil-ripened cheeses was stable during maturation because the foil prevented evaporation of water. In the nature-ripened cheeses, a decline in the average water activity was observed during ripening, concomitant with a reduction of the water-in-cheese content. Immediately after brining for 0.33, 2.1 and 8.9 d, the water activity in the rind was 0.96, 0.93 and 0.90, respectively, in the nature-ripened cheeses. The water activity in the core of these cheeses was 0.99. The average water activity decreased from 0.98 to 0.93 in the cheeses that were brined for 0.33 d, from 0.97 to 0.91 in the cheeses that were brined for 2.1 d, and from 0.95 to 0.85 in the cheeses that were brined for 8.9 d between 0 and 26 wk of ripening. The differences in water activity values observed after brining between the cheeses that were brined for 0.33, 2.1 and 8.9 d persisted during maturation (Fig. 4.4B).

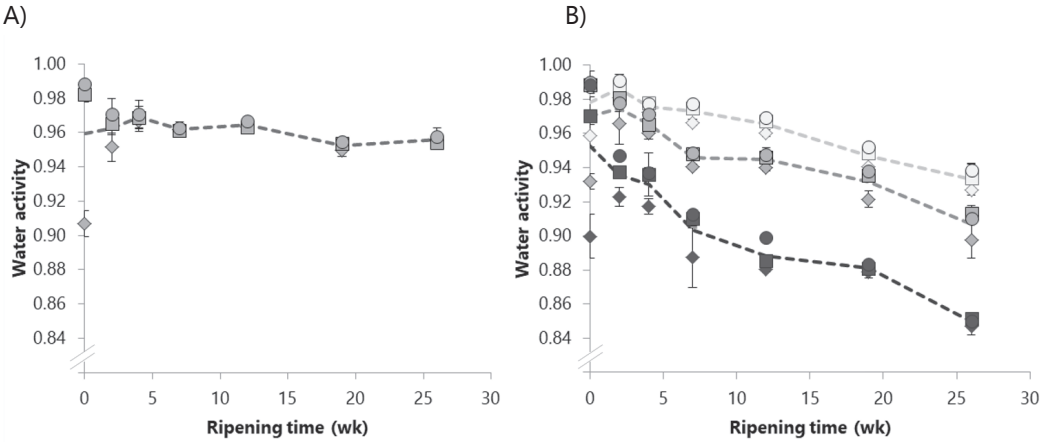

Fig. 4.4 Water activity (a_w) of Gouda cheese at different time points during ripening, showing the water activity (mean ± SD) in samples taken at different distances from the rind at each time point: 0.005 m from the rind (diamonds; n = 4), 0.025 m from the rind (squares; n = 4), and 0.045 m from the rind (circles; n = 2). In addition, the average water activity is plotted (dashed lines) for each brining time in (A) block-type foil-ripened cheese of 15.7 kg brined for 3.8 d; and (B) wheel-type nature-ripened cheese of 4.5 kg brined for 0.33 d (open white symbols), 2.1 d (grey symbols), or 8.9 d (black symbols).

4.3.4 Empirical model for the water activity of Gouda cheese

An empirical model (R^2 = 0.89, regression coefficient -0.00721 ± -0.0003) for the water activity of Gouda cheese as a function of the NaCl-in-moisture content was derived (based on the data presented in Fig. 4.5):

$$a_w = 0.995 - 0.00721 \cdot NaCl_{H_2O} \qquad \text{(Eq. 4.7).}$$

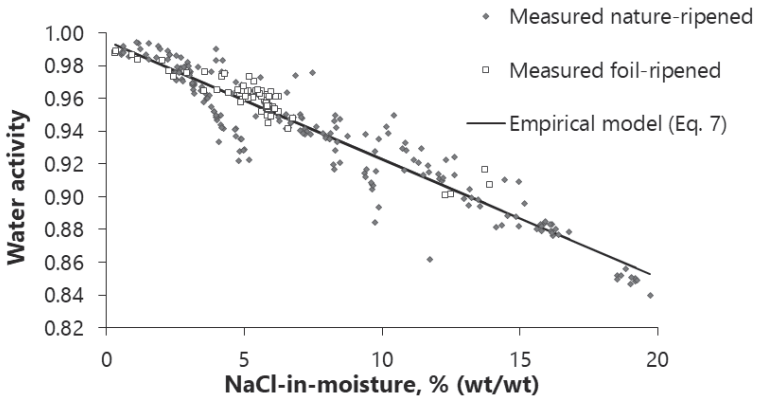

Fig. 4.5 Empirical model (R^2 = 0.89) for the water activity (a_w) of Gouda cheese as a function of the NaCl-in-moisture content ($NaCl_{H_2O}$) in %, wt/wt: $a_w = 0.995 - 0.00721 \cdot NaCl_{H_2O}$ (with -0.00721 ± -0.0003).

Freshly pressed but unsalted cheese already contains some solutes, rendering a water activity of 0.995 (water activity of pure water is 1.000). Therefore, the latter value was used as intercept in the empirical model. The model presented here is independent of time and is valid during brining and ripening. It incorporates both the NaCl content and the water content, which are the main factors that change during brining and ripening of Gouda cheese. The model presented here is similar to the empirical model for water activity of Emmental cheese during brining, as derived by Saurel et al. (2004) in Equation [4.5]. That model likely resembles the empirical model for Gouda, because part of the $a_w/NaCl_{H_2O}$ intervals of Gouda were similar to that of Emmental cheese (the a_w and $NaCl_{H2O}$ of aged Gouda cheese seem similar to those of fresh Emmental). The R^2 (0.89) and the regression coefficient (-0.00721) in this empirical model are lower than those in the model of Saurel et al. (2004; R^2 of 0.98 and a regression coefficient of -0.00604), but that model was based only on samples taken during brining, whereas it does not include samples during ripening. The regression coefficient of -0.00721 that we established (see Equation [4.7]) was smaller than that determined by Marcos et al. (1981, 1983). This implies that the effect of the $NaCl_{H_2O}$ factor on the water activity is greater than expected based on the data of the latter reports. Moreover, our empirical model has a broader scope as it also includes cheeses with a water-in-cheese content <40% and a water activity <0.96.

4.3.5 Implications of reduced brining time and foil-ripening for *L. monocytogenes*

Immediately after brining, the water activities were 0.93 and 0.90 in the outer layer of the cheeses that were brined for 2.1 and 8.9 d, respectively. These values are close to or lower than the a_w growth limit for *L. monocytogenes*. Such low water activities in the outer layer of the cheese can likely hinder outgrowth of *L. monocytogenes* in Gouda cheese following a contamination after curd formation (e.g. by the brine bath or conveyors). Such conditions may even lead to inactivation of the pathogen, as was observed by Wemmenhove et al. (2014).

Food producers that use foil-ripening or that shorten their brining times (e.g., to produce low-salt cheeses) must be aware that a lower salt content and a higher water content can have a negative impact on inhibition of bacterial growth. A lower salt content or a higher water content results in a reduced water activity hurdle for microbial pathogens. According to the Gamma model described in Equation [4.2], a shortened brining time will lead to a higher a_w and a smaller effect on inhibition of growth. The water activity was 0.96 in the outer layer of the cheese immediately after brining when cheeses were brined for only 0.33 d. In addition to water activity, other microbial hurdles (e.g., pH and the presence of

organic acid) are present in cheese that can contribute to full inhibition of growth of *L. monocytogenes* or other pathogens, even at higher water activity values, such as occurring in foil-ripened cheeses and in cheeses with shortened brining times. Fig. 4.6 displays the effect of water activity (γ_{a_w}) on growth inhibition of *L. monocytogenes* during ripening, as predicted from the Gamma model. The effect of water activity on growth inhibition of *L. monocytogenes* in foil-ripened cheese seems stable during ripening (Fig. 4.6A), as the average water activity during ripening is stable within this type of cheese (Fig. 4.4A). Full growth inhibition of *L. monocytogenes* as a result of low water activity can be expected after 26 wk of ripening in Gouda cheese that was brined for 2.1 d, as in the nature-ripened cheeses, the a_w decreases to values below the critical a_w limit for growth of *L. monocytogenes* (Fig. 4.6C).

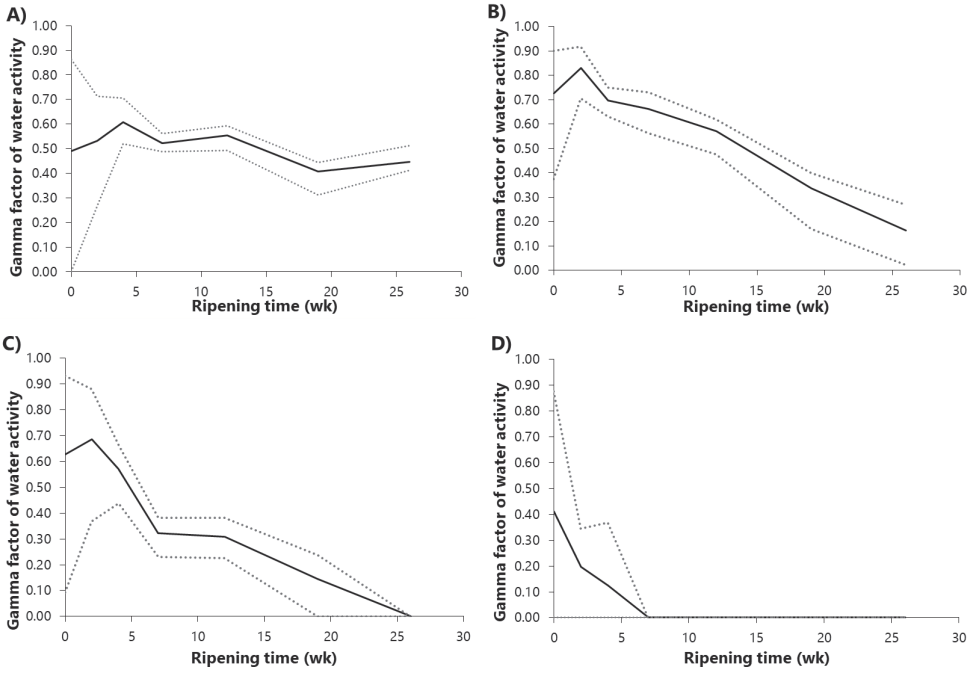

Fig. 4.6 The effect of water activity (γ_{a_w}) on growth inhibition of *L. monocytogenes* during ripening as predicted from the Gamma model, with (A) foil-ripened block-type Gouda cheeses that were brined for 3.8 d at 12 °C and with nature-ripened wheel-type Gouda cheeses that were brined for (B) 0.33 d, C) 2.1 d or D) 8.9 d at 12 °C. Dotted lines present the minimum and maximum gamma factors; straight lines are the average gamma factors of water activity within Gouda cheese at the indicated ripening time.

We recently demonstrated absence of growth of *L. monocytogenes* in and on Gouda cheese (Wemmenhove et al. 2013, 2014), and furthermore showed that the concentrations of undissociated lactate that are present in Gouda cheese contribute significantly to the inhibition of growth of this pathogen (Wemmenhove et al., 2016). The current study shows

that low water activity can also contribute significantly to inhibition of growth of this bacterium in cheese. Conditions of low water activity may completely suppress growth of *L. monocytogenes*. In fact, inactivation of *L. monocytogenes* in model Gouda cheeses was seen after 28 wk of ripening when a_w decreased below 0.92; that is, the minimum a_w required for growth of *L. monocytogenes* (Wemmenhove et al., 2013). These findings are in line with the study of Koutsoumanis & Sofos (2005), who showed that growth of *L. monocytogenes* was inhibited at an a_w lower than 0.93 in combination with a pH lower than 5.0 in tryptic soy broth.

Overall, our a_w study has delivered an empirical model for water activity as a function of the NaCl and water content and underlines the importance of the a_w as a factor that can lead to inhibition of growth of *L. monocytogenes* in Gouda cheese.

Acknowledgements

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Chapter 5

Minimal inhibitory concentrations of undissociated lactic, acetic, citric and propionic acid for *Listeria monocytogenes* under conditions relevant to cheese

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ABSTRACT

Minimal inhibitory concentrations (MICs) of undissociated lactic acid were determined for six different *Listeria monocytogenes* strains at 30 °C and in a pH range of 4.2–5.8. Small increments in pH and acid concentrations were used to accurately establish the growth/no growth limits of *L. monocytogenes* for these acids. The MICs of undissociated lactic acid in the pH range of 5.2–5.8 were generally higher than at pH 4.6 for the different *L. monocytogenes* strains. The average MIC of undissociated lactic acid was 5.0 (SD 1.5) mM in the pH range 5.2–5.6, which is relevant to Gouda cheese. Significant differences in MICs of undissociated lactic acid were found between strains of *L. monocytogenes* at a given pH, with a maximum observed level of 9.0 mM. Variations in MICs were mostly due to strain variation. In the pH range 5.2–5.6, the MICs of undissociated lactic acid were not significantly different at 12 °C and 30 °C. The average MICs of undissociated acetic acid, citric acid, and propionic acid were 19.0 (SD 6.5) mM, 3.8 (SD 0.9) mM, and 11.0 (SD 6.3) mM, respectively, for the six *L. monocytogenes* strains tested in the pH range 5.2–5.6. Variations in MICs of these organic acids for *L. monocytogenes* were also mostly due to strain variation. The generated data contribute to improved predictions of growth/no growth of *L. monocytogenes* in cheese and other foods containing these organic acids.

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5.1 Introduction

Listeria monocytogenes is a Gram-positive foodborne pathogen (Vazquez-Boland et al., 2001). Growth of this bacterium can be inhibited effectively with short-chain organic acids in their undissociated form; these can pass through the bacterial cell membrane. The acid subsequently dissociates in the cytoplasm, resulting in an increased hydrogen ion concentration in the cell. To restore the intracellular pH, the hydrogen ions are pumped out, but this disturbance of the proton motive force is an energetically unfavorable process for bacteria (Mitchell, 1961).

The concentration of undissociated acid in watery solutions is determined by the total concentration of the acid, the pH, and the dissociation constant pK_a. Minimal inhibitory concentrations (MICs) of the undissociated forms of lactic acid, acetic acid, citric acid and propionic acid for *L. monocytogenes* have been reported in various studies (Ahamad & Marth, 1989; Aryani, den Besten, Hazeleger, & Zwietering, 2015; Chen & Shelef, 1992; Conner, Scott, & Bernard, 1990; Coroller et al., 2005; Houtsma, de Wit, & Rombouts, 1993; van der Veen, Moezelaar, Abee, & Wells-Bennik, 2008; Vasseur, Baverel, Hébraud, & Labadie, 1999; Young & Foegeding, 1993). However, the data on MICs of undissociated acids for *L. monocytogenes* available in literature are limited, varying from 2 to 46 data points per acid. In a number of studies available in literature, relatively large intervals between undissociated acid concentrations were used, resulting in quite rough estimations of the actual lowest concentrations that inhibit growth (Ahamad & Marth, 1989; Conner et al., 1990; Coroller et al., 2005; van der Veen et al., 2008). Furthermore, a large variability in MIC values between different strains of *L. monocytogenes* has been observed in previous studies (Coroller et al., 2005; van der Veen et al., 2008).

To allow for prediction of growth/no growth of *L. monocytogenes* in foods that contain short-chain organic acids, more comprehensive datasets with MICs of these acids (using small increments in total acid concentrations and pH values) are needed for a variety of *L. monocytogenes* strains. For instance, in the case of Dutch-type cheeses like Gouda, Edam and Maasdam, made from pasteurized milk with rennet-induced curd formation, it is known that a low pH and the presence of short-chain organic acids are important factors contributing to inhibition of growth of *L. monocytogenes* (Millet, Saubusse, Didienne, Tessier, & Montel, 2006).

The aim of this study was to establish accurate minimum inhibitory concentrations of undissociated lactic acid, acetic acid, citric acid and propionic acid for six different strains of *L. monocytogenes*. The inhibitory effects of the undissociated acids were established in a range of pH values from 4.2 to 5.8, thus including conditions that are relevant to Dutch-type cheeses. In addition, the effects of different salts that are present in cheese were

tested in the presence of lactate. MICs of the different acids for *L. monocytogenes* were determined at 30 °C, reflecting normal processing temperatures for cheese-making, and in addition, MICs of lactate were established at 12 °C, reflecting cheese ripening temperatures.

5.2 Materials and methods

5.2.1 *Listeria monocytogenes* strain selection and cultivation

Six strains of *L. monocytogenes* were selected from the NIZO culture collection (containing 138 *L. monocytogenes* strains) based on the origin of the strains, the serotype and the resistance to lactic acid, which were previously established (van der Veen et al., 2008). The following strains were selected, based on reported MICs of undissociated lactic acid in van der Veen et al. (2008): *L. monocytogenes* strain 1F (1/2a, cheese isolate, reported MIC 7.5 mM), 2F (1/2a, cheese isolate, reported MIC 4.0 mM), 6E (1/2a, isolate from wall in cheese factory, reported MIC 7.5 mM), EGDe (1/2a, rabbit isolate, reported MIC 2.5 mM), L4 (1/2b, milk isolate, reported MIC 7.5 mM) and Scott A (4b, outbreak isolate linked with milk, reported MIC 2.5 mM); see also Tables S1 and S3 in van der Veen et al. (2008). The strains were cultured individually in Brain Heart Infusion (BHI, Merck, Darmstadt, Germany) for ~19 h and the initial OD of the individual strains was normalized by varying the culturing time at 30 °C before exposure to acid.

5.2.2 Generation of datasets to determine MICs of organic acids for *L. monocytogenes*

Four datasets were generated to determine the MICs of organic acids in BHI (Supplementary Table S1).

Dataset 1 was generated to establish the MICs of undissociated lactic acid for six different *L. monocytogenes* strains (1F, 2F, 6E, EGDe, L4, Scott A), using final total concentrations of 0.009, 0.013, 0.020, 0.030, 0.040, 0.046, 0.059, 0.071, 0.088, 0.11, 0.13, 0.18, 0.21, 0.32, 0.50, or 0.79 M, at nine different pH values (ranging from 4.2 to 5.8 with intervals of 0.2), and one temperature in two independent experiments with three replicates per experiment. Incubation was performed at 30 °C up to one month, reflecting normal processing temperatures during cheese-making.

As Dutch-type cheese contains sodium chloride and calcium chloride, dataset 2 was generated to determine the effect of addition of salt on the MIC of lactate for *L. monocytogenes*. The MIC of lactate was determined for the same concentration interval of lactate and for the same six *L. monocytogenes* strains as in dataset 1, but using pH values ranging from 5.2 to 5.6 with intervals of 0.2, and in the absence or in the presence of salts (0.20 M sodium chloride, 0.36 M calcium chloride and 0.20 M sodium chloride + 0.36 M calcium chloride in dataset 2).

Dataset 3 was generated to establish the MICs of undissociated acetic acid, citric acid and propionic acid for *L. monocytogenes*. The same six *L. monocytogenes* strains were used as in dataset 1, using pH values ranging from 5.2 to 5.6 with intervals of 0.2, and the following final total acid concentrations: acetic acid, 0.006, 0.014, 0.029, 0.032, 0.043, 0.057, 0.071, 0.081, 0.086, 0.16, 0.24, 0.32, 0.40, or 0.49 M; citric acid, 0.014, 0.14, 0.35, 0.71, 2.13 M; propionic acid 0.0077, 0.015, 0.031, 0.062, 0.093, 0.12, 0.15, 0.19 M.

Dataset 4 was generated to investigate whether lowering the temperature to that of cheese ripening would influence the MIC of organic acids. In dataset 4, the MICs of lactate were determined as in dataset 1, but generated in one single experiment with three replicates and incubated at 12 °C (cheese ripening temperature) up to three months. The MICs of lactate of dataset 4 were generated simultaneously with the first duplicate determination of the MICs of lactate of dataset 1, incubated at 30 °C up to one month. The simultaneous assessment at 12 °C and 30 °C allowed for a comparison of the MICs at those temperatures.

5.2.3 Determination of the MICs of undissociated organic acids for *L. monocytogenes*

Culture medium with different pH values and concentration ranges of organic acids were prepared as follows. Equimolar stock solutions of an organic acid and of its potassium salt (both dissolved in water) were made at 2.1 times the final target concentrations (as listed above in paragraph 5.2.2) and mixed to obtain the desired pH (i.e. 4.2 to 5.8 with intervals of 0.2 for datasets 1 and 4, and 5.2 to 5.6 with intervals of 0.2 for dataset 2 and 3). The 2.1-fold concentrated organic acid solutions (with different organic acid concentrations and pH values) were filter-sterilized using Nalgene 0.2 µm filters (Sigma Aldrich, Seele, Germany) and 95 µL aliquots were added to wells of 96-well plates containing 95 µL of two-fold concentrated filter sterilized BHI that was adjusted with hydrochloric acid (Sigma Aldrich) to the same pH as the organic acid solution that was added. The final pH was recorded prior to incubation. Finally, 10 µL of a bacterial inoculum in BHI was added. The bacterial inoculum was obtained by culturing six strains of *L. monocytogenes* individually

overnight at 30 °C to an OD_{630} of approximately 1.0 (~$3.6 \cdot 10^9$ cfu mL^{-1}). Cells from 100 µL of the culture were concentrated by centrifugation (15 min, 5000x g), and the cell pellet was resuspended in 10 mL BHI of which the pH was adjusted to match the pH used in the experiments to determine the MICs. The volume of 10 µL of the bacterial inoculum was suspended in the 96-well plates (in wells with corresponding pH values) to obtain final concentrations of individual *L. monocytogenes* strains of ~$1.8 \cdot 10^6$ cfu mL^{-1} as determined by plate counts on BHI agar (Sigma Aldrich, Seele, Germany) in 200 µL final volumes. Incubation was performed at 30 °C and growth of *L. monocytogenes* was monitored in triplicate in two independent experiments by measurements of the optical density at 630 nm (OD_{630}) using an EL808 IU-PC spectrophotometer (Bio-Tek, Winooski, Vermont, USA) throughout a 1-month incubation period. Growth was defined as an increase in OD_{630} greater than 0.05 compared with the negative control. To confirm the growth/no growth limits that were determined based on optical density readings, counts in the wells with an OD_{630} increase less than 0.05 were determined by plating on BHI agar and compared with the initial inoculum level.

MICs of undissociated organic acid for *L. monocytogenes* were calculated based on equation [5.1], which was derived from the Henderson-Hasselbalch equation.

$$[Undissociated\ acid] = \frac{[Total\ acid]}{1 + 10^{pH - pK_a}} \quad \text{(Eq. 5.1).}$$

The molarities of the total acid solutions were pre-set, and the pH values used in these calculations were determined prior to incubation. The equilibrium constants K for ionization of the undissociated form of the different acids were established previously (Dawson, Elliott, Elliott, & Jones, 1986), i.e. pK_a 3.86 for lactic acid, 4.76 for acetic acid, 3.13 for citric acid and 4.86 for propionic acid.

5.2.4 Literature search

Previously reported MICs of undissociated lactic acid, acetic acid, citric acid and propionic acid were obtained from literature (first 200 hits in Scopus, sorted on relevance). The following keywords were used in the search: (*L. monocytogenes* AND MIC AND [undissociated acid OR organic acid OR lactic acid OR acetic acid OR citric acid OR propionic acid OR lactate OR acetate OR citrate OR propionate]). Furthermore, a literature search was performed using Web of Science and Google Scholar.

5.2.5 Statistical analysis

A general linear model ANOVA (between subjects design, pairwise comparison, Product Tukey test at 95%) was constructed in Minitab (Minitab Inc., Pine Hall, Pennsylvania, USA) to determine if the MIC was determined by the fixed factors of study or by random variation. Datasets 1, 2 and 3 consisted of data from two independent experiments. For those datasets, 'experiment' and 'replicates' were defined as random factors. Fixed factors were 'pH' and 'strain' in dataset 1, 'pH', 'strain' and 'type of salt' in dataset 2, and 'pH', 'strain' and 'type of organic acid' in dataset 3. In dataset 4, the MICs of undissociated lactic acid were determined at pH values ranging from 4.2 to 5.8 at 12 °C and compared with the MICs at 30 °C of the first duplicate determination of dataset 1. 'Replicates' were treated as random factor and 'pH', 'strain' and 'temperature' were treated as fixed factors in the ANOVA analysis of dataset 4. Residual plots and boxplots were constructed to check for normality and outliers. Post-hoc ANOVA tests (Product Tukey test at 95%) were performed using Fizz software (Biosystèmes, Couternon, France) to determine the factors significantly affecting the MIC.

5.3 Results and discussion

In this study, the MIC values of undissociated lactic acid, acetic acid, citric acid and propionic acid were established for the six *L. monocytogenes* strains at pH 4.2–5.8. No growth was observed at pH values 4.2 or 4.4 for any of the strains, independent of the absence or presence of organic acids (Fig. 5.1). This is in line with the lowest pH growth limit reported by ICMSF (1996), which is pH 4.4 for *L. monocytogenes*.

The MICs of undissociated lactic acid for *L. monocytogenes* showed a pH dependency, with significantly lower values at pH 4.6 than at pH 5.2, 5.6 and 5.8 for strains 1F, EGDe, L4 and Scott A and significantly higher values at pH 5.8 than at pH 4.6–5.6 for strains 2F and 6E (Fig. 5.1 and Supplementary Table S2). Between strains, significant differences in MICs of undissociated organic acid at a given pH were seen in all four datasets generated (Supplementary Table S1). More specifically, the MICs of undissociated lactic acid showed significant differences between strains of *L. monocytogenes* at pH 4.8, 5.0, 5.2, 5.4 and 5.6, but not at pH 4.6 and pH 5.8 (Supplementary Table S3).

Relatively high standard deviations were observed for MICs at low pH values (Fig. 5.1, Supplementary Tables S2 and S3). This likely results from a heterogeneous response to severe acid stress of cells within a population, with only certain cells able to overcome this stress and grow (this phenomenon was recently reviewed by Abee, Koomen, Metselaar,

Zwietering, & den Besten, 2016). In addition, some high standard deviations were found for MICs at pH 5.6 in the presence of high concentrations of undissociated acids; here, the variation is likely due to increasing intervals between the concentrations at the top end of the range.

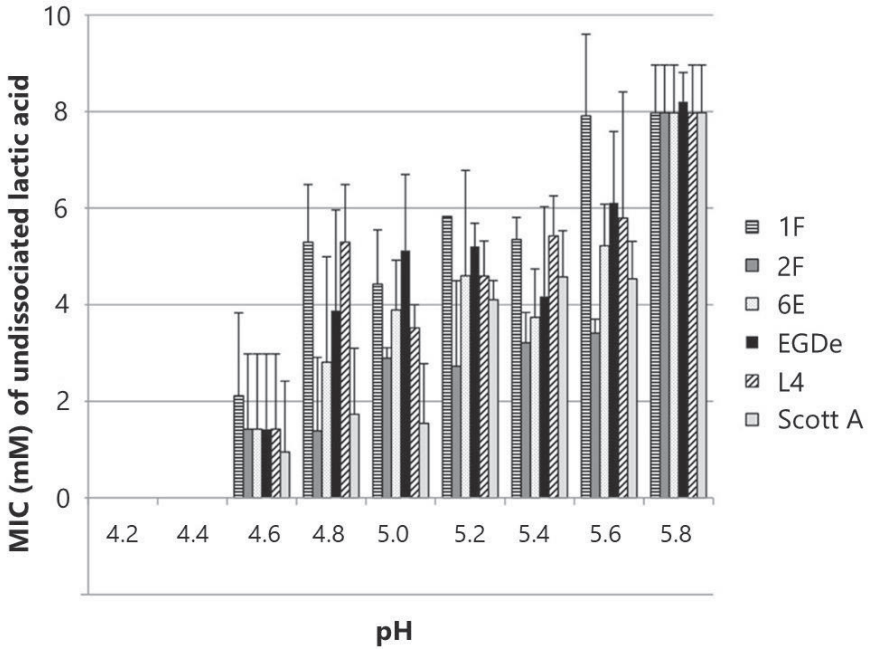

Fig. 5.1 Average MIC of undissociated lactic acid (mM) for *L. monocytogenes* strains 1F, 2F, 6E, EGDe, L4 and Scott A at pH values ranging from 4.2 to 5.8, determined in BHI at 30 °C. Growth of *L. monocytogenes* was only observed at pH values higher than 4.4. At pH 4.2 and 4.4 growth was not observed in the absence or presence of lactic acid. Upper error bars are used to show the standard deviation of six measurements (n=6 for each strain and for each pH).

