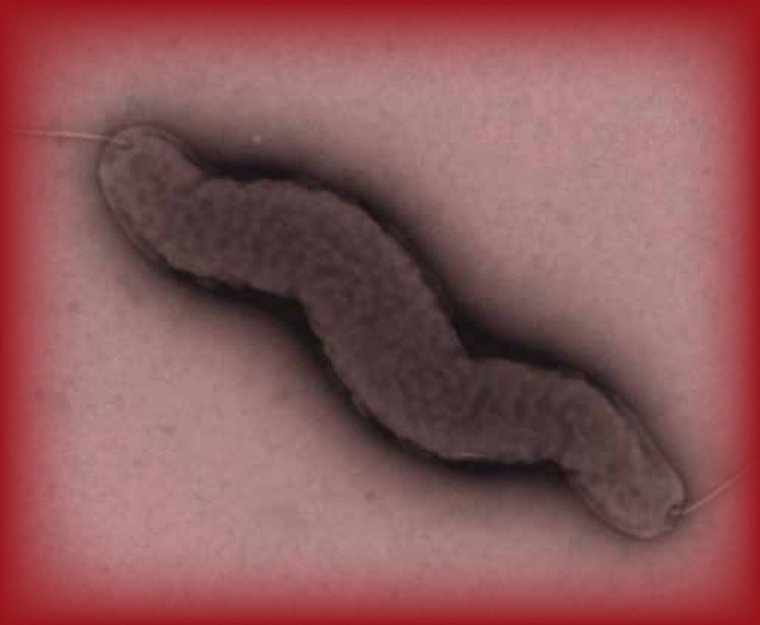
Campylobacter jejuni: exposure assessment and hazard characterization

Growth, survival and infectivity of *Campylobacter jejuni*



Liesbeth Verhoeff-Bakkenes

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Growth, survival and infectivity of *Campylobacter jejuni*

Liesbeth Verhoeff-Bakkenes

Thesis

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To my four beloved men: Gerjan, Pieter, Rutger and Jesper

To my parents

Contents

Abstract

Campylobacter jejuni, a small, curved or spirally shaped highly motile microorganism, is identified as a major cause of bacterial gastroenteritis throughout the world. Serious complications such as the Guillain-Barré syndrome and reactive arthritis might occasionally follow infection. In this thesis data were generated in the fields of hazard characterization and exposure assessment, which were used to identify risk factors for *Campylobacter*.

Although *C. jejuni* is generally seen as an obligate micro-aerophile, in this thesis it was shown that it can grow aerobically in the presence of pyruvate, and growth might also be possible in the presence of other antioxidants. However, as result of the strict minimal growth temperature (30ºC), *C. jejuni* is still assumed to be generally unable to grow in foods and therefore growth is not considered as a large risk for campylobacteriosis.

C. jejuni can, and does, survive effectively for long periods of time under non-growing conditions in various environments. Temperature is the most influencing factor in survival and survival will be optimal at low temperatures (around 4ºC). As many foods are stored chilled, this will prolong the survival of *C. jejuni*. Furthermore survival is enhanced at low oxygen conditions, as often present in packaged foods. While the effects of environmental conditions on the survival of *C. jejuni* have been studied extensively, the knowledge of the effect of environmental factors on the infectivity was scarce. Therefore, the effect of environmental conditions (temperature, medium and atmosphere) on both the survival of *C. jejuni* and the infectivity of the surviving cells was investigated. We revealed that culturability and infectivity are linearly related. Furthermore, our study on the effect on the infectivity of adding nonculturable *C. jejuni* cells to culturable cell suspensions, showed nonculturable cells not to be infective (*in vitro*). Therefore, absence of culturable *C. jejuni* cells indicates that a product can be regarded as representing a very low risk with respect to campylobacteriosis.

Food products can be contaminated with *Campylobacter* during production or afterwards at the consumer's home. Once contaminated the risk is high that *Campylobacter* will survive on food products until the moment of consumption, except if the product undergoes an elimination step during preparation, such as heating. The growth requirements for *Campylobacter* are met in the gastrointestinal tract of warm-blooded animals. As a result especially products of animal origin, like chicken meat and raw milk, are often contaminated by *Campylobacter*, but also products of non-animal origin, like vegetables and fruits, can be contaminated. Within the exposure assessment cross-contamination in the home, the importance of raw vegetables and fruits as a risk factor for *Campylobacter*, and the relative importance of chicken meat, raw milk and raw vegetables and fruits were studied.

