Persistence of foodborne pathogens and their control in primary and secondary food production chains

Larsen, Marianne Halberg; Dalmasso, Marion; Ingmer, Hanne; Langsrud, Solveig; Malakauskas, Mindaugas; Mader, Anneluise; Møretø, Trond; Mozina, Sonja Smole; Rychli, Kathrin; Wagner, Martin; Wallace, R. John; Zentek, Jurgen; Jordan, Kieran

Published in:
Food Control

DOI:
10.1016/j.foodcont.2014.03.039

Publication date:
2014

Document version
Early version, also known as pre-print

Citation for published version (APA):
Review

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Marianne Halberg Larsena, Marion Dalmasob, Hanne Ingmera, Solveig Langsrudc, Mindaugas Malakauskasd, Anneluise Madere, Trond Møretrøc, Sonja Smole Mo/C20, Kathrin Rychlii, Martin Wagnerf, R. John Wallaceh, Jurgen Zentekm, Kieran Jordanc

a Department of Veterinary Disease Biology, University of Copenhagen, Denmark
b Teagasc Food Research Centre, Moorepark, Fermoy, Co. Cork, Ireland
c Nofima, The Norwegian Institute of Food, Fisheries and Aquaculture Research, Aas, Norway
d Veterinary Academy, Lithuanian University of Health Sciences, Kaunas, Lithuania
e Institute of Animal Nutrition, Freie Universität Berlin, Germany
f Department of Food Science and Technology, Biotechnical Faculty, University of Ljubljana, Slovenia
g Institute for Milk Hygiene, Milk Technology and Food Science, University of Veterinary Medicine Vienna, Austria
h Rowett Institute of Nutrition and Health, University of Aberdeen, Bucksburn, UK

A R T I C L E   I N F O

Article history:
Received 14 June 2013
Received in revised form 19 March 2014
Accepted 25 March 2014
Available online 3 April 2014

Keywords:
Foodborne pathogen
Persistence
Control
Escherichia coli
Listeria monocytogenes
Salmonella

A B S T R A C T

This review highlights factors involved in the persistence of foodborne pathogens in selected food chains and covers aspects of the basis for persistence, the consequences of persistence in terms of food safety implications, and the strategies that can be employed to combat persistence. The examples selected are Escherichia coli O157 and Salmonella at primary production of cattle and pigs, respectively, Listeria monocytogenes and Cronobacter spp. at secondary production, while persistence of Campylobacter spp. represents both primary and secondary production.

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Contents

1. Introduction to persistence in the food chain ......................................................... 93
2. Persistence in primary production — verocytotoxin producing E. coli in cattle ................................. 94
   2.1. Food safety consequences of shedding of VTEC from cattle ................................. 94
   2.2. Evidence of persistence ..................................................................................... 94
3. Persistence in primary production — Salmonella in pigs and pig feed ................................. 95
   3.1. Role of feed contamination and persistence mechanisms involved ......................... 95
   3.2. Contamination in farm environments and persistence mechanisms involved ......................... 96
4. Persistence in secondary production — L. monocytogenes ................................. 96
   4.1. L. monocytogenes in food processing facilities and food safety consequences ......................... 96
   4.2. Mechanisms of persistence ..................................................................................... 96
      4.2.1. Disinfectant tolerance ................................................................................. 97
      4.2.2. Attachment to surfaces and biofilm formation ................................................. 97
      4.2.3. Mixed species biofilms ............................................................................... 97
      4.2.4. Other factors ................................................................................................. 98

* Corresponding author. Tel.: +353 25 42451; fax: +353 25 42340.
E-mail address: kieran.jordan@teagasc.ie (K. Jordan).

http://dx.doi.org/10.1016/j.foodcont.2014.03.039
0956-7135/ © 2014 Elsevier Ltd. All rights reserved.
1. Introduction to persistence in the food chain

In recent years, the awareness of the persistence of foodborne pathogens in primary and secondary food processing environments has attracted much scientific interest in microbiology. Persistence means that particular types of microorganisms survive for prolonged periods of time in certain habitats. Persistence of a pathogen relies on many factors, such as the physical and microbial natural habitat, transmission routes and genetic determinants (Fig. 1). Persistence can cause repeated isolation of the same strain for months or even years at the same sites (Unnerstad et al., 1996). Persistence always refers to a particular matrix or environment, either soil, feed, animals, farm production environments, food processing environments or food itself. If looking to transmission of food-borne pathogens, one may easily understand that pathogens travel through a sequence of ecological niches before they pose a threat to humans. A very simple example is *Listeria monocytogenes* that is believed to be ubiquitously spread in nature, and can colonize food processing environments through inappropriate or inadequate hygiene, from where it can contaminate food and finally infect humans who have been exposed to contaminated food (Khelef et al., 2006). Thus, scientific disciplines such as microbial ecologists and food microbiologists should work together in a multidisciplinary approach to address the issue.

Most microbial communities are highly complex and subject to reorganisation. That some microbial communities are more stable than others implies that exposure to stress and reorganisation can lead to a more resistant population. Even under very harsh conditions, such as in the animal stomach or during high heat treatments, a surviving microbiota exists that may proliferate and pose new hazards. In contrast, some environments such as the animal intestine may be carriers of human pathogens, although they are not pathogenic to the carriers (Naylor et al., 2003). Such zoonotic strains include verocytotoxigenic *Escherichia coli* or shiga toxin producing *Escherichia coli* (VTEC or STEC, in this review we used VTEC) in cattle and *Campylobacter* in chickens. Understanding factors that foster creation of stable microbial communities will allow manipulation of these factors. With respect to environmental contaminants, the goal should be to understand persistence and therefore enable the development of a stable commensal microbiota not inducing persistence of pathogens, rather than placing an over-emphasis on sanitisation.

For the purposes of this review, persistence will be defined broadly and differently at primary and secondary production. At primary production, colonization of the animal is in this review perceived as persistence as it may result in shedding of the same strain of for example *E. coli* O157:H7 from cattle for weeks or months, or repeated isolation of the same *Salmonella* strain from pigs. In addition, bacterial isolates may persist in the farm environment such as in the stable or the feed. At secondary production in the processing environment, persistence of bacterial strains refers to repeated isolation of the same strain for months or even years at the same sites (Unnerstad et al., 1996).

For the purposes of understanding the basis of persistence, it is usual to compare the behaviour and properties of persistent (also called permanent or resident) and non-persistent strains. In order to do this, the term non-persistent needs to be defined. This is more difficult as failure to isolate a strain may be due to a sampling issue.
or simple absence, rather than to an inherent property of the strain. Therefore, strains should be termed ‘presumed’ non-persistent or sporadic or transient. In primary production, shedding of a pathogen would be considered non-persistent if it occurred only for 1–2 days, and environmental non-persistence would be disappearance of the strain in a week, while in secondary production a ‘presumed’ non-persistent strain is one that is isolated only once in the process of a monthly sampling plan of 12 months.

Infections with *Campylobacter* spp. and *Salmonella* spp. are among the most often reported zoonotic diseases in humans in the EU (EFSA, 2013). Poultry and fresh pig meats are among the most important food vehicles involved in foodborne *Campylobacter* and *Salmonella* outbreaks, and much of the disease burden originates from contamination at primary production. Cases of human infections caused by VTEC or *L. monocytogenes* are less numerous, but due to the serious nature of the diseases caused and the high mortality rates, these infections are also very important to control. *Cronobacter* spp. (formerly *Enterobacter sakazakii*) are opportunistic pathogenic bacteria that can cause serious infections such as necrotizing enterocolitis, bacteremia and meningitis in vulnerable neonates and infants (Hunter & Bean, 2013; Lai, 2001; Mullane et al., 2008). However, recent reports describing cases of *Cronobacter* infections in immuno compromised adults including the elderly, suggest that older individuals may also be susceptible to *Cronobacter* infections. Within the susceptible population, the number of cases is approximately 6 per year worldwide (Healy et al., 2010). Disease caused by *Cronobacter* spp. is not frequent but as the mortality rate has been estimated to be up to 80% (Lai, 2001) and the main source of neonatal infections has been rehydrated powdered infant formula (Iversen, Lane, & Forsythe, 2004; van Acker et al., 2001; Yan et al., 2012), monitoring and dealing with its occurrence and persistence in the powder-processing environment is a major concern for the dry food industry, including the infant formula industry. Thus, the most important foodborne infections either in numbers or severity are due to pathogens that have the ability to persist in the primary production or at secondary production facilities.