At pH values relevant to Gouda cheese, namely pH 5.2, 5.4 and 5.6, no significant differences were observed between the MICs of undissociated lactic acid per individual strain at these three pH values, except in the case of strain 1F (Supplementary Table S2). For the individual strains, the MIC data generated at pH 5.2, 5.4 and 5.6 were subsequently combined in further analyses.

Differences between strains were observed: MIC values of undissociated lactic acid were significantly higher for *L. monocytogenes* strain 1F (average MIC of 6.1 mM) than for strains 2F, 6E and Scott A (average MICs of 3.0, 5.1 and 4.3 mM, respectively), but not significantly higher or lower than the MICs for strains EGDe and L4 (average MICs of 5.5 and 5.6 mM) (Supplementary Table S4). These findings indicate that strain variation contributes significantly to the observed variation in the concentrations of lactic acid that are required to inhibit growth. The average MIC of undissociated lactic acid determined at

pH 5.2–5.6 for all six strains combined was 5.0 mM with a standard deviation of 1.5 mM, as seen in dataset 1 and 2 (Supplementary Table S4). The highest value observed in this pH range was 9.0 mM (Fig. 5.1).

The cumulative frequency plot of the MICs of undissociated lactic acid at pH 5.2–5.6 obtained in this study and MICs reported in literature are presented in Fig. 5.2A. The previously reported MICs are in the same range as the ones determined in this study. The small increments in consecutive concentrations of undissociated lactic acid used in this study resulted in more accurate estimations of the actual MICs for *L. monocytogenes* (Fig. 5.2A).

At a temperature commonly used during cheese ripening, namely 12 °C, the MICs of undissociated lactic acid for *L. monocytogenes* after one month of incubation were not significantly different ($p > 0.05$) from those determined after incubation for one month at 30 °C in the pH range 5.2–5.6. When taking all data over the full pH range tested into account*, i.e.* at pH 4.6–5.8, the average MICs were slightly lower at 12 °C (3.2 mM on average) than at 30 °C (3.5 mM on average) (see Supplementary Table S1, $p = 0.02$). Similar effects were seen by Houtsma (1996) who reported lower MICs of undissociated lactic acid for *L. innocua* at 4 °C after three weeks than at 10, 20, or 30 °C.

MICs of undissociated lactic acid for strains EGDe and Scott A were significantly lower in the presence of sodium chloride together with calcium chloride at concentrations relevant to cheese (0.20 M and 0.36 M, respectively) than in the absence of these added salts or in the presence of these salts added individually. For the other four strains, no significant differences were observed (results not shown). Hence, the effect of sodium chloride and calcium chloride on the MIC of undissociated lactic acid for *L. monocytogenes* in cheese appears overall limited, but may be strain dependent.

For undissociated acetic acid, the average MIC for all six *L. monocytogenes* strains tested in pH range 5.2–5.6 was 19.0 mM with a standard deviation of 6.5 mM. The highest MIC observed was 30.2 mM (Fig. 5.2B). The average MIC at pH 5.6 was 20.7 mM, which was significantly higher than the average MICs of undissociated acetic acid at pH 5.2 and 5.4 (18.0 and 17.7 mM). The consecutive MIC intervals of acetic acid were larger at pH 5.6 than at pH 5.2 and 5.4 (data not shown), which might result in greater deviations between the determined and the true MICs at pH 5.6 than at pH 5.2 and 5.4. The MIC for *L. monocytogenes* strain Scott A (11.6 mM) was significantly lower than the MICs for strains 1F, 2F, 6E, EGDe and L4 (18.3 - 21.8 mM; see Supplementary Table S4). The MICs of undissociated acetic acid are in line with the MICs in previous studies for *L. monocytogenes* (Fig. 5.2B), which varied from 6.2 to 43.9 mM, with an average of 19.7 mM (Ahamad & Marth, 1989; Conner et al., 1990; Coroller et al., 2005; Vasseur et al., 1999; Young and Foegeding, 1993).

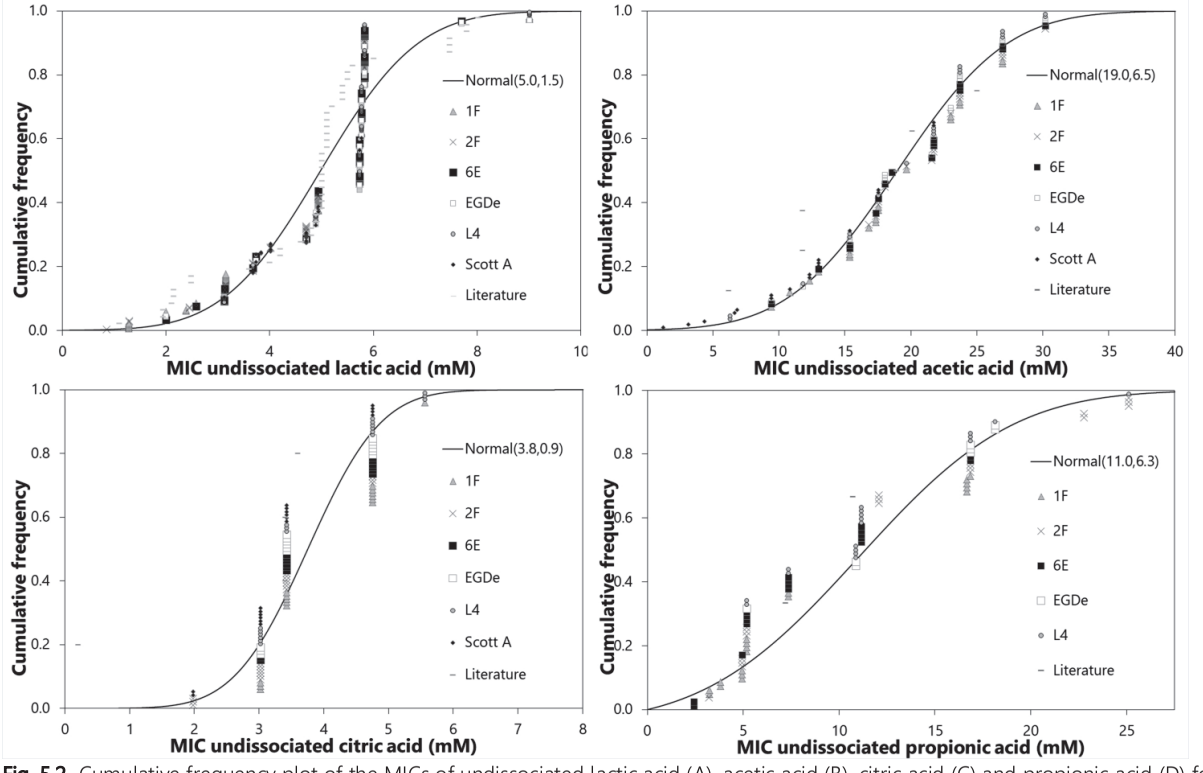

Fig. 5.2 Cumulative frequency plot of the MICs of undissociated lactic acid (A), acetic acid (B), citric acid (C) and propionic acid (D) for *L. monocytogenes* strains 1F, 2F, 6E, EGDe, L4 and Scott A determined at pH 5.2, 5.4 and 5.6 and plotted individually. Furthermore, literature values of the MICs of undissociated acids for *L. monocytogenes* strains have been included, as reported by Ahamad and Marth (1989), Aryani et al. (2015), Chen and Shelef (1992), Conner et al. (1990), Coroller et al. (2005), Houtsma et al. (1993), van der Veen et al. (2008), Vasseur et al. (1999) and Young and Foegeding (1993). In the study by van der Veen et al. (2008), MICs of undissociated lactic acid were obtained for 138 strains of *L. monocytogenes*, but only the MICs of strains 1F, 2F, 6E, EGDe, L4, Scott A were incorporated in this figure to avoid a skew in the literature dataset due to one study. The experimental data generated in this study have been fitted with Normal distributions (average with standard deviations) and were 5.0 (SD 1.5) mM for undissociated lactic acid, 19.0 (SD 6.5) mM for undissociated acetic acid, 3.8 (SD 0.9) mM for undissociated citric acid and 11.0 (SD 6.3) mM for undissociated propionic acid.

For undissociated citric acid, the average MIC value for all six strains of *L. monocytogenes* tested in pH range 5.2–5.6 was 3.8 mM with a standard deviation of 0.9 mM (Supplementary Table S4) and a highest MIC of 5.6 mM (Fig. 5.2C). The MIC for strain 2F (3.3 mM on average) was significantly lower than that of strains 1F and L4 (4.0 and 4.1 mM on average). For strains 6E, EGDe and Scott A, the MIC values of undissociated citric acid (3.4 to 3.9 mM) did not differ significantly from the MICs for 1F, 2F and L4 (Supplementary Table S4). The MICs determined for undissociated citric acid in this study are similar to or higher than the previously reported MICs of 3.4 mM (Ahamad & Marth, 1989), 0.2, and 3.6 mM (Coroller et al., 2005). The MIC reported by Ahamad and Marth (1989) was determined at pH 4.5, which is near the pH limit for growth of *L. monocytogenes*. This could explain the lower MIC observed in that study compared with those found in this study. Coroller et al. (2005) determined the MICs of undissociated citric acid at a concentration range of 0–300 mM total citric acid and at pH 5.0 to 7.0. Only the lowest and highest MICs of undissociated citric acid were reported, but not the consecutive concentrations of undissociated citric acid tested. This complicates a direct comparison between MIC values of undissociated citric acid obtained by Coroller et al. (2005) and the ones established in this study.

For undissociated propionic acid, the average MIC for the *L. monocytogenes* strains tested in pH range 5.2–5.6 was 11.0 mM with a standard deviation of 6.3 mM (Supplementary Table S4). The highest value observed was 25.1 mM (Fig. 5.2D). The MICs for strains 1F and 6E (8.2 and 8.3 mM on average) were consistent with the MICs previously determined by Coroller et al. (2005) of 7.2 and 10.7 mM (Fig. 5.2D), but were significantly different from the MICs for strains 2F and EGDe (average MICs of 13.4 and 15.3 mM, respectively). The MIC of undissociated propionic acid for strain L4 (average MIC of 12.2 mM) was not significantly different from the other strains (Supplementary Table S4).

Growth of *L. monocytogenes* has not been observed in challenge studies using Gouda and Swiss-type cheese; in fact, inactivation was observed after extended ripening times (Bachmann & Spahr, 1995; Buazzi, Johnson, & Marth, 1992; Northolt, Vecht, Toepoel, Soentoro, & Wisselink, 1988; Wemmenhove, Stampelou, van Hooijdonk, Zwietering, & Wells-Bennik, 2013; Wemmenhove, Beumer, van Hooijdonk, Zwietering, & Wells-Bennik, 2014). In Dutch-type cheeses, several short-chain organic acids can be found, such as lactic acid, acetic acid, citric acid and propionic acid. Cow's milk naturally contains 0.05-0.8 mM acetate, 9 mM citrate and <0.4 mM lactate (total acid) and has a pH of around 6.5 to 6.7 (Walstra & Jenness, 1984). It also contains lactose, which starter lactic acid bacteria used in cheese manufacturing can convert to lactic acid (Fox, McSweeney, Cogan, & Guinee, 2004a). In Gouda cheese ripened for 4 weeks or longer, concentrations of total lactic acid and acetic acid were 13.9 and 1.1 g kg^{-1}, respectively, whereas citric acid and propionic acid were absent (Wemmenhove et al., 2013). The estimated concentration of

undissociated lactic acid in the water phase of Gouda cheese is 9.2 mM (Supplementary Table S5), thus higher than the average and highest MICs needed for full inhibition of growth of *L. monocytogenes* (i.e. 5.0 mM and 9.0 mM, respectively). The estimated concentration of undissociated acetic acid in Gouda is 9.0 mM (Supplementary Table S5), which is lower than the average MIC (19.0 mM) needed for full inhibition of growth of *L. monocytogenes*. Therefore, lactic acid appears to be a critical factor for inhibition of growth of this pathogen in Gouda cheese.

In semi-hard cheeses with propionic acid fermentation, such as Maasdam and Swiss-type cheese, propionic acid bacteria convert the lactic acid produced by lactic acid bacteria to other organic acids during ripening, resulting in an increase of the pH from 5.3 to 5.8 (Fox, McSweeney, Cogan, & Guinee, 2004b). Total concentrations of 3.5 g lactic acid per kg cheese, 2.0 g acetic acid per kg cheese and 5.0 g propionic acetic acid per kg cheese have been reported in a 35-day old Swiss-type cheese (Fox et al., 2004a). This results in estimated concentrations of 0.95 mM undissociated lactic acid, 8.1 mM undissociated acetic acid, and 17.3 mM undissociated propionic acid in the water phase of the cheese, respectively (Supplementary Table S5). Given the MICs of these acids (average 5.0 mM, 19.0 mM and 11.0 mM undissociated acid, respectively), propionic acid is thought to play an important role in inhibition of growth of *L. monocytogenes* during ripening of such cheeses.

5.4 Conclusions

MICs of undissociated lactic acid for six strains of *L. monocytogenes* were generally lower at pH values approaching the pH limit for growth of this bacterium (*i.e.* pH 4.4) than at pH values above 5.2. MICs of undissociated lactic acid, acetic acid, citric acid and propionic acid for *L. monocytogenes* at pH values relevant to cheese (in the range 5.2 to 5.6) showed variations for each of the acids, which could mostly be attributed to strain variation. Undissociated lactic acid is expected to have a substantial inhibitory effect on growth of *L. monocytogenes* in Gouda cheese, whereas propionic acid is expected to play an important role in semi-hard cheeses that undergo propionic acid fermentation. The generated data on MICs of undissociated organic acids in Dutch-type cheeses for different strains of *L. monocytogenes* can be used to improve risk assessments.

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Supplementary Table S1 Statistical analysis of the parameters in the different datasets affecting the minimal inhibitory concentration of undissociated acids for *L. monocytogenes*, based on the general model ANOVA

Data-set		Independent experiments	Replicates	Strain	pH	Type of salt K = potassium, NaCl = sodium chloride, $CaCl_2$ = calcium chloride	Type of acid	T (°C)
1	Parameters	1; 2	1; 2; 3	1F; 2F; 6E; EGDe; L4; Scott A	4.2; 4.4; 4.6; 4.8; 5.0; 5.2; 5.4; 5.6; 5.8	K	Lactic acid	30
	F-value	1.4	0.72	14.6	152	-	-	-
	p-value	0.23	0.49	*0.000*	*0.000*	-	-	-
2	Parameters	1; 2	1; 2; 3	1F; 2F; 6E; EGDe; L4; Scott A	5.2; 5.4; 5.6	K; K+ NaCl; K+ $CaCl_2$; K+ NaCl + $CaCl_2$	Lactic acid	30
	F-value	2.4	0.32	74.3	1.3	6.9	-	-
	p-value	0.12	0.72	*0.000*	0.28	*0.000*	-	-
3	Parameters	1; 2	1; 2; 3	1F; 2F; 6E; EGDe; L4; Scott A	5.2; 5.4; 5.6	K	Lactic acid (data from dataset 1 and 2); acetic acid; citric acid; propionic acid	30
	F-value	1.1	0.57	7.2	5.7	-	370	-
	p-value	0.29	0.56	*0.000*	*0.004*	-	*0.000*	-
4	Parameters	1	1; 2; 3	1F; 2F; 6E; EGDe; L4; Scott A	4.2; 4.4; 4.6; 4.8; 5.0; 5.2; 5.4; 5.6; 5.8	K	Lactic acid	12
	F-value	-	0.74	24.3	138	-	-	5.5
	p-value	-	0.48	*0.000*	*0.000*	-	-	*0.02*

linear

Dataset 1, 2 and 4 contained MICs of undissociated lactic acid; dataset 3 contained MICs of different types of organic acids potentially present in Dutch-type cheeses. Significant differences within each dataset were determined with a general linear model (between subjects design, pair-wise comparison, Product Tukey Test at 95%). In the GLM analysis, independent experiments and replicates were treated as random variables. Per dataset, factors that significantly differed ($p < 0.05$) are displayed in italics. Each experimental MIC value was determined in triplicate under different conditions for different strains. For the three replicates of the same condition (pH, acid, temperature, strain), no significant differences ($p > 0.05$) were observed in all four datasets. Datasets 1, 2 and 3 contained two independent experiments, and no significant differences in MIC values were observed between the duplicates of the same condition (Supplementary Table S1).

Supplementary Table S2 MICs (average±stdev, based on 6 replicates) of undissociated lactic acid for *L. monocytogenes* at pH values ranging from 4.2 to 5.8, as tested for significant differences between pH values for each of the *L. monocytogenes* strains listed

pH	MIC of undissociated lactic acid (mM) for 6 strains of *L. monocytogenes* (n=6)					
	1F	2F	6E	EGDe	L4	Scott A
4.2*	–	–	–	–	–	–
4.4*	–	–	–	–	–	–
4.6	2.1±1.7 D	1.4±1.6 B	1.4±1.6 C	1.4±1.6 C	1.4±1.6 C	0.9±1.5 C
4.8	5.3±1.2 C	1.4±1.5 B	2.8±2.2 BC	3.9±2.1 BC	5.3±1.2 B	1.7±1.4 C
5.0	4.4±1.1 C	2.9±0.2 B	3.9±1.0 BC	5.1±1.6 B	3.5±0.5 BC	1.5±1.2 C
5.2	5.8±0.0 BC	2.7±1.8 B	4.6±2.2 B	5.2±0.5 B	4.6±0.7 B	4.1±0.4 B
5.4	5.4±0.5 C	3.2±0.6 B	3.7±1.0 BC	4.2±1.9 B	5.4±0.8 B	4.6±1.0 B
5.6	7.9±1.7 AB	3.4±0.3 B	5.2±0.9 B	6.1±1.5 AB	5.8±2.6 AB	4.5±0.8 B
5.8	8.0±1.0 A	8.0±1.0 A	8.0±1.0 A	8.2±0.6 A	8.0±1.0 A	8.0±1.0 A
F-value	18.4	20.8	11.1	11.8	12.7	28.9
p-value	*<0.0001*	*<0.0001*	*<0.0001*	*<0.0001*	*<0.0001*	*<0.0001*

To establish whether MICs of undissociated lactic acid significantly differed between different pH values for each of the *L. monocytogenes* strains, a post-hoc ANOVA test (Product Tukey test at 95%) was performed for the MIC of undissociated lactic acid, as determined for 6 different strains of *L. monocytogenes* (1F, 2F, 6E, EGDe, L4 and Scott A) at pH values ranging from 4.2 to 5.8 in BHI medium at 30 °C. The measurements at pH 4.2 and 4.4 were excluded from analysis, as all *L. monocytogenes* strains tested did not grow at those pH values (indicated by a –), even without lactic acid present. MIC values are expressed as concentration of undissociated lactic acid (average ± standard deviation in mM), based on n = 6 determinations. Statistical analysis was performed for each strain of *L. monocytogenes* and significant differences were listed (verified with both the F-value and p-value). In case of significant differences ($p < 0.05$, indicated in italics in the table), those differences were indicated by the letters A, B, C, D (results in the table should be read vertically, as the MIC values were compared for pH for each strain of *L. monocytogenes*). The further the letter in the alphabet, the lower the MIC of undissociated lactic acid determined for that pH and for the strain of *L. monocytogenes* given. The absence of an identical letter behind the scores at different pH values for a certain strain of *L. monocytogenes* means that the MIC is significantly different between those pH values for that particular strain. The F-value is calculated by dividing the between-group variation (SST) by the within-group variation (MSE). The F-value is expected to have a value close to 1.0 when there are no significant differences between groups. A large F-value means that there is more variation among group means than is expected by chance.

* at pH 4.2 and pH 4.4, growth was not observed in the absence and presence of lactic acid

Supplementary Table S3 MICs (average±stdev, based on 6 replicates) of undissociated lactic acid for *L. monocytogenes* at pH values ranging from 4.2 to 5.8 as described in Table 3.2 have been tested for significant differences between different strains of *L. monocytogenes* at a given pH

MIC of undissociated lactic acid (mM) for 6 strains of *L. monocytogenes* (n=6)									
	pH								
Strain	4.2*	4.4*	4.6	4.8	5.0	5.2	5.4	5.6	5.8
1F	-	-	2.1±1.7 !	5.3±1.2 A	4.4±1.1 AB	5.8±0.0 A	5.4±0.5 A	7.9±1.7 A	8.0±1.0 !
2F	-	-	1.4±1.6 !	1.4±1.5 B	2.9±0.2 BC	2.7±1.8 B	3.2±0.6 B	3.4±0.3 C	8.0±1.0 !
6^E	-	-	1.4±1.6 !	2.8±2.2 AB	3.9±1.0 AB	4.6±2.2 AB	3.7±1.0 AB	5.2±0.9 ABC	8.0±1.0 !
EGDe	-	-	1.4±1.6 !	3.9±2.1 AB	5.1±1.6 A	5.2±0.5 A	4.2±1.9 AB	6.1±1.5 AB	8.2±0.6 !
L4	-	-	1.4±1.6 !	5.3±1.2 A	3.5±0.5 AB	4.6±0.7 AB	5.4±0.8 A	5.8±2.6 ABC	8.0±1.0 !
Scott A	-	-	0.9±1.5 !	1.7±1.4 B	1.5±1.2 C	4.1±0.4 AB	4.6±1.0 AB	4.5±0.8 BC	8.0±1.0 !
F-value	-	-	0.32	6.7	8.1	4.4	3.9	6.0	0.06
p-value	-	-	0.90	*0.0003*	*<0.0001*	*0.004*	*0.008*	*0.0007*	0.997

To establish whether MICs of undissociated lactic acid shown in Table 3.2 significantly differed between strains of *L. monocytogenes* for each given pH, a post-hoc ANOVA test (Product Tukey test at 95%) was performed for the MIC of undissociated lactic acid, as determined for 6 different strains of *L. monocytogenes* (1F, 2F, 6E, EGDe, L4 and Scott A) at each of given pH values ranging from 4.6 to 5.8 in BHI medium at 30 °C. The measurements at pH 4.2 and 4.4 were excluded from analysis, as all *L. monocytogenes* strains tested did not grow at those pH values, even without lactic acid present. MIC values are expressed as concentration of undissociated lactic acid (average ± standard deviation in mM), based on n = 6 determinations. Statistical analysis was performed for each pH and significant differences were listed (verified with both the F-value and p-value). In case of significant differences ($p < 0.05$, indicated in italics), those differences were indicated by the letters A, B, C (results in the table should be read vertically, as the MIC values of the 6 strains of *L. monocytogenes* were compared at each pH). The further the letter in the alphabet, the lower the MIC of undissociated lactic acid was for that strain at the given pH. The absence of an identical letter behind the scores for different strains of *L. monocytogenes* at a certain pH means that the MIC at that particular pH significantly differed between those strains. An exclamation mark (!) was used instead of a letter when no significant differences ($p > 0.05$) were observed for the different strains at a given pH such as at pH 4.6 and 5.8. The F-value is calculated by dividing the between-group variation (SST) by the within-group variation (MSE). The F-value is expected to have a value close to 1.0 when there are no significant differences between groups. A large F-value means that there is more variation among group means than is expected by chance.

* at pH 4.2 and pH 4.4, growth was not observed in the absence and presence of lactic acid

Supplementary Table S4 MICs (average±stdev, based on 18 replicates) of undissociated lactic acid, acetic acid, citric acid and propionic acid for *L. monocytogenes* at pH values 5.2 to 5.6

Type of organic acid	MIC of undissociated acid (mM) for 6 strains of *L. monocytogenes* (n=18 per strain)							
	1F	2F	6E	EGDe	L4	Scott A	F-value	p-value
Lactic acid dataset 1	6.4±1.5 A	3.1±1.1 C	4.5±1.5 B	5.2±1.5 AB	5.3±1.6 AB	4.4±0.7 B	12.3	*<0.0001*
Lactic acid dataset 2	5.8±1.1 A	3.0±0.6 C	5.8±0.04 A	5.8±0.5 A	6.0±0.8 A	4.1±0.8 B	54.3	*<0.0001*
Lactic acid (average MIC of dataset 1 and 2)	6.1±1.3 A	3.0±0.9 D	5.1±1.2 B	5.5±1.2 AB	5.6±1.3 AB	4.3±0.8 C	34.9	*<0.0001*
Acetic acid	18.3±5.3 A	21.8±4.2 A	20.4±5.3 A	21.1±5.8 A	20.5±7.2 A	11.6±5.9 B	10.0	*<0.0001*
Citric acid	4.0±0.9 A	3.3±0.8 B	3.9±0.7 AB	3.9±0.8 AB	4.1±1.0 A	3.4±0.8 AB	3.3	*0.009*
Propionic acid	8.2±5.5 B	13.4±8.2 A	8.3±4.3 B	15.3±5.5 A	12.2±5.0 AB	ND	5.5	*0.0007*

Post-hoc ANOVA tests for the MICs of different undissociated organic acids for 6 different strains of *L. monocytogenes* (1F, 2F, 6E, EGDe, L4 and Scott A) in BHI medium at 30 °C. Per type of organic acid and strain of *L. monocytogenes*, data on MIC values were determined at pH 5.2, 5.4 and 5.6. As no significant differences in MIC values were observed in this pH range for undissociated lactic acid for 4 out of 6 strains of *L. monocytogenes* and as this pH range is typical for Dutch-type cheeses, data at this pH interval were merged, resulting in n=18 per strain of *L. monocytogenes*. Statistical analysis was performed for the MIC for each strain of *L. monocytogenes* per type of organic acid and significant differences were listed (verified with both the F-value and p-value). In case of significant differences ($p < 0.05$, indicated in italics), those differences were indicated by the letters A, B, C, D (results in the table should be read horizontally, as the MIC values of strains of *L. monocytogenes* were compared per type of organic acid). The further the letter in the alphabet, the lower the MIC of that acid was for this strain. The absence of an identical letter behind the MIC of a certain type of organic acid for different strains of *L. monocytogenes* means that the MIC for that particular acid was significantly different between those strains. For undissociated lactic acid, the average MIC was calculated, as the MIC for this acid was generated in 2 individual datasets (dataset 1 and 2). The average MICs per type of acid were 5.0±1.5 mM for undissociated lactic acid, 19.0±6.5 mM for undissociated acetic acid, 3.8±0.9 mM for undissociated citric acid and 11.0±6.3 mM for undissociated propionic acid. The MIC of undissociated propionic acid was not determined (ND) for *L. monocytogenes* strain Scott A. The F-value is calculated by dividing the between-group variation (SST) by the within-group variation (MSE). The F-value is expected to have a value close to 1.0 when there are no significant differences between groups. A large F-value means that there is more variation among group means than is expected by chance.