The effect of different cross-contamination routes during the preparation of a chicken fruit salad was investigated. It was shown that the mean transfer rate by cross-contamination was 0.12% of the initial number of *C. jejuni* on the chicken fillet, and that the different tested cross-contamination routes; cutlery, cutting board and hands, were equally important. The high prevalence and concentration of *C. jejuni* on chicken meat, the high percentage of consumers who exhibit improper hygienic and cleaning behaviour, and the significant transfer of *Campylobacter* during improper food preparation, indicate cross-contamination from raw foods, such as chicken, to other ready-to-eat foods as a large risk factor for *Campylobacter* infection.

Fresh produce can become contaminated with *C. jejuni* during cultivation or processing. Especially vegetables and fruits which are eaten raw may present a risk. In this thesis Dutch data on the prevalence of *Campylobacter* on raw vegetables and fruits were newly analysed. Thirteen of the 5640 vegetable and fruit samples were *Campylobacter* positive, resulting in a prevalence of 0.23% (95% confidence interval (Cl): 0.12-0.39%). The prevalence on packaged products was significantly higher than on unpackaged products.

Finally, the newly acquired data and quantitative literature data on the prevalence and concentration of *Campylobacter* entering the consumer phase were summarized for three food groups; vegetables and fruit, chicken, and raw milk. These data were used in a quantitative risk assessment model to estimate the exposure and the number of illnesses as result of the consumption of these foods and to compare their relative importance. The prevalence and concentration are low on raw vegetables and fruit, but the quantity of raw vegetables and fruits consumed is very high. In contrast, raw chicken and raw milk show relative high prevalence and concentration levels, but the consumption is low. Despite the differences in parameters, the exposure calculated out of the combination of parameters indicates all these three foods as high risk factors for *Campylobacter*. So foods which are not heavily contaminated with *Campylobacter*, but are consumed in large quantities can constitute a similar high risk on *Campylobacter* infection as foods which are consumed in small quantities but are heavily contaminated with *Campylobacter*.

1

Introduction and outline

Introduction

The first description of bacteria now recognized as members of the genus *Campylobacter* probably was in 1886, when "vibrios" in the large intestines of a child were observed by Dr Theodor Escherich (1886). Next, McFaydean and Stockman (1913) were the first who were able to culture "a vibrio" which caused abortion in a sheep and probably is now known as *Campylobacter fetus*.

From the start *Campylobacter* was mainly considered to be a veterinary pathogen and only few reports on human infections were published. Levy (1946) described a milk borne outbreak of diarrhea, which is now regarded as the first well-documented instance of human infection caused by *Campylobacter jejuni* (or *C. coli*). Vinzent et al. (1947) reported *Vibrio fetus* as a cause of septic abortion in woman, and King (1957) isolated *V. fetus* and "related vibrios" from blood samples of children with diarrhea. The genus name *Campylobacter* (campylo - Greek adjective meaning curved; bacter - Greek noun meaning rod) was officially introduced in 1963, when Sebald and Veron (1963) showed that there were certain fundamental differences in DNA base content between the micro-aerophilic *V. fetus* group and *Vibrio* spp.

A breakthrough in *Campylobacter* research was the development of proper isolation techniques in the 1970s (Dekeyser et al. 1972; Butzler et al. 1973; Skirrow 1977), enabling clinical laboratories to easily isolate this pathogen from human faeces, which elucidated its significance as a human pathogen and a major cause of bacterial diarrhea.

Currently 26 species and 11 subspecies of *Campylobacter* are recognized and named (On 2001; Euzéby 2011). This thesis focuses on the thermotolerant *Campylobacter jejuni* subsp. *jejuni* (further called *C. jejuni*), responsible for 80 to 99% of all human *Campylobacter* infections (Lindblom et al. 1990; Tauxe 1992; Allos 2001). However, various other *Campylobacter* species, including *C. coli*, *C. upsaliensis*, *C. hyointestinalis* and *C. lari* can also infect humans (Mishu et al. 1992; Jones 2001).

Campylobacter nowadays is the leading cause of bacterial gastroenteritis worldwide, with approximately 59,000 estimated cases per year in the Netherlands (Kemmeren et al. 2006), but despite several decades of research many questions remain. In this thesis data were generated in the field of hazard characterization and exposure assessment. The outcomes can be used to identify risk factors for *Campylobacter* and to give better education on the safety and handling of food, leading to a decrease in the cases of campylobacteriosis.