### 2. Persistence in primary production – verocytotoxin producing *E. coli* in cattle

Many different animal species are reservoirs for VTEC, but by far the most important animal source of human cases of infections with zoonotic VTEC is cattle (EFSA, 2012; Nataro & Kaper, 1998). VTEC strains are categorised into a number of serogroups of which O157 is the most widely known. Other important serogroups are O26, O91, O103, O111, and O145. Serogroup O157 is the cause of approximately half of the VTEC associated diseases in the EU and commonly associated with the most severe cases of disease i.e. haemorrhagic colitis (HC) and the potentially life threatening haemolytic uremic syndrome (HUS) (EFSA, 2008a, 2013). However, the epidemiology of VTEC may differ in different geographical regions (EFSA, 2013). In addition, the role of the pathogenic non-O157 strains in human disease and the prevalence of them in animals has begun to emerge as more laboratories begin to use detection methods for non-O157 strains (EFSA, 2012; Thomas et al., 2012).

#### 2.1. Food safety consequences of shedding of VTEC from cattle

The potential routes for contamination with VTEC at farm level are shown in Fig. 2. Faecal shedding of *E. coli* O157:H7 from cattle may result in contaminated food of bovine or ovine origin including meat and raw milk and derived dairy products. In addition, produce and water may become contaminated with VTEC from animal faeces. Environmental contamination and direct contact with animals play a role in human VTEC infections (Fig. 2) (Chapman, 2000; O’Brien, Adak, & Gilham, 2001; Ogden et al., 2002). The level of excretion is important as this will affect the risk of faecal contamination in all potential contamination routes. Thus, cattle persistently shedding VTEC, particularly at high numbers, give rise to a higher prevalence of VTEC in the farm environment, creating a higher risk of contaminated food products than cattle with occasional shedding (Chapman, 2000; O’Brien et al., 2001; Ogden et al., 2002). The incidence of *E. coli* O157:H7 infections in man and shedding of VTEC from cattle have both been well established to be seasonal (Chapman, 2000; Edrington et al., 2006; Hancock, Besser, Rice, Herriott, & Tarr, 1997). At least part of the seasonality may be due to the greater survival/proliferation of *E. coli* O157:H7 in faeces when the temperature increases (Bach, Selinger, Stanford, & McAlister, 2005). The prevalence of *E. coli* O157 displays striking variability across the cattle population (Matthews et al., 2006a). On 78% of Scottish farms, in a cross-sectional survey of 952 farms, no shedding of *E. coli* O157 was detected. However, on a small proportion of farms, ca. 2%, a very high prevalence of infection was found (with 90–100% of faeces samples being positive). The numbers of VTEC in the faeces of VTEC-positive cattle also varies from very low to very high. Thomas et al. (2012) found that in 130 VTEC positive faecal samples only two samples had numbers above the detection levels of 200 CFU/g. These two samples had counts of 1000 and 1300 CFU/g. The numbers of VTEC in faeces will affect the risk of faecal contamination of the carcasses either directly or indirectly via a contaminated hide. Lairage at the slaughterhouse (holding pens used immediately before slaughter) will increase the risk of cross contamination to other animals and subsequently onto the carcasses. The hide removal process during slaughter makes it difficult to prevent transfer of bacteria from the hide to the carcass and several studies show correlation between the incidence of pathogens on hides and on derived carcasses (Arthur et al., 2004; Baird, Lucia, Acuff, Harris, & Savell, 2006; Barkocy-Gallagher et al., 2003; Elder et al., 2000; Jacob, Paddock, Renter, Lechтенberg, & Nagaraja, 2010).

#### 2.2. Evidence of persistence

The human health risk is, as described above, associated with the level of excretion of VTEC in the faeces. Some cattle shed very high numbers of *E. coli* O157:H7 in their faeces and are known as ‘super-shedders’ (Chase-Topping, Gally, Low, Matthews, & Woolhouse, 2008; Matthews et al., 2006a, 2006b). According to the definitions by Lim et al. (2007) and Carlson et al. (2009) cows are defined as super-shedders if they shed more than 10^6 CFU/g but these so-called super-shedders may excrete as many as 10^9 CFU/g of faeces (Fukushima & Seki, 2004), which corresponds to >10^11 *E. coli* O157:H7 per day. However, the concentration of VTEC in the faeces is not only associated to the levels of excretion but also to the duration of shedding (Davis et al., 2006) and super-shedders have also been defined as those animals that shed VTEC O157:H7 for more than three consecutive months or extended periods (Lim et al., 2007). The duration of shedding varies between animals with most animals being culture positive for less than one week but few animals are culture positive for several weeks or months and these colonized animals have been suggested to contribute significantly to the pathogen load on farms (Carlson et al., 2009; Davis et al., 2006). Carlson et al. (2009) have shown that shedding of a strain with an indistinguishable pulsed field gel electrophoresis (PFGE) profile (indicating a very similar strain) can persist for up to 110 days in cattle. It was shown that 1% of feedlot cattle persistently shed strains with indistinguishable PFGE profiles. However, there has been little study on the molecular ecology of strain persistence of *E. coli* O157:H7 and non-O157 in cattle and further studies are...
needed to elucidate pathogen factors involved in persistence with VTEC strains.

E. coli O157:H7 can survive in cattle slurry for at least several weeks (Kudva, Hatfield, & Hovde, 1995), and in soil for at least as long. Survival in soil depends on temperature and moisture and is considered to be a key aspect of on-farm persistence of E. coli O157:H7. Survival in soil depends on temperature and moisture and is considered to be a key aspect of on-farm persistence of E. coli O157 in soil. In a study of 36 Dutch arable manure-amended soils (Franz et al., 2008) concluded that E. coli O157 populations declined faster under more oligotrophic soil conditions, which can be achieved by the use of organic fertilizer with a relatively high carbon/nitrogen ratio and consequently a relatively low rate of nutrient release. Mubiru, Coyne, and Grove (2000) stated that enhancing moisture retention and fine soil particles could increase bacterial survival because of an increased ability to retain nutrients. Organic farms were not different to conventional farms. Ma et al. (2011) added different mutants of E. coli O157 to soils. Virulence played no part in persistence, but the different soils did: the calculated time needed to reach the detection limit for loamy sand, sandy loam, and silty clay was 32, 80, and 110 days, respectively. It has been speculated that free-living protozoa may play a role in harbouring the pathogen, particularly via the dissemination of amoeba cysts contaminated with the bacteria (Barker, Humphrey, & Brown, 1999).

3. Persistence in primary production — Salmonella in pigs and pig feed

Pig meat is a significant source of human infections of Salmonella (EFSA, 2012). The Salmonella load of animals and animal products can be minimized by controlling all potential entry pathways. Food animal may acquire Salmonella infection on the farm from wild birds and rodents, but the main sources are either other animals, which may be symptomless excreters, or contaminated feed (Wierup & Haggblom, 2010). The relative importance of different sources of Salmonella infections in animals varies. In regions with a low prevalence status of Salmonella, contaminated feed is the major source of Salmonella in the animal food production. In regions with a high prevalence in animals, the relative importance of feed compared to other sources of Salmonella may be lower (EFSA, 2008b) (Tables 1 and 2).

3.1. Role of feed contamination and persistence mechanisms involved

There are numerous examples of outbreaks of Salmonella infections in pigs that were traced to contaminated feed, and published data from 2010 (EFSA, 2012) indicate an occurrence of 0.5–0.7% of Salmonella in feed for pigs, poultry and cattle in the EU (Table 1). The prevalence of Salmonella in feedstuffs depends on the nature and origin of the feed material, its primary hygienic quality, the treatment and other factors during the production process.

The primary mode of contamination of feed materials is from animals defecating in the farm environment, by fertilization of crops with manure (Maciorowski, Herrera, Jones, Pillai, & Ricke, 2007) or by recontamination with Salmonella that persist in the production and transport facilities. In addition, wild birds, rodents and insects may carry Salmonella, but the significance of these species as sources of contamination in feed factories is unclear (Davies & Wray, 1997; Nesse et al., 2005). Salmonella can persist in the feed processing facilities, where some serovars can be repeatedly isolated for many years. The clones may persist on parts of the factory equipment or environment (Nesse et al., 2003). If long-lasting contaminants are present, it can be assumed that the routines for decontamination have been inadequate or that the prevention of bacteria from entering the factories, or from the raw material part of the plant to the finished product has not been successful (Nesse et al., 2003).