Supplementary Table S5 Estimation of the concentrations of undissociated acids in cheese, based on the following equation: $[HA]_{water} = \frac{[HA]_{total}}{\varphi_{v_{water}} + 10^{pH-pKa.} \cdot \varphi_{v_{water}} + P_{ow} \cdot \varphi_{v_{fat}}}$ as derived by Wemmenhove et al. (in preparation [a]). The total concentration of acid (M) was calculated from total concentration (g kg^{-1}), the molecular mass of the acid and a cheese density of 1070 kg m^{-3}. Parameters $\varphi_{v_{fat}}$ and $\varphi_{v_{water}}$ were calculated from the moisture and fat content of cheese, and a cheese density of 1070 kg m^{-3}, water density of 998 kg m^{-3}, a milk fat density of 940 kg m^{-3}

Acid	Molecular weight (g mol^{-1})	Log Pow	pK$_a$	Concentration organic acid in Gouda cheese					Concentration organic acid in Swiss-type cheese				
				Total [d] (g kg^{-1})	Total (M)	$\varphi_{v_{fat}}$ [e]	$\varphi_{v_{water}}$ [e]	Undissociated [e] in water phase (mM) at pH 5.3	Total [f] (g kg^{-1})	Total (M)	$\varphi_{v_{fat}}$ [g]	$\varphi_{v_{water}}$ [g]	Undissociated [g] in water phase (mM) at pH 5.8
Lactic	90.08	-1.5 [a]	3.71 [a]	13.9	0.17	0.32	0.45	9.2	3.5	0.042	0.34	0.35	0.95
Acetic	60.05	-0.31 [b]	4.76 [c]	1.1	0.020	0.32	0.45	9.0	2.0	0.036	0.34	0.35	8.1
Citric	192.12	-1.7 [b]	3.13 [c]	-	-	0.32	0.45	-	-	-	0.34	0.35	-
Propionic	74.08	0.33 [b]	4.86 [c]	-	-	0.32	0.45	-	5.0	0.072	0.34	0.35	17.3

[a] Wemmenhove, E., van Valenberg, H.J.F., Zwietering, M.H., Wells-Bennik, M.H.J., van Hooijdonk, A.C.M. (2018). Factors that inhibit growth of *Listeria monocytogenes* in nature-ripened Gouda cheese: A major role for undissociated lactic acid. Food Control 84:413-418.

[b] CDC, 2016. The National Institute for Occupational Safety and Health, International Chemical Safety Cards, http://www.cdc.gov/niosh/ipcs Date last accessed: 02-09-2018.

[c] Dawson et al. 1986

[d] Wemmenhove et al. 2013

[e] Calculated for Gouda cheese using the above equation, $[HA]_{water} = \frac{[HA]_{total}}{\varphi_{v_{water}} + 10^{pH-pKa.} \cdot \varphi_{v_{water}} + P_{ow} \cdot \varphi_{v_{fat}}}$, taking into account dissociation and partition of the organic acids and based on a moisture content of 42% and a fat content of 48% dry matter for Gouda

[f] Fox et al. 2004a

[g] Calculated for Swiss-type cheese using the above equation, $[HA]_{water} = \frac{[HA]_{total}}{\varphi_{v_{water}} + 10^{pH-pKa.} \cdot \varphi_{v_{water}} + P_{ow} \cdot \varphi_{v_{fat}}}$, taking into account dissociation and partition of the organic acids and based on a moisture content of 33% for 6-month ripened Swiss-type cheeses according to Fox et al. 2004b, and a fat content of 45% dry matter

Chapter 6

Factors that inhibit growth of *Listeria monocytogenes* in nature-ripened Gouda cheese: A major role for undissociated lactic acid

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ABSTRACT

In this study, factors relevant to nature-ripened Gouda cheese were evaluated for their potential to inhibit growth of *Listeria monocytogenes*. Factors included water activity, pH, undissociated acetic and lactic acid, diacetyl, free fatty acids, lactoferrin, nitrate, nitrite and nisin. In addition, the effect of temperature was evaluated. For each factor, the actual concentrations and values relevant to Gouda cheese were obtained and the inhibitory effect of these individual factors on growth of *L. monocytogenes* was assessed. This evaluation revealed that undissociated lactic acid is the most important factor for growth inhibition of *L. monocytogenes* in Gouda cheese and that, additionally, low water activity as present in the cheese rind and after prolonged ripening times can also cause full growth inhibition. Gouda cheeses have a typical total lactic acid content of 1.47% w/w. In a 2-week old Gouda cheese, with a pH value of 5.25 and a moisture content of 42% w/w, the concentration of undissociated lactic acid in the water phase is 10.9 mM. Growth of *L. monocytogenes* is not supported when the undissociated lactic acid concentration is >6.35 mM. Concentrations of undissociated lactic acid in the water phase of Gouda cheese will be higher than this value when the total lactic acid content is >0.86% w/w at a pH < 5.25 (relevant to young Gouda cheese), or >1.26% w/w at a pH < 5.50 for mature Gouda cheese (moisture content of 35% w/w). This study underlines the importance of undissociated lactic acid as growth inhibitor for *L. monocytogenes* in Gouda cheese.

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6.1 Introduction

Listeria monocytogenes is a foodborne pathogen and the causative agent of listeriosis (Lou & Yousef, 2000). *L. monocytogenes* is able to form biofilms (Pan, Breidt, & Kathariou, 2006), can grow at low temperatures and can adapt to highly acidic or saline conditions (Cole, Jones, & Holyoak, 1990). This pathogen is a concern to the food industry, in particular in ready-to-eat (RTE) products.

Nature-ripened Dutch-type Gouda cheese is cheese that is coated after production and then dried during ripening. This study focused on the vast majority of Dutch-type Gouda cheeses which are produced using mesophilic starter cultures that primarily consist of strains of *Lactococcus* lactis subsp. *cremoris* and *Lactococcus lactis* subsp. *lactis*, but may also contain *Leuconostoc* species and *Lactococcus lactis* subsp. *lactis* var. *diacetylactis* (Stadhouders, 1974). Nature-ripened Dutch-type Gouda cheese as produced on an industrial scale by the Dutch dairy industry is made from pasteurized cow's milk and is a RTE food. To avoid contamination of cheese with *L. monocytogenes*, raw milk is subjected to minimal pasteurization conditions of 15 s 72 °C as one of the options according to regulation (EC) No 1662/2006 (European Commission, 2006) amending regulation (EC) No 853/2004 laying down specific hygiene rules for food of animal origin (European Commission, 2004). The estimated concentration of *L. monocytogenes* in raw milk is low: Meyer-Broseta, Diot, Bastian, Rivière, & Cerf (2003) reported maximum concentrations of 0.1 cfu mL^{-1} in 7.7% of the raw milk samples (<0.04 cfu mL^{-1} for the remaining 92.3% of samples), and Ruusunen et al. (2013) reported maximum concentrations of 30 cfu mL^{-1} for 5.5% of the raw milk samples (<1 cfu mL^{-1} for the remaining 94.5% of samples). Minimum pasteurization of 15 s at 72 °C leads to a reduction of *L. monocytogenes* with 10.4 log units based on the average $D_{72°C}$ (and a minimum inactivation of 2.7 logs based on the 95% prediction interval of $D_{72°C}$ [den Besten & Zwietering, 2012; ILSI, 2012]). Contamination of cheese during further processing and storage is furthermore limited by applying good manufacturing processing conditions, and sampling schemes are in place to verify absence of *L. monocytogenes* in processing areas, on equipment and on finished products.

Microbiological food safety criteria have been set in European Union regulation EC 2073/2005, which includes criteria for *L. monocytogenes* in RTE foods for three different RTE food categories (European Commission, 2005). For food category 1.1, "Ready-to-eat foods intended for infants and ready-to-eat foods for special medical purposes" this is absence in 25 g (n = 10) for products placed on the market during shelf life. For food category 1.2, "Ready-to-eat foods able to support the growth of *L. monocytogenes*, other than those intended for infants and for special medical purposes" the maximum limit is 100 cfu g^{-1} (n = 5) for products placed on the market during their shelf-life. This criterion

shall apply if the manufacturer is able to demonstrate, to the satisfaction of the competent authority, that the product will not exceed the limit 100 cfu g^{-1} throughout the shelf-life. The operator may fix intermediate limits during the process that must be low enough to guarantee that the limit of 100 cfu g^{-1} is not exceeded at the end of shelf-life. For food category 1.2, an alternative criterion may apply, namely, absence in 25 g (n = 5) before the food has left the immediate control of the food business operator, who has produced it; this criterion shall apply to products before they have left the immediate control of the producing food business operator, when he is not able to demonstrate, to the satisfaction of the competent authority, that the product will not exceed the limit 100 cfu g^{-1} throughout the shelf-life. Lastly, for food category 1.3, "Ready-to-eat foods unable to support the growth of *L. monocytogenes*, other than those intended for infants and for special medical purposes" the maximum limit is 100 cfu g^{-1} (n = 5) for products placed on the market during their shelf-life.

When a RTE product intended for consumption by the general population has a pH ≤ 4.4 or $a_w \leq 0.92$, a pH ≤ 5.0 and $a_w \leq 0.94$, or a shelf life <5 days, the product falls in category 1.3. If this is not the case, evidence for no growth potential can be obtained by predictive mathematical modelling, durability tests and/or challenge tests (European Commission, 2005).

In the case of Dutch-type Gouda cheese, the pH is > 5.0 and $a_w > 0.94$. Nevertheless, various challenge studies have shown that *L. monocytogenes* does not grow within or on this cheese (Northolt, Vecht, Toepoel, Soentoro, & Wisselink, 1988; Wemmenhove, Beumer, van Hooijdonk, Zwietering, & Wells-Bennik, 2014; Wemmenhove, Stampelou, van Hooijdonk, Zwietering, & Wells-Bennik, 2013). In this study, the impact of growth-inhibiting factors that are present in Gouda cheese or relevant to storage of Gouda cheese was evaluated for growth inhibition of *L. monocytogenes*, and the predictions were compared with the fate of this bacterium in Gouda as determined in previous challenge studies. Factors with potential to inhibit growth in this evaluation included temperature (T), pH, water activity (a_w), undissociated acetic acid, undissociated lactic acid, diacetyl, free fatty acids, lactoferrin, nitrate, nitrite and nisin. Data were obtained from literature, or experimentally obtained in absence of data in the literature. This evaluation leads to a better understanding of the most important factors in Gouda cheese that result in growth inhibition of *L. monocytogenes.*

6.2 Materials and methods

6.2.1 Comparison of growth-inhibiting potentials

Components that are present in Gouda cheese were evaluated individually for their potential to inhibit growth of *L. monocytogenes* and ranked in order of importance for growth inhibition. The evaluation was based on the concentration present in cheese, the concentration needed for inhibition of growth of *L. monocytogenes* in culture medium and/or cheese, and the Gamma factor formula describing the relationship between the concentration in cheese and the concentration needed for inhibition of the pathogen.

6.2.2 Data search

For each component that had the potential to inhibit growth, the scientific literature was evaluated for data on concentrations in Gouda cheese, critical growth limits and Gamma factor formulas. Data on concentrations in Gouda cheese were obtained using a cheese handbook (Fox, McSweeney, Cogan, & Guinee, 2004) and various literature databases (Web of Science, Scopus, PubMed) by using the search terms [cheese] AND [temperature OR water activity OR pH OR diacetyl OR lactoferrin OR nitrate OR nitrite OR nisin OR acetic acid OR lactic acid]. Data on concentrations that are minimally needed to inhibit growth of *L. monocytogenes* were obtained from publications and by using the same search terms as for data on concentrations in Gouda cheese, but by replacing the term [cheese] with [*Listeria monocytogenes*]. In addition, data on concentrations needed for growth inhibition were obtained from Combase (www.combase.cc) using terms ([*Listeria monocytogenes*]; [cheese]; minimum pH OR minimum water activity OR minimum temperature]). The obtained results were sorted on relevance, and data were extracted from the first 500 hits. Critical growth limits for T, a_w and pH in culture medium were obtained from ICMSF (1996). Additional experimental data were generated (see sections 6.2.3 and 6.2.4) when no literature data were available.

Additional data on the pH of Gouda after two weeks of ripening and on the total lactic acid content of Gouda ripened during 3–26 weeks were supplied by four Dutch cheese-producing companies (BelLeerdammer, DOC kaas, FrieslandCampina and Rouveen Kaasspecialiteiten), resulting in n = 24502 data points for pH and n = 89 data points for the total lactic acid content. The data on pH as supplied by different companies were clustered, following a Posthoc test (Duncan, alpha = 0.05, harmonized mean sample size) with SPSS Statistics (IBM, NY, US) showing alpha > 0.05. All available data on the total

lactic acid content obtained from cheeses of different ages were clustered, as it has previously been observed that the total lactic acid content did not change in Gouda cheese ripened for two weeks until six months (Wemmenhove et al., 2013).

Gamma factor formulas were available in literature for T, a_w, pH, undissociated lactic acid and acetic acid (Supplemental Material S1), but not for diacetyl, free fatty acids, lactoferrin, nitrate, nitrite, and nisin. For these potential factors, we assumed a linear relation between the concentration of the factor in the cheese and the degree of inhibition of growth of *L. monocytogenes*. The Gamma factors were calculated by dividing the concentration of the inhibiting factor in cheese by the maximum concentration needed to inhibit growth of *L. monocytogenes* and subtracting this fraction from 1 (as in Equation [6.5] from Supplemental Material S1).

6.2.3 Experimental determination of concentrations of free fatty acids in Gouda cheese

Data on the concentrations of free fatty acids present in Gouda cheese were not available in the literature. The concentrations of free fatty acids were analyzed in 2-week, 2-month and 6-month ripened Gouda cheeses (n = 12 data points) produced by four different Dutch producers. Analysis of free fatty acids was performed in duplicate according to the method described by de Jong & Badings (1990).

6.2.4 Experimental assessment of concentrations of factors present in cheese with inhibitory potential

No literature data were available on growth inhibition of *L. monocytogenes* by nitrate and nitrite, and insufficient data were available for growth inhibition by free fatty acids, therefore additional data were generated for these factors. The experimental design to determine the effects of additional nitrate and nitrite in Brain Heart Infusion (BHI) is described in Supplemental Material S2. Experiments to determine concentrations of free fatty acids with potential to inhibit growth of *L. monocytogenes* were performed with capric acid and lauric acid, as these free fatty acids reportedly have a higher inhibitory potential than other free fatty acids in culture medium (Chen, Nummer, & Walsh, 2014; Petrone et al., 1998; Sprong, Hulstein, & van der Meer, 2001; Wang & Johnson, 1992;). Additional experiments were performed to investigate whether growth inhibition by free fatty acids was impaired in cheese compared with their presence in culture medium due to the presence of calcium, which may form complexes with the fatty acids (Galbraith,

Miller, Paton, & Thompson, 1971), and casein, which may complex the fatty acids and therewith limit the available concentrations to inhibit *L. monocytogenes* (Wang & Johnson, 1992). The experiments in this study were performed in BHI with and without calcium chloride ($CaCl_2$) and Tween 80, and in model Gouda cheeses (experimental design also described in Supplemental Material S2).

6.2.5 Calculation of growth inhibition by lactic and acetic acid

The Gamma factors of lactic and acetic acid were calculated according to Equations [6.5] and [6.6] in Supplemental Material S1, with the highest and lowest $[HLac]_{MIC}$ and $[HAcet]_{MIC}$ in culture medium as input values, in comparison to the average $[HLac]_{water}$ and $[HAcet]_{water}$ as calculated in Supplemental Material S3. Additional experiments (Supplemental Material S4) were performed for lactic acid to obtain the dissociation constant (pK_a) in cheese, which contains high concentrations of Ca^{2+} and Na^+ that compete with H^+ for complexation with Lac^-, and to obtain the lactic acid logarithmic partition coefficient ($\log P_{fw}$) in cheese using milk fat, as cheese contains milk fat instead of octanol, which is normally the apolar phase when determining the logarithmic partition coefficient ($\log P_{ow}$).

$[HAcet]_{water}$ was also calculated according to Equation [6.12], but with pK_a = 4.76 (Dawson, 1986) and $\log P_{ow}$ = -0.31 (CDC, 2016).

The contribution of undissociated lactic acid to the overall inhibition of growth of *L. monocytogenes* in Gouda cheese was assessed by computational predictions, taking into account the variation in undissociated lactic acid concentrations in Gouda cheese and the concentrations of undissociated lactic acid needed to inhibit *L. monocytogenes.* Hereby, it was assessed in how many cases out of 1,000,000 produced cheeses undissociated lactic acid alone ensures full inhibition of growth of *L. monocytogenes*, starting with a hypothetical assumption that all cheeses were contaminated with *L. monocytogenes*. A Monte Carlo simulation with 1,000,000 iterations was run using @Risk 7 (Palisade Corporation, Ithaca, USA) and in each iteration the calculated concentration of undissociated lactic acid present in the water phase of Gouda ($[HLac]_{water}$) was compared with the minimal inhibitory concentration (MIC) of undissociated lactic acid for *L. monocytogenes* ($[HLac]_{MIC}$). A Kolmogorov-Smirnov test was used to find the best fits describing the variation in $[HLac]_{water}$ and $[HLac]_{MIC}$. The $[HLac]_{water}$ was calculated according to Equation [6.12], by using the variation in total concentrations of lactic acid from data provided by Gouda cheese manufacturing companies (total lactic acid content of 1.44% w/w cheese as displayed in Supplementary

Table S1) and own measurements in Gouda cheeses (total lactic acid content of 1.50% w/w cheese as displayed in Supplementary Table S2). Its variation was described with a Normal distribution: Normal (8.32, 0.69) mM for data provided by companies as presented in Supplementary Table S1 and Normal (11.48, 3.91) mM for the dataset as presented in Supplementary Table S2. $[HLac]_{MIC}$ was described by a Normal distribution with an average value of 5.11 mM and a standard deviation of 0.31 mM according to Aryani, den Besten, Hazeleger, & Zwietering (2015). In case that in an iteration $[HLac]_{water}$ was higher than $[HLac]_{MIC}$, undissociated lactic acid was indicated as able to inhibit growth of *L. monocytogenes*, so no growth of the pathogen was predicted.

6.3 Results and discussion

6.3.1 The effect of T, a_w and pH

An overview of growth inhibition by the individual factors present in Gouda cheese is given in Fig. 6.1. The factors are ranked in order of magnitude of growth inhibition. The most generic growth-inhibiting factors, namely, T, a_w and pH had a relevant effect on growth of *L. monocytogenes*. These three factors are often included in growth models (Augustin, Zuliani, Cornu, & Guiller, 2005; Mataragas, Stergiou, & Nychas, 2008; Schvartzman et al., 2011).

During curd formation T is favorable for growth of *L. monocytogenes*, but it is relatively low during ripening of Gouda (e.g. 12-13 °C), resulting in a large growth-inhibiting effect on *L. monocytogenes*. Nevertheless, T is not expected to fully inhibit growth of *L. monocytogenes,* as growth can occur at lower temperatures (ICMSF, 1996). In addition, ripening times can be very long. The a_w of Gouda cheese depends highly on the brining and ripening time and the location within a cheese (Wemmenhove, Wells-Bennik, Stara, van Hooijdonk, & Zwietering, 2016b). As a result, the Gamma factor for a_w may vary from values close to 0 (especially on the cheese rind after brining which makes a_w a very relevant growth inhibiting factor if a cheese would be contaminated on the surface, and inside a cheese after prolonged ripening times) to values close to 1 (for example initially in the centre of a cheese). The pH is also an important factor for growth inhibition of *L. monocytogenes*. The minimum pH limit for growth is 4.4 in culture medium (ICMSF, 1996) and 4.32 in acidified milk (El-Shenawy & Marth, 1990). The pH of a 2-week old Gouda cheese is 5.25 (SD 0.06) (Supplementary Table S3). In this range, the pH contributes to growth inhibition of *L. monocytogenes* (Gamma factor 0.55 in culture medium and 0.57 in cheese). In addition, pH affects the degree of dissociation of organic acids. Gouda cheese

contains lactic acid and acetic acid, and lower pH values result in increased concentrations of their undissociated forms, which have growth-inhibiting effects.

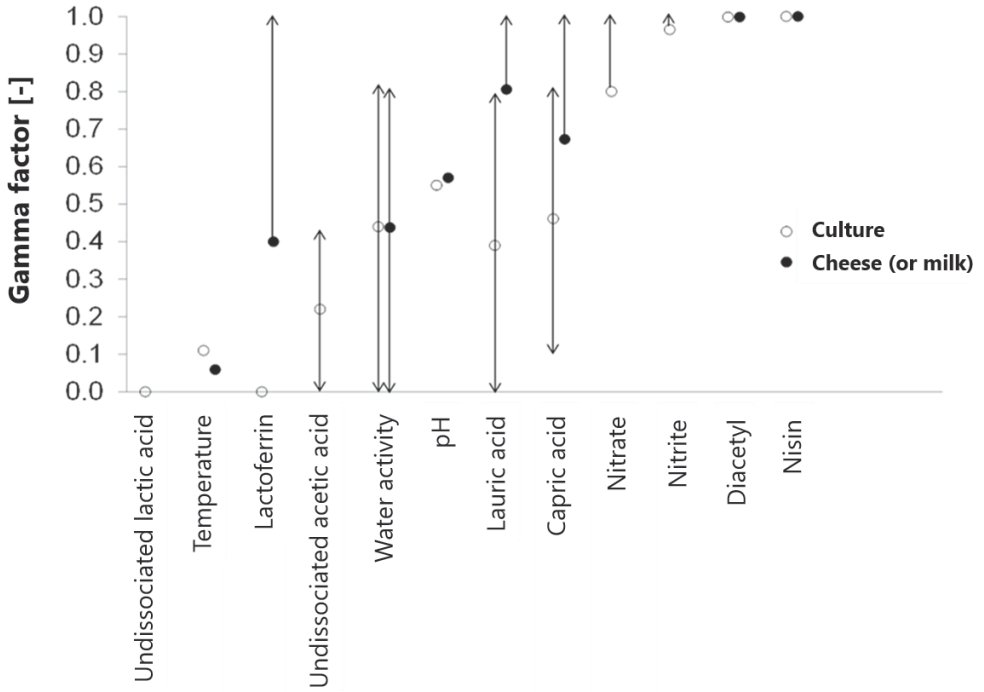

Fig. 6.1 The growth-inhibiting potential (Gamma factor) of undissociated lactic acid, T, lactoferrin, undissociated acetic acid, a_w, pH, lauric acid, capric acid, nitrate, nitrite, diacetyl and nisin for *L. monocytogenes*. Average Gamma factors were calculated for each inhibitor based on the concentration as prevailing in cheese, which was compared with the concentration needed to inhibit growth in culture medium (○) according to the calculations presented in Supplementary Table S3. The concentration as prevailing in cheese was also compared with the concentration needed to inhibit growth in cheese or milk (●), if data were available. The Gamma factor is indicated with an arrow with two ends if the Gamma factor spans a range, in case in the literature minimum and maximum values were available for the concentration in cheese and/or for the concentration needed to inhibit growth of *L. monocytogenes*. An arrow with only an upper end is used for an inhibitor in case growth of *L. monocytogenes* was not inhibited at the highest concentration tested, implicating that for that inhibitor, the actual Gamma factor is higher than the Gamma factor indicated with ○ or ●.

6.3.2 The effect of lactic and acetic acid

In addition to pH, T, and a_w, undissociated lactic acid was evaluated to be the main growth-inhibiting factor for *L. monocytogenes* in Gouda cheese. This factor can explain why the three tested *L. monocytogenes* strains Scott A, 2F and 6E did not grow in a challenge study with Gouda model cheeses. After two weeks of ripening, these cheeses contained 1.40%

lactic acid w/w cheese and had a pH of 5.3 and a moisture content of 48%, equaling $[HLac]_{water}$ of 8.1 mM; after 26 weeks of ripening, when the a_w has approached the critical level for growth of *L. monocytogenes* due to water loss, these cheeses contained 1.40% lactic acid w/w cheese and had a pH of 5.50 and moisture content of 43%, resulting in $[HLac]_{water}$ of 5.8 mM. The MIC values of undissociated lactic acid of these strains at pH 5.4 were 4.3 (SD 0.96) mM for Scott A, 4.1 (SD 1.1) mM for 2F and 4.8 (SD 1.3) mM for 6E (Wemmenhove, van Valenberg, Zwietering, & Wells-Bennik, 2016a) with a maximum determined MIC of 5.8 mM for these strains at pH 5.4 in BHI. Wemmenhove et al. (2016a) determined $[HLac]_{MIC}$ for six strains of *L. monocytogenes* as 4.98 (SD 1.48) mM with a maximum $[HLac]_{MIC}$ of 9.00 mM based on 216 measurements (3 pH values, measurement in 6-fold, individual replicate measurements). Out of the 216 measurements, the $[HLac]_{MIC}$ of 9.00 mM published by Wemmenhove et al. (2016a) was found in eight instances: four times for strain 1F, once for strain EGDe and three times for strain L4, but only at pH 5.6. At pH 5.6, the interval between the determined concentrations of undissociated lactic acid was relatively large (from 5.8 mM to 9.0 mM), resulting in a quite rough estimation of the $[HLac]_{MIC}$ (the true $[HLac]_{MIC}$ is thus between 5.8 and 9.0 mM in this setting).

The $[HLac]_{MIC}$ data from Aryani et al. (2015) were used to evaluate the growth-inhibiting potential of undissociated lactic acid. The small intervals between the determined concentrations of undissociated lactic acid in their study rendered an accurate determination of the true MIC of strains, and a large number of strains of *L. monocytogenes* was studied, permitting an accurate estimation of strain variation. In total, Aryani et al. (2015) established $[HLac]_{MIC}$ for 20 strains of *L. monocytogenes* with an average $[HLac]_{MIC}$ of 5.11 (SD 0.31) mM and a maximum $[HLac]_{MIC}$ of 6.35 mM at pH 5.50. EGDe was one of the strains studied by Aryani et al. (2015) and the maximum determined $[HLac]_{MIC}$ was 5.2 mM. In an additional challenge study with Gouda model cheeses as described by Wemmenhove et al. (2013), no growth of EGDe was observed during ripening of Gouda (unpublished work).

The concentration of undissociated lactic acid present in Gouda cheese was calculated based on the pH, total lactic acid content and moisture content (Fig. 6.2): these parameters affect the concentration of undissociated lactic acid, and thereby its potential to inhibit growth of *L. monocytogenes*. This study focused on nature-ripened Gouda cheese. In such cheeses, an increase in pH and decrease in moisture content is observed during ripening (Wemmenhove et al., 2013; 2016b). The concentration and growth-inhibiting effect of undissociated lactic acid may be slightly different in other types of Gouda cheeses. For instance, in a foil-ripened cheese the moisture content will not decrease during ripening, and certain variants of Gouda cheese are produced using adjunct cultures in addition to the mesophilic starter culture. Such adjunct cultures may have proteolytic, lipolytic or lactate-converting activity, and under such circumstances the pH after ripening may be

slightly higher than in a Gouda that is produced without adjunct cultures. If the concentration of undissociated lactic acid is insufficient to ensure complete growth inhibition, other growth-inhibiting factors become more important.