Campylobacteriosis, the disease

Clinical features

Most typically, an infection with *C. jejuni* results in acute gastrointestinal illness characterized by profuse diarrhea, fever, and abdominal cramps. However, clinical manifestations are extremely diverse, ranging from a complete absence of symptoms to severe inflammatory diarrhea (Butzler and Skirrow 1979; Walker et al. 1986). The incubation period usually ranges from 1 to 7 days (Skirrow 1994; Van Vliet and Ketley 2001), although the exact timing of infection is often difficult to establish (Ketley 1997). Patients are likely to be out of action for about one to two weeks, but the duration is highly variable, up to one month or more (Butzler and Skirrow 1979; Havelaar et al. 2000).

Acute enteritis due to a *Campylobacter* infection begins in about half the patients with a prodrome characterized by acute abdominal pain (70-90% of cases), headache, myalgia, and general malaise. Fever is reported by more than 90% of patients and can be low-grade to above 40°C. After 12 to 24 h the symptoms progress into a profuse diarrhea (90-100% of cases), which might contain blood (35% of cases); nausea and vomiting are also documented symptoms (25-40% of cases). After 2 to 3 days bowel actions gradually become fewer and the stools semi-formed, but characteristically the abdominal pain and discomfort persist even after the diarrhea has stopped (Butzler and Skirrow 1979; Walker et al. 1986; Butzler and Oosterom 1991; Kapperud and Aasen 1992; Havelaar et al. 2000; De Wit et al. 2001a; Butzler 2004). The disease is usually self-limiting and only incidentally treatment with antimicrobial agents is needed (Skirrow and Blaser 1992). Bacterial shedding often persists for about two to five weeks after clinical symptoms have ended (Butzler and Skirrow 1979). For example, Taylor et al. (1993) were able to isolate high numbers of *Campylobacter* (10^6 to 10^8 per gram) from human faeces for two to three weeks. However, family studies have shown that inter-human transmission is low, probably on account of the short survival of *C. jejuni* outside the body (Butzler and Skirrow 1979). Inter-human transmission has been described infrequently in young children (Butzler 2004).

Complications are uncommon, but may follow infections with *Campylobacter*. The most serious complication is the Guillain-Barré syndrome (GBS), an acute demyelinating disorder of the peripheral nervous system resulting in acute neuromuscular paralysis. The estimated occurrence ranges from 1 in 1000 *Campylobacter* infection cases in the United States (Allos 1997; Nachamkin et al. 1998) to 1 in 5000 cases in the Netherlands (Havelaar et al. 2000). Approximately 20% of patients with GBS are left with some disability, and approximately 5% die despite advances in respiratory care (Havelaar et al. 2000). The most frequent complication associated with *Campylobacter* infection is reactive arthritis (ReA)

in about 1 to 3% of cases. ReA is an immune-mediated inflammation of the joints. Multiple joints can be affected, particularly the knee joint. In general symptoms last for two to ten weeks, and for almost all cases full recovery is reported (Peterson 1994; Skirrow 1994; Havelaar et al. 2000). Other complications associated with *C. jejuni* are inflammatory bowel disease (IBD) (Mangen et al. 2004), Miller Fisher syndrome (Jacobs et al. 1995), bacteraemia (Butzler et al. 1992), and peritonitis (Crushell et al. 2004). Deaths from *C. jejuni* infection are rare and occur primarily in infants, the elderly, and patients with underlying illnesses (Tauxe 1992). The median number of fatal cases in the Netherlands was estimated to be up to 25 cases per year, with a range between a minimum of 18 and a maximum of 90 cases (Kemmeren et al. 2006).

Pathogenesis

In association with food or water, *C. jejuni* orally enters the host. The mechanism of how the pathogen responds to acid stress in the stomach has not been well studied. Cells adapted to acid-, aerobic-, and starvation-conditions were able to withstand further acid challenges in comparison to non-stressed cells in some strains, which is probably the result of an adaptive tolerance response (Reid et al. 2008; Ma et al. 2009). Phospholipid modification and hydrogenase activity also may be important for acid resistance in the stomach (Reid et al. 2008).