Several mechanisms that may be the basis of persistence of Salmonella have been investigated. These include resistance to disinfection and desiccation and biofilm formation (Moretro, Midtgaard, Nesse, & Langsrud, 2003; Vestby, Moretro, Langsrud, Heir, & Nesse, 2009). Earlier studies have shown that Salmonella

Table 1

<table>
<thead>
<tr>
<th>Year</th>
<th>2002</th>
<th>2004</th>
<th>2006</th>
<th>2008</th>
<th>2010</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cattle feed</td>
<td>1.3</td>
<td>0.4</td>
<td>0.7</td>
<td>0.5</td>
<td>0.7</td>
</tr>
<tr>
<td>Pig feed</td>
<td>0.7</td>
<td>0.7</td>
<td>0.6</td>
<td>0.6</td>
<td>0.5</td>
</tr>
<tr>
<td>Poultry feed</td>
<td>0.3</td>
<td>2.0</td>
<td>0.8</td>
<td>0.9</td>
<td>0.5</td>
</tr>
</tbody>
</table>
are not particularly resistant to disinfection or air-drying at surfaces (Moret et al., 2003). Because biofilm protects bacteria against environmental stress, e.g. disinfection (Ronner & Wong, 1993), one hypothesis is that biofilm formation facilitates persistence. A correlation between persistence and biofilm formation has been described, which suggests that biofilm forming ability may be an important factor for increasing tolerance against drying processes and persistence of Salmonella in the factory environment (Vestby et al., 2009).

3.2. Contamination in farm environments and persistence mechanisms involved

Salmonella can survive in feed for prolonged periods of time and contaminate farm animals. In several studies, Salmonella could readily be isolated from several samples that had been stored at room temperature for up to 3 years (D’Aoust, 1977; D’Aoust & Sewell, 1986; Ray, Jezeski, & Busta, 1971; Rayman, D’Aoust, Aris, Maishment, & Wasik, 1979). In another study, broth culture of S. Typhimurium (5.6–9.8×10^8 CFU/ml) was added to 70 g of pelleted poultry feed and the samples were stored at 11, 25, and 38 °C, with a relative humidity of 68, 51, and 27%, respectively. The survival times in days were 552, 495, and 40 days, respectively (Williams & Benson, 1978).

Salmonella can also survive and persist in soil and on pasture at the farm (Cote & Quessy, 2005; Sinton, Braithwaite, Hall, & Mackenzie, 2007). Various studies were conducted to determine the survival times of different Salmonella serovars (including Salmonella Typhimurium, Salmonella Dublin and Salmonella Senftenberg) and their potential to persist in slurry. Storage of inoculated samples at temperatures ranging from 1 to 20 °C or room temperature resulted in survival for at least 65 days and up to almost a year, whereas the survival time decreased with the temperature (Burrows & Rankin, 1970; Jones, 1976; Rankin & Taylor, 1969; Tannock & Smith, 1971; Thunegard, 1975). If the temperature was increased to >45 °C the moist heat resistance of various Salmonella serovars could be reduced to 2–48 h (Mitscherlich & Marth, 1984).

Taken together the data indicate that Salmonella may become persistent in the farm environment. The mechanism(s) by which this occurs remain to be elucidated. It has been well established that bacteria from colonized food animals can be transmitted to humans through the food supply. Cattle, poultry, pigs, and other food animals can be colonized with Salmonella, which have multiple routes into the food supply. Consumption of meat or poultry products contaminated with Salmonella can lead to human salmonellosis (EFSA, 2007).

4. Persistence in secondary production – L. monocytogenes

4.1. L. monocytogenes in food processing facilities and food safety consequences

L. monocytogenes is a high risk in processed ready-to-eat foods such as dairy products made from unpasteurized and pasteurized milk, ready-to-eat meat and fish products and raw vegetables and fruit (EFSA, 2013; Ferreira, Wiedmann, Teixeira, & Stasiewicz, 2014). The bacterium is ubiquitous in the environment and may therefore be transmitted into the processing facilities by the raw materials, the workers, trucks, tools, cleaning materials or machines (Reij, Den Aantrekker, & ILSI Europe Risk Analysis in Microbiology Task Force, 2004). The most important contamination route is from niches in equipment colonized by persistent L. monocytogenes that survive the cleaning and disinfection procedures and can then cross contaminate the food products (Fig. 3). Persistence of L. monocytogenes isolates has been shown at food processing facilities, often for many years (Tompkin, 2002). In addition to strains persisting at larger scale cheese production facilities (Lomonaco et al., 2009), persistence has also been documented at smaller artisan facilities (Fox, Leonard, & Jordan, 2011a), in the salmon industry (Rorvik, Caugant, & Yndestad, 1995; Vogel, Huss, Ojeniyi, Ahrens, & Gram, 2001; Wulff, Gram, Ahrens, & Vogel, 2006), in meat processing plants (Giovannacci et al., 1999; Neshkken, Kapperud, & Caugant, 1996) and poultry production plants (Lawrence & Gilmour, 1995; Ojeniyi, Christensen, & Bisgaard, 2000; Ojeniyi, Wegen, Jensen, & Bisgaard, 1996).

4.2. Mechanisms of persistence

Various factors have been studied for their role in the persistence of L. monocytogenes strains, including disinfectant and desiccation resistance (Aase, Sundheim, Langsrud, & Rorvik, 2000; Holah, Taylor, Dawson, & Hall, 2002; Kastbjerg & Gram, 2009; Vogel, Hansen, Mordhorst, & Gram, 2010), differences in gene expression (Fox, Leonard, & Jordan, 2011b) and biofilm formation (Djordjevic, Wiedmann, & McLandsborough, 2002; Lunden, Miettinen, Autio, & Korkeala, 2000; Norwood & Gilmour, 1999). Studies relating to these, however, have not always been conclusive and strong evidence describing mechanisms of persistence remains unclear. Persistent L. monocytogenes have been shown to be involved in listeriosis infections and product recalls (Table 3).

### Table 2

<table>
<thead>
<tr>
<th>Year</th>
<th>2002</th>
<th>2004</th>
<th>2006</th>
<th>2008</th>
<th>2010</th>
<th>Total positives (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fish meal</td>
<td>2.1</td>
<td>1.1</td>
<td>1.9</td>
<td>2.1</td>
<td>9.1</td>
<td></td>
</tr>
<tr>
<td>Meat and bone meal</td>
<td>2.9</td>
<td>1.7</td>
<td>2.3</td>
<td>1.0</td>
<td>0.6</td>
<td></td>
</tr>
<tr>
<td>Cereals</td>
<td>1.2</td>
<td>0.7</td>
<td>0.3</td>
<td>0.2</td>
<td>0.9</td>
<td></td>
</tr>
<tr>
<td>Oil seeds and products</td>
<td>5.3</td>
<td>5.7</td>
<td>2.5</td>
<td>1.8</td>
<td>1.5</td>
<td></td>
</tr>
</tbody>
</table>

### Table 3
Partial list of listeriosis outbreaks and product recalls associated with persistent L. monocytogenes.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Source</th>
<th>Year</th>
<th>Years of persistence</th>
<th>Country</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>FG584</td>
<td>Turkey franks, Deli turkey meat</td>
<td>1989</td>
<td>12</td>
<td>USA</td>
<td>MMWR (1989); Orsi et al. (2008)</td>
</tr>
<tr>
<td>En2</td>
<td>Ice cream plant</td>
<td>1990</td>
<td>7</td>
<td>Finland</td>
<td>Miettinen, Bjorkroth and Korkeala (1999)</td>
</tr>
<tr>
<td>Li111</td>
<td>Cold smoked salmon</td>
<td>1996</td>
<td>6</td>
<td>Denmark</td>
<td>Wulff et al. (2006)</td>
</tr>
<tr>
<td>FSL.N1-449</td>
<td>Smoked fish processing environment</td>
<td>1998</td>
<td>11</td>
<td>USA</td>
<td>Vongkamjan, Roof, Stasiewicz, and Wiedmann (2013)</td>
</tr>
<tr>
<td>6179</td>
<td>Cheese</td>
<td>2000</td>
<td>8</td>
<td>Ireland</td>
<td>Fox et al. (2011b)</td>
</tr>
<tr>
<td>4423</td>
<td>Cheese smear water</td>
<td>2004</td>
<td>7</td>
<td>Austria</td>
<td>Szejtli et al. (2014)</td>
</tr>
<tr>
<td>QMP-L1-070</td>
<td>Milk</td>
<td>2005</td>
<td>3</td>
<td>USA</td>
<td>Latorre et al. (2011)</td>
</tr>
</tbody>
</table>

* Related to outbreak and sporadic cases of listeriosis.
4.2.1. Disinfectant tolerance