The distributions of $[HLac]_{water}$ (based on input parameters of Supplementary Table S1 and S2) and $[HLac]_{MIC}$ (obtained from Aryani et al., 2015) are based on a Monte Carlo simulation with 1,000,000 iterations and presented in Fig. 6.3 In the unlikely case that Gouda cheeses were contaminated with *L. monocytogenes*, we predict that undissociated lactic acid will inhibit growth of *L. monocytogenes* in 100% of cases, based on a $[HLac]_{water}$ of 8.32 (SD 0.69) mM from process data of 89 Gouda cheeses obtained from Dutch manufacturers of these cheeses (Fig. 6.3A and dataset of Supplementary Table S1). For another set of Gouda cheeses, $[HLac]_{water}$ was calculated as 11.48 (SD 3.91) mM (Fig. 6.3B and Supplementary Table S2). For this dataset, we predict that undissociated lactic acid will inhibit growth of *L. monocytogenes* in 98.3% of cases. In one out of 20 cheeses, $[HLac]_{water}$ was 5.25 mM, which is below the maximum $[HLac]_{MIC}$ of 6.35 mM. At such a condition, there will be a need for growth-inhibiting factors other than undissociated lactic acid to inhibit growth of *L. monocytogenes* in case of a contamination. The data of Supplementary Table S2 only consisted of 20 measurements and in one out of these 20 cases $[HLac]_{water}$ was <6.35 mM. The standard deviation was much larger for this dataset than for the dataset of process data provided by companies, for which the lowest $[HLac]_{water}$ was 7.07 mM. The food industry can ensure growth inhibition of *L. monocytogenes* in Gouda by controlling the amount of lactic acid and pH and its variation.

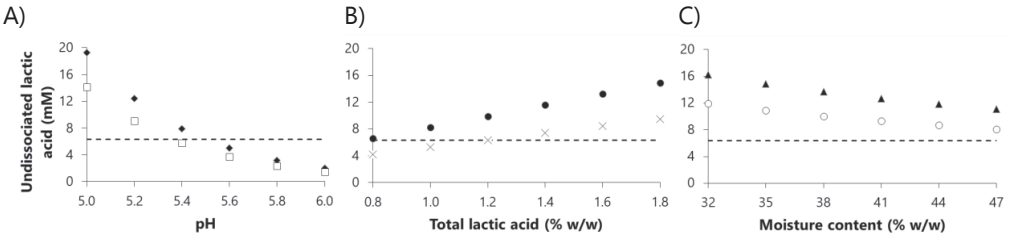

Fig. 6.2 Concentration (mM) of undissociated lactic acid in the water phase of cheese ($[HLac]_{water}$), as dependent on A) pH (based on a total lactic acid content of 1.1 (□) and 1.5 % w/w cheese (◆) and a moisture content of 42% w/w cheese), B) total content of lactic acid (% w/w cheese) (based on a pH of 5.2 (●) and 5.4 (x) and a moisture content of 42% w/w cheese) and C) moisture content (taking a total lactic acid content of 1.1 (○) and 1.5 % w/w cheese (▲) and an average pH of 5.2). The concentration of undissociated lactic acid was calculated as described in Supplementary Material S3, taking into account the effects of dissociation and ion associations and partition in milk fat/water. Calculations were based on a fat content of 48% w/w dry matter, a cheese density of 1070 kg m^{-3}, pK_a of 3.71 and log P_{fw} of -1.5. The dotted line indicates the highest minimum concentration of 6.35 mM of undissociated lactic acid needed to inhibit growth of *L. monocytogenes* in BHI, based on previous results from Aryani et al. (2015).

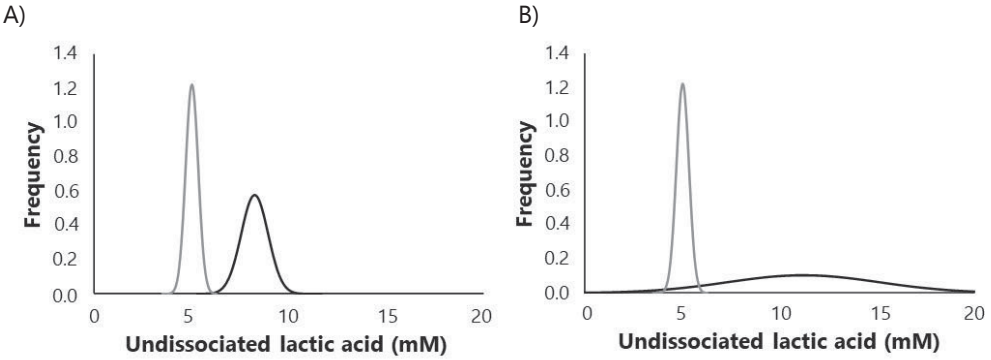

Fig. 6.3 Frequency distribution of the minimal inhibitory concentration of undissociated lactic acid $[HLac]_{MIC}$ for *L. monocytogenes* (indicated with a solid grey line) and of the concentration of undissociated lactic acid in the water phase of cheese $[HLac]_{water}$ (indicated with a solid black line). $[HLac]_{MIC}$, as obtained from Aryani et al. (2015) for 20 *L. monocytogenes* strains, was 5.11 (SD 0.31) mM). $[HLac]_{water}$ is displayed in A) as 8.32 (SD 0.69) mM based on process data of Dutch Gouda cheese-producing companies (n=89) and in B) as 11.48 (SD 3.91) mM based on own measurements (n=20).

When taking $[HLac]_{water}$ above 6.35 mM as a basis for no growth of *L. monocytogenes* in Gouda cheese (based on the maximum $[HLac]_{MIC}$ of Aryani et al., 2015), then full growth inhibition of *L. monocytogenes* is expected at a minimum total lactic acid content of 0.86% w/w for a young cheese with 48% w/w fat in dry matter (pH 5.25 and moisture content 42% w/w) and a minimum total lactic acid content of 1.26% w/w for mature cheese with 48% w/w fat in dry matter (pH 5.50 and moisture content 35%).

Acetic acid is present in Gouda cheese at concentrations of 0.11% w/w cheese, which is around 10 times lower than those of lactic acid (Wemmenhove et al., 2013). Overall, growth inhibition of *L. monocytogenes* by undissociated acetic acid in Gouda with 48% w/w fat in dry matter seems limited, as the calculated concentration of undissociated acetic acid is 9.82 mM at pH 5.25 (based on pK_a = 4.76 and $log\ P_{ow}$ = -0.31 and 42% w/w moisture). The $[HAcet]_{MIC}$ of undissociated acetic acid for *L. monocytogenes* is 19.0 (SD 6.5) mM with a maximum value of 30.2 mM (Wemmenhove et al., 2016a).

6.3.3 The effect of other factors

Other factors with a potential to inhibit growth of *L. monocytogenes* (e.g. diacetyl, free fatty acids, lactoferrin, nitrate, nitrite and nisin) may contribute to growth inhibition in Gouda cheese, but these are less important than T, a_w, pH and lactic acid (Fig. 6.1 and Supplementary Table S3). The free fatty acids capric and lauric acid inhibited growth of

L. monocytogenes in culture medium, but their inhibition efficacy decreased in the presence of emulsifier (as shown for Tween 80) and $CaCl_2$ (Supplementary Table S3), suggesting a poor growth inhibition by capric and lauric acid in a cheese matrix. Nevertheless, free fatty acids may have the potential to inhibit growth of *L. monocytogenes* in other types of cheese (e.g. Emmental, Roquefort), given the fact that free fatty acid concentrations can be 4-150 times higher in those cheese types than in Dutch-type cheeses (Woo, Kollodge, & Lindsay, 1984). Lactoferrin is naturally present in milk and can be present in the cheese as it can withstand pasteurization (Dupont et al., 2006). However, lactoferrin does not contribute to growth inhibition of *L. monocytogenes* to a large extent, considering Gamma factors higher than 0.4 in milk (Fig. 6.1). Conesa et al. (2010) suggested that growth inhibition by lactoferrin can be counteracted by proteins and divalent cations such as Ca^{2+} in rich growth media. The growth-inhibiting effect of lactoferrin can be further reduced during ripening because the protein may be degraded as a result of proteolysis. Gamma factors for nitrate and nitrite were at least 0.8 and 0.97 in BHI (Fig. 6.1), suggesting a non-relevant or very limited growth inhibition for *L. monocytogenes*. The growth-inhibiting effect of diacetyl is even smaller, which is reflected by a Gamma factor of 1.0. A typical Gouda cheese does not contain nisin, reflected by Gamma factor 1.0, but nisin-producing starter bacteria are sometimes applied. In such cases, nisin can inhibit growth of *L. monocytogenes* in cheese (Maisnier-Patin, Deschamps, Tatini, & Richard, 1992; Samelis et al., 2017), but due to proteolysis during ripening, its effect can be reduced over time (Davies, Bevis, & Delves-Broughton, 1997).

6.4 Conclusion

This study provides quantitative insight in the factors that potentially inhibit growth of *L. monocytogenes* in Gouda cheese. Undissociated lactic acid was evaluated as the most important factor for growth inhibition of *L. monocytogenes*. Gouda cheeses have a typical total lactic acid content of 1.47% w/w (and 10.9 mM undissociated lactic acid in 2-week old Gouda with pH 5.25). No growth of *L. monocytogenes* is predicted in Gouda cheese with 48% w/w fat in dry matter and with a total lactic acid content ≥ 0.86% w/w for young cheese (pH < 5.25) and with a total lactic acid content ≥ 1.26% w/w for mature cheese (pH < 5.50). For *L. monocytogenes* in cheeses, it is very relevant that undissociated lactic acid is included as a factor, in addition to T, a_w and pH, in both predictive models and in setting criteria for growth/no growth.

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Supplementary Table S1 $[\mathrm{HLac}]_{\mathrm{water}}$ and its Gamma factors for *L. monocytogenes*, as calculated from data of 89 Gouda cheeses

Cheese no.	Total lactic acid content (% w/w)	Ripe-ning time (weeks)	Moisture content, % (w/w) (assumed)	pH (assu-med)	$[HLac]_{water}$ (mM)	Gamma factor of undissociated lactic acid for *L. monocytogenes* with maximum $[HLac]_{MIC}$ = 6.35 mM
1	1.30	4	40.0	5.30	9.02	0
2	1.50	13	35.0	5.50	7.57	0
3	1.40	13	35.0	5.50	7.07	0
4	1.50	26	31.5	5.50	8.41	0
5	1.30	26	31.5	5.50	7.29	0
6	1.56	15	35.0	5.50	7.85	0
7	1.52	13	35.0	5.50	7.65	0
8	1.57	13	35.0	5.50	7.90	0
9	1.40	8	36.0	5.45	7.67	0
10	1.41	8	36.0	5.45	7.73	0
11	1.39	7	36.0	5.40	8.57	0
12	1.40	6	37.0	5.40	8.39	0
13	1.36	5	39.0	5.35	8.62	0
14	1.33	5	39.0	5.35	8.47	0
15	1.40	4	40.0	5.30	9.70	0
16	1.34	4	40.0	5.30	9.31	0
17	1.35	3	41.0	5.25	10.19	0
18	1.65	14	35.0	5.50	8.31	0
19	1.49	6	37.0	5.40	8.90	0
20	1.41	6	37.0	5.40	8.45	0
21	1.40	6	37.0	5.40	8.38	0
22	1.54	13	35.0	5.50	7.79	0
23	1.43	5	39.0	5.35	9.07	0
24	1.42	5	39.0	5.35	9.02	0
25	1.52	10	35.5	5.50	7.56	0
26	1.70	16	35.0	5.50	8.59	0
27	1.45	9	36.0	5.50	7.11	0
28	1.40	4	40.0	5.30	9.68	0
29	1.56	12	35.5	5.50	7.79	0
30	1.46	11	35.5	5.50	7.24	0
31	1.37	6	37.0	5.40	8.22	0
32	1.62	15	35.0	5.50	8.20	0
33	1.41	7	36.0	5.40	8.70	0
34	1.36	6	37.0	5.40	8.14	0
35	1.43	8	36.0	5.45	7.83	0
36	1.55	10	35.5	5.50	7.73	0
37	1.60	13	35.0	5.50	8.06	0
38	1.34	7	36.0	5.40	8.25	0
39	1.43	8	36.0	5.45	7.83	0
40	1.45	9	36.0	5.50	7.14	0
41	1.36	7	36.0	5.40	8.38	0
42	1.57	14	35.0	5.50	7.91	0
43	1.52	15	35.0	5.50	7.65	0
44	1.49	15	35.0	5.50	7.51	0
45	1.43	15	35.0	5.50	7.24	0
46	1.48	11	35.5	5.50	7.36	0
47	1.60	11	35.5	5.50	7.97	0
48	1.54	12	35.5	5.50	7.67	0

49	1.78	17	34.5	5.50	9.13	0
50	1.51	11	35.5	5.50	7.51	0
51	1.42	6	37.0	5.40	8.47	0
52	1.40	6	37.0	5.40	8.41	0
53	1.42	6	37.0	5.40	8.52	0
54	1.42	6	37.0	5.40	8.53	0
55	1.43	6	37.0	5.40	8.58	0
56	1.40	6	37.0	5.40	8.37	0
57	1.41	6	37.0	5.40	8.41	0
58	1.41	6	37.0	5.40	8.46	0
59	1.34	4	40.0	5.30	9.30	0
60	1.47	6	37.0	5.40	8.80	0
61	1.39	6	37.0	5.40	8.29	0
62	1.43	6	37.0	5.40	8.57	0
63	1.40	6	37.0	5.40	8.37	0
64	1.47	7	36.0	5.40	9.07	0
65	1.25	7	36.0	5.40	7.66	0
66	1.37	4	40.0	5.30	9.47	0
67	1.42	4	40.0	5.30	9.84	0
68	1.36	4	40.0	5.30	9.43	0
69	1.35	4	40.0	5.30	9.39	0
70	1.35	7	36.0	5.40	8.32	0
71	1.40	7	36.0	5.40	8.60	0
72	1.40	7	36.0	5.40	8.60	0
73	1.40	7	36.0	5.40	8.60	0
74	1.45	7	36.0	5.40	8.94	0
75	1.41	7	36.0	5.40	8.65	0
76	1.37	7	36.0	5.40	8.46	0
77	1.46	7	36.0	5.40	9.00	0
78	1.43	7	36.0	5.40	8.77	0
79	1.45	7	36.0	5.40	8.94	0
80	1.47	7	36.0	5.40	9.03	0
81	1.48	9	36.0	5.50	7.28	0
82	1.52	12	35.5	5.50	7.57	0
83	1.43	12	35.5	5.50	7.10	0
84	1.43	12	35.5	5.50	7.10	0
85	1.37	7	36.0	5.40	8.42	0
86	1.36	7	36.0	5.40	8.35	0
87	1.40	7	36.0	5.40	8.64	0
88	1.35	7	36.0	5.40	8.30	0
89	1.36	7	36.0	5.40	8.39	0
Average	1.44	8.65	36.46	5.42	8.32	
S.D.	0.089	4.33	1.73	0.067	0.69	

Data were provided by Dutch companies, all from cheeses with 48 % w/w fat in dry matter. $[HLac]_{water}$ was calculated according to Equation [6.12] from Supplementary Material S3, using a cheese density of 1070 kg m^{-3}, a water density of 998 kg m^{-3}, a milk fat density of 940 kg m^{-3}, pK_a of 3.71, a $log\ P_{fw}$ of -1.5, a molar mass of 90.08 g mol^{-1} for lactic acid. No data on the pH and moisture content were available for the cheeses, only the total lactic acid content and the age of the cheese analyzed were provided by the companies. The age of the cheese was used for assumptions on the pH and moisture content. The Gamma factor of undissociated lactic acid for *L. monocytogenes* was calculated according to Equation [6.5] from Supplemental Material S1, making a worst-case comparison by comparing to the highest MIC determined for *L. monocytogenes* (with maximum $[HLac]_{MIC}$ = 6.35 mM (the upper CI for 1 out of 20 strains of *L. monocytogenes* according to Aryani et al. (2015)). A Gamma factor of 0 indicates full inhibition of growth of *L. monocytogenes* by undissociated lactic acid, even without the need for other growth inhibitors.

References Supplementary Table S1

Aryani, D.C., den Besten, H.M.W., Hazeleger, W.C., & Zwietering, M.H. (2015). Quantifying strain variability in modeling growth of *Listeria monocytogenes*. International Journal of Food Microbiology 208:19-29.

Supplementary Table S2 $[HLac]_{water}$ and its Gamma factor for *L. monocytogenes*, as calculated from 20 Gouda cheeses

Cheese no.	Total lactic acid content, % (w/w)	Moisture content, % (w/w)	pH	$[HLac]_{water}$ (mM)	Gamma factor of undissociated lactic acid for *L. monocytogenes* with $[HLac]_{MIC}$ = 6.35 mM
1	1.42	42	5.22	11.2	0
2	1.43	42	5.22	11.3	0
3	1.14	42	5.14	10.8	0
4	2.10	42	5.26	15.2	0
5	1.48	37	5.22	13.3	0
6	1.43	31	5.10	19.9	0
7	1.55	42	5.34	9.39	0
8	1.43	37	5.51	6.66	0
9	1.25	31	5.42	8.57	0
10	1.32	42	5.44	6.39	0
11	1.20	37	5.54	5.25	0.17
12	1.38	31	5.56	6.84	0
13	1.13	42	5.24	8.57	0
14	1.32	37	5.18	12.9	0
15	1.49	31	5.21	16.3	0
16	1.55	40	5.30	10.9	0
17	1.69	37	5.26	14.0	0
18	1.77	34	5.41	11.3	0
19	1.93	30	5.42	13.6	0
20	1.99	28	5.36	17.3	0
Average	1.50	36.75	5.32	11.48	
S.D.	0.266	4.83	0.13	3.91	

Data on 15 Gouda cheeses were obtained from our previous study (Wemmenhove et al., 2013) and determined in five additional Gouda cheeses for this study. All cheeses contained 48 % w/w fat in dry matter. $[HLac]_{water}$ was calculated according to Equation [6.12] from Supplementary Material S3, using a cheese density of 1070 kg m^{-3}, a water density of 998 kg m^{-3}, a milk fat density of 940 kg m^{-3}, pK_a of 3.71, a $log\ P_{fw}$ of -1.5, a molar mass of 90.08 g mol^{-1} for lactic acid. The Gamma factor of undissociated lactic acid for *L. monocytogenes* was calculated according to Equation [6.5] from Supplemental Material S1, making a worst-case comparison by comparing to the highest MIC value determined for *L. monocytogenes* (with maximum $[HLac]_{MIC}$ = 6.35 mM (the upper CI for 1 out of 20 strains of *L. monocytogenes* according to Aryani et al. (2015)). A Gamma factor of 0 indicates full inhibition of growth of *L. monocytogenes* by undissociated lactic acid, even without the need for other growth inhibitors.

References Supplementary Table S2

Aryani, D.C., den Besten, H.M.W., Hazeleger, W.C., & Zwietering, M.H. (2015). Quantifying strain variability in modeling growth of *Listeria monocytogenes*. International Journal of Food Microbiology 208:19-29.

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Supplementary Table S3 Overview of the growth-inhibiting potentials calculated for the inhibitors

Factor	Concentration in Gouda cheese (X)	Concentration needed to inhibit growth of *L. monocytogenes* (X_{mic}) in culture medium	Concentration needed to inhibit growth of *L. monocytogenes* (X_{mic}) in cheese	Gamma factors	
				Based on $\boldsymbol{X_{mic}}$ in culture medium	Based on $\boldsymbol{X_{mic}}$ in cheese
T	12 °C (Fox et al., 2004)	-0.4 °C (ICMSF, 1996)	4 °C in Graviera cheese of pH 5.3 and a_w 0.95 ripened for 90 days (Giannou, Kakouri, Matija, Rogelj, & Samelis, 2009) 4 °C in Queso Blanco cheese of pH 5.25 incubated for 42 days (Glass et al., 1995)	0.11	0.059
a_w	0.91-0.99 in Gouda cheese aged 0-26 wk (Wemmenhove et al., 2016b)	0.92 (ICMSF, 1996)	0.948 in Graviera cheese of pH 5.3 and a_w 0.95 ripened for 90 days (Giannou et al., 2009)	0-0.88	0-0.81
pH	5.25 (s.d. 0.060) in 2-week old Gouda (based on n=24502 input data from Dutch cheese-producing companies)	4.4 (ICMSF, 1996)	4.32 in milk acidified with 1.5% GDL incubated for five days, whereas growth at pH 4.81 (El-Shenawy & Marth, 1990)	0.55	0.57
Undisso-ciated acetic acid	0.110 % w/w (Wemmenhove et al., 2013), $[HAcet]_{water}$ calculated to be 9.82 mM in the water phase of Gouda cheese at pH 5.25 and 42% w/w moisture, $log\ P_{fw}$ of -0.31 and pK_a 4.76 (this study)	1.2-30.2 mM (Wemmenhove et al., 2016a)	NA	0-0.43	NA
Undisso-ciated lactic acid	1.47 % w/w (see Supplementary Table S1 and S2) of this study), $[HLac]_{water}$ calculated to be 10.9 mM in the water phase of Gouda cheese at pH 5.25 and 42% w/w moisture, $log\ P_{fw}$ of -1.5 and pK_a 3.71 (this study)	4.30-6.35 mM (Aryani et al., 2015)	NA	0	NA

Supplementary Table S3 (continued)

Diacetyl	0.00004 % w/w (Calbert & Price, 1949)	>0.03 % w/v (Lanciotti, Patrignani, Bagnolini, Guerzoni, & Gardini, 2003)	0.025-0.034 % w/w (Jay, 1982)	1.00	1.00
Capric acid	0.0016-0.0018 % w/w (this study)	0.002 – 0.009 % w/v BHI (Petrone et al., 1998); 0.017 % w/v BHI with $CaCl_2$ (this study); 0.086 % w/v BHI with Tween 80 (this study)	> 0.0052 % w/w in Gouda cheese (this study)	0.10-0.82 (without $CaCl_2$ and Tween 80) 0.90 ($CaCl_2$) 0.98 (Tween 80)	>0.67
Lauric acid	0.0022-0.0027 % w/w (this study)	0.0002 – 0.01 % w/v BHI (Wang & Johnson, 1992; Petrone et al., 1998; Sprong et al., 2001; Chen et al., 2014), 0.10 % w/v BHI with $CaCl_2$ (this study); >0.10 % w/v BHI with Tween 80 (this study)	> 0.0126 % w/w in Gouda cheese (this study)	0-0.78 (without $CaCl_2$ and Tween 80) 0.98 ($CaCl_2$) >0.98 (Tween 80)	>0.81
Lactoferrin	0.12 % w/w, but decrease in time (Dupont et al., 2006)	0.010-0.10 % w/v (Murdock, Cleveland, Matthews, & Chikindas, 2007; Ripolles et al., 2015)	> 0.2 % w/v in UHT milk with 2% fat (Branen & Davidson, 2004)	0	>0.40
Nitrate	< 0.020 % w/w in Edam (Goodhead, Gough, Webb, Stadhouders, & Elgersma, 1976)	>0.10 % w/v in BHI (this study)	>0.015 % w/w in Edam (Luukkonen et al., 2005)	>0.80	NA
Nitrite	< 0.00007 % w/w (Renner, 1999)	>0.0020 % w/v in BHI (this study)	NA	>0.97	NA
Nisin	Usually 0.00 % w/w, but if nisin-producing starters are used, nisin concentrations up to 0.044 % w/w can be achieved (van den Heuvel & Smit, 1996), but drastic decrease during ripening.	0.000005-0.013 % w/v (Benkerroum & Sandine, 1988; Bouksaim, Lacroix, Audet, & Simard, 2000; Branen & Davidson, 2004; Martínez, Bravo, & Rodríguez, 2005; Neetoo, Ye, & Chen, 2008)	0.0013-0.040 % w/w (Maisnier-Patin et al., 1992)	1.00 (but can be 0 when nisin-producing starters are used)	1.00 (but can be 0 when nisin-producing starters are used)

The growth-inhibiting potential of T, a_w pH, undissociated acetic and lactic acid, diacetyl, capric acid, lauric acid, lactoferrin, nitrate, nitrite and nisin for *L. monocytogenes* was established by comparing the concentrations needed for inhibition with the concentrations in Gouda cheese. NA indicates that data were not available. The symbol < is used if the concentration of a potential growth-inhibiting factor in Gouda was smaller than listed; the symbol > indicates that growth of *L. monocytogenes* was not inhibited at the highest concentration of the inhibitors tested. No concentrations but levels were established for T, a_w and pH, as the growth-inhibiting potentials of T, a_w and pH rely on levels instead of concentrations.

References Supplementary Table S3

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Supplemental Material S1 Description of the inhibitory effects of T, a_w, pH, lactic and acetic acid, using Gamma factors.

The Gamma factors for T, a_w and pH were described in the literature according to the multiplicative approach established by Zwietering, Wijtzes, de Wit, & van 't Riet (1992):

$$\mu_{max} = \mu_{opt} \cdot \gamma(T) \cdot \gamma(a_w) \cdot \gamma(pH) \cdot \gamma(i) \quad \text{(Eq. 6.1)},$$

with $\gamma(T)$, $\gamma(a_w)$ and $\gamma(pH)$ as the Gamma factors for T, a_w and pH, and with $\gamma(i)$ as the Gamma factors for the other growth-inhibiting factors identified.

The conditions for the factors T, a_w and pH in Gouda cheese are suboptimal for growth of *L. monocytogenes* (e.g. pH < 7.0, a_w < 1.00 and T < 37 °C). The potential of these growth inhibitors on *L. monocytogenes* was calculated from the Gamma factors of T, a_w and pH defined previously by te Giffel and Zwietering (1999):

$$\gamma(T) = \left(\frac{(T - T_{min})}{(T_{opt} - T_{min})}\right)^2 \quad \text{(Eq. 6.2)},$$

with T_{min} = -1.5 °C and T_{opt} = 37 °C.

$$\gamma(a_w) = \frac{a_w - a_{w,min}}{1 - a_{w,min}} \quad \text{(Eq. 6.3)},$$

with $a_{w,min}$ = 0.920.

$$\gamma(pH) = \frac{(pH - pH_{min}) \cdot (2 \cdot pH_{opt} - pH_{min} - pH)}{(pH_{opt} - pH_{min})^2} \quad \text{(Eq. 6.4)},$$

with pH_{min} = 4.4 and pH_{opt} = 7.0.

The Gamma factor for undissociated lactic acid was obtained from the literature:

$$\gamma(x)(undissociated\ lactic\ acid) = 1 - \frac{HLac_{water}}{HLac_{MIC}} \quad \text{(Eq. 6.5)},$$

with $HLac_{water}$ as the concentration of the undissociated acid present in the water phase of the cheese and $HLac_{MIC}$ as the undissociated concentration of the acid found to be needed to inhibit growth of *L. monocytogenes* in culture medium.