After passage through the stomach, it reaches the intestines where it colonizes the mucous layer covering the epithelium of the distal ileum and colon (Ketley 1997). After colonizing, it perturbs the normal absorptive capacity of the intestine by damaging epithelial cell function either directly, by cell invasion or the production of toxin(s), or indirectly, following the initiation of an inflammatory response (Ketley 1997).

To cross the mucous layer during colonization of the intestine, *C. jejuni* needs a high degree of motility (Morooka et al. 1985). It usually possesses a polar flagellum at one or both ends. Together with the spiral shape, these flagella give a high degree of motility to the cell with the characteristic ‘cork-screw’ motion (Griffiths and Park 1990; Ketley 1995). Also chemotaxis, the ability to move along chemical concentration gradients, is essential for *Campylobacter* infections, as non-chemotactic mutants were unable to colonize the intestines in animal models (Takata et al. 1992; Van Vliet and Ketley 2001).

After crossing the mucous layer covering the epithelial cells, *C. jejuni* adheres to the cells, and a subpopulation invades the epithelial cells (Van Vliet and Ketley 2001). Inflammation and occasional bacteraemia strongly suggest that cell invasion is an important pathogenic mechanism for *Campylobacter*. Invasion has been demonstrated *in vivo* and *in vitro*, although the invasive ability of strains differs (Everest et al. 1992; Konkel et al. 1992).

The ability to adhere and invade cells has been attributed to a variety of surface components, including flagella (Wassenaar et al. 1991; Grant et al. 1993; Yao et al. 1994), the major outer-membrane constituents lipooligosaccharides and lipopolysaccharides (Newell et al. 1985a; McSweegan and Walker 1986; Fry et al. 2000; Jin et al. 2001; Van Vliet and Ketley 2001) and the outer-membrane adhesion proteins (De Melo and Pechère 1990; Konkel et al. 1997; Pei et al. 1998; Ziprin et al. 1999). Microfilaments, microtubules and receptor mediated endocytosis play a role in invasion, but the exact mechanism of invasion is not clear yet (De Melo et al. 1989; Konkel and Joens 1989; Oelschlaeger et al. 1993; Russell and Blake 1994; Biswas et al. 2000). Once colonization has been established, oxidative stress defense and iron acquisition are important for intracellular growth and survival, whereby toxins may be produced or not (Ruiz-Palacios et al. 1983; McCardell et al. 1984; Johnson and Lior 1986; Fricker and Park 1989).

Incidence and disease burden

In the last decade of the 20th century *Campylobacter* became the most frequently reported cause of bacterial gastrointestinal illness in many industrialized countries (Friedman et al. 2000; WHO 2001; EFSA 2005; EFSA 2009). As shown in Fig. 1.1, from that time the reported incidence of campylobacteriosis in the EU has stabilized, except the increase from 2003 to 2004 which can be explained by several new member states with high reported incidence of campylobacteriosis which joined the EU in 2004 (EFSA 2005; EFSA 2009).

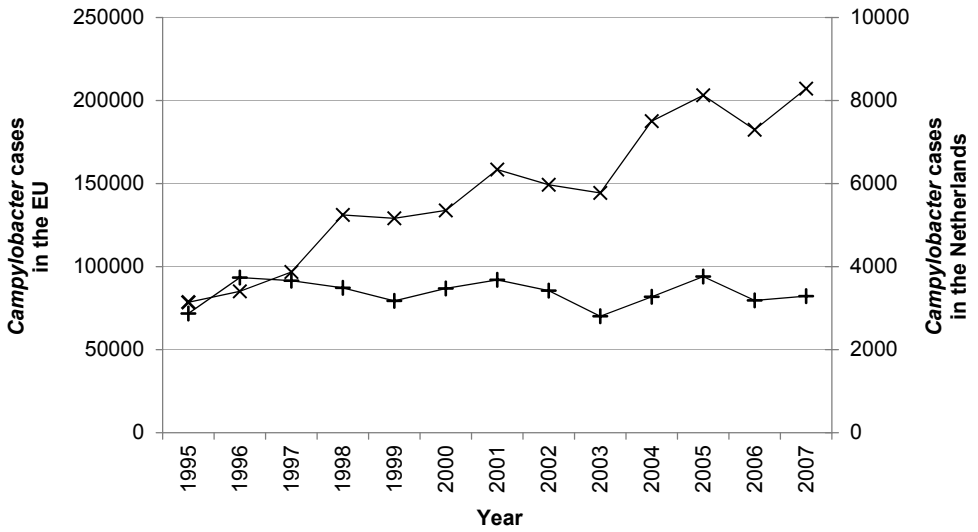

Figure 1.1. Reported confirmed *Campylobacter* cases in the EU (x) and in the Netherlands (+) from 1995-2007 (EFSA 2005; EFSA 2009).