Using disinfectants at concentrations recommended for the food industry, *L.* monocytogenes in suspension will be completely inactivated (Bridier, Briandet, Thomas, & Dubois-Brissoulet, 2011). Also, even after adaptation to a quaternary ammonium compound (QAC) based disinfectant, *L.* monocytogenes EGD were still sensitive at a concentration far below in-use concentrations (Kastbjerg & Gram, 2012). Tolerance to disinfectants has been studied among groups of persistent and presumed non-persistent strains but results from different studies are contradictory. Some studies have demonstrated that persistent strains showed higher resistance than presumed non-persistent strains (Aase et al., 2000; Fox et al., 2011b; Lunden, Autio, Markkula, Hellstrom, & Korkeala, 2003). However, even in persistent isolates a resistance-level far below user-concentrations was found (Aase et al., 2000; Fox et al., 2011b). Also, a clear link between persistence and increased disinfectant resistance was not recorded in some other studies (Holah et al., 2002; Kastbjerg & Gram, 2009). Elhanafi, Dutta, and Kathariou (2010) identified a plasmid-based gene cassette that conferred increased resistance to benzalkonium chloride, a widely used QAC disinfectant in the food industry. In addition, a novel transposon carrying a transporter (QacH), which is responsible for benzalkonium chloride tolerance, has been described (Muller et al., 2013). Genetic elements such as these may have significance in terms of strain persistence in some strains. It should be emphasised that measuring minimum inhibitory concentrations was used to determine the level of resistance in these studies and the results cannot be directly extrapolated to survival after disinfection. On the other hand, it cannot be ruled out that mechanisms involved in a low-level enhanced tolerance contribute to survival at sub-optimal disinfection conditions. Examples could be when the disinfectant is partly neutralised by organic matter from food debris or biofilms (González-Fandos, Sanz, García-Fernández, & García-Arias, 2005; Grönholm, Wirtanen, Ahlgren, Nordström, & Sjöberg, 1999; Pan, Breidt, & Kathariou, 2006).

4.2.2. Attachment to surfaces and biofilm formation

A number of studies have investigated attachment/biofilm formation of persistent and presumed non-persistent strains of *L.* monocytogenes to surfaces, with varying results. The varying results may be as a result of different methods used, and the fact that many studies refer to biofilm formation as occurring in a few hours (which is really attachment) whereas actual biofilm formation may take several days. Differences in strains, culture medium, physicochemical properties of the environmental surfaces and time of the experiment could impact these results and explain the different results as pointed out by Carpentier and Cerf (2011). Norwood and Gilmour (1999) found statistically greater mean adherence ability among persistent strains compared to presumed non-persistent strains. However, the results were not entirely consistent as some individual non-persistent strains showed high adherence. Using a microtitre plate assay method, Djordjevic et al. (2002) did not find higher adherence among persistent strains. In a study by Lunden et al. (2000), it was shown that persistent strains showed enhanced attachment over short periods of time, however, some presumed non-persistent strains matched, or in some cases surpassed, the levels of attachment of persistent strains after 72 h. A possible explanation for this observation may involve differences in flagella between different strains; flagella have been shown to facilitate early attachment to stainless steel (Vatanyoopaisarn, Nazli, Dodd, Rees, & Waites, 2000). A recent study found better adherence of persistent strains from dairy environment than sporadic strains (Latorre et al., 2011). Higher biofilm formation among persistent compared to non-persistent strains from bulk milk samples was also described (Borucki, Peppin, White, Loge, & Call, 2003). It has been speculated that attachment affects the susceptibility to disinfectants, but Kastbjerg and Gram (2009) found no difference between attached and planktonic cells. However, other studies showed increased resistance to disinfection of *L.* monocytogenes in biofilm compared to in suspension and in several studies resistance was observed to user-concentrations of disinfectants (Annous, Fratamico, & Smith, 2009). However, it should be pointed out that in most of these studies, biofilms are disinfectated without a prior cleaning step, which is included in the normal routine in the food industry. Cleaning is supposed to remove the majority of the biofilm. Typically, thick biofilms will not form on smooth food contact surfaces subjected to daily cleaning and disinfection, but are more likely to form in areas not as thoroughly cleaned (like floors and walls where there can be crevices or cracks), or on equipment difficult to clean (Holah & Gibson, 2000). Latorre et al. (2009) conducted a study monitoring the epidemiology of *L.* monocytogenes strains on a dairy farm, in which they postulated that biofilm formation was responsible for repeated contamination events during the study period. The work, including typing of *L.* monocytogenes strains isolated from bulk milk and milking equipment, and examination of biofilm on the milking equipment, supported the view that the ability of *L.* monocytogenes to form biofilm is important in persistence of strains.

4.2.3. Mixed species biofilms

The examples above focus on the link between persistence and biofilm formation examined for pure bacterial cultures. However, bacteria dominating in the food production environment are typically non-pathogenic species of *Pseudomonas*, *Acinetobacter* and *Staphylococcus* (Bagge-Ravn et al., 2003; Marouani-Gadri, Augier, & Carpentier, 2009), thus pathogenic bacteria in a biofilm will most likely be a part of a multispecies biofilm and the ability of a strain to co-exist in multispecies biofilms may be important for its persistence in the production environment. It has been shown that biofilm formation of *L.* monocytogenes can both be inhibited (Jeong & Frank,
5. Persistence in secondary production — Cronobacter spp. in powder processing facilities

Cronobacter spp. are widespread in the environment, although a specific reservoir remains to be identified. They have been detected from a wide variety of sources, including in several dry food processing facilities, in retail bakeries, different types of mainly dry foods and powdered ingredients, animal feed, different vegetables, herbs and spices, rice and in the environment (Beuchat et al., 2009; Kandhai, Reij, Gorris, Guillaume-Gentil, & van Schothorst, 2004; Walsh et al., 2011). However, the frequency with which they are isolated is generally low, except in the case of dried food matrices (Molloy et al., 2009), including powdered infant formula (Osaii & Forsythe, 2009). Dairy cattle and raw milk do not, however, seem to be a dissemination route (Molloy et al., 2009).

5.1. Persistence in a dry environment and food safety consequences

The majority of cases of disease due to Cronobacter are susceptible infants becoming infected following consumption of contaminated reconstituted powdered infant formula (Norberg et al., 2012). Cronobacter spp. appear to be adapted to survive in low water activity matrices and environments, sometimes extending over considerable time periods. In general the level of contamination is low but after rehydration Cronobacter may rapidly grow to critical levels if the reconstituted product is temperature abused. Survival in dehydrated powdered infant formula, where the water activity is about 0.2, for up to two years and in infant cereal for up to 24 weeks has been shown (Edelson-Mammel, Porteous, & Buchanan, 2005; Gurtler & Beuchat, 2007a, 2007b; Lin & Beuchat, 2007a, 2007b). Persistence of desiccated Cronobacter depends on water activity and temperature with less survival with increasing temperature (30 °C compared to 4 °C) and higher water activities (a_w 0.43–0.5 compared to a_w 0.25–0.3). The unknown reservoir of this pathogen challenges the control of its entry into food products. Cronobacter can survive the actual spray drying process (Arku, Mullane, Fox, Fanning, & Jordan, 2008), but is unlikely to survive heat treatments prior to that and thus its presence in the final formula may be due to its presence in un-heat treated raw material or recontamination from the processing environment. Cronobacter spp. can persist in dry food processing and preparation environments such as powdered infant formula processing environments (Hein et al., 2009; Mullane et al., 2008; Proudy, Bougle, Leclercq, & Vergnaud, 2008), but also in retail confectionery shops, and other dry food facilities for example chocolate, potato and pasta factories (Baumgartner, Grand, Liniger, & Iversen, 2009; Kandhai et al., 2004). Proudy et al. (2008) subtyped 200 Cronobacter isolates from a powdered infant formula factory and found that 70% of the isolates were clonally identical. Epidemiologically, powdered infant formula and the environment in which it is produced have been linked with transmission of disease to neonates (Himelright et al., 2002; Nzarowec-White & Farber, 1999) where among other things, air filters were shown to be a source of contamination (Mullane et al., 2008).

5.2. Mechanisms of persistence

The prevalence of Cronobacter in these low water activity products and corresponding processing environments also during prolonged storage shows that Cronobacter has the capacity to adapt to, survive and persist under desiccated environmental conditions (Beuchat et al., 2013; Walsh et al., 2011). The mechanism behind the unique ability of Cronobacter to survive desiccation is not fully understood and studies investigating the mechanism are limited. However, Cronobacter has cross-resistance to dry and thermal stresses (Dancer, Mah, Rhee, Hwang, & Kang, 2009). Proteomic analysis showed that genes upregulated during desiccation stress included proteins that serve some structural or protective role and down-regulation of the motility-associated proteins (Riedel & Lehner, 2007). Survival in a dry environment was also studied by Breuwer, Lardeau, Peterz, and Joosten (2003), who concluded that uptake of ions (calcium, for example) and compatible solutes (for...
example, trehalose) can increase intracellular osmolarity (Kempf & Bremer, 1998) and thus survival. The sequences of the recently sequenced Cronobacter sakazakii BAA-894 and of sixteen other strains show a number of genes associated with neonatal infection, including copper and silver resistance mechanisms known to be important for invasion of the blood–brain barrier (Grím et al., 2013; Joseph et al., 2012; Kucerova et al., 2010; Shin, Lee, Choi, & Ryu, 2012; Shin, Lee, Kim, & Ryu, 2012; Stephan, Lehner, Tischler, & Rattei, 2011; Yan et al., 2013). Subsequent mining of the genome data will facilitate an understanding of the persistence of Cronobacter spp. and the development of methods to inactivate persistent clones.