Equation [6.5] was slightly altered for undissociated acetic acid based on observations of Le Marc et al. (2002):

$$\gamma(x)(undissociated\ acetic\ acid) = 1 - \sqrt{\frac{HAcet_{water}}{HAcet_{MIC}}} \quad \text{(Eq. 6.6)}.$$

References Supplemental Material S1

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Supplemental Material S2 Experimental design for determination of growth inhibition of *L. monocytogenes* by nitrate and nitrite in BHI and by capric acid and lauric acid in BHI and in model cheeses.

Growth inhibition by nitrate and nitrite in BHI

Inhibitory effects of nitrate (VWR, Amsterdam, The Netherlands) and nitrite (VWR) were established in Brain Heart Infusion (BHI, Merck, Darmstadt, Germany) set at pH 7.0. Inhibition was investigated for three individual strains of *L. monocytogenes*: Scott A (serotype 4b, human isolate from a listeriosis outbreak with pasteurized milk), 2F (serotype 1/2a, cheese isolate) and 6E (serotype 1/2a, cheese equipment isolate). Strains were grown overnight in BHI before exposure and added at a final initial concentration of ~$2.5 \cdot 10^6$ cfu mL^{-1}. The exposure experiment included a positive and negative control and was performed in 96-well microtiter plates (Greiner Bio-One, Frickenhausen, Germany) in which successive concentrations of nitrate (5 times serially diluted, concentrations varying from 0.063 g L^{-1} to 1.0 g L^{-1}) and nitrite (5 times serially diluted, concentrations varying from 0.0013 g L^{-1} to 0.020 g L^{-1}) were incubated at 30 °C for 24 h. The growth/no growth limits were established based on optical density measured at 630 nm using an EL808 IU-PC spectrophotometer (Bio-Tek, Winooski, Vermont, USA). No growth was defined as the concentration of the component where no increase in optical density greater than 0.05 (compared to the negative control) was observed in three separate wells in two independent experiments. Growth/no growth limits were confirmed by pour-plating on PALCAM-*Listeria* selective agar (VWR).

Capric acid and lauric acid in BHI

The experiments in BHI (Merck) were performed with and without 4.4 g L^{-1} $CaCl_2$ (Merck) and 11 g L^{-1} emulsifier Tween 80 (Merck). The individual inhibitory effects of capric and lauric acid on growth of *L. monocytogenes* were evaluated at 0, 0.2, 1.0 and 5.0 mM in 96-well microtiter plates containing filter sterilized BHI of pH 5.2 or 6.6. Stock solutions (200 mM) of capric acid (Fluka, Buchs, Switzerland) and lauric acid (Fluka) were prepared by dissolving capric and lauric acid in 0.1 M KOH (Scharlau Chemie SA, Barcelona, Spain). The stock solutions of capric and lauric acid were dissolved at 45 °C and were then kept for 24 h at 37 °C. Prior to the exposure, the stock solutions were diluted in sterile physiological peptone solution (Biotrading Benelux, Mijdrecht, The Netherlands) warmed at 37 °C to keep the free fatty acids dissolved but prevent exposure of *L. monocytogenes* to a high concentration of KOH in BHI. Each well was inoculated with an overnight culture of *L. monocytogenes* Scott A (initially ~10^7 cfu mL^{-1}) and then continuously stirred at 60 rpm for 24 h at 30 °C. Each experiment included a negative control and all experiments were performed in triplicate.

Capric acid and lauric acid in model Gouda cheeses

The inhibitory potential of free fatty acids on *L. monocytogenes* in Gouda cheese was established using the microcheese protocol (Bachmann, Kruijswijk, Molenaar, Kleerebezem, & van Hylckama Vlieg, 2009; Wemmenhove et al., 2013). Lipase from a non-pathogenic, non-toxigenic strain of *A. niger* (Sigma-Aldrich, St Louis, MO, USA) was added to part of the microcheeses to obtain elevated concentrations of free fatty acids (e.g. 47 (SD 5) mM capric acid and 119 (SD 10) mM lauric acid instead of 17 (SD 2) mM capric acid and 24 (SD 4) mM lauric acid, as determined in two individual experiments according to de Jong & Badings (1990). The lipase was prediluted in a phosphate buffer of pH 7.0, kept overnight refrigerated and then added at 0.8 g L^{-1} cheese milk just prior to addition of rennet and DL-starter culture. Simultaneously, *L. monocytogenes* Scott A cultured overnight in BHI (Merck) was added at ~10^7 cfu mL^{-1} to simulate a worst-case scenario with a severe recontamination immediately after pasteurization of the cheese milk. Samples were taken from the inoculated cheese milk, the first and second whey and from the curds 0.7, 4 and 24 h after renneting. *L. monocytogenes* was enumerated in duplicate by serial dilution in peptone physiological salt solution and pour-plating on PALCAM-*Listeria* selective agar, including a negative control.

References Supplemental Material S2

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Supplemental Material S3 Calculation of the concentration of undissociated lactic acid in the water phase of Gouda cheese.

Assuming that Gouda cheese is a homogeneous product in which organic acid is evenly distributed, the total amount of organic acid present in cheese in % w/w cheese can be converted to the total organic acid concentration (mol L^{-1}) in the cheese. Then the concentration of undissociated acid can be calculated using the Henderson-Hasselbalch equation:

$$pK_a = -\log\frac{[H^+][A^-]}{[HA]} \quad \text{(Eq. 6.7).}$$

Following this equation and the previous assumptions, the following relationship applies:

$$[HA] = \frac{[Total\ acid]}{1 + 10^{pH-pK_a}} \quad \text{(Eq. 6.8).}$$

However, Gouda cheese is actually an inhomogeneous product, consisting of water, fat, and water bound to proteins and non-protein/non-fat dry matter. The concentration of undissociated acid should actually be calculated in the water phase, as acids inhibit growth of bacteria mainly through their undissociated form and when present in the water phase (Mitchell, 1961) and we believe only organic acid in the water phase will access *L. monocytogenes*, which is primarily located on interface of the fat /protein structure of cheese (Kalab, 1979).

Taking lactic acid, being the major organic acid in Gouda cheese, as an example for this calculation, cheese was divided into three different phases:

proteins+minerals+added salt;

water, containing $HLac$, Lac^- and H^+;

-and fat, containing $HLac$.

Organic acids may be present in both the water phase (in the undissociated or dissociated form) and the fat phase (in the undissociated form) of cheese. This yields in:

$$Total\ lactic\ acid = HLac_{water} + Lac^-_{water} + HLac_{fat} \quad \text{(Eq. 6.9).}$$

in which $Total\ lactic\ acid$ is the total amount of lactic acid formed in the cheese.

The concentration of undissociated lactic acid that is present in the water phase strongly depends on the volume (V) of water, which decreases during brining and ripening of the cheese:

$$V_{cheese} = V_{water} + V_{fat} + V_{protein+minerals+salt} \quad \text{(Eq. 6.10),}$$

with V_{water} representing the volume of water that can contain undissociated lactic acid.

The logarithmic partition coefficient ($\log P_{fw}$) of lactic acid between milk fat and water (fw) can be established by the following equation:

$$\log P_{fw} = \log\frac{[HLac]_{fat}}{[HLac]_{water}} \quad \text{(Eq. 6.11).}$$

Equations [6.7], [6.9], [6.10] and [6.11] can be rewritten:

$$Total\ lactic\ acid = HLac_{water} + Lac^{-}{}_{water} + HLac_{fat}$$

$$1 = \varphi_{v\_water} + \varphi_{V\_fat} + \varphi_{v_{proteins,minerals,salt}}$$

$$V_{cheese} \cdot [HLac]_{Total} = [HLac]_{water} \cdot V_{water} + [Lac^{-}] \cdot V_{water} + [HLac]_{fat} * V_{fat}$$

$$[HLac]_{Total} = [HLac]_{water} \cdot \varphi_{v\_water} + \frac{K_a \cdot [HLac]_{water}}{[H^{+}]} \cdot \varphi_{v\_water} + P_{fw} \cdot [HLac]_{water} \cdot \varphi_{V\_fat}$$

$$[HLac]_{Total} = [HLac]_{water} \left(\varphi_{v\_water} + \frac{K_a}{H^{+}} \cdot \varphi_{v\_water} + P_{fw} \cdot \varphi_{V\_fat}\right).$$

This leads to an overall expression for $[HLac]_{water}$:

$$[HLac]_{water} = \frac{[HLac]_{Total}}{\varphi_{v\_water} + 10^{pH-pKa} \cdot \varphi_{v\_water} + P_{fw} \cdot \varphi_{V\_fat}} \qquad \text{(Eq. 6.12),}$$

in which $[HLac]_{Total}$ = total lactic acid present within the cheese, $[HLac]_{water}$ = undissociated lactic acid present in the watery fraction of the cheese, $[Lac^{-}]_{water}$ = dissociated lactic acid present in the watery fraction of the cheese, $[HLac]_{fat}$ = undissociated lactic acid present in the fat fraction of the cheese. Components surrounded by square brackets are expressed as concentrations (M) and components not surrounded by square brackets are expressed as content (mol). Furthermore, φ_{v_water}, φ_{V_fat} and $\varphi_{v_{proteins,minerals,salt}}$ indicate the volume fractions of water, fat and proteins/minerals/salt, and V_{water} and V_{fat} indicate the volumes of water and fat. pK_a is the logarithmic constant of the dissociation constant for lactic acid and P_{fw} describes the partition of undissociated lactic acid between fat and water (Eq. 6.11).

References Supplemental Material S3

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Supplemental Material S4 Determination of pK_a and $log\ P_{fw}$ for undissociated lactic acid in Gouda cheese.

pK_a

The pK_a for lactic acid is 3.86 in water containing only $HLac$, H^+ and Lac^- ions (Lide, 2006). The pK_a of lactic acid in cheese is lower, as Ca^{2+} and Na^+ Ca^{2+} and Na^+ are present in cheese at high concentrations (Holland, Unwin, & Buss, 1989), allowing them to complex with Lac^- and therewith limit complexation of Lac^- with H^+ (Martell & Smith, 1977; Morris, Holt, Brooker, Banks, & Manson, 1988; Gao, 2010), lowering the actual pK_a in the water phase of cheese and thus also lowering the actual concentration of $HLac$ present in cheese. Morris et al. (1988) analyzed juice from a 4-week old Cheddar cheese and determined $HLac$ of 10.2 mM, Lac^- of 337.7 mM and H^+ of 5.89·10-3 mM, resulting in a calculated pK_a of lactic acid of 3.71. We assumed the same pK_a for lactic acid in Gouda as in Cheddar, as Gouda resembles Cheddar in pH, ion composition and ionic strength (Holland et al., 1989).

$log\ P_{fw}$

The logarithmic octanol/water partition coefficient for $HLac$ is -0.6. We determined the logarithmic partition coefficient of $HLactate$ between milk fat and water ($logP_{fw}$), as cheese contains no octanol but milk fat, which could be more apolar than octanol, and therefore a lower logarithmic partition coefficient of $HLac$ can be expected for milk fat than for octanol. The $logP_{fw}$ for $HLactate$ was established based on $[HLac]_{total}$ and $[HLac]_{water}$ by making samples containing known amounts of anhydrous milk fat, L-lactic acid and HCl and rotating these samples for 24 h before analysis of the concentration of lactic acid in the water phase ($[HLac]_{water}$) and to the initial concentration of lactic acid that was added to the sample ($[HLac]_{total}$), as the following accounts in the milk fat sample:

$$HLac_{total} = HLac_{milk\ fat} + HLac_{water} \quad \text{(Eq. 6.13).}$$

Anhydrous milk fat (AMF) was obtained from pasteurized cream containing 99.99% milk fat (FrieslandCampina, Noordwijk, The Netherlands). L-lactic acid (Alfa Aesar, Karlsruhe, Germany) was diluted to an initial concentration of 0.01 M. AMF and L-lactic acid were carefully weighed before mixing in different ratios (25/75, 50/50 and 75/25). AMF was liquefied at 40 °C before L-lactic acid was added. A known amount of 1.0 M HCl (Merck) was then added to the samples to lower the pH to 2.8 to make sure all lactic acid was undissociated. Subsequently, the samples were rotated around the transverse axis (20 rpm) on a PTR-60 mechanical shaker (Grant Instruments Ltd., Shepreth, United Kingdom). Rotation for 24 h was followed by equilibration of the samples for 1 h at 21 °C and centrifugation (4000 g x 15 min) at 5 °C to ensure the separation of the water and fat phase and to prevent inclusion of fat in the water phase. After centrifugation, the water phase of each sample was obtained by use of a syringe and the concentration of lactic acid was analyzed at a wavelength of 210 nm by RP-HPLC using a Diode Array Detector with an eluent of a 20 mM potassium phosphate buffer of pH 2.5 and a flow of 1 mL min^{-1}. The $\log P_{fw}$ was determined in duplicate. The determination included negative controls (1.0 M HCl and the water phase from demineralised water that was mixed with AMF) and a calibration curve for lactic acid (0, 1, 5, 10 and 50 mM). No lactic acid was detected in the negative controls, so the concentration of lactic acid present in milk fat by nature was negligible, and lactic acid was only introduced in the sample by addition of 0.010 M L-lactic acid.

Logarithmic partition coefficient ($\log P_{fw}$) of lactic acid between milk fat and water, as determined in three different mass fractions of AMF and water containing 0.01 M lactic acid (75% AMF and 25% water, 50% AMF and 50% water, 25% AMF and 75% water) and rotating these samples for 24 h at 21 °C. The concentration of lactic acid was analyzed in the water phase ($[\mathrm{HLac}]_{\mathrm{water}}$) and compared to the concentration of lactic acid that was initially added to the sample ($[\mathrm{HLac}]_{\mathrm{total}}$). The $\log P_{fw}$ of lactic acid was determined in duplicate.

	$\log P_{fw}$ **(duplo 1)**	$\log P_{fw}$ **(duplo 2)**	$\log P_{fw}$ **(average)**
75% AMF/25% water:	-1.4	-1.3	-1.4
50% AMF/50% water:	-1.6	-1.4	-1.5
25% AMF/75% water:	-1.9	-1.5	-1.7
Average of all $\log P_{fw}$ values determined:			-1.5

References Supplemental Material S4

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Chapter 7

General discussion

7.1 Introduction

Ensuring the safety of foods that are placed on the market is paramount to the food industry. This relates to many different hazards, for instance, chemical contaminants, microbial contaminants, foreign bodies and allergens. Amongst the microbial contaminants, many different pathogenic foodborne bacteria can pose a threat to the health of consumers when present in foods. One of these is the pathogen *Listeria monocytogenes*, which can cause listeriosis. This pathogen is of particular concern to ready-to-eat foods, *i.e.* products that do not undergo a heat treatment prior to consumption. Food product categories that have been associated with listeriosis outbreaks include meat, seafood and dairy products. In cases of listeriosis that have been associated with dairy products, especially consumption of raw milk and soft cheeses that were contaminated with *L. monocytogenes* have been linked with the disease. Listeriosis has only sporadically been linked with consumption of semi-hard and hard cheeses, and in cases in which such cheeses were involved, those cheeses were possibly made from raw milk. *L. monocytogenes* may be present in raw milk at low concentrations, but pasteurization is an effective and widely used procedure to reduce the concentrations of the pathogen in finished products. *L. monocytogenes* may be persistent in the processing environment and thus, potential contamination of finished products must be controlled as well.

An important cheese type for the Dutch dairy industry is Gouda cheese. Gouda cheese has been categorized in various ways by scientists and legislators with respect to its risk related to *L. monocytogenes*. The Codex General Standard for Cheese (Codex, 2013) classifies cheese based on the moisture on a fat free basis (MFFB). Gouda, with an MFFB between 53 and 63% (van den Berg, Meijer, Düsterhöft, & Smit, 2004), is considered a firm/semi-hard cheese. The classification of cheese by the Food and Drug Administration (FDA, 2003) of the USA is based on the moisture on whole cheese content (moisture%). Gouda, having a moisture% of 39 to 50%, is put in the group of semi-soft cheeses by the FDA, together with blue, brick, Monterey and Münster cheese. For the group of semi-soft cheeses, the relative risk of association with *L. monocytogenes* causing listeriosis is classified as low (FDA, 2003). In the European food safety criteria for RTE foods EC 2073/2005 (European Commission, 2005), products are categorized based on their ability to support growth of *L. monocytogenes*.

Gouda has a pH > 5.0 and a_w > 0.94. Taking only pH and a_w criteria into account, Gouda falls into category 1.2 of products that may be able to support the growth of *L. monocytogenes* (with a maximum limit of *L. monocytogenes* 100 cfu g^{-1} with n=5 for products placed on the market during their shelf-life*, or absence in 25 g with n=5 before the food has left the control of the food business operator, who has produced it**), unless it can be proven that the product does not support growth.

Gouda can be made from pasteurized or raw milk. Gouda cheese, when made from pasteurized milk, has never been linked to listeriosis and the only published challenge study of *L. monocytogenes* in Gouda (Northolt, Vecht, Toepoel, Soentoro, & Wisselink, 1988) showed survival but no growth of *L. monocytogenes* during 6 weeks of ripening.

The aim of the work presented in this thesis was to gain a better understanding of factors that inhibit outgrowth of *L. monocytogenes* in Gouda cheese and to establish criteria related to Gouda cheese composition and properties that warrant safety with respect to *L. monocytogenes*.

The thesis encompasses five studies (**Chapters 2**-**6**). The fate of *L. monocytogenes* in and on Gouda was assessed by performing two challenge studies. In the first challenge study, *L. monocytogenes* was added to cheese milk after pasteurization and the fate of the pathogen in Gouda cheese was assessed using micro Gouda cheeses (**Chapter 2**). The second challenge study was performed using whole Gouda cheeses that were contaminated with *L. monocytogenes* on the outside during brining (**Chapter 3**). The variation in a_w during the production and ripening of Gouda cheese was evaluated in **Chapter 4**, and the variation in growth inhibition of *L. monocytogenes* by organic acid under conditions relevant to Gouda cheese was assessed in **Chapter 5**. In **Chapter 6**, the most important factors that determine growth / no growth of *L. monocytogenes* in Gouda cheese were evaluated based on the minimal inhibitory concentrations (MIC) of antimicrobial substances relevant to Gouda cheese and the actual concentration present in Gouda. Additionally, a criterion was established for growth inhibition of *L. monocytogenes* in Gouda cheese due to the undissociated lactic acid concentration.

In the following paragraph [7.2] the factors that contribute to the absence of growth of *L. monocytogenes* in Gouda cheese are addressed. Some insights in the fate of *L. monocytogenes* in curd are described in paragraph 7.3. To understand the importance of the identified factors that have potential to inhibit growth of *L. monocytogenes* in various cheeses other than Gouda, growth / no growth of *L. monocytogenes* was evaluated in 10

*This criterion applies if the manufacturer is able to demonstrate, to the satisfaction of the competent authority, that the product will not exceed the limit 100 cfu g^{-1} throughout the shelf-life. The operator may fix intermediate limits during the process that must be low enough to guarantee that the limit of 100 cfu g^{-1} is not exceeded at the end of shelf-life.
**This criterion applies to products before they have left the immediate control of the producing food business operator, when he is not able to demonstrate, to the satisfaction of the competent authority, that the product will not exceed the limit of 100 cfu g^{-1} throughout the shelf-life.

different types of cheeses in section 7.4. The focus of the work described in this thesis was on nature-ripened whole Gouda cheeses with 48% w/w fat in dry matter, made from pasteurized milk. The expected fate of *L. monocytogenes* in other Gouda cheeses (e.g. low-salt, low-fat, slices) is discussed in section 7.5. In the subsequent section 7.6, the importance of undissociated lactic acid as a safety factor in cheese to control *L. monocytogenes* and implications for applying legislation are discussed. The overall conclusions and recommendations are described in section 7.7.

7.2 Gouda cheese does not support growth of *L. monocytogenes*

In the studies described in this thesis, it was demonstrated that growth of *L. monocytogenes* is not supported during ripening of Gouda cheese made from artificially contaminated milk. In the studies described, no growth but only survival and inactivation of *L. monocytogenes* was observed during 2-52 weeks in Gouda cheese that was contaminated with *L. monocytogenes* during curd formation (**Chapter 2**). The results are in line with the study of Northolt et al. (1988), who reported survival but no growth of *L. monocytogenes* in Gouda cheese throughout ripening for 1-6 weeks. In a follow-up study described in **Chapter 3**, Gouda cheese was contaminated during brining, and inactivation of *L. monocytogenes* was observed after 2-12 weeks of ripening. The most important factors that are present in Gouda cheese and have an impact on growth of *L. monocytogenes* were identified as undissociated lactic acid, temperature, pH and a_w (**Chapter 6)**. From these four factors, undissociated lactic acid and a_w specifically can lead to full growth inhibition of *L. monocytogenes* in Gouda. Subsequent limits that do not allow for growth of *L. monocytogenes* were established for lactic acid: no growth is predicted at a total lactic acid content of 0.86% w/w for young Gouda with pH 5.25 and 42% moisture, and 1.26% w/w for matured Gouda with pH 5.50 and 35% moisture. Full inhibition of growth of *L. monocytogenes* by a_w in Gouda can be expected if $a_w < 0.92$.

The work presented in this thesis focused on the fate of *L. monocytogenes* in finished product, *i.e.* ready-to-eat Gouda cheese. This product leaves the production facilities of the manufacturer at the earliest 2 weeks after production and is subsequently ripened for at least 2 more weeks. The European food safety criteria for RTE foods (EC 2073/2005) apply to finished ready-to-eat products.

7.3 Fate of *L. monocytogenes* during curd formation of Gouda

In the microcheese study presented in **Chapter 2**, an increase of 0.5 log cfu g^{-1} was observed during curd formation. This increase was not caused by growth of *L. monocytogenes*, but by

entrapment of *L. monocytogenes* cells in the curd during whey removal. During curd formation of Gouda, the lactic acid and salt concentrations are relatively low and the temperature is favorable for growth of *L. monocytogenes*. An increase in the concentration of *L. monocytogenes* during curd formation was also reported in other challenge studies (Buazzi, Johnson, & Marth, 1992a; Dominguez, Garayzabal, Vazquiz, Blanco, & Suarez, 1987; Northolt et al., 1988). Unfortunately, these authors did not specify whether the observed increases resulted from growth or from entrapment in the curd. An increase of *L. monocytogenes* in the curd of -0.5 to 0.4 log cfu g^{-1} was reported for high-moisture Mozzarella (Buazzi et al., 1992a); for semi-hard cheese an increase of 0.2 to 0.7 log cfu g^{-1} was found (Dominguez et al., 1987), and for Gouda cheese, a 1.6 log cfu g^{-1} has been reported before (Northolt et al., 1988). The highest increase of *L. monocytogenes* of 1.6 log cfu g^{-1} reported by Northolt et al. (1988) is difficult to interpret, as the measuring point was 6 h after inoculation of the starter culture and the measurement was only performed in singular. Details on curd processing, e.g. amounts of whey and curd, and acidification rate, were lacking in that study. If the time between inoculation and cheese pressing would be 6 h and if the pH would only be 5.5 after this time, the acidification rate may have been lower than the acidification rate that is common in current practice. Nowadays, the time between addition of the starter culture to the cheese milk and transformation into a curd that is ready to be pressed is only 3.3 h. At the low acidification rate that appeared to occur during production of Gouda cheese in the study of Northolt et al. (1988), an increase of *L. monocytogenes* of 1.6 log cfu g^{-1} in the curd during curd formation could theoretically be expected (Table 7.1). When considering both growth and entrapment in the curd, an increase of *L. monocytogenes* of 2.7 log cfu g^{-1} is predicted from milk to curd when using μ_{opt} = 1.69 h^{-1} (Table 7.1 and Fig. 7.1a), which is the maximum optimal growth rate of *L. monocytogenes* as extracted from www.combase.cc for milk at temperatures ranging from 30 to 37 °C. An increase of 1.7 log cfu g^{-1} is predicted when using μ_{opt} = 0.73 h^{-1}, which is the average optimal growth rate as extracted from www.combase.cc. When not considering entrapment of *L. monocytogenes* in the curd but only growth, increases of 1.7 and 0.74 log cfu are predicted during curd formation when using μ_{opt} = 1.69 h^{-1} and μ_{opt} = 0.73 h^{-1}, respectively (Table 7.1 and Fig. 7.1b). The predicted increases did not include lag times or other stress factors that may reduce growth of *L. monocytogenes*. In practice, a lag time may be present, which Buazzi et al. (1992a) suggested earlier when studying the fate of *L. monocytogenes* in high-moisture Mozzarella cheese. The μ_{opt} values for *L. monocytogenes* extracted from www.combase.cc were based on milk. The actual μ_{opt} values may be lower for cheese and curd, as these are semi-solid liquids that could immobilize *L. monocytogenes* in the matrix. Other reasons for the limited growth *'in situ'* as shown in **Chapter 2** may be growth competition with starter cultures (Beresford, Fitzsimons, Brennan, & Cogan, 2001) and competitive exclusion due to the presence of lactic acid bacteria (Zhao, Doyle, & Zhao, 2004).