Since 2004 approximately 200,000 confirmed cases have been reported in the EU annually, of which around 3200 from the Sentinel system in the Netherlands with an estimated coverage of 52% of the Dutch population. The estimated rate (number of confirmed cases/100,000 individuals) differs strongly around the word, with New Zealand as the country with the highest rate, 396/100,000 (Baker et al. 2007), compared to e.g. the EU with 45.2/100,000 (EFSA 2009), the Netherlands with 38.6/100,000 (EFSA 2009), and the United States with 12.7/100,000 (Centers for Disease Control and Prevention 2007). Table 1.1 shows the number of reported confirmed *Campylobacter* cases per 100,000 inhabitants per country in the EU in 2007. However, a comparison between countries should be carried out with caution, as different reporting systems and microbiological methods employed do influence the outcomes. For instance the very low number of reported cases in Poland is the result of limited surveillance as routine diagnosis is limited to certain regions of the country (EFSA 2009).

Table 1.1. Reported *Campylobacter* cases per 100,000 inhabitants per country in the EU in 2007 (EFSA 2009)

Country	Cases per 100,000	Country	Cases per 100,000	Country	Cases per 100,000
Poland	0.5	Ireland	43.7	Finland	77.8
France	4.8	Belgium	55.8	Sweden	78.0
Estonia	8.5	Hungary	57.7	Switzerland	79.5
Spain	11.4	Norway	60.6	Germany	80.3
Lithuania	16.7	Slovakia	62.7	United Kingdom	95.0
Iceland	30.2	Austria	70.1	Czech Republic	234.6
Netherlands	38.6	Denmark	71.0	Total EU	45.2

The true population incidence of campylobacteriosis is estimated to be 7.6 to 100 times higher than the number of reported cases (Skirrow 1991; Mead et al. 1999; Wheeler et al. 1999; Van Pelt et al. 2003; Samuel et al. 2004), as corrections have to be made for several factors like the percentage of cases visiting a physician and the number of cases tested for *Campylobacter*.

In the Netherlands (16 million inhabitants) an incidence of about 59,000 gastroenteritis cases was estimated in 2004, of which 12,000 (20%) required medical help, 25 were fatal, 60 resulted in GBS, 1050 in ReA and 22 in IBD (Kemmeren et al. 2006). For public health measures, the sum of years of life lost by premature mortality and years lived with disability, weighted with a factor for the severity of illness, can be expressed in disability adjusted life years (DALYs). The disease burden of *Campylobacter*-associated illness in the

Netherlands was equal to about 1300 DALYs per year, similar to diseases such as meningitis and upper respiratory infections. The associated cost-of-illness, including health care costs and paid employment lost due to work absence, was estimated to about 22 million euros per year for the Netherlands (Havelaar et al. 2000; Kemmeren et al. 2006).

Epidemiology of infection

Outbreaks account only for a small part of human campylobacteriosis cases, since most cases are sporadic (Tauxe 1992; Frost 2001). Blaser (1997) even estimated over 95% of all cases as being endemic. However, the number of outbreaks might be under-reported as result of the set-up of surveillance studies and the long incubation period of *Campylobacter* when compared to *Salmonella* spp. or *E. coli*. Gillespie et al. (2003) reported for 333 of 3489 cases of apparently sporadic *Campylobacter* infections knowledge of an individual outside the household with a similar coincident illness, which suggests that these cases might be part of an outbreak instead of sporadic. Moreover, Pearson et al. (2000) reported a large outbreak which was only recognized after genotyping isolates.