6. Persistence of Campylobacter spp. in the food chain

Campylobacter spp., especially Campylobacter jejuni and Campylobacter coli, are the most commonly reported bacterial causes of food-borne gastroenteritis in humans worldwide, usually transmitted to humans via consumption of undercooked food, unpasteurized milk, or contaminated RTE food or via water. Besides their widespread occurrence, Campylobacter strains have become increasingly resistant to antibiotics (Smole Mozina, Kurincic, Mozina, & Mavri, 2011), including the major drugs of choice for treatment of clinical campylobacteriosis. This could reduce the effectiveness of treatments, but could also influence their persistence in food production chains.

6.1. Evidence of persistence and food safety consequences

Molecular approaches, such as PFGE, have shown persistence of Campylobacter spp. in the whole food production chain from the farm to the retail product (Damjanova et al., 2011; Eberle & Kiss, 2012; Lienau, Ellerbroek, & Klein, 2007; Melero, Juntunen, Hanninen, Jaime, & Rovira, 2012). Persistence was reported on farms and in the well-water supplied to the farms (Perez-Boto et al., 2010). Characteristic prevalent clones have been found in the majority of poultry flocks and their transmission between consecutive flocks demonstrated (Damjanova et al., 2011; Petersen & Wedderkopp, 2001). Persistent strains were shown to be transferred from the flock to carcasses after slaughter (Wirz, Overesch, Kuhnert, & Korczak, 2010) or even from the farm to retail products (Melero et al., 2012). Besides this, cross-contamination at different points, e.g. including poultry transportation crates, different slaughtering steps, especially scalding, defeathering and evisceration and also at retail (e.g. market places), was reported despite periodic sanitation procedures at all these steps (Hastings, Colles, McCarthy, Maiden, & Sheppard, 2011; Melero et al., 2012; Peyrat, Soumet, Maris, & Sanders, 2008; Zorman, Heyndrickx, Uzunovic-Kamberovic, & Mozina, 2006). Contamination of working surfaces and equipment during slaughter of a positive flock can persist and lead to contamination of negative flocks even after cleaning and disinfection. A similar situation was described for broiler (Peyrat et al., 2008) and turkey (Perko-Makela et al., 2009) production chains, mainly with C. jejuni, but also for the transmission of C. coli in pig breeding (Jensen, Dalsgaard, Baggesen, & Nielsen, 2006) and pork meat processing environments both in conventional and organic and/or antibiotic free production systems (Abley, Wittum, Funk, & Gebreyes, 2012; Quintana-Hayashi & Thakur, 2012).

6.2. Mechanisms of persistence

Many reports illustrate persistence of at least some strains at specific points in the food chain. Whether certain clonal groups, and which ones repeatedly contaminate and have the potential to persist on farms, slaughterhouses or even market places and kitchen, needs further investigation. Similar to L. monocytogenes, some studies with Campylobacter have proposed that persistence could be related to disinfectant resistance (Mavri, Kurincic, & Smole Mozina, 2012; Mavri & Smole Mozina, 2012; Peyrat et al., 2008) or to antibiotic resistance, which has been recognized as an emerging risk in chicken (Habib, Miller, Uttendaelae, Houf, & De Zutter, 2009) and pork meat processing (Luangtongkum et al., 2009; Quintana-Hayashi & Thakur, 2012). Antibiotic resistance acquired through chromosomal mutations could confer survival ability on the bacteria in the absence of antibiotic selection pressure, which could result in the spread and persistence of antimicrobial-resistant Campylobacter (Zhang, Sahin, McDermott, & Payot, 2006).

Although many stress response genes are noticeably absent in C. jejuni (Murphy, Carroll, & Jordan, 2006), they can survive hurdles during food processing and preparation. The differences in survival ability were linked to the strain diversity, revealed by multi locus sequence typing (MLST) (Habib, Uttendaelae, & De Zutter, 2010), to antibiotic resistance mutations (González & Hänninen, 2012) as well as to protective environment, like chicken meat juice (Ligowska, Cohn, Stabler, Wren, & Brondsted, 2011) and combinations of stresses (Klancnik et al., 2009; Klancnik, Vuckovic, Planik, Abram, & Smole Mozina, 2013). However, a direct link of a stress response and subsequent enhanced survival in persistence in the food chain is not clear and should be further studied.

Beside the reasons already mentioned, Campylobacter persistence may also be linked to its biofilm forming potential. It has been suggested that the presence of biofilms in water distribution systems is responsible for the colonization of the bacteria in poultry flocks and that C. jejuni can persist in these aquatic environments. Thus, biofilms are likely to represent an important reservoir for C. jejuni (Ica et al., 2012). In different studies, multispecies biofilms were shown to be more robust and protective for Campylobacter persistence (Ica et al., 2012; Sanders, Bothe, Frank, & Arnold, 2007; Teh, Flint, & French, 2010), similar to that reported by many authors for other types of multispecies biofilms (Smole Mozina, Klancnik, & Rasper, 2013).

Similar to L. monocytogenes, protozoan cells are suspected to serve as persistent reservoirs for other bacterial pathogens, including campylobacters (Snelling, McKenna, Lecky, & Dooley, 2005). The most recent reports suggest that depletion of dissolved oxygen by Acanthamoeba castellani is a major factor in the observed amoeba-mediated growth enhancement of Campylobacter (Bui et al., 2012). However, the interactions of C. jejuni and protozoan cells such as A. castellani are complex and multifactorial, but pre-exposure to various stresses does not confer a competitive advantage on C. jejuni for survival within A. castellani. Further research on Campylobacter–protozoan interactions is required in order to gain a full understanding of this relationship and how it influences persistence.

7. Strategies to overcome/prevent persistence

At both primary and secondary production there are many strategies that attempt to reduce the pathogen load of feed and food. Some of these strategies are aimed at a general reduction in pathogens, while others are specifically aimed at particular pathogens. In general, control procedures will reduce numbers of all pathogens, and not necessarily only persistent strains.

7.1. Control strategies at farm level

7.1.1. Management practices

Farm management practices, including hygiene, are an important part of a pathogen control strategy on the farm. General
management practices include maintenance of equipment, upkeep of buildings, a sanitation plan, pest control and general hygiene. Overall, it is important to control possible entries of pathogens into the farm and stables and prevent transmission and persistence within the farm with a good biosecurity plan. One example of a site with high risk of contamination is purchase, transport and mixing of feed on farms depending on the equipment and its sanitary status (Binter et al., 2011). Feed storage is also important because of the potential of contamination under improper conditions. Ideally, ingredients and compound feeding stuffs used on farms should be stored in sealed containers. It was shown on Scottish farms, that approximately 80 rodent and 25 bird faeces were deposited per m² of stored feed per month over the winter period (Daniels, Hutchings, & Greig, 2003). Wild birds, pets, rodents and insects in the farm environment can be carriers of pathogens like *Salmonella* and *Campylobacter* and downstream contamination can result in an elevated prevalence of infection with these bacteria (Hald, Sommer, & Skovgaard, 2007; Warnick, Crofton, Pelzer, & Hawkins, 2001). Effective cleaning of stables including feeding and water equipment between batches of animals/pigs is another important factor in controlling *Salmonella* on pig farms, but is difficult and requires special procedures (EFSA, 2008a). Also the occurrence of *L. monocytogenes* can be reduced with increased farm hygiene (Fox et al., 2009). Similarly, the occurrence of *Campylobacter* in poultry houses can be prevented by application of enhanced hygiene and biosecurity measures together with complementary interventions (feed additives, etc.) (Newell et al., 2011). However, there is still no one effective strategy available to prevent or to reduce *Campylobacter* prevalence in poultry flocks (Hermans et al., 2011; Lin, 2009).

### 7.1.2. Vaccination

*E. coli* O157:H7 colonizes its host by producing several proteins such as the translocated intimin receptor (Tir) and the needle elongation filament protein, EspA, that are secreted by a type III secretion system (Naylor et al., 2005). These proteins play a role in colonization of the intestine, suggesting that they might be useful targets for the development of a vaccine to reduce numbers of this organism in cattle. Vaccination of cattle with proteins secreted by *E. coli* O157:H7 significantly reduced the numbers of bacteria shed in faeces, the numbers of animals that shed, and the duration of shedding in an experimental challenge model (Potter et al., 2004). Subsequent trials, however, with the same vaccine proved less successful in eliminating *E. coli* O157:H7 from feedlot pens (Van Donkersgoed, Hancock, Rogan, & Potter, 2005). Vaccination against the H7 flagellin may be an alternative strategy to control *E. coli* O157:H7 numbers (McNeillly et al., 2008). Localized mucosal antibody responses at the rectum may also be useful as a control mechanism (Nart et al., 2008). Vaccination against *Salmonella* is another strategy that has been used in chicken (Desin, Koster, & Potter, 2013), pigs (De Busser, De Zutter, Dewulf, Houf, & Maes, 2013), and cattle (Nielsen, 2013).