Table 7.1 Concentration (log N) of *L. monocytogenes* during curd formation, following predictions and challenge data. The initial inoculum concentration was 7.8, 7.7 and 7.4 log cfu g^{-1} for strains Scott A, 2F and 6E in the challenge study of Wemmenhove et al. (2013), and 2.7 log cfu g^{-1} in the challenge study of Northolt et al. (1988). These initial concentrations were normalized in the table to 7.7 log cfu g^{-1} equaling the average initial inoculum concentration according to Wemmenhove et al. (2013). This was done to simplify the comparison between the challenge studies and predictions. Values of temperature (T), pH, a_w and undissociated lactic acid (HLac) are based on expert knowledge. For certain values, no knowledge was available and values have been assumed (indicated with **). Log N values are presented in log cfu g^{-1} when including entrapment of *L. monocytogenes* in the curd and growth of *L. monocytogenes* during curd formation, and in log cfu (originally from 1 mL or g) to correct for the effect of entrapment of the curd and thus only look at growth / no growth of *L. monocytogenes* during curd formation. Predicted log N values, using μ_{opt} = 1.69 h^{-1} and 0.73 h^{-1} (as extracted from Combase, www.combase.cc, when searching for growth rates of *L. monocytogenes* in milk at 30-37 °C), were based on $\gamma_T = \left(\frac{T--1.5}{37--1.5}\right)^2$, $\gamma_{pH} = \frac{(pH-4.4)\cdot(2\cdot7-4.4-pH)}{(7-4.4)^2}$, $\gamma_{a_w} = \frac{a_w-0.92}{1-0.92}$ and $\gamma_{HLac} = 1 - \frac{[HLac]}{6.35}$. Predictions were compared to results from challenge studies, in which a curd before pressing was obtained 3.3 h after inoculation of *L. monocytogenes* into the milk according to Wemmenhove et al. (2013) and 6 h after inoculation according to Northolt et al. (1988)

Process stage			Milk	Renneted milk	First curd	First curd before whey separation	Second curd, 3.3 h after inoculation according to Wemmenhove et al. (2013)	Cheese 6 h after inoculation according to Northolt et al. (1988)
T	value (°C)		30.5	30.5	30.5	35.5	35.5	21**
	Gamma factor		0.69	0.69	0.69	0.92	0.92	0.34
pH	value		6.7	6.6	6.4	6.1	5.6	5.5**
	Gamma factor		0.99	0.98	0.95	0.88	0.71	0.66
a_w	value		0.995	0.994	0.994	0.994	0.994	0.992**
	Gamma factor		0.94	0.93	0.93	0.93	0.93	0.90
HLac	value (mM)		0	0.0025**	0.015**	0.024**	1.4**	3.0**
	Gamma factor		1.0	1.0	1.0	1.0	0.78	0.53
Total t (h) after inoculation of cheese milk			0	0.33	1.0	2.17	3.33	6.0
Log N calculated based on μ_{opt} = 1.69 h^{-1}		log cfu g^{-1}	7.7	7.8	9.1	9.7	10.1	10.4
		log cfu	7.7	7.8	8.1	8.7	9.1	9.4
Log N calculated based on μ_{opt} = 0.73 h^{-1}		log cfu g^{-1}	7.7	7.7	8.8	9.1	9.3	9.4
		log cfu	7.7	7.7	7.8	8.1	8.3	8.4
Log N for Scott A (Wemmenhove et al., 2013)		log cfu g^{-1}	7.7	7.3	7.8	7.6	8.0	ND
		log cfu	7.7	7.3	6.8	6.6	7.0	ND
Log N for 2F (Wemmenhove et al., 2013)		log cfu g^{-1}	7.7	7.1	7.6	7.5	8.1	ND
		log cfu	7.7	7.1	6.6	6.5	7.1	ND
Log N for 6E (Wemmenhove et al., 2013)		log cfu g^{-1}	7.7	6.9	7.9	8.0	8.5	ND
		log cfu	7.7	6.9	6.9	7.0	7.5	ND
Log N for cocktail 1, 13 & 669 (Northolt et al., 1988)		log cfu g^{-1}	7.7	ND	ND	ND	ND	9.2
		log cfu	7.7	ND	ND	ND	ND	8.2

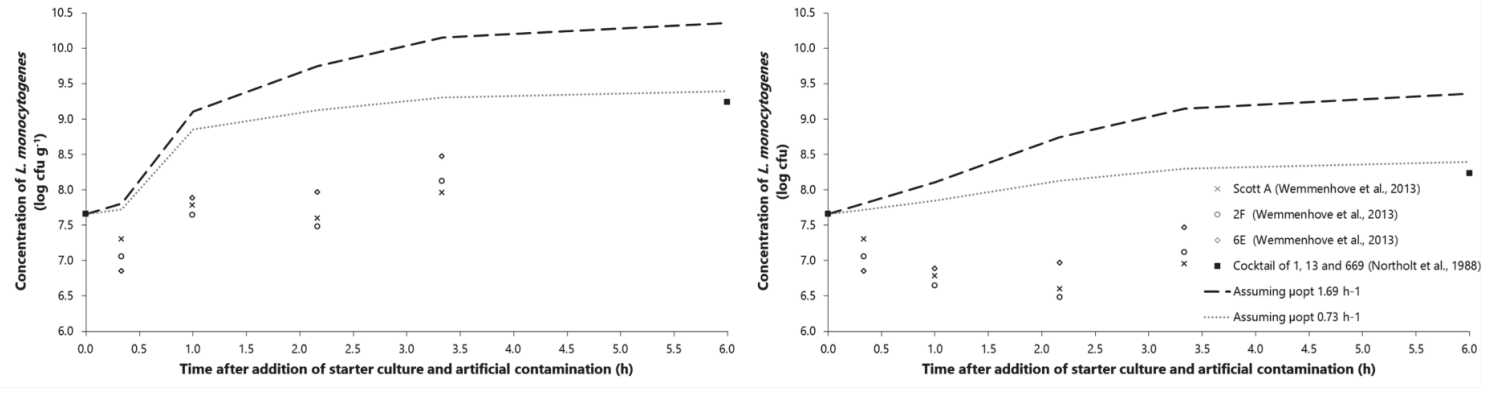

Fig. 7.1 Predicted and actual fate of *L. monocytogenes* during curd formation, indicating the importance of the factors undissociated lactic acid, temperature, a_w and pH for growth inhibition of *L. monocytogenes* during curd formation of Gouda cheese. Data are presented in A) log cfu g^{-1} when including entrapment of *L. monocytogenes* in the curd and growth of *L. monocytogenes* during curd formation, and B) log cfu to correct for the effect of entrapment of the curd and thus only look at growth / no growth of *L. monocytogenes* during curd formation. Symbols indicate the data extracted from the challenge study with model Gouda cheeses of **Chapter 2** and Northolt et al. (1988). The lines indicate the predicted fate of *L. monocytogenes* in Gouda cheese using the Gamma model without interaction as described in **Chapter 6**, assuming an initial concentration of *L. monocytogenes* of $5 \cdot 10^7$ cfu g^{-1} as chosen in **Chapter 2**, a MIC of undissociated lactic acid of 6.35 mM according to Aryani et al. (2015), and using μ_{opt} = 1.69 h^{-1} (the maximum μ_{opt} depicted with dark stripes) and μ_{opt} = 0.73 h^{-1} (average μ_{opt} depicted with grey dots) as extracted from Combase (www.combase.cc) when searching for growth rates of *L. monocytogenes* in milk at 30-37 °C.

7.4 The importance of undissociated lactic acid, temperature, pH and a_w in other types of cheeses

The contribution of undissociated lactic acid, temperature, pH and a_w toward the control of *L. monocytogenes* during ripening of Gouda cheese and nine other cheese types is evaluated below. Per cheese type, available data were extracted from published challenge studies with respect to the fate of *L. monocytogenes*. In these studies, cheeses were artificially contaminated with *L. monocytogenes* via the surface/crust, or via the milk/curd. In addition, the growth rates were calculated based on the average values of the aforementioned factors that are relevant to the respective cheeses, whilst using the maximum and average μ_{opt} values extracted from www.combase.cc for milk at 30–37 °C, the Gamma factors described in Supplementary Material S1 of **Chapter 6**, and the concentration of undissociated lactic acid calculated as described in Supplementary Materials S3 and S4 of **Chapter 6**. The growth rates calculated from the challenge studies and the growth rates of *L. monocytogenes* as predicted based on the characteristics of the different cheeses were compared per cheese type (Fig. 7.2). Similar to Gouda cheese, the same four factors (undissociated lactic acid, temperature, pH and a_w) appeared to be important for growth inhibition of *L. monocytogenes* in/on the other types of cheeses. The cheese types selected for the comparison represented all ready-to-eat (RTE) cheese categories identified by the FDA (FDA 2003): fresh soft cheese (Queso Fresco), soft unripened cheeses with >50% moisture (Cottage cheese and Ricotta), soft ripened cheeses with >50% moisture (Camembert, Feta and high-moisture Mozzarella), semi-soft cheeses with 39-50% moisture (Blue and Gouda) and hard cheeses with <39% moisture (Cheddar and Emmental). For 9 (namely, Ricotta, Queso Fresco, Camembert, high-moisture Mozzarella, Cottage, Blue, Feta, Cheddar and Gouda) out of the above mentioned 10 cheese types, correct predictions of growth / no growth are obtained when including the factors undissociated lactic acid, temperature, pH and a_w in the prediction. Growth was correctly predicted for Ricotta, Queso Fresco, Camembert, high-moisture Mozzarella, Cottage and Blue), and no growth was correctly predicted for Feta, Cheddar and Gouda. These results show the importance of undissociated lactic acid, temperature, pH and a_w for cheese in general. These four factors do not fully explain why growth of *L. monocytogenes* is inhibited in practice upon inoculation in cheese or on the surface of Emmental. In the case of Emmental, acetic acid, propionic acid and free fatty acids are possibly important factors for inhibition of growth of *L. monocytogenes*, in addition to temperature, pH and a_w. Inhibition of growth of *L. monocytogenes* by acetic and propionic acid is described in **Chapter 5**, and by free fatty acids in **Chapter 6**. The actual growth rates of *L. monocytogenes* in and on high-moisture Mozzarella that have been reported are considerably higher than the predicted growth rates, possibly because the actual growth rates were established at worst-case conditions for pH, whereas the predicted growth rates were based on average conditions for undissociated lactic acid, pH and a_w.

The model predictions as presented in this thesis for cheese are based on the Gamma model without interaction between the individual factors (**Chapter 6, Supplemental Material S1, S3 and S4**), but interactions may exist. Gadotti, Nelson, & Diez-Gonzalez (2014) reported that a combination of nisin with free fatty acids can fully inhibit growth of *L. monocytogenes* in Queso Fresco. In another study it was shown that lactoferrin alone did not inhibit growth of *L. monocytogenes* in UHT milk, but in combination with nisin some growth inhibition was observed (Branen & Davidson, 2004). Proper understanding of the mechanism of action of each growth-inhibiting factor is necessary to be able to select effective combinations of factors that inhibit growth and to establish an effective control strategy.

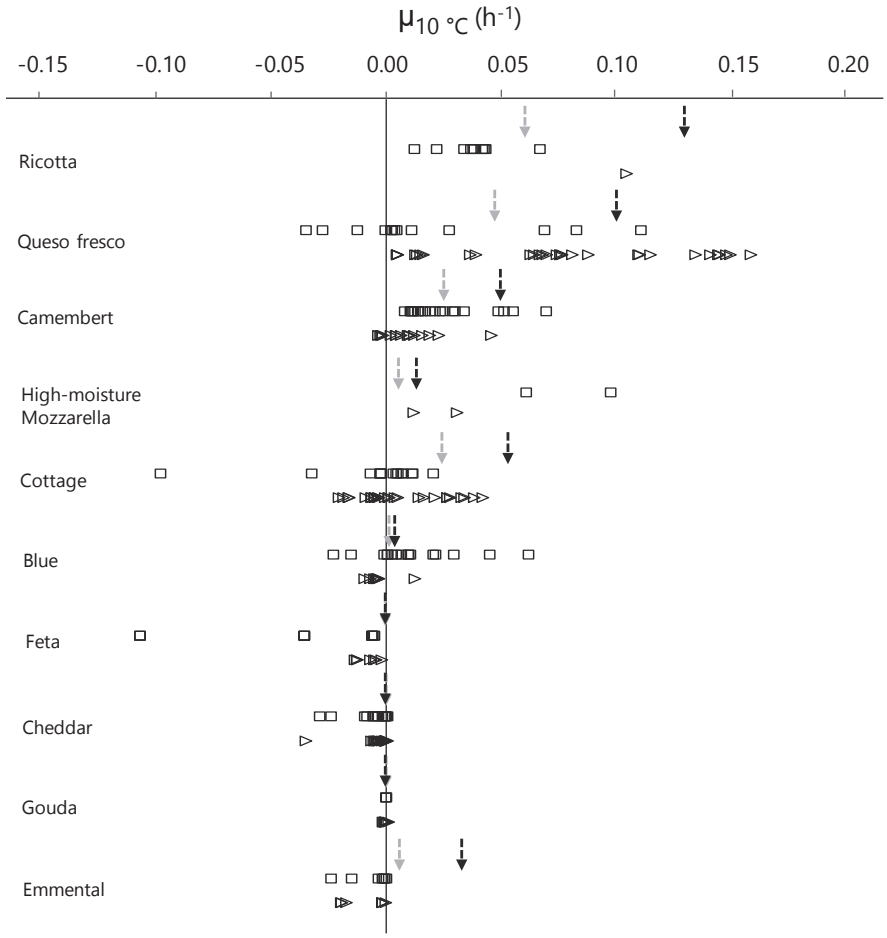

Fig. 7.2 Comparison of the actual and predicted growth rates at 10 °C, to validate the importance of the factors undissociated lactic acid, a_w and pH on growth inhibition of *L. monocytogenes* for 10 different RTE cheese types. The actual growth rates of *L. monocytogenes* were extracted from 45 challenge studies (n = 249 for 10 cheese types, data described in **Supplementary Table S2**) were compared to predicted growth rates (**Table 7.2 and Supplementary Table S1**). Per cheese type, the squares display the growth rates for *L. monocytogenes* after artificial contamination of the surface or crust, and the triangles display the growth after artificial contamination of the cheese curd or milk. For the predicted growth rates, μ_{max} values were calculated for each type of cheese using $\frac{\Delta \log N \cdot \ln(10)}{t}$, with Δlog N as the log concentration of *L. monocytogenes* at the end of sampling minus the log concentration after pressing, and with t as the time between the end of sampling and after pressing. The predicted growth rates ($\mu_{10\ °C}$ values) were based on μ_{opt} = 1.69 h^{-1} and μ_{opt} = 0.73 h^{-1} as extracted from Combase (www.combase.cc) when searching for growth rates of *L. monocytogenes* in milk at 30-37 °C. The $\mu_{10\ °C}$ values calculated with μ_{opt} = 1.69 h^{-1} were indicated with black arrows, and those calculated with μ_{opt} = 0.73 h^{-1} were indicated with grey arrows. The $\mu_{10\ °C}$ values were based on Gamma factors for undissociated lactic acid, a_w, temperature and pH calculated according to **Chapter 6 Supplemental Material S1, S3 and S4**), and a MIC of undissociated lactic acid for *L. monocytogenes* of 6.35 mM according to Aryani et al. (2015). Average conditions were chosen for undissociated lactic acid, a_w and pH (according to **Table 7.2**). The temperature was normalized to 10 °C by use of the square-root function of McMeekin, Olley, Ross, & Ratkowsky (1993): $\mu_T = \mu_{ref} \cdot \frac{(T-T_{min})^2}{(T_{ref}-T_{min})^2}$, with T_{min} = -1.5 °C and T_{ref} = 10 °C. The application of the square-root function by McMeekin et al. (1993) for temperature is only validated for positive actual growth rates. As for the positive actual growth rates, the negative growth rates were normalized to 10°C using the function, but the latter was not scientifically substantiated in this thesis.

Table 7.2 Average values for total lactic acid content, moisture content, fat content, pH, a_w, temperature (T) and the calculated undissociated lactic acid and Gamma factors (as calculated from these average values and from the formulas in Supplementary Table S1). The Gamma factor for undissociated lactic acid was calculated using a MIC of 6.35 mM (the maximum MIC of undissociated lactic acid for *L. monocytogenes* as established by Aryani et al. (2015)). The predicted growth rates were calculated based on the calculated Gamma factors, using μ_{opt} = 1.69 h^{-1} and 0.73 h^{-1}, as extracted from Combase (www.combase.cc) when searching for growth rates of *L. monocytogenes* in milk at 30-37 °C. The Gamma factors are displayed in italics. In addition to the predicted growth rate μ_{max}, the growth rate $\mu_{10\,°C}$ was calculated, which is the growth rate μ_{max} using the average Gamma factors for pH, a_w and undissociated lactic acid but normalizing the temperature to 10 °C by use of the square-root function of McMeekin et al. (1993): $\mu_T = \mu_{ref} \cdot \frac{(T-T_{min})^2}{(T_{ref}-T_{min})^2}$, with T_{min} = -1.5 °C and T_{ref} = 10 °C

Type of cheese	Cheese category, as grouped by FDA (2003).	Total lactic acid content	Moisture content	Fat content	pH		a_w		T		Undissociated lactic acid		μ_{max} (h^{-1}) calculated based on μ_{opt} = 1.69 h^{-1}	μ_{max} (h^{-1}) calculated based on μ_{opt} = 0.73 h^{-1}	$\mu_{10\,°C}$ (h^{-1}) calculated based on μ_{opt} = 1.69 h^{-1}	$\mu_{10\,°C}$ (h^{-1}) calculated based on μ_{opt} = 0.73 h^{-1}
		Value (g per kg cheese)	Value (g per 100 g cheese)	Value (g per 100 g cheese)	Value	*Gamma factor*	Value	*Gamma factor*	Value (°C)	*Gamma factor*	Value (mM)	*Gamma factor*				
Blue	Semi-soft	10.8	40.5	32.6	5.75	*0.77*	0.925	*0.06*	11.5	*0.11*	2.7	*0.58*	0.0054	0.0023	0.0042	0.0018
Camembert	Soft ripened	10.0	52	23.7	5.8	*0.79*	0.97	*0.62*	8	*0.06*	1.7	*0.73*	0.037	0.016	0.054	0.023
Cheddar	Hard	15.0	37.5	34.4	5.2	*0.52*	0.95	*0.37*	10	*0.09*	13.9	***0***	**0**	**0**	**0**	**0**
Cottage	Soft unripened	0.29	79.5	3.9	5.05	*0.44*	0.99	*0.88*	17	*0.23*	0.17	*0.97*	0.15	0.063	0.056	0.024
Feta	Soft ripened	14.0	53.5	20.2	4.65	*0.18*	0.96	*0.50*	5.5	*0.03*	29.8	***0***	**0**	**0**	**0**	**0**
Gouda	Semi-soft	14.7	38.5	31.0	5.25	*0.55*	0.945	*0.31*	12.5	*0.13*	11.8	***0***	**0**	**0**	**0**	**0**
High-moisture Mozzarella	Soft ripened	8.25	47.2	21.0	5.5	*0.67*	0.94	*0.25*	4	*0.02*	3.09	*0.51*	0.0030	0.0013	0.013	0.0056
Queso Fresco	Fresh soft	1.0	48.3	19.0	6.03	*0.86*	0.985	*0.81*	10	*0.09*	0.11	*0.98*	0.10	0.045	0.10	0.045
Ricotta	Soft unripened	1.93	60.3	30.5	6.5	*0.96*	0.995	*0.94*	11	*0.11*	0.057	*0.99*	0.16	0.069	0.13	0.058
Emmental	Hard	9.0	36.7	29.7	5.7	*0.75*	0.97	*0.62*	14	*0.16*	2.8	*0.57*	0.015	0.064	0.040	0.017

7.5 Perspectives for low-fat, low-salt and sliced Gouda

This thesis focused on nature-ripened whole Gouda cheese of 48% w/w fat in dry matter made from pasteurized milk. In addition to this type of Gouda cheese, the Dutch cheese industry produces low-salt cheese, low-fat cheese, foil-ripened cheese and pre-packed Gouda cheese, such as cheese slices or cheese wedges. The expected fate of *L. monocytogenes* in Gouda produced with such characteristics or stored under such conditions is described below.

The a_w can be an important factor contributing to the inhibition of growth of *L. monocytogenes* in Gouda, and inactivation of *L. monocytogenes* was seen when the a_w drops to values near or below the minimal a_w limit of 0.92 (**Chapter 4 and 6**). Such low a_w values can occur after extended ripening times or immediately after brining in the crust. In a low-salt cheese, the Gamma factor of a_w for *L. monocytogenes* is higher than in standard Gouda cheeses, so the growth-inhibiting effect of a_w on *L. monocytogenes* in Gouda cheese is smaller. Nevertheless, growth of *L. monocytogenes* at a low salt content was not observed in the challenge studies described in **Chapter 3** (*L. monocytogenes* on Gouda, artificial contamination of the brine), and **Chapter 2** (*L. monocytogenes* in Gouda model cheeses). Inhibition of growth is primarily controlled by lactic acid. After an extended period of time, inactivation of *L. monocytogenes* seemed to occur which can likely be attributed to the low a_w present. The inactivation of *L. monocytogenes* seemed slower in low-salt cheeses in which the average a_w is higher than in cheeses with normal salt contents. Furthermore, the contribution of salt to inactivation of *L. monocytogenes* may be smaller within the core of the cheese than the inactivation as observed in the model cheese study of **Chapter 2**. In industrially produced cheeses, salt diffuses during ripening from the crust to the core (**Chapter 4**), but in such cheeses that are made from pasteurized milk, the presence of *L. monocytogenes* inside the cheese is unlikely. In the challenge study with microcheeses described in **Chapter 2**, *L. monocytogenes* was added to the cheese milk after pasteurization, and salt was added directly after pressing in the microcheeses; therewith, no salt gradient between crust and core was present at the start of ripening.

In low-fat Gouda cheeses, the risk of outgrowth of *L. monocytogenes* is not expected to be higher than in standard Gouda cheese, primarily because there is enough undissociated lactic acid to suppress the growth of *L. monocytogenes*, as explained below. No differences were observed with respect to the fate of *L. monocytogenes* during curd formation in additional experiments with model Gouda cheeses with 48% and 30% w/w fat in dry matter (results not shown). Fat (in a cheese with equal dry matter) only minimally affects the concentration of undissociated lactic acid, as can be deduced from Equation [6.12] in **Chapter 6**. In Gouda cheeses with 30% w/w fat in dry matter, the moisture content is often increased to compensate for texture changes due to the lowered fat content. An increased moisture content leads to an increase in the a_w (**Chapter 4**) and a decrease in the concentration of undissociated lactic acid

(**Chapter 6**, Fig. 6.3C)**.** In a Gouda cheese with 30% w/w fat in dry matter, full growth inhibition can be expected when the pH <5.25 and moisture <50.5%, because the concentration of undissociated lactic acid in the water phase of the cheese would then still be above the critical concentration of 6.35 mM that leads to full inhibition of growth of *L. monocytogenes*. Leong et al. (2014) studied the fate of *L. monocytogenes* in normal-fat versus low-fat cheese with high moisture content for Cheddar, Colby Jack and Provolone slices that were artificially contaminated, vacuum packaged and stored at room temperature. No significant differences were observed with respect to the fate of *L. monocytogenes* after 15-day storage of normal-fat and low-fat cheeses (-0.73 vs 0.41 log cfu g^{-1} for normal-fat vs low-fat Cheddar, -0.32 vs -0.27 log cfu g^{-1} for normal-fat vs low-fat Colby Jack, and -0.92 vs -1.68 log cfu g^{-1} for normal-fat vs low-fat Provolone). In Cheddar cheese, reduction of the fat in dry matter content from 48 to 36% did not alter the fate of *L. monocytogenes* according to Mehta & Tatini (1994), even when the moisture content of the low-fat Cheddar was 42.9% instead of 39.7% in the normal-fat Cheddar. In both studies, the concentration of lactic acid was not determined.
The risk of presence of *L. monocytogenes* may be different in pre-packed sliced Gouda cheese. On slices of Gouda cheese that were artificially contaminated with *L. monocytogenes* and subsequently vacuum packed, Leong et al. (2014) and Kapetanakou, Gkerekou, Vitzilaiou, & Skandamis (2017) observed survival but no growth, and subsequent inactivation of *L. monocytogenes*, thus showing a fate that is similar to the one seen during ripening of whole Gouda cheeses (Northolt et al., 1988; **Chapter 2**). Further assessment of the data displayed in Fig. 7.2 showed that the fate of *L. monocytogenes* after contamination via the surface was in general similar to the fate after contamination via the cheese curd or milk. Based on these observations, the fate of *L. monocytogenes* in cheese slices is expected to be similar to the one in or on whole Gouda cheese, if the microbial hurdles present (e.g. concentration of undissociated lactic acid, temperature, pH and a_w) are not altered due to cutting, packaging or other treatments.

7.6 Implications for legislation and undissociated lactic acid as a classification criterion

This thesis focused on nature-ripened whole Gouda cheese made from pasteurized milk with 48% w/w fat in dry matter. This cheese should currently be classified as category 1.2 RTE product according to EC 2073/2005 based on pH > 5.0 and a_w > 0.94, in absence of further evidence that the product does not support the growth of *L. monocytogenes*. Gouda made from pasteurized milk can be categorized as an RTE product that does not support growth of *L. monocytogenes*, based on the evidence presented in this thesis, namely, two challenge studies that showed no growth, but only survival and inactivation, and predictive modelling demonstrating the factors that explain growth inhibition of *L. monocytogenes* in Gouda cheese.

Overall, Gouda has an average total lactic acid content of 1.47 % w/w and pH 5.25 (**Chapter 6**). Undissociated lactic acid can fully inhibit growth when the concentration of undissociated lactic acid in cheese is ≥ 6.35 mM on the basis of the highest MIC observed for *L. monocytogenes* (Aryani, den Besten, Hazeleger, & Zwietering, 2015). In an average 2-week old Gouda (moisture content of 42%, a total lactic acid content of 1.47 % w/w cheese and a pH of 5.25), the concentration of undissociated lactic acid was calculated to be 10.9 mM. A concentration of undissociated lactic acid of 6.35 mM is reached when the total lactic acid concentration is >0.86% w/w at a pH <5.25 (relevant to young Gouda cheese with moisture content 42% w/w), or >1.26% w/w at a pH <5.50 (relevant to a mature Gouda cheese with moisture content of 35% w/w) (**Chapter 6**).

Undissociated lactic acid is thus very important for growth inhibition of *L. monocytogenes* in Gouda cheese and therefore it seems justified that RTE cheeses are classified based on their undissociated lactic acid concentration, and not only on pH and a_w. The importance of undissociated lactic acid is underlined by the calculated Gamma factors for 10 cheese types using a MIC of undissociated lactic acid for *L. monocytogenes* of 6.35 mM and average values for total lactic acid content, moisture content, fat content, pH, a_w and temperature (Table 7.2). This calculation showed that in 3 out of 10 cheese types (namely, Feta, Cheddar and Gouda), the Gamma factor for undissociated lactic acid is 0, implying that undissociated lactic acid alone fully inhibits growth of *L. monocytogenes* in these three cheeses.

Temperature and pH can be important growth inhibiting factors for *L. monocytogenes*, but typical pH values and ripening temperatures of Gouda cheese will not lead to full inhibition of growth of *L. monocytogenes* (**Chapter 6**). The pH has a large effect on growth inhibition of *L. monocytogenes*, as it indirectly affects the concentration of undissociated lactic acid. Spoilage by yeasts and molds can result in an increase of the pH and subsequently a reduced inhibition of growth as a result of pH and undissociated lactic acid. A low temperature can delay or completely prevent spoilage by yeasts and molds. However, in the case of RTE cheeses, the T_{min} of -0.4 °C for *L. monocytogenes* is not reached and therefore it is advised to not include temperature as a criterion for inhibition of growth of *L. monocytogenes* in RTE cheeses.

In Table 7.3, a suggested categorization according to EU regulation EC 2073/2005 for 10 RTE cheese types is presented. The categorization is based on whether or not the consumption of the cheese was associated with identified cases of listeriosis in the past, and whether or not growth rates extracted from challenge studies and those predicted based on growth-inhibiting factors were positive or not. The data presented lend support to categorization of Gouda, Feta and Cheddar as a category 1.3 food with ≤ 100 cfu g^{-1} (n = 5) for products placed on the market during their shelf-life. Feta has an average pH of 4.65 and a total lactic acid content 14 g per kg cheese; the undissociated lactic acid concentration is thus calculated to be 29.8 mM (Table 7.2). Cheddar has an average pH of 5.2 and a total lactic acid content of 15 g per kg cheese, resulting in an undissociated lactic acid concentration of 13.9 mM (Table 7.2). In all

challenge studies with Cheddar and Feta, no growth of *L. monocytogenes* was observed (Fig. 7.2). For high-moisture Mozzarella, growth was both observed and predicted, therefore categorization as a 1.2 food is suggested, especially in high-moisture Mozzarella-type cheeses with pH >5.18 (then <6.35 mM undissociated lactic acid is calculated). The cheeses Queso Fresco and Camembert can be categorized as category 1.2 foods, requiring absence of *L. monocytogenes* in 25 g (n = 5) before the food has left the immediate control of the food business operator, who has produced it. In Queso Fresco and Camembert, average pH values are 6.0 and 5.8 and average total lactic acid contents are 1 and 10 g per kg cheese, respectively, corresponding with undissociated lactic acid concentrations of 0.11 mM and 1.7 mM, respectively. As the calculated concentrations of undissociated lactic acid are much lower than the average and maximum MICs of 5.11 and 6.35 mM, respectively, for *L. monocytogenes* (**Chapter 5**), it is unlikely that undissociated lactic acid is an important growth-inhibiting factor in these cheeses. In practice, Queso Fresco and Camembert cheeses have been linked with listeriosis cases on several occasions, and growth of *L. monocytogenes* was supported in challenge studies that were performed with these cheeses. When a food is categorized as an RTE food product able to support growth of *L. monocytogenes*, more stringent sampling guidelines need to be in place. In addition, the food safety risk of cheeses that can support growth of *L. monocytogenes* can be decreased by applying additional hurdles and different storage regimes (e.g. shorter shelf life, refrigerated storage) (Maisnier-Patin, Deschamps, Tatini, & Richard, 1992; Davies, Bevis, & Delves-Broughton, 1997; Soni, Nannapaneni, Schilling, & Jackson, 2010; Soni, Desai, Oladunjoye, Skrobot, & Nannapaneni, 2012; Spanu et al. 2012). In the case of cottage cheese, sorbic acid may be added which inhibits growth of *L. monocytogenes* (Østergaard, Eklöw, & Dalgaard, 2014; Østergaard, Christiansen, & Dalgaard, 2015).