The epidemiology of outbreaks differs from that of sporadic infections. Main risk factors for human *Campylobacter* outbreaks are the consumption of raw milk, contaminated water from a municipal supply or untreated surface water, and to a lesser extent undercooked poultry (Tauxe 1992; Blaser 1997; Pebody et al. 1997). Whereas, the most important factor for sporadic *Campylobacter* infections is the handling or consumption of poultry products. Drinking raw milk or untreated surface water, living in a household with a cat or a dog, and foreign travel are other dominant vectors in the transmission of sporadic *Campylobacter* infections as identified by case-control studies (Hopkins et al. 1984; Deming et al. 1987; Kapperud and Aasen 1992; Tauxe 1992; Adak et al. 1995; Blaser 1997). Although handling or consumption is depicted as most important factor, several studies showed (frequent) handling or consumption of poultry at home to be protective (Adak et al. 1995; Eberhart-Phillips et al. 1997; Friedman et al. 2000; Effler et al. 2001). This might be the result of immunity, which is in agreement with the reduced susceptibility to campylobacteriosis after long-term exposure of poultry abattoir workers (Christenson et al. 1983; Cawthraw et al. 2000).

The incidence of campylobacteriosis is influenced by demographics, the season, and the degree of urbanization. In industrialized nations *Campylobacter* infections occur in all age groups, but two age-peaks occur. The first peak is in the youngest age group (0-5 years) and a second surge occurs during young adulthood (15-29 years) (Skirrow 1991; Kapperud and Aasen 1992; Tauxe 1992; Adak et al. 1995; Blaser 1997; Friedman et al. 2000; Studahl and Andersson 2000; De Wit et al. 2001b; Van Pelt et al. 2003). The first peak for infants can probably be explained by their greater vulnerability, higher exposure to pets and the

environment, and a low threshold for seeking medical attention (Sibbald and Sharp 1985; Tauxe 1992; Saeed et al. 1993). The peak in young adults might be explained by poor food handling (Tauxe 1992). Some studies found higher infection rates among males (Tauxe et al. 1988; Friedman et al. 2000), while in the Netherlands the high peak for young adults was attributed mainly to women (De Wit et al. 2001a; Van Pelt et al. 2003).

Human *Campylobacter* infections have demonstrated a marked seasonal distribution with peaks during the late spring and summer (Friedman et al. 2000; Sopwith et al. 2003; Van Pelt et al. 2003). This seasonal distribution might be the result of differences in food preparation and consumption patterns during the year, e.g. more raw products and barbequed food consumed during the summer, or by the influence of temperature, directly or indirectly as contamination sources in the farm environment like migratory birds, beetles, rodents and flies are also temperature dependent (Hald et al. 2004; Patrick et al. 2004; Ekdahl et al. 2005; Louis et al. 2005; Nichols 2005). Furthermore the *Campylobacter* incidence in poultry flocks which also peaks during this period might be related to the number of human infections (Stanley et al. 1998; Van Pelt and Valkenburg 2001).

The level of urbanization also correlates with the incidence. Lower incidence rates were shown in rural than in urbanized areas in the Netherlands (Van Pelt et al. 2003; Van Hees et al. 2006), which might be explained by differences in food handling and consumption patterns (Van Hees et al. 2006). On the other hand, a Danish study showed increased risk of *Campylobacter* infections, especially in children, in rural areas, which was explained by the increased exposure to farm animals and natural environments in the countryside in comparison with the city (Ethelberg et al. 2005).

Campylobacter jejuni, the microorganism

C. jejuni is a small (1.5-6.0 μm long and 0.2-0.5 μm wide), curved or spirally shaped, highly motile, Gram-negative bacterium with a single polar flagellum at one or both ends of the cell (Ketley 1997). It is catalase and oxidase positive, and urease negative (Griffiths and Park 1990; Van Vliet and Ketley 2001).
C. jejuni has a genome of 1.6-1.7 Mb, which is relatively small when compared to that of other enteropathogens such as *E. coli*, which have a genome of approximately 4.5 Mb. The G+C content of their DNA is on average 28 to 38% (Park et al. 1991).

Table 1.2. Limits for the growth of *Campylobacter* spp. (ICMSF 1996)

Parameter	Minimum	Optimum	Maximum
Temperature (ºC)	30	42-43	45
Oxygen (%)	3	5	15
Carbondioxide (%)	-	10	-
pH	4.9	6.5-7.5	Ca. 9.0
Water activity (a_w)	0.987	0.997	-
NaCl (%)	-	0.5	1.5