Currently, there is no vaccine designed to control *Campylobacter* spp. in poultry. Live attenuated vaccines, killed whole-cell vaccines and subunit vaccines have been investigated for effective control of this pathogen in humans and chickens. One of the reasons for the absence of commercially available vaccines is that *Campylobacter* spp., unlike other foodborne pathogens, are easily transformed and show a high level of genetic diversity (Jaguszyn-Krynicka, Lamiewski, & Wysznyska, 2009). Recent studies on the use of small immunodominant peptide epitopes of Cj0113 inserted into the genomic DNA of the *Salmonella* vector revealed that it triggers a strong immune response what leads to a decreased *Campylobacter* numbers up to below detectable levels following challenge with *C. jejuni* (Layton et al., 2011). However, the mechanism of treatment was not examined in that study and further investigations are needed for a better understanding of the protection mechanisms afforded by vaccination with attenuated *Salmonella*-vectored Cj0113. Another approach used in the study done by Clark et al. (2012) revealed a promising up to 91% immune protection against *C. jejuni* challenge in comparison to unvaccinated and wild-type *Eimeria tenella* vaccinated controls when specific pathogen free chickens were vaccinated by single or multiple oral inoculation of *E. tenella*-CjAA oocysts. Promising results were also obtained after vaccination of chickens with an *Salmonella enterica* serovar Typhimurium ΔaroA vaccine expressing the *C. jejuni* amino acid binding protein CjAA as a plasmid-borne fusion to the C-terminus of fragment C of tetanus toxin (Buckley et al., 2010). Protection induced by CjAA-specific serum IgY and biliary IgA significantly reduced the caecal number of *C. jejuni* by 1.4 log CFU/g at three and four weeks post-challenge relative to age-matched unvaccinated birds. Despite promising results from most of such type studies, an effective vaccine able to reduce *Campylobacter* colonization in poultry and available commercially is still at the development stage.

#### 7.1.3. Use of beneficial bacteria

For reasons of safety and convenience, probiotics would be an ideal solution to the problem of pathogens at farm level (Roselli et al., 2005). Probiotics can be defined as viable micro-organisms which, after sufficient oral intake, lead to beneficial effects for the host by modifying the intestinal microbiota. Strains of *Lactobacillus acidophilus*, *L. salivarius* subsp. *salivarius*, *L. pentosus*, *L. murinus* and *Pediococcus pentosaceus* as well as yeast strains have been used as probiotics (Brashears, Galyean, Loneragan, Mann, & Killinger-Mann, 2003; Brashears, Jaroni, & Trimble, 2003; Casey et al., 2007; Stephens, Loneragan, Chichester, & Brashears, 2007; Sowers, Carlson, Nightingale, Belk, & Archibeque, 2011). At present, documentation for the effect of using probiotics to inhibit pathogens are lacking and therefore use of probiotics for reduction and/or elimination of pathogens is not yet widely used.

Several studies have shown that the use of undefined or pre-defined bacterial mixtures can effectively prevent *Campylobacter* colonization of chicken intestines. Such competitive exclusion can be used as a control measure against colonisation of birds by *Campylobacter* spp. (Hakkinen & Schneitz, 1999; Soerjadi, Snoeyenbos, & Weinack, 1982; Stern, Cox, Musgrove, & Park, 2001). However, a few studies propose that this prophylactic measure does not reduce colonization rates for *C. jejuni* in poultry (Lainsley, Gillard, & Salvat, 2004; Stern, Bailey, Blankenship, Cox, & McHan, 1988) but application of this measure can efficiently reduce count of campylobacters in poultry intestines. The study of Hakkinen and Schneitz (1999) showed that even colonisation rate was not reduced considerably by application of a commercial lyophilised competitive exclusion product for prevention of *Salmonella* in broilers but was very efficient by reducing the *Campylobacter* counts by up to 9-log, depending on the trial. If a 2-log reduction of the *Campylobacter* counts is achieved on the chicken carcasses it further leads to the reduced incidence of human campylobacteriosis associated with consumption of chicken meals (Rosenquist, Nielsen, Sommer, Norring, & Christensen, 2003). Competitive exclusion in combination with other preventive means may explain the low prevalence of *Campylobacter* in broiler flocks in Finland (Perko-Makela, Hakkinnen, Honkanen-Buzalski, & Hanninen, 2002).

#### 7.1.4. Bacteriophages

Several studies have been conducted to examine the possible effectiveness of bacteriophage treatment in suppressing *Salmonella* numbers in pigs and *E. coli* O157:H7 numbers in the ruminants. In pre-processing of pigs, cecal numbers of *Salmonella* were significantly (*P* < 0.05) reduced when a bacteriophage cocktail was...
administered to pigs along with a strain of *S. Typhimurium* (Wall, Zhang, Rostagno, & Ebner, 2010), thus potentially reducing the occurrence of *Salmonella* during processing.

Similarly, bacteriophages can be used to reduce *E. coli* O157:H7 in cattle. In a rumen model, Rivas et al. (2010) showed that bacteriophage significantly (P < 0.05) reduced the numbers of *E. coli* O157:H7. However, further work is required to demonstrate a reduction of *E. coli* O157:H7 shedding in bacteriophage animal trials (Rivas et al., 2010). In a different approach, Coffey et al. (2011) showed that bacteriophages can be effective in reduction of *E. coli* O157:H7 on cattle hides, thus potentially reducing the numbers of *E. coli* O157:H7 during slaughter and subsequently reducing cross contamination and improving food safety. Three lytic bacteriophages active against *E. coli* O157:H7 were also isolated (Kudva, Jelacic, Tarr, Youderian, & Hovde, 1999), but their activity only under highly aerobic conditions would limit their usefulness in vivo. This may explain the apparently contradictory results obtained for in vivo animal trials and trials showing reduction on hides. Sheng, Knecht, Kudva, and Hovde (2006) isolated a broad host-range bacteriophage that, in combination with other bacteriophage, eliminated *E. coli* O157:H7 in experimental infections in mice and lowered shedding when applied rectally to cattle. Thus, further work is required to definitively study the efficacy of bacteriophage for *E. coli* O157:H7 reduction in cattle. One of the main issues to be resolved is that bacteriophage resistance can set in rapidly, so many different bacteriophages will be needed for lasting efficacy, which in turn can lead to interactions between bacteriophages making some of them ineffective (Raya et al., 2011).

Bacteriophage application was reported to be successful also against *Campylobacter* in chickens (Loc Carrillo et al., 2005; Wagenaar, Van Bergen, Mueller, Wassenaar, & Carlton, 2005). The reductions observed in the *Campylobacter* levels of the colonized birds following bacteriophage administration were between 1.5 and 5 log CFU/g of intestinal contents compared with controls. Similar reductions in *Campylobacter* numbers were observed later using a different group III bacteriophage (Scott et al., 2007), and with group II bacteriophages (Carvalho et al., 2010; El-Shibiny et al., 2009).

The efficacy of bacteriophages against *Campylobacter* was also tested also on meat surfaces (El-Shibiny et al., 2009; Orquera, Golz, Sparborth, Joldic, & Alter, 2012; Wagenaar et al., 2005) and against *Campylobacter* biofilms. The treatments led to a 1 to 3 log reduction of the viable cell counts under microaerobic conditions and dispersal of biofilm matrix (Siringan, Connerton, Payne, & Connerton, 2011). However, quick evolution of phage resistance could limit practical applications, and in addition, the mechanism(s) of action need to be understood.

### 7.1.5. Feed additives

Many early studies investigated the possibility of feed additives to control numbers of *E. coli* O157:H7 in the rumen. Conventional feed additives such as the ionophores, which selectively inhibit growth of Gram-positive bacteria (Chen & Wolin, 1979), are ineffective against *E. coli* (Edrington et al., 2003). Antimicrobials, including oxytetracycline and neomycin, were found to lower numbers (Alali, Sargeant, Nagaraja, & DeBey, 2004), but their widespread application would lead to concerns about introducing transmissible antibiotic resistance to human pathogens.