The μ_{max} values were calculated for the different cheeses based on average values of undissociated lactic acid, temperature, pH and a_w. For most cheeses, only average values were reported in literature. For some cheeses, the variation was assessed for some of the factors (Supplementary Table S1). In studies available in literature, however, it is not clear for each type of cheese whether the variation occurred during processing or whether it occurred between batches. Because of this unclarity, the μ_{max} values were only calculated from the average values as stated above. The estimation of the μ_{max} values can be improved further by considering the variation and worst-case conditions (e.g. low undissociated lactic acid, and high temperature, pH and a_w) in addition to average conditions for each type of cheese.

7.7 Conclusion and recommendations

Gouda cheese does not support growth of *L. monocytogenes* as demonstrated in all three challenge studies published so far. The most important factors for growth inhibition were

identified as undissociated lactic acid, temperature, pH and a_w. From these four factors, undissociated lactic acid and a_w can specifically in Gouda lead to full growth inhibition of *L. monocytogenes*. Undissociated lactic acid alone can explain absence of growth of *L. monocytogenes* in Gouda cheese. Additionally, low a_w in the cheese rind and after prolonged ripening times can cause full growth inhibition. Even though temperature considerably affects the actual growth rate of *L. monocytogenes* in Gouda cheese, low temperature alone does not result in full growth inhibition in Gouda cheese, as temperature does not approximate a value at which the growth of *L. monocytogenes* is completely halted in Gouda. As undissociated lactic acid is evaluated as the primary factor determining growth / no growth factor Gouda and many other RTE cheese types, it is recommended to include undissociated lactic acid in future safety criteria for RTE cheeses, together with pH and a_w. The cheese-making industry can monitor the total lactic acid content, the moisture content, and the pH of cheese to calculate the undissociated lactic acid concentration in the water phase of Gouda cheese. If the latter is ≥6.35 mM, this can be applied as a criterion for non-growth of *L. monocytogenes*.

This work focused on nature-ripened whole Gouda cheeses. According to studies performed with cheese slices and foil-ripened cheeses, the fate of *L. monocytogenes* on slices of Gouda and in foil-ripened Gouda cheeses is expected to be similar to that in whole cheeses. In future challenge studies, it is recommended to additionally measure pH, temperature, a_w, and the content of lactic acid so that the impact of the levels of these factors on the fate of *L. monocytogenes* can be established. It is also recommended to generate more data on pH, temperature, lactic acid content, other organic acids and a_w during manufacturing, ripening and storage of cheeses other than Gouda. Such data contribute to a better understanding on the variation of these factors during manufacturing and storage and thereby to a better understanding of the effect of manufacturing, ripening and storage steps on growth/no growth of *L. monocytogenes* in cheese. For Emmental, further study of growth inhibition by organic acids other than lactic acid and/or free fatty acids is recommended.

In this thesis, challenge tests, literature data and predictive models have been used to perform a risk assessment of *L. monocytogenes* in relation to Gouda cheese, ultimately leading to practical advice to the cheese industry and legislators.

Overall, the work presented in this thesis confirmed that Gouda cheese does not support growth of *L. monocytogenes*. The factors with inhibitory potential toward *L. monocytogenes* in Gouda cheese were evaluated, and it was shown that undissociated lactic acid is the primary factor that determines growth/no growth of *L. monocytogenes* in Gouda cheese. Furthermore, it was determined that the levels of undissociated lactic acid that are present in Gouda are sufficiently high to ensure complete inhibition of growth. The knowledge generated in this thesis can be applied by manufacturers of Gouda cheese and authorities.

Table 7.3 Suggestion for classification of different types of cheese. Suggestion is based on the association with listeriosis in the past (+ when linked), the minimum and maximum value for $\frac{\Delta \log N \cdot \ln(10)}{t}$ as calculated from data from challenge studies (+ when positive values were obtained; +/- when sometimes positive values and – when no positive values were obtained for $\frac{\Delta \log N \cdot \ln(10)}{t}$) and the predicted growth rates (μ_{max}) using the Gompertz equation and the Gamma model with average values of the individual factors undissociated lactic acid, temperature (T), pH and a_w in the cheese as listed in Table 7.2, with t_{lag} = 0 h and μ_{opt} = 1.69 h^{-1} and 0.73 h^{-1} (equaling the maximum and average optimum growth rates for *L. monocytogenes* in milk as extracted from Combase (www.combase.cc) when searching for growth rates of *L. monocytogenes* in milk at 30-37 °C, and a MIC of undissociated lactic acid for *L. monocytogenes* of 6.35 mM according to Aryani et al. (2015)

Type of cheese	Cheese category and risk ranking according to FDA (2003)	Associated with identified cases of listeriosis in the past (based on Table 1.4)? If 'Yes', this results in a '+'.	Minimum and maximum $\frac{\Delta logN \cdot \ln(10)}{t}$ (h^{-1}) extracted from challenge studies. If positive, this results in a '+'.	μ_{max} (h^{-1}) calculated with the Gamma model taking the average values for undissociated lactic acid, temperature, pH and a_w, each growth inhibitor from Table 7.2, and μ_{opt} = 0.73 h^{-1} or μ_{opt} = 1.69 h^{-1}. If > 0, this results in a '+'.	Suggestion for category (based on association with listeriosis, maximum $\frac{\Delta logN \cdot \ln(10)}{t}$ and μ_{max} values
Ricotta	Soft unripened -> moderate risk	Yes +	0.0049 to 0.32 +	0.069 or 0.16 +	1.2, absence in 25 g (n = 5) before the food has left the immediate control of the food business operator, who has produced it
Queso Fresco	Fresh soft -> low risk	Yes +	-0.0089 to 0.038 +/-	0.045 or 0.10 +	1.2, absence in 25 g (n = 5) before the food has left the immediate control of the food business operator, who has produced it
Camembert	Soft ripened -> low risk	Yes +	-0.0062 to 0.084 +/-	0.016 or 0.037 +	1.2, absence in 25 g (n = 5) before the food has left the immediate control of the food business operator, who has produced it
Cottage	Soft unripened -> low risk	No -	-0.13 to 0.029 +/-	0.063 or 0.15 +	1.2, absence in 25 g (n = 5) before the food has left the immediate control of the food business operator, who has produced it
Blue	Semi-soft -> low risk	Yes +	-0.011 to 0.042 +/-	0.0023 or 0.0054 +	1.2, absence in 25 g (n = 5) before the food has left the immediate control of the food business operator, who has produced it

Table 7.3 (continued)

High-moisture Mozzarella	Soft ripened -> low risk	No -	0.017 to 0.086 +	0.0013 or 0.0030 +	1.2, absence in 25 g (n = 5) before the food has left the immediate control of the food business operator, who has produced it
Emmental	Hard -> low risk	No -	-0.029 to -0.00085 -	0.0064 or 0.015 +	1.2, absence in 25 g (n = 5) before the food has left the immediate control of the food business operator, who has produced it But possibly 1.3 with ≤100 cfu g^{-1} (n = 5) for products placed on the market during their shelf-life, if there is enough scientific proof that factors that are present in this type of cheese but that are not included in the predictions presented in this thesis (e.g. propionic acid; acetic acid; free fatty acids), can inhibit growth of *L. monocytogenes* in this type of cheese
Gouda	Semi-soft -> low risk	No -	-0.0033 to 0.0010 -	0 or 0 -	1.3, ≤ 100 cfu g^{-1} (n = 5) for products placed on the market during their shelf-life
Cheddar	Hard -> low risk	No -	-0.067 to 0.000 -	0 or 0 -	1.3, ≤ 100 cfu g^{-1} (n = 5) for products placed on the market during their shelf-life
Feta	Soft ripened -> low risk	No -	-0.049 to -0.00055 -	0 or 0 -	1.3, ≤ 100 cfu g^{-1} (n = 5) for products placed on the market during their shelf-life

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Supplementary Table S1 Overview of data on parameters in cheese types selected, as extracted from literature. Calculation of undissociated lactic acid is described in **Chapter 6**

Type of cheese	pH	a_w	Ripening temperature (°C)	Moisture content (g 100 g^{-1} cheese)	Fat content (g 100 g^{-1} cheese)	Total lactic acid content (g kg^{-1} cheese)	Undissociated lactic acid (mM) calculated	References
Blue	4.7-6.8	0.91-0.94	8-15	37-44	29.6-35.5	0.1-21.4	2.7	Madkor, Fox, Shalabi & Metwalli (1987); Schaffer, Tatini & Baer (1995); Fox et al. (2004); Wolf, Perotti & Zalazar (2011); Rosshaug, Detmer, Ingmer & Larsen (2012); Diezhandino, Fernández, González, McSweeney & Fresno (2015)
Camembert	4.6-7.0	0.97	8	52	23.7	10.0 on average (25.0 in fresh cheese; 1.0 in ripened cheese)	1.7	Fox, Law, McSweeney & Wallace (1993); Engel, Niklaus, Septier, Salles & Le Quéré (2001); Fox et al. (2004)
Cheddar	5.2	0.95	10	37-38	34.4	15	13.9	Fox et al. (1993); Schaffer et al. (1995); Fox et al. (2004)
Cottage	4.6-5.5	0.99	17	76-83	3.9	0.12-0.45	0.17	Brocklehurst & Lund (1985); Chen & Hotchkiss (1993); Fox et al. (2004)
Feta	4.5-4.8	0.96	5.5	53-54	20.2	14.0	29.8	Michaelidou (1997); Fox et al. (2004); Manolakia, Katsiarib & Alichanidis (2006); Belessi, Papanikolaou, Drosinos & Skandamis (2008)
Gouda	5.05-5.45	0.92-0.97	12.5	32-45	31	14.7	11.8	Northolt et al. (1988); Fox et al. (2004); **Chapter 2**; **Chapter 3**; **Chapter 5**
High-moisture Mozzarella	4.9-6.2	0.94	4	47.2	21	8.25	11.7	Esti et al. (1996); Fox et al. (2004); Kapetanakou et al. (2017)
Queso Fresco	5.25-6.8	0.97-1.00	10	40-56.5	19	<1.0 (Queso blanco)	0.11	Torres & Chandan (1981); Fox et al. (1993); Engel et al. (2001), van Hekken & Farkye (2003); Fox et al. (2004); Uhlich et al. (2006); Guo, van Hekken, Tomasula, Shieh & Tunick (2011); Soni et al. (2012); van Hekken, Tunick, Leggett & Tomasula (2012)
Ricotta	6.1-6.9	1.00	10-12	50-70.69	28-33	1.20-2.66	0.06	Brocklehurst & Lund (1985); Genigeorgis, Carniciu, Dutulescu & Farver (1991); Chen & Hotchkiss (1993); Fox et al. (2004); Giangolini et al. (2009); Spanu et al. (2012); Spanu et al. (2013); Asensio, Gallucci, de las Mercedes, Demo & Grosso (2014)
Emmental	5.7	0.97	6-22 (average of 10)	35-38.3	29.7	14.0 after manufacture; 4.0 in ripened cheese	2.8	Buazzi, Johnson & Marth (1992b); Fox et al. (1993); Weimer (2007)

Supplementary Table S2 Overview of growth rates extracted from challenge studies for 10 different cheese types. The growth rates μ (h^{-1}) extracted from challenge studies were calculated according to $\mu = \frac{\Delta \log N \cdot \ln(10)}{t}$, with t as the time after ripening minus the time immediately after pressing the cheese, and Δlog N as the log concentration of *L. monocytogenes* after ripening the cheese (N_t) minus the log concentration of *L. monocytogenes* immediately after pressing the cheese (N_0). The growth rates μ_{max} were assessed by calculating $\frac{\Delta \log N \cdot \ln(10)}{t}$ from each of the 45 challenge studies. Linear regression was applied to data points taken from the exponential phase. The lag phase and stationary phase were omitted, taking a worst-case approach. To determine μ_{max} in the exponential phase, the time interval was studied at which a clear increase of growth could be observed. The search on growth rates from challenge studies resulted in total in 45 studies containing 249 growth rates for 10 types of cheeses. Data from challenge studies were obtained through a literature search (searching Scopus and Web of Science for: monocytogenes AND cheese AND (Blue OR Camembert OR Cheddar OR Cottage OR Emmental OR Feta OR Gouda OR Mozzarella OR Queso Fresco OR Ricotta OR Swiss), sorted on relevance) and Combase (search on www.combase.cc for Listeria monocytogenes AND cheese, data from all scientific article records were incorporated for each of the 10 cheese types). The growth rate $\mu_{10\,°C}$ is the actual growth rate μ_{max} normalized for a temperature of 10 °C by use of the square-root function of McMeekin et al. (1993): $\mu_T = \mu_{ref} \cdot \frac{(T-T_{min})^2}{(T_{ref}-T_{min})^2}$, with T_{min} = -1.5 °C and T_{ref} = 10 °C. The application of the square-root function by McMeekin et al. (1993) for temperature is only validated for positive actual growth rates. As for the positive actual growth rates, the negative growth rates were normalized to 10 °C using the function, but the latter was not scientifically substantiated in this thesis

Cheese type	Reference	Inoculation target	Tempe-rature (°C)	Δlog N (log cfu g^{-1})	Δt (h)	μ_{max} (h^{-1})	$\mu_{10\,°C}$ (h^{-1})
Ricotta	Genigeorgis, Carniciu, Dutulescu, & Farver et al., 1991	Surface	30	3.33	24	0.32	0.043
Ricotta	Genigeorgis et al., 1991	Surface	8	2.11	192	0.025	0.037
Ricotta	Genigeorgis et al., 1991	Surface	4	1.53	720	0.0049	0.021
Ricotta	Genigeorgis et al., 1991	Surface	30	2.83	24	0.27	0.036
Ricotta	Genigeorgis et al., 1991	Surface	8	1.75	144	0.028	0.041
Ricotta	Genigeorgis et al., 1991	Surface	4	3.58	864	0.010	0.042
Ricotta	Genigeorgis et al., 1991	Surface	30	3.67	96	0.088	0.012
Ricotta	Genigeorgis et al., 1991	Surface	8	1.88	192	0.023	0.033
Ricotta	Genigeorgis et al., 1991	Surface	4	1.97	528	0.0086	0.038
Ricotta	Kapetanakou et al., 2017	Cheese blend	7	5.90	240	0.057	0.10
Ricotta	Martins, Cerqueira, Souza, Carmo Avides, & Vicente, 2010	Surface	4	1.10	168	0.0151	0.066
Queso Fresco	Brown, Kozak, & D'Amico, 2018	Surface	7	4.1	648	0.015	0.027
Queso Fresco	Gadotti et al., 2014	Curd	4	5.20	480	0.025	0.11
Queso Fresco	Gadotti et al., 2014	Curd	4	4.50	288	0.036	0.16
Queso Fresco	Gadotti et al., 2014	Curd	4	4.10	288	0.033	0.14
Queso Fresco	Gadotti et al., 2014	Curd	4	4.20	288	0.034	0.15
Queso Fresco	Gadotti et al., 2014	Curd	4	4.00	288	0.032	0.14
Queso Fresco	Gadotti et al., 2014	Curd	4	3.20	480	0.015	0.067
Queso Fresco	Genigeorgis et al., 1991	Surface	30	0.39	72	0.012	0.0017
Queso Fresco	Genigeorgis et al., 1991	Surface	8	-1.30	336	-0.0089	-0.013
Queso Fresco	Genigeorgis et al., 1991	Surface	4	-2.00	720	-0.0064	-0.028
Queso Fresco	Genigeorgis et al., 1991	Surface	30	0.95	144	0.015	0.0020
Queso Fresco	Genigeorgis et al., 1991	Surface	8	-0.05	192	-0.00060	-0.0009
Queso Fresco	Genigeorgis et al., 1991	Surface	4	-0.84	240	-0.0081	-0.035
Queso Fresco	Genigeorgis et al., 1991	Surface	30	0.74	72	0.024	0.0032
Queso Fresco	Genigeorgis et al., 1991	Surface	8	0.45	144	0.0072	0.011
Queso Fresco	Genigeorgis et al., 1991	Surface	4	0.28	720	0.00090	0.004

Queso Fresco	Ibarra-Sanchez, van Tassell, & Miller, 2018	Curd	4	1.1	168	0.015	0.066
Queso Fresco	Ibarra-Sanchez et al., 2018	Curd	4	1.15	168	0.016	0.069
Queso Fresco	Ibarra-Sanchez et al., 2018	Curd	4	1.25	168	0.017	0.075
Queso Fresco	Ibarra-Sanchez et al., 2018	Curd	4	1.25	168	0.017	0.075
Queso Fresco	Ibarra-Sanchez et al., 2018	Curd	4	3.8	336	0.026	0.11
Queso Fresco	Leggett et al., 2012	Curd	4	3.50	480	0.017	0.073
Queso Fresco	Leggett et al., 2012	Curd	10	4.00	240	0.038	0.038
Queso Fresco	Leggett et al., 2012	Curd	4	3.60	480	0.017	0.076
Queso Fresco	Leggett et al., 2012	Curd	10	4.50	288	0.036	0.036
Queso Fresco	Lin, Zhang, Doyle, & Swaminathan, 2016	Cheese blend	4	2.67	336	0.018	0.080
Queso Fresco	Lin et al., 2016	Cheese blend	4	2.07	336	0.014	0.062
Queso Fresco	Lin et al., 2016	Cheese blend	4	2.9	336	0.020	0.087
Queso Fresco	Lin et al., 2016	Cheese blend	4	2.12	336	0.015	0.064
Queso Fresco	Lin et al., 2016	Cheese blend	12	2.91	336	0.020	0.014
Queso Fresco	Lin et al., 2016	Cheese blend	12	2.46	336	0.017	0.012
Queso Fresco	Lin et al., 2016	Cheese blend	12	3.14	336	0.022	0.016
Queso Fresco	Lin et al., 2016	Cheese blend	12	2.58	336	0.018	0.013
Queso Fresco	Lin et al., 2016	Cheese blend	21	2.33	336	0.016	0.0042
Queso Fresco	Lin et al., 2016	Cheese blend	21	2.38	336	0.016	0.0043
Queso Fresco	Lin et al., 2016	Cheese blend	21	2.47	336	0.017	0.0044
Queso Fresco	Lin et al., 2016	Cheese blend	21	2.44	336	0.017	0.0044
Queso Fresco	Lourenço, Kamnetz, Gadotti, & Diez-Gonzalez, 2017	Curd	4	4.8	336	0.033	0.14
Queso Fresco	Lourenço et al., 2017	Curd	4	4.6	312	0.034	0.15
Queso Fresco	Lourenço et al., 2017	Curd	4	4.4	408	0.025	0.11
Queso Fresco	Lourenço et al., 2017	Curd	4	3.5	264	0.031	0.13
Queso Fresco	Soni et al., 2010	Surface	4	3.40	312	0.025	0.11
Queso Fresco	Soni et al., 2012	Surface	4	3.40	504	0.016	0.068
Queso Fresco	van Hekken, Tunick, Leggett, & Tomasula, 2012	Surface	4	4.10	504	0.019	0.082
Camembert	Back, Langford, & Kroll, 1993	Milk	8 on average (14d at 13 °C, then at 3 °C)	1.20	672	0.0041	0.0060
Camembert	Back et al., 1993	Milk	8 on average (14d at	1.80	672	0.0062	0.0090

			13 °C, then at 3 °C)				
Camembert	Back et al., 1993	Milk	8 on average (14d at 13 °C, then at 3 °C)	3.00	672	0.010	0.015
Camembert	Back et al., 1993	Milk	8 on average (14d at 13 °C, then at 3 °C)	3.80	696	0.013	0.018
Camembert	Back et al., 1993	Milk	8 on average (14d at 13 °C, then at 3 °C)	-0.50	672	-0.0017	-0.0025
Camembert	Back et al., 1993	Milk	8 on average (14d at 13 °C, then at 3 °C)	-0.40	672	-0.0014	-0.0020
Camembert	Back et al., 1993	Milk	8 on average (14d at 13 °C, then at 3 °C)	0.30	672	0.0010	0.0025
Camembert	Back et al., 1993	Milk	8 on average (14d at 13 °C, then at 3 °C)	2.40	696	0.0079	0.012
Camembert	Back et al., 1993	Surface	3	0.70	360	0.0045	0.029
Camembert	Back et al., 1993	Surface	6	2.20	360	0.014	0.033
Camembert	Back et al., 1993	Surface	3	1.30	360	0.0083	0.054
Camembert	Back et al., 1993	Surface	6	1.00	192	0.012	0.028
Camembert	Genigeorgis et al., 1991	Surface	30	1.75	48	0.084	0.011
Camembert	Genigeorgis et al., 1991	Surface	8	2.05	528	0.0089	0.013
Camembert	Genigeorgis et al., 1991	Surface	4	0.64	864	0.0017	0.0075
Camembert	Kapetanakou et al., 2017	Slices	7	4.70	288	0.038	0.069
Camembert	Lahou et al., 2017	Cheese rind	7	1.92	336	0.013	0.024
Camembert	Lahou et al., 2017	Cheese rind	7	1.20	336	0.0082	0.015
Camembert	Lahou et al., 2017	Cheese rind	7	1.30	336	0.0089	0.016
Camembert	Lahou et al., 2017	Cheese rind	14	3.55	336	0.024	0.013
Camembert	Lahou et al., 2017	Cheese rind	14	2.66	336	0.018	0.010
Camembert	Lahou et al., 2017	Cheese rind	14	2.74	336	0.019	0.010
Camembert	Lahou et al., 2017	Surface	7	3.82	336	0.026	0.048
Camembert	Lahou et al., 2017	Surface	7	4.01	336	0.027	0.050
Camembert	Lahou et al., 2017	Surface	7	1.82	336	0.012	0.023
Camembert	Lahou et al., 2017	Surface	14	5.46	336	0.037	0.021
Camembert	Lahou et al., 2017	Surface	14	4.93	336	0.034	0.019

Camembert	Lahou et al., 2017	Surface	14	2.82	336	0.019	0.011
Camembert	Larson et al., 1996	Cheese blend	4	1.93	432	0.010	0.045
Camembert	Larson et al., 1996	Cheese blend	12	3.53	264	0.031	0.022
Camembert	Linton, Mackle, Upadhyay, Kelly, & Patterson, 2008	Milk	13	-0.67	336	-0.0046	-0.0029
Camembert	Linton et al., 2008	Milk	13	-0.91	336	-0.0062	-0.0039
Camembert	Maisnier-Patin et al., 1992	Milk	11	5.00	1008	0.011	0.010
Camembert	Sulzer & Buzze et al., 1991	Milk	17	4.40	1068	0.0095	0.0037
Camembert	Sulzer & Buzze et al., 1991	Milk	17	4.20	936	0.010	0.0040
Camembert	Wan et al., 1997	Milk	15	4.36	504	0.020	0.010
Mozzarella	Kapetanakou et al., 2017	Slices	7	4.40	192	0.053	0.097
Mozzarella	Menon et al., 2001	Cheese	7	2.07	288	0.017	0.030
Mozzarella	Menon et al., 2001	Cheese	30	4.51	120	0.086	0.012
Mozzarella	Stecchini, Aquili, & Sarais, 1995	Surface	5	4.20	504	0.019	0.060
Cottage	Chen & Hotchkiss, 1993	Cheese blend	4	3.09	1512	0.0047	0.021
Cottage	Chen & Hotchkiss, 1993	Cheese blend	7	2.94	384	0.018	0.032
Cottage	Chen & Hotchkiss, 1993	Cheese blend	4	0.07	1512	0.00011	0.00048
Cottage	Chen & Hotchkiss, 1993	Cheese blend	7	1.27	1176	0.0025	0.0045
Cottage	El-Ziney & Debevere, 1998	Cheese blend	7	0.40	504	0.0018	0.0033
Cottage	Genigeorgis et al., 1991	Surface	30	-1.95	192	-0.023	-0.0031
Cottage	Genigeorgis et al., 1991	Surface	8	0.59	432	0.0031	0.0046
Cottage	Genigeorgis et al., 1991	Surface	4	0.39	576	0.0016	0.0068
Cottage	Genigeorgis et al., 1991	Surface	30	-1.87	192	-0.022	-0.0030
Cottage	Genigeorgis et al., 1991	Surface	8	-1.87	864	-0.0050	-0.0073
Cottage	Genigeorgis et al., 1991	Surface	4	0.34	576	0.0014	0.0059
Cottage	Genigeorgis et al., 1991	Surface	30	-1.87	192	-0.022	-0.0030
Cottage	Genigeorgis et al., 1991	Surface	8	0.42	576	0.0025	0.0025
Cottage	Genigeorgis et al., 1991	Surface	4	0.41	384	0.002	0.011
Cottage	Genigeorgis et al., 1991	Surface	30	1.19	96	0.029	0.0038
Cottage	Genigeorgis et al., 1991	Surface	8	1.13	192	0.014	0.020
Cottage	Genigeorgis et al., 1991	Surface	4	0.94	864	0.0025	0.011
Cottage	Genigeorgis et al., 1991	Surface	30	-1.87	192	-0.022	-0.0030
Cottage	Genigeorgis et al., 1991	Surface	8	-1.87	192	-0.022	-0.033
Cottage	Genigeorgis et al., 1991	Surface	4	-1.87	192	-0.022	-0.098
Cottage	Kapetanakou et al., 2017	Cheese blend	7	-0.20	240	-0.0019	-0.0035
Cottage	Larson et al., 1996	Cheese blend	4	2.22	672	0.0076	0.033
Cottage	McAuliffe, Hill, & Ross, 1999	Curd	4	-0.35	168	-0.0048	-0.021
Cottage	McAuliffe et al., 1999	Curd	18	-0.40	48	-0.019	-0.0067
Cottage	McAuliffe et al., 1999	Curd	30	-3.95	72	-0.13	-0.017
Cottage	Piccinin & Shelef, 1995	Cheese blend	5	-0.75	576	-0.0030	-0.0094
Cottage	Piccinin & Shelef, 1995	Cheese blend	5	-0.08	576	-0.00032	-0.0010