Growth

Compared to other foodborne bacterial pathogens, the growth requirements of *C. jejuni* as shown in Table 1.2 are very strict, although they differ significantly per strain.
As a member of the thermotolerant campylobacters, its optimal temperature is 42-43°C (Butzler and Skirrow 1979; Griffiths and Park 1990; Nachamkin 1995, ICMSF 1996), and it can multiply within a growth range from 30°C to 45°C (Doyle and Roman 1981; Griffiths and Park 1990; Park et al. 1991; Grant et al. 1993; ICMSF 1996; Hazeleger et al. 1998). Unlike other microorganisms, which show a gradual reduction in growth rate near the minimal growth temperature, *C. jejuni* shows a dramatic and sudden growth rate decline near the lower temperature limit of 30°C (Hazeleger et al. 1998). This might be explained by the absence of cold shock proteins, which are associated in many other bacteria with the ability to grow at temperatures below the optimum for growth (Hazeleger et al. 1998; Park 2002). Furthermore, transitions in the structure of important enzymes or regulatory compounds might play a role (Hazeleger et al. 1998).
C. jejuni is normally characterized as an obligate micro-aerophile with a respiratory metabolism based on the use of oxygen as a terminal electron acceptor (Griffiths and Park 1990; Hazeleger et al. 1995; Kelly 2001), and can normally grow at partial oxygen tensions from 3 to 15% oxygen (On 1996; Altekruse et al. 1999). The genome sequence of *C. jejuni*

11168 also encodes a number of reductases, which are predicted to allow the bacterium to use several alternative electron acceptors besides oxygen, including fumarate, nitrate, nitrite, and N- or S-oxides. However, *C. jejuni* is seemingly unable to grow under strict anaerobic conditions (Veron et al. 1981; Parkhill et al. 2000; Kelly 2001; Sellars et al. 2002), which might be explained by an oxygen requirement for DNA synthesis (Sellars 2002). Nevertheless, alternative respiratory pathways can contribute to growth under micro-aerobic conditions, as adding fumarate, nitrate, nitrite, TAO, and DMSO was shown to significantly increase both the growth rate and final cell density under oxygen-limited conditions (Sellars et al. 2002).

Although *C. jejuni* requires oxygen as a terminal electron acceptor to respire, it is sensitive to high oxygen tensions, such as normal atmospheric concentration (Hoffman et al. 1979a, b; Humphrey 1988; Hodge and Krieg 1994). However, the tolerance to oxygen varies greatly between strains (Griffiths and Park 1990), and for some strains the development of aerotolerance has been described (Jones et al. 1993; Chynoweth et al. 1998). During respiration, as result of the partial reduction of oxygen to water, reactive oxygen intermediates (ROI) are formed. ROI, such as the superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2), can cause lethal damage to bacterial cells (Fridovich 1978; Imlay and Linn 1988). Three oxidative defense mechanisms against ROI play an important role in the growth and survival of *C. jejuni*. Superoxide dismutase (SOD) removes superoxide anions by their dismutation into hydrogen peroxide and oxygen. *C. jejuni* possesses a single iron cofactored SOD which plays a key role in the defense against oxidative stress and aerotolerance (Pesci et al. 1994; Purdy and Park 1994; Purdy et al. 1999). Catalase (KatA) degrades hydrogen peroxide to water and oxygen (Grant and Park 1995). Alkyl hydroperoxide reductase (AhpC) removes hydroperoxide intermediates and can play a role in detoxification as it repairs damaged molecules that have been peroxidised (Baillon et al. 1999). As *C. jejuni* is not able to grow in air, SOD, KatA and AhpC activities appear to give limited protection to the toxic effects of oxygen derivates. Another explanation for the oxygen sensitivity of *C. jejuni* might be the possession of some enzymes which are particularly sensitive to oxygen or to a high oxidation-reduction potential (Lascelles and Calder 1985; Kelly 2001).

To minimize oxidative damage, *C. jejuni* is generally cultured in complex media with additional growth supplements, like blood, charcoal, ferrous sulphate, sodium metabisulfite, and sodium pyruvate (Hoffman et al. 1979a; Bolton et al. 1984; Kelly 2001). These supplements might act by reducing the concentration of free oxygen radicals in the medium or by preventing accumulation of oxygen derivates (Bolton and Coates 1983; Bolton et al. 1984; Hodge and Krieg 1994).