Essential oils are widely used in ruminant nutrition as feed additives (Benschard et al., 2008; Wallace, 2004), and various essential oils have been shown to be toxic to *E. coli* O157:H7 in vitro (Burt & Reinders, 2003), and *S. Typhimurium* (Si et al., 2006). Feed with essential oils was shown to reduce intestinal *Salmonella* colonization in broilers (Alali, HoFacre, Mathis, & Falty, 2013). Natural products such as colostrum were identified as toxic to *E. coli* O157:H7 (Duncan et al., 2004), but none have found widespread acceptance for various reasons, including safety and efficacy.

Short chain organic acids such as formic, propionic or sorbic acid and their salts can have antimicrobial effects and have been used for a long time for the preservation of feed on farms (Wales, Allen, & Davies, 2010). It is well known, that *Salmonella* are sensitive to organic acids and that those additives can reduce numbers and therefore prevent re-contamination of feed during storage. Another reason to use organic acids as feed additives is the positive influence on animal health and performance. Some epidemiological studies show that pig farms using organic acids have a lower prevalence of *Salmonella* (Kamphues et al., 2007). There are however some contradictory results of epidemiological studies probably due to the fact that organic acids are dosed differently and the efficacy is dependent on the time factor, the duration of application and feed composition. Liquid feed may contain ingredients such as corn cob mix with a higher acid concentration and in addition the buffering capacity of different types of feed may differ significantly. Potassium diformate was shown to have the potential to reduce *Salmonella* excretion in pigs (Dennis & Blanchard, 2004). Coarse feed structure and potassium diformate were able to reduce *Salmonella* excretion of infected piglets, some combined effects could be observed (Pappenbrock, Stensie, Amtsberg, Verpoehl, & Kamphues, 2005). Medium chain fatty acids might also be of interest for the reduction of *Salmonella* shedding due to their potential of antimicrobial activity in the upper gastrointestinal tract (Deschepper, Bruggeman, & Molly, 2005; Skanseng et al., 2010).

Similarly, organic acids could also be used as feed additives to reduce *Campylobacter* prevalence in poultry flocks with different level of efficiency. The studies showed that the high level of lactic acid in combination with a low pH and caprylic acid can reduce susceptibility of poultry to *Campylobacter* infection and reduce colonization rates within broiler flock (Heres, Engel, Urlings, Wagenaar, & van Knape, 2004; Solis de Los Santos et al., 2008). Also, the addition of a medium-chain fatty acid mixture to the feed increased resistance of broilers to *Campylobacter* colonization (Van Gerwe et al., 2010). However, there are contradictory studies including discrepant in vitro and in vivo results (Skanseng et al., 2010; Van Deun, Haesebrouck, Van Immerseel, Ducatelle, & Pasmans, 2008) and further studies are required for the development of useful tools for the reduction of *Campylobacter* colonization in broiler flocks (Van Gerwe et al., 2010).

### 7.1.6. Feed: particle size of feed, coarse or finely ground feed, pelleted feed

Feed structure is an important parameter that affects the prevalence of *Salmonella*. The majority of studies indicate that finely ground feed and pelleting can increase the risk of *Salmonella* prevalence in fattening pigs (Kamphues et al., 2007). The reason for this may be that feeding a coarsely ground meal to pigs changed the physicochemical and microbial properties of the digesta and the adherence capacity of *Salmonella* (Mikkelsen, Naughton, Hedemann, & Jensen, 2004).

Compare to other types of feed, liquid feed (such as whey) has been reported as a protective factor in some studies (Farzan et al., 2006; Lo Fo Wong et al., 2004; Poljak, Dewey, Friendship, Martin, & Christensen, 2008) and alternatively, some studies demonstrated a higher risk for farms using dry feed (Beloel et al., 2004; Von Altrick, Schutte, & Hildebrandt, 2000). Farms using liquid feeding of by-products, such as whey, had a lower risk of a *Salmonella* infection than herds not using such a system. Herds that used a trough feeding system had a four times higher risk of a *Salmonella* infection than herds not using this feeding system (Van der Wolf et al., 1999). The lower pH might be the relevant protective factor in liquid feeding systems. Piggeries with liquid feeding and...
addition of whey had a 2.6-times lower odds ratio to be test sero-positive than pigs not getting whey (Lo Fo Wong et al., 2004). Because whey is high in lactose, it could cause diarrhea in animals with a low tolerance for lactose, although pigs are relatively lactose tolerant and if used at appropriate concentrations does not cause diarrhea. Lactose stimulates the growth of certain bacteria in the intestinal tract of pigs, especially lactobacilli and an inverse correlation seems to exist between the fecal shedding of Salmonella and the fecal concentrations of lactobacilli (Wells, Yen, & Miller, 2005). In recent years the use of oilseeds, especially soya, has been critically discussed. Tracing from a pig herd positive for S. Cuba discovered the origin in a Swedish feed plant from which 77 pig farms had received feed (Osterberg, Vagsholm, Boqvist, & Lewerin, 2000). Soya was identified as major risk factor. Epidemiological studies showed that the use of fermented feed could significantly reduce Salmonella prevalence in pigs compared to the use of normal feed (Van der Wolf et al., 2001; Van Winsen et al., 2002).

7.1.7. Other control measures

The development of new genetic poultry lines to control measure of Campylobacter colonization in poultry for improved safety of poultry meat is only beginning. As there are two recently published comprehensive reviews on the genomics of genetic resistance to pathogens and microorganisms including Campylobacter (Cullen & Beaumont, 2012; Swaggerty et al., 2009), this specific measure is not discussed in detail in this review.

7.2. Control measures at secondary production

In general, as with measures used to control pathogens in primary production, strategies aimed at controlling one pathogen in the processing environment often also apply to other bacteria. In the following, L. monocytogenes and Cronobacter are used as examples on how to control persistence at secondary production.

7.2.1. Hygiene measures

L. monocytogenes is ubiquitous in the environment and may therefore be transmitted into the processing facility by the raw material, the workers, tools, trucks etc, where it may or may not persist. This may cause problems of persistence within the processing facility causing re-contamination of food. The challenge for food manufacturers is thus to direct efforts to prevent the entry and establishment of L. monocytogenes within the processing environment. Good Manufacturing Practices (GMP) and employee training will facilitate this. In addition, an adequate Hazard Analysis Critical Control Plan (HACCP), or similar type plan, is necessary. The focus of such a plan should be on the production environment, the people and the facility, minimizing the opportunity for entry of contamination and spread of contamination within the production plant. One example is the introduction of L. monocytogenes and Enterobacteriaceae from operators moving between different zones. The production facilities and equipment should fulfill international regulations and standards for hygienic design, such as ISO (International Organization for Standardisation, 2002) and the EC regulation food hygiene and machinery (European parliament, 2004, 2006), so that if contamination does enter the facility, there are limited opportunities for persistence. It is also very important to maintain equipment and protect production lines during any building work or reconstruction. Proper zoning is important to prevent contamination of the food produced with bacteria from the processing environment. Zoning starts with defining wet and dry zones and within these define levels of basic, medium and high hygiene zones. Dry zones should be kept dry to prevent growth of bacteria as these will not multiply in the absence of water. One example is that hand-washing creates an increase in the humidity in the dry zone environment and thus the need for hand washing facilities within or even in the air locks should be carefully evaluated.

7.2.2. Limit the opportunities for contamination

In order to limit the opportunities for contamination and persistence, cleaning routines of facilities and equipment should be designed to avoid selection of pathogenic bacteria with enhanced survival capacity. It is important to:

1. Prevent attachment: Some studies have shown that persistent bacteria may have a greater ability to attach to surfaces and form biofilm (Aase et al., 2000; Borucki et al., 2003; Lunden et al., 2000; Norwood & Gilmour, 1999). It is not possible to identify one single material which is resistant to bacterial attachment and suitable for all purposes (Failie & Carpentier, 2009). However, in comparative studies, stainless steel has been shown to be the material with lowest adherence of microorganisms or as easiest to clean (Midelet & Carpentier, 2002; Rodriguez & McLandsborough, 2007; Somers & Wong, 2004). Persistence has been linked to use of porous materials (Mead & Scott, 1994) and cleanliness is therefore crucial when choosing materials. Porous materials should therefore be avoided.

2. Prevent possibilities of growth and biofilm formation. This can be achieved by use of antifouling or antibacterial materials in production facilities and using a cleaning routine with a short time between production and cleaning/disinfection. There are many materials that have incorporated bacteriostatic or bactericidal agents, e.g. silver containing AgiON stainless steel (Campoccia, Montanaro, & Arciola, 2013; Hasan, Crawford, & Ivanova, 2013), or titanium nitride coating (Skovager et al., 2013). A common problem with these materials is neutralization of the antimicrobial component by proteins and therefore their efficacy in practical use can be limited (Chaitinemwong, Hazeleger, & Beumer, 2010; Moretrø, Hoiby-Pettersen, Habimana, Heir, & Langsrud, 2011; Rodrigues, Teixeira, Oliveira, & Azeredo, 2011). Their use to eradicate or avoid persisters is therefore questionable. A better strategy is to avoid contamination in the first place.