Cottage	Piccinin & Shelef, 1995	Cheese blend	5	-1.31	576	-0.0052	-0.016
Cottage	Piccinin & Shelef, 1995	Cheese blend	5	-1.52	576	-0.0061	-0.019
Cottage	Piccinin & Shelef, 1995	Cheese blend	5	-0.42	576	-0.0017	-0.0053
Cottage	Piccinin & Shelef, 1995	Cheese blend	5	0.06	576	0.00024	0.00075
Cottage	Piccinin & Shelef, 1995	Cheese blend	5	-0.42	576	-0.0017	-0.0053
Cottage	Piccinin & Shelef, 1995	Cheese blend	5	-0.54	576	-0.0022	-0.0068
Cottage	Piccinin & Shelef, 1995	Cheese blend	5	-0.33	576	-0.0013	-0.0041
Cottage	Piccinin & Shelef, 1995	Cheese blend	5	-0.42	576	-0.0017	-0.0053
Cottage	Ryser, Marth, & Doyle, 1985	Milk	3	1.04	605	0.0040	0.026
Cottage	Østergaard et al., 2014	Cheese blend	5	2.7	470	0.013	0.041
Cottage	Østergaard et al., 2014	Cheese blend	10	2.4	350	0.016	0.016
Cottage	Østergaard et al., 2014	Cheese blend	15	1.7	140	0.028	0.014
Cottage	Østergaard et al., 2015	Cheese blend	7.5	2.10	290	0.017	0.027
Cottage	Østergaard et al., 2015	Cheese blend	7.5	1.50	150	0.023	0.038
Cottage	Østergaard et al., 2015	Cheese blend	7.5	2.00	290	0.016	0.026
Blue	Back et al., 1993	Surface	4	0.00	336	0.00	0.000
Blue	Back et al., 1993	Surface	8	0.00	600	0.00	0.000
Blue	Bernini, Bottari, Dalzini, & Gatti, 2013	Surface	4	2.40	840	0.0066	0.029
Blue	Bernini et al., 2013	Surface	8	3.50	1320	0.0061	0.0089
Blue	Bernini et al., 2013	Surface	4	0.50	1320	0.00087	0.0038
Blue	Bernini et al., 2013	Surface	8	2.30	1320	0.0040	0.0059
Blue	Dalzini et al., 2017	Milk	4	2.2	1848	0.0027	0.012
Blue	Dalzini et al., 2017	Slices	8	3.40	576	0.014	0.020
Blue	Dalzini et al., 2017	Slices	8	1.50	504	0.0069	0.010
Blue	Dalzini et al., 2017	Slices	8	3.10	504	0.0142	0.021
Blue	Dalzini et al., 2017	Slices	8	3.70	1344	0.0063	0.0093
Blue	Dalzini et al., 2017	Slices	8	3.90	216	0.042	0.061
Blue	Dalzini et al., 2017	Slices	8	4.40	336	0.030	0.044
Blue	Genigeorgis et al., 1991	Surface	30	-2.00	432	-0.011	-0.0014
Blue	Genigeorgis et al., 1991	Surface	8	-2.00	432	-0.011	-0.016
Blue	Genigeorgis et al., 1991	Surface	4	-2.00	864	-0.0053	-0.023
Blue	Papageorgiou & Marth, 1989a	Milk	10.5	-2.80	600	-0.011	-0.010
Blue	Papageorgiou & Marth, 1989a	Milk	10.5	-2.80	1200	-0.0054	-0.0049
Blue	Papageorgiou & Marth, 1989a	Milk	10.5	-2.50	1440	-0.0040	-0.0037
Blue	Papageorgiou & Marth, 1989a	Milk	10.5	-2.50	1200	-0.0048	-0.0044
Blue	Papageorgiou & Marth, 1989a	Milk	10.5	-2.70	1440	-0.0043	-0.0040

Blue	Papageorgiou & Marth, 1989a	Milk	10.5	-2.50	720	-0.0080	-0.0073
Blue	Schaffer, Tatini, & Baer, 1995	Milk	13	-2.80	720	-0.0090	-0.0056
Blue	Schaffer et al., 1995	Milk	13	-4.40	1440	-0.0070	-0.0044
Blue	Whitley, Muir, & Waites, 2000	Surface	5	0.30	1008	0.00069	0.0021
Feta	Genigeorgis et al., 1991	Surface	30	-2.04	96	-0.049	-0.0065
Feta	Genigeorgis et al., 1991	Surface	8	-2.04	192	-0.024	-0.036
Feta	Genigeorgis et al., 1991	Surface	4	-2.04	192	-0.024	-0.107
Feta	Genigeorgis et al., 1991	Surface	30	-2.04	96	-0.049	-0.007
Feta	Genigeorgis et al., 1991	Surface	8	-2.04	192	-0.024	-0.036
Feta	Genigeorgis et al., 1991	Surface	4	-2.04	192	-0.024	-0.107
Feta	Genigeorgis et al., 1991	Surface	30	-2.04	96	-0.049	-0.0065
Feta	Genigeorgis et al., 1991	Surface	8	-2.04	192	-0.024	-0.036
Feta	Genigeorgis et al., 1991	Surface	4	-2.04	192	-0.024	-0.107
Feta	Genigeorgis et al., 1991	Surface	30	-2.04	96	-0.049	-0.0065
Feta	Genigeorgis et al., 1991	Surface	8	-2.04	192	-0.024	-0.036
Feta	Konteles, Sinanoglou, Batrinou, & Sflomos, 2009	Milk	4	-0.40	720	-0.0013	-0.0056
Feta	Leong et al., 2014	Slices	25	-4.58	360	-0.029	-0.0055
Feta	Leong et al., 2014	Slices	25	-4.89	360	-0.031	-0.0059
Feta	Papageorgiou & Marth, 1989b	Milk	4	-0.80	1680	-0.0011	-0.0048
Feta	Papageorgiou & Marth, 1989b	Milk	4	-0.40	1680	-0.00055	-0.0024
Feta	Papageorgiou & Marth, 1989b	Milk	4	-1.50	2040	-0.0017	-0.0074
Feta	Papageorgiou & Marth, 1989b	Milk	4	-3.00	2160	-0.0032	-0.014
Feta	Papageorgiou & Marth, 1989b	Milk	4	-2.80	2160	-0.0030	-0.013
Feta	Papageorgiou & Marth, 1989b	Milk	4	-2.80	2160	-0.0030	-0.013
Cheddar	Buyong, Kok, & Luchansky, 1998	Milk	8	-1.75	168	-0.0240	-0.035
Cheddar	Genigeorgis et al., 1991	Surface	30	-1.26	96	-0.030	-0.0040
Cheddar	Genigeorgis et al., 1991	Surface	30	-2.09	168	-0.029	-0.0038
Cheddar	Genigeorgis et al., 1991	Surface	8	-2.09	720	-0.0067	-0.010
Cheddar	Genigeorgis et al., 1991	Surface	4	-2.09	720	-0.0067	-0.029
Cheddar	Genigeorgis et al., 1991	Surface	30	-0.38	96	-0.0091	-0.0012
Cheddar	Genigeorgis et al., 1991	Surface	8	-0.06	96	-0.0014	-0.0021
Cheddar	Genigeorgis et al., 1991	Surface	30	-2.09	72	-0.067	-0.0089
Cheddar	Genigeorgis et al., 1991	Surface	8	-1.31	864	-0.0035	-0.0051
Cheddar	Genigeorgis et al., 1991	Surface	4	-2.09	864	-0.0056	-0.0244
Cheddar	Leong et al., 2014	Slices	25	-0.70	360	-0.0045	-0.00084
Cheddar	Leong et al., 2014	Slices	25	-0.76	360	-0.0049	-0.00092
Cheddar	Leong et al., 2014	Slices	25	-0.35	360	-0.0022	-0.00042
Cheddar	Leong et al., 2014	Slices	25	0.00	360	0.00	0.0000
Cheddar	Limjaroen, Ryser, Lockhart, & Harte, 2005	Surface (cubes)	4	-0.10	168	-0.0014	-0.0060
Cheddar	Limjaroen et al., 2005	Surface (cubes)	4	-0.50	840	-0.0014	-0.0060
Cheddar	Mehta et al., 1994	Milk	7	-0.30	288	-0.0024	-0.0044
Cheddar	Ryser et al., 1987	Milk	13	-1.40	1320	-0.0024	-0.0015
Cheddar	Ryser et al., 1987	Milk	13	-1.80	3000	-0.0014	-0.00087
Cheddar	Ryser et al., 1987	Milk	13	-2.20	5400	-0.00094	-0.00059

Cheddar	Ryser et al., 1987	Milk	6	-1.80	1680	-0.0025	-0.0058
Cheddar	Ryser et al., 1987	Milk	6	-1.45	1560	-0.0021	-0.0050
Cheddar	Ryser et al., 1987	Milk	6	-2.00	3000	-0.0015	-0.0036
Cheddar	Ryser et al., 1987	Milk	13	-2.00	3720	-0.0012	-0.00078
Cheddar	Ryser et al., 1987	Milk	13	-1.70	2400	-0.0016	-0.0010
Cheddar	Ryser et al., 1987	Milk	13	-0.40	4680	-0.00020	-0.00012
Cheddar	Ryser et al., 1987	Milk	6	-2.00	4320	-0.0011	-0.0025
Cheddar	Ryser et al., 1987	Milk	6	-1.50	2160	-0.0016	-0.0038
Cheddar	Ryser et al., 1987	Milk	6	-1.00	8640	-0.00027	-0.00063
Cheddar	Schaffer et al., 1995	Milk	7	-2.20	2160	-0.0023	-0.0043
Cheddar	Schaffer et al., 1995	Milk	7	0.00	2872	0.00	0.0000
Cheddar	Shrestha et al., 2011	Cheese blend	4	-0.56	1080	-0.0012	-0.0052
Cheddar	Shrestha et al., 2011	Cheese blend	4	-0.40	1080	-0.00085	-0.0037
Cheddar	Shrestha et al., 2011	Cheese blend	4	-0.35	1080	-0.00075	-0.0033
Cheddar	Shrestha et al., 2011	Cheese blend	4	-0.73	1080	-0.0016	-0.0068
Cheddar	Shrestha et al., 2011	Cheese blend	10	-0.55	2168	-0.00058	-0.00058
Cheddar	Shrestha et al., 2011	Cheese blend	10	-0.30	2168	-0.00032	-0.00032
Cheddar	Shrestha et al., 2011	Cheese blend	10	-0.37	2168	-0.00039	-0.00039
Cheddar	Shrestha et al., 2011	Cheese blend	10	-0.76	2168	-0.00081	-0.00081
Gouda	Leong et al., 2014	Slices	25	-0.51	360	-0.0033	-0.00061
Gouda	Leong et al., 2014	Slices	25	-0.44	360	-0.0028	-0.00053
Gouda	Kapetanakou et al., 2017	Slices	7	-0.2	1200	-0.00038	-0.00070
Gouda	Northolt et al., 1988	Milk	13	-1.07	1008	-0.0025	-0.0015
Gouda	Northolt et al., 1988	Milk	13	0.43	1008	0.0010	0.0006
Gouda	Wemmenhove, Stampelou, van Hooijdonk, Zwietering, & Wells-Bennik, 2013	Milk	12.5	-1.26	1008	-0.0029	-0.0019
Gouda	Wemmenhove et al., 2013	Milk	12.5	-0.59	1008	-0.0013	-0.00091
Gouda	Wemmenhove et al., 2013	Milk	12.5	-0.45	1008	-0.0010	-0.00069
Gouda	Wemmenhove et al., 2013	Milk	12.5	-3.77	5846	-0.0015	-0.0010
Gouda	Wemmenhove et al., 2013	Milk	12.5	-1.30	1702	-0.0018	-0.0012
Gouda	Wemmenhove et al., 2013	Milk	12.5	-1.54	3450	-0.0010	-0.00069
Gouda	Wemmenhove et al., 2013	Milk	12.5	-1.80	4704	-0.00088	-0.00059
Gouda	Wemmenhove et al., 2013	Milk	12.5	-0.40	4704	-0.0002	-0.00013
Gouda	Wemmenhove et al., 2013	Milk	12.5	-1.00	4704	-0.00049	-0.00033
Emmental	Bachmann & Spahr, 1995	Milk	12	-0.80	2160	-0.00085	-0.00062
Emmental	Buazzi et al., 1992b	Milk	7	-3.04	722	-0.010	-0.018
Emmental	Buazzi et al., 1992b	Milk	7	-3.36	722	-0.011	-0.020
Emmental	Buazzi et al., 1992b	Milk	7	-3.41	722	-0.011	-0.020
Emmental	Buazzi et al., 1992b	Milk	24	-3.04	722	-0.010	-0.0020

Emmental	Buazzi et al., 1992b	Milk	24	-3.36	722	-0.011	-0.0022
Emmental	Buazzi et al., 1992b	Milk	24	-3.41	722	-0.011	-0.0022
Emmental	Genigeorgis et al., 1991	Surface	30	-2.09	168	-0.029	-0.0038
Emmental	Genigeorgis et al., 1991	Surface	8	-2.09	456	-0.011	-0.015
Emmental	Genigeorgis et al., 1991	Surface	4	-2.09	864	-0.0056	-0.024
Emmental	Leong et al., 2014	Slices	25	-1.20	360	-0.0077	-0.0014
Emmental	Leong et al., 2014	Slices	25	-0.93	360	-0.0059	-0.0011
Emmental	Leong et al., 2014	Slices	25	-0.43	360	-0.0028	-0.00052
Emmental	Leong et al., 2014	Slices	25	-1.83	360	-0.012	-0.0022

Summary

Listeria monocytogenes is known as an important bacterial pathogen and is the causative agent of foodborne listeriosis, a disease that can be fatal, especially for people with a weak immune system. *L. monocytogenes* is a robust bacterium that can be present in ingredients and production environments due to its ability to survive in highly acidic, salty and low-temperature environments and due to its ability to form biofilms. The pathogen is a particular concern to ready-to-eat (RTE) food products, i.e., food products that do not undergo heat processing prior to consumption. Strict microbiological criteria for *L. monocytogenes* are applicable to RTE products as specified in regulation (EC) No 2073/2005. Three different categories of RTE products with different criteria for *L. monocytogenes* are defined therein.

Dutch-type Gouda cheese is a semi-hard cheese that is made from bovine milk that is pasteurized when produced at an industrial scale. It is a RTE food with a pH >5.0 and water activity (a_w) >0.94. Without further scientific evidence that the product does not support the growth of *L. monocytogenes*, it belongs to category 1.2 food products as specified in regulation (EC) No 2073/2005, namely, 'a ready-to-eat food able to support growth of *L. monocytogenes*'. Category 1.2 products must comply with the criterion 'guarantee that the level does not exceed the limit of 100 cfu g^{-1} throughout the shelf life (n=5)' for products placed on the market during their shelf-life* or with 'absence in 25 g (n=5) before the food has left the immediate control of the food business operator, who has produced it'**. If a ready-to-eat food is unable to support the growth of *L. monocytogenes* (other than those intended for infants and for special medical purposes) it belongs to category 1.3 and the criterion maximally 100 cfu g^{-1} (n=5) applies. Scientific evidence for no growth potential can be obtained by predictive mathematical modelling, durability tests and/or challenge tests and must be to the satisfaction of the competent authority.
The aims of this study were to establish whether *L. monocytogenes* can grow in or on Gouda cheese, to investigate the most important factors present in Gouda cheese that determine growth/no growth of *L. monocytogenes* and the variation of these factors in Gouda, and to provide criteria with respect to the most important growth inhibitors in Gouda cheese that prevent growth of *L. monocytogenes*.

* This criterion applies if the manufacturer is able to demonstrate, to the satisfaction of the competent authority, that the product will not exceed the limit 100 cfu g^{-1} throughout the shelf-life. The operator may fix intermediate limits during the process that must be low enough to guarantee that the limit of 100 cfu g^{-1} is not exceeded at the end of the shelf-life.

** This criterion applies to products before they have left the immediate control of the producing food business operator, when he is not able to demonstrate, to the satisfaction of the competent authority, that the product will not exceed the limit of 100 cfu g^{-1} throughout the shelf-life.

This research comprises two challenge studies investigating the fate of *L. monocytogenes* in and on Gouda cheese, two studies focusing on the variation in growth inhibition of *L. monocytogenes* by organic acids and a_w at conditions relevant to Dutch-type Gouda cheese, and an overall review of the factors that are most important for inhibition of growth of *L. monocytogenes* in Gouda cheese.

In the first challenge study, cheese milk was artificially contaminated with *L. monocytogenes* before curd formation. It was demonstrated that *L. monocytogenes* does not grow in Gouda cheese. No growth of *L. monocytogenes* was observed during the first eight weeks of ripening, and viable numbers declined significantly between 8 and 52 weeks in a Gouda microcheese system. During curd formation, viable numbers of *L. monocytogenes* increased by 0.5 log cfu g^{-1}, resulting from entrapment in the curd and removal of whey (**Chapter 2**).

In the second challenge study, the fate of *L. monocytogenes* was studied on and within factory-scale Gouda cheeses made from pasteurized cheese milk that had been submerged in artificially contaminated brine and then ripened at 12.5 °C for up to 26 weeks. The fate of the pathogen in brine was established as well, showing that viable numbers in the brine were stable or decreased during brining. *L. monocytogenes* was enumerated on the surface of Gouda cheese immediately after brining and during ripening. This showed that transfer of *L. monocytogenes* from brine to cheese during brining was limited, and that *L. monocytogenes* was detected in the outer layer of Gouda cheese but not inside the cheese directly after brining or during ripening. Throughout the ripening period, the viable numbers of *L. monocytogenes* on the outer layer declined significantly (**Chapter 3**).

Profiles of NaCl and water and the resulting a_w were determined in nature-ripened and foil-ripened Gouda cheese during brining and ripening to assess the variation in a_w in Gouda cheese over time, as input for risk assessments. Immediately after brining, gradients of NaCl and water were observed throughout both types of cheese. During ripening, these gradients disappeared, except for the water gradient in nature-ripened cheeses. An empirical model was derived for Gouda cheese, in which the a_w is expressed as a function of the NaCl-in-moisture content, based on the data collected for cheeses with different brining times, at different positions in the cheeses and at different ripening times. Moreover, the effect of a reduced a_w on inhibition of growth of *L. monocytogenes* in Gouda cheese was calculated. In addition to the presence of undissociated lactic acid, the reduced a_w as seen in Gouda cheese can substantially contribute to inhibition of microbial growth, and even to inactivation of *L. monocytogenes* when cheeses are brined and nature-ripened for extended periods of time (**Chapter 4**).

In **Chapter 5**, the minimal inhibitory concentrations (MICs) of organic acids relevant to cheese for *L. monocytogenes* are reported. The MICs of undissociated lactic acid for *L. monocytogenes* were determined for six different strains that were cultured in the presence of different lactate concentrations at 30 °C and in a pH range of 4.2-5.8. No growth was

observed at pH 4.2 and 4.4, and the MICs of undissociated lactic acid in the pH range of 5.2-5.8 were generally higher than at pH 4.6 for the different *L. monocytogenes* strains. The average MIC of undissociated lactic acid was 5.0 (SD 1.5) mM in the pH range 5.2-5.6. Significant differences in MICs of undissociated lactic acid were found between strains of *L. monocytogenes* at a given pH. Variations in MICs were mostly due to strain variation. For the strains tested in the pH range 5.2-5.6, the MICs of undissociated lactic acid were not significantly different at 12 °C and 30 °C. The MICs of undissociated acetic acid, citric acid, and propionic acid for the six *L. monocytogenes* strains were also determined, showing average values of 19.0 (SD 6.5) mM, 3.8 (SD 0.9) mM, and 11.0 (SD 6.3) mM, respectively. The MIC values that were established in this study improve the understanding of the growth-inhibiting effect of organic acids for *L. monocytogenes*, and offer more input data for predictive growth models that incorporate organic acids as growth-inhibiting factors for *L. monocytogenes* in cheese.

The known factors that are relevant to nature-ripened Gouda cheese were subsequently evaluated for their potential to inhibit growth of *L. monocytogenes* in this cheese. Factors included a_w, pH, undissociated acetic and lactic acid, diacetyl, free fatty acids, lactoferrin, nitrate, nitrite and nisin. In addition, the effect of temperature was evaluated. For each factor, the actual concentrations and values relevant to Gouda cheese were obtained and the inhibitory effect of these individual factors on growth of *L. monocytogenes* was assessed based on literature data or on experimental data if data were not available in the scientific literature. This evaluation revealed that undissociated lactic acid is the primary factor for growth inhibition of *L. monocytogenes* in Gouda cheese. In a 2-week ripened Gouda cheese, which has a typical total lactic acid content of 1.47% w/w, moisture content of 42% w/w, and pH 5.25, the concentration of undissociated lactic acid is 10.9 mM. Under such conditions, full growth inhibition of *L. monocytogenes* can be expected. Based on the minimal inhibitory concentrations of undissociated lactic acid for *L. monocytogenes* of 6.35 mM, full growth inhibition of *L. monocytogenes* can be expected in a young Gouda cheese when the total lactic acid concentration is >0.86% w/w at a pH <5.25, and in a mature Gouda cheese (moisture content of 35% w/w) when the total lactic acid concentration is >1.26% w/w at a pH <5.5. In addition to undissociated lactic acid, the a_w was identified as a factor that can cause full growth inhibition after prolonged ripening times (**Chapter 6**).

In the challenge studies that have been performed with *L. monocytogenes* in Gouda cheese, growth was not supported. The most important factors contributing to growth inhibition were identified as undissociated lactic acid, temperature, pH and a_w. These four factors determined full growth inhibition of *L. monocytogenes* during ripening of Gouda cheese. These factors were also the main determinants to correctly predict growth/no growth of *L. monocytogenes* in several other RTE cheeses (e.g. Queso fresco, Camembert, Feta, Cheddar, Ricotta, Mozzarella and Blue cheese). For Gouda, the factors undissociated lactic acid and a_w can lead to full growth inhibition. Temperature and pH only delay growth of *L. monocytogenes* in Gouda cheese, as

the critical values for growth of *L. monocytogenes* are not reached for these factors in this cheese type.

In this thesis, it was concluded that Dutch-type Gouda cheese does not support growth of *L. monocytogenes*. The assessment of potential inhibitory factors in this cheese revealed the importance of undissociated lactic acid as a growth-inhibiting factor, together with temperature, pH and a_w. This thesis lends support to categorizing Gouda as a ready-to-eat food product that does not support growth of *L. monocytogenes*. Furthermore, it is justifiable to include undissociated lactic acid (together with pH and a_w) in future food safety criteria for ready-to-eat products related to absence of growth of *L. monocytogenes*.

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Writing a PhD thesis is not an overnight activity. It is a process in which complex challenges pop up, and for which creativity, consistency and perseverance are required. I hope that the insights from this thesis will be used in the sector, and that the research results will contribute to assurance of food safety of Gouda cheese.

Doing a PhD research could be perceived as a solitary occupation, however my PhD has enabled me to collaborate with great people whom I would like to thank.

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List of publications

Wemmenhove, E., Van Valenberg, H.J.F., Van Hooijdonk, A.C.M., Wells-Bennik, M.H.J., Zwietering, M.H. (2018) Factors that inhibit growth of *Listeria monocytogenes* in nature-ripened Gouda cheese: A major role for undissociated lactic acid. Food Control 84, 413-418.

Wemmenhove, E., Stara, A., Wells-Bennik, M.H.J., Van Hooijdonk, A.C.M., Zwietering, M.H. (2016) How NaCl and water content determine water activity during ripening of Gouda cheese, and the predicted effect on inhibition of *Listeria monocytogenes.* Journal of Dairy Science 99, 5192-5201.

Wemmenhove, E., Beumer, R.R., Van Hooijdonk, A.C.M., Zwietering, M.H., Wells-Bennik, M.H.J. (2016) Minimal inhibitory concentrations of undissociated lactic, acetic, citric and propionic acid for *Listeria monocytogenes* under conditions relevant to cheese. Food Microbiology 58, 63-67.

Wemmenhove, E., Beumer, R.R., Van Hooijdonk, A.C.M., Zwietering, M.H., Wells-Bennik, M.H.J. (2014) The fate of *Listeria monocytogenes* in brine and Gouda cheese after an artificial contamination during brining: no growth, and inactivation in cheese during ripening. International Dairy Journal, 39, 253-258.

Wemmenhove, E., Stampelou, I., Van Hooijdonk, A.C.M., Zwietering, M.H., Wells-Bennik, M.H.J. (2013) Fate of *Listeria monocytogenes* in Gouda microcheese: no growth, and substantial inactivation after extended ripening times. International Dairy Journal, 32, 192-198.

Curriculum vitae

Ellen Wemmenhove was born in 1985 in Den Ham, The Netherlands. She finished her secondary education (VWO) at Reggesteijn in Nijverdal in 2002. That same year, she moved to Wageningen to do a BSc in Food Technology, followed by an MSc in Food Safety in 2005, focusing on food microbiology and toxicology. After graduating in 2007, Ellen started working as an Assistant Approvals Manager at the Safety and Environmental Assessment Center in Unilever Colworth, United Kingdom. In 2008, she started her PhD in employment of the Dutch Dairy Association (NZO), working as a Scientist at NIZO food research. In 2012 she decided to continue her career in cheese technology by working for Royal FrieslandCampina as a Technology Developer. In 2015, Ellen moved to Denmark to work for Arla Foods, to extend her knowledge on fermentation and cheese technology. Since 2016, she works as a Research Scientist, developing Mozzarella in the Global Cheese team of Arla Foods in Aarhus.

VLAG graduate school - Overview of completed training activities

Discipline- specific activities

Courses:

Basic laboratory pathogens training, NIZO, Ede, NL, 2008
Management of microbiological hazards in foods, VLAG, Wageningen, NL, 2008
Nutrient density of milk, VLAG, Wageningen, NL, 2009
Reaction kinetics in food, VLAG, Wageningen, NL, 2009
Listeria challenge tests, Nieuwsbrief Voedselveiligheid, Maarssen, NL, 2010
Genetics and physiology of food-associated m.o. VLAG, Wageningen, NL, 2010

Conferences/symposia/workshops:

Food Micro 2008 (oral presentation), ICFMH, Aberdeen, UK, 2008
Safety issues of raw milk cheeses conference, SAFE, Brussels, BE, 2008
Food Micro 2010, ICFMH, Copenhagen, DK, 2010
Workshop Listeria modeling, DTU, Copenhagen, DK, 2010
ICPMF 7, ICPMF, Dublin, IE, 2011
NVVM workshop food microbiology (oral presentation), NVVM, Wageningen, NL, 2012
Food safety & spoilage seminar NIZO (oral lecture), NIZO, Ede, NL, 2012
Workshop Modeling of *Listeria monocytogenes* in Gouda cheese (organization and oral lecture), NZO/NIZO, Ede, NL, 2012

General courses

VLAG PhD week (Introduction course), VLAG, Wageningen, NL, 2008
Communicative skills, NIZO, Wageningen, NL, 2008
PhD competence assessment, WGS, Wageningen, NL, 2009
Scientific writing, WGS, Wageningen, NL, 2010
Presentation skills, WGS, Wageningen, NL, 2010
Philosophy & ethics of food science and technology, VLAG, Wageningen, NL, 2011
Communication with the media & the general public, WGS, Wageningen, NL, 2011
Career perspectives, WGS, Wageningen, NL, 2011

Optional courses

Preparation of research proposal, WUR, Wageningen, NL, 2008
PhD trip Australia Dairy Science & Technology, WUR, Wageningen, NL, 2010
TIFN (molecular) microbiology meetings, NIZO, Ede, NL, 2008-2012
Half-yearly meetings of industrial taskforce Listeria in cheese (oral presentations), NIZO, Ede, NL, 2008-2012

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