C. jejuni is unable to metabolize externally supplied glucose, as the key glycolytic enzyme phosphofructokinase is absent (Parkhill et al. 2000). Instead, energy is derived from either

amino acids (Mansfield and Abner 2000; Kelly 2001) or tricarboxylic acid (TCA) cycle intermediates (Rowe and Madden 2000). The amino acids are deaminated to provide TCA intermediates for subsequent oxidation, but no complex molecules such as proteins are utilized (Cools 2005). In the presence of amino acids or TCA cycle intermediates, *C. jejuni* has the capacity to operate a complete TCA cycle, with all of the key enzymes present (Kelly 2001). Genes for catabolism of a limited range of amino acids are present (Parkhill et al. 2000) and cysteine, serine, aspartate, glutamate, asparagine and glutamine are shown to be utilized, however the ability to utilize these amino acids differed per strain (Tenover et al. 1985; Tenover and Patton 1987; Leach et al. 1997; Mohammed et al. 2004). The TCA cycle intermediates, succinate, fumarate, aspartic acid, and α-ketoglutarate are oxidized by *C. jejuni,* but the ability to oxidize these intermediates differed per strain (Mohammed et al. 2004). Alternatively, another metabolic pathway might be employed to operate the TCA cycle, the anaplerotic pathway as the genome encodes homologues of both pyruvate carboxylase and phosphoenol pyruvate (PEP) carboxykinase (Parkhill et al. 2000), which function in oxaloacetate and PEP synthesis respectively (Velayudhan and Kelly 2002).
The optimum pH for growth of *Campylobacter* is between pH 6.5 and 7.5 (ICMSF 1996; Stern and Line 2000). The organism is very sensitive to low pH and organic acids (Doyle and Roman 1981; Waterman and Small 1998; Altekruse et al. 1999). Growth is not observed below pH 4.9, where *Campylobacter* is inactivated quickly (Blaser et al. 1980; ICMSF 1996).
C. jejuni is particularly sensitive to drying (Lowrie et al. 1974; Doyle and Roman 1982a; Fernandez et al. 1985; Altekruse et al. 1999) and osmotic stress (Doyle and Roman 1982a; Abram and Potter 1984; Altekruse et al. 1999). It grows at a water activity above 0.987 (ICMSF 1996), and will not grow in concentrations of sodium chloride above 1.5% (Doyle and Roman 1982a; ICMSF 1996). This is likely to be because of the limited capacity for the accumulation and transport of compatible solutes (Park 2002).
Concluding, the thermotolerant and micro-aerophilic nature and the sensitivity to environmental stress place severe restrictions on the ability of *C. jejuni* to multiply outside of an animal host (Park 2002). Consequently, unlike most other bacterial foodborne pathogens, *Campylobacter* is generally regarded as not normally capable of multiplication in food during either processing or storage.

Survival

Although incapable of growth outside of an animal host as result of its strict growth requirements, *C. jejuni* survives for prolonged times in a broad range of environments. The ability to survive of *C. jejuni* is largely dependent on temperature, but also on medium,

atmosphere, pH and a_w. Furthermore a large variation was shown in the survival kinetics obtained with different strains (Chan et al. 2001; Cools et al. 2003).

Survival, defined as the conservation of culturability, of *C. jejuni* is optimal at low temperatures, as shown in Table 1.3. At 4°C, the organism is still motile and metabolically active and able to perform respiration and protein synthesis (Hazeleger et al. 1995; Lazaro et al. 1999; Park 2002; Murphy et al. 2006). Generally, as temperatures increase so does the decline to nonculturability, with links being made to an increase in the metabolic rate and rapidity of substrate utilization at the expense of other functions, including culturability (Svedhem et al. 1981; Rollins and Colwell 1986; Hazeleger et al. 1995; Buswell et al. 1998).

Most survival studies were conducted under aerobic conditions and *C. jejuni*, although micro-aerophilic, survives well under these conditions, especially at low temperatures as depicted in Table 1.3. Low temperatures, the absence of oxygen, or micro-aerobic conditions prolong the survival (Koidis and Doyle 1983; Phebus et al. 1991; Chynoweth et al. 1998), but at ~25°C no effect of oxygen was measured (Phebus et al. 1991; Chynoweth et al. 1998). For survival under aerobic stress, SOD, KatA and AhpC activities are important. An SOD-deficient *C. jejuni* mutant was less able to survive in milk and on poultry meat under aerobic conditions than the parental strain (Purdy et al. 1999). Furthermore, *C. jejuni* endures unfavourable atmospheres by their ability to move to favourable places by aerotaxis and chemotaxis (Hazeleger et al. 