3. Eliminate bacteria at critical sites. Microbial biofilms can be removed using regular cleaning agents based on chlorinated alkaline detergents and mechanical energy, although an additional cleaning step with an acid cleaner may be included on periodically to remove layers of inorganic salts that may protect bacteria (Stanga, 2010).

7.2.3. Cleaning, disinfection and biofilm removal

Disinfectants should meet efficacy criteria as determined in standardized tests both in suspension and on surfaces (CEN, 1997, 2002). Since biofilm can be managed by a cleaning process that disperses the cells, a prerequisite for disinfection should be effective cleaning that suspends biofilm cells. Several studies have been done to determine which types of disinfectants are most effective against biofilms. The results from these studies are contradictory, probably due to differences in the biofilms, methods and disinfectants tested (Belessi, Gounadaki, Psomas, & Skandamis, 2011; Cruz & Fletcher, 2012).

Bacteria resistant to several disinfectants have been isolated (Bore & Langsrud, 2005), but in most cases, failure in disinfection is not due to resistance (Carpentier & Cerf, 2011; Meyer, 2006). A robust disinfection routine should involve alternation between chemical disinfectants with different properties and mechanisms of action, or between chemical and physical disinfection such as heat or UV.

The strategy that has contributed to the most dramatic reduction in Cronobacter spp. occurrence has been the concept of ‘dry
cleaning’ being applied to the facility. Traditionally, wet cleaning, foaming with sanitizer and rinsing have been practiced. The concept of ‘dry cleaning’ is to keep dry zones dry. Thus, spray dryers, dry mixers, storage silos packaging machines should be dry cleaned. Vacuum cleaners are the most common tools for dry cleaning. For more information on cleaning strategies in dry food processing environments we refer to expert guidelines (Beuchat et al., 2011). This has led to lower levels of Cronobacter spp. in the environment, no risk from water contamination of the lines and increased product quality and consistency.

Alternative prevention strategies aiming at limiting the growth of L. monocytogenes and Cronobacter in foods and on surfaces include the use of bacteriophages as processing aids (Coffey, Mills, Coffey, McAuliffe, & Ross, 2010; Endersen et al., 2014; Kim, Klumpp, & Loesner, 2007; Mahony, McAuliffe, Ross, & van Sinderen, 2011).

7.2.4. Sampling to verify control

Assuming compliance with HACCP or the HACCP-type protocol in use, an important tool in prevention of persistence within the facility is an appropriate sampling plan to verify such compliance. To make a meaningful sampling plan it is important to identify the critical sampling sites and the pathogens of concern. Thus, in monitoring the production environment by sampling for L. monocytogenes it is important to focus sampling to sites where occurrence of L. monocytogenes is expected and where contamination is likely to occur. In this regard, the European Union Reference Laboratory for L. monocytogenes has published guidelines for processing environment sampling (European Union Reference Laboratory for Listeria monocytogenes, 2012). Trend analysis of the results from such sampling should be undertaken, enabling a rapid response to changes that may indicate a potential loss of control and thus enable timely corrective actions. Because L. monocytogenes is ubiquitous, it is important to remember that finding a positive result should be viewed as a success of the monitoring program rather than a failure of hygiene measures. Being aware of the occurrence of L. monocytogenes in the processing environment is important as this occurrence can be dealt with. Having an action plan in the case of a positive result from sampling as part of the hygiene procedures is essential. In this way, food contamination can be avoided and food safety improved. Instead of sampling for the presence of specific pathogens, indicator microorganisms may be preferential given that it is possible to find a suitable indicators for the pathogen in the given processing environment.

In order to mitigate diseases caused by Cronobacter spp., the Food and Agricultural Organisation (FAO) and World Health Organisation (WHO) have developed a risk assessment model for Cronobacter species in powdered infant formula (FAO/WHO, 2008). This model can facilitate control measures aimed at reducing the occurrence of Cronobacter spp. in powdered infant formula, and it can be implemented along with specific microbiological criteria. It focuses on the elimination of batches of powdered infant formula that are deemed unacceptable (in accordance with the specified sampling plan), and estimates the risk reduction that can be obtained following removal of product that fails to meet the criteria. The model can also be used to assess the impact of sampling in terms of risk reduction through sampling alone, or in association with other risk reducing strategies such as preparation and storage strategies in the home. The model allows risk managers to explore the impact on relative risk and is freely available; interested food producers are encouraged to make use of this analytical tool.

8. Future perspectives

Despite the knowledge gained on persistence of foodborne pathogens, persistence is not fully understood. The exciting prospect afforded by new sequencing technologies has implications for future studies on the ecology and pathology of these pathogens. Such technologies enable the study of changes in the composition of microbial communities, including non-culturable microorganisms, over time and space and should lead to a better understanding of the mechanisms of persistence. Furthermore, community-analysis methodology and metagenomics will enable the acquisition of microbiomes to be analysed comprehensively, adding to the body of knowledge of persistence.

For E. coli O157:H7 there is an obvious lack of data on strain comparisons in super-shedders. The assumption is that persistent shedding is shedding of strains of the same genotype, but there is limited evidence for this. Additionally, there are limited studies on the time of persistent shedding or on the transfer of persistently shed strains to the animal hide then to the carcass during processing and to the final product. Understanding the mechanisms whereby animals show the characteristics of super-shedders are vital; it was demonstrated very elegantly by Matthews et al. (2006a) that targeting these animals specifically would have a very large effect on the incidence of E. coli O157 infection.

More knowledge on the Listeria strains contaminating processing facilities and the genetic and physiological factors that allow for persistence is needed. To obtain this, more relevant models simulating niches in the food production environments combined with analytical tools to investigate the composition as well as genetic and physiological responses of complex microbiota should be developed (Malley et al., 2013). This will provide more relevant background information needed to develop new eradication strategies. Examples of new principles for combating biofilms that could be further investigated include the use of bacteriophages, targeting cell-to-cell communication, or the iron pathway or enzymes attaching specific biofilm components or new antibacterial materials. With new and fast techniques for strain characterization, differentiation of L monocytogenes strains with respect to virulence will be possible. Thus, virulence of a particular isolate may be taken into account in risk assessment.

With systematic studies, the current knowledge gaps on persistence of Salmonella at various levels of the farm-to-fork approach can be reduced. Therefore, molecular biological methods that allow fast strain characterization and differentiation of the pathogen in various matrices need to be improved. The aim should be to enhance the scientific understanding of the behaviour and persistence of Salmonella under the multi-faceted farm-to-fork environments of feed matrices, compound feed production, and farm level as well as the processing steps slaughtering, dissection, packaging, and transport.

The current knowledge of the virulence and epidemiology of Cronobacter spp. is limited. The virulence traits are uncertain, yet it is important to distinguish between pathogenic and nonpathogenic strains. Future research should focus on an improved understanding of the diversity of the genus in order to define its ecology, epidemiology, virulence and role in human infections. In addition, there should be a focus on the management of this increasingly important bacterium in the production environment. Publication of the genome of C. sakazakii BAA-894 (Kucerova et al., 2010) will contribute to these studies.

Despite intensive research over the last two decades, there is still no single effective measure that can be applied to prevent or to reduce colonization of broiler flocks by Campylobacter spp. Therefore, neither the prevalence of campylobacters in broiler flocks, nor contamination of broiler products at the retail level have been reduced in Europe in recent years. In fact, the trend in the number of poultry meat consumption-related human campylobacteriosis cases has increased over recent years (EFSA, 2013). There is still a need for fundamental and applied research with a focus on
bacterial persistence that will lead to development of more effective measures for Campylobacter control in commercial poultry settings and therefore ensure consumer protection.

9. Conclusions

Whether at primary production or secondary processing of food, contamination by persistent foodborne pathogens is a risk to human health. This risk has been demonstrated for the major foodborne pathogens Escherichia coli O157:H7, Salmonella spp. L. monocytogenes, Cronobacter spp. and Campylobacter spp., all of which can persist. Understanding the mechanisms of persistence at the different levels in the farm to fork continuum is important in combating such persistence and by doing this improving food safety. Future concepts of prevention should not be based on the control of the production chain endpoint, but on process control throughout the chain.

Acknowledgements

This work was supported by the 7th Framework Programme projects FOODSEG (project number 266061) and PROMISE (project number 265877), and by the Danish Research Council for Strategic Research (project number 2101-08-0030; HI, MHL) and The Foundation for Research Levy on Agricultural Products in Norway (SL, TM (project number 224921/F40)).

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chemical disinfectants and antiseptics for use in food, industrial, domestic and institutional areas – test methods and requirements (phase 2-step 2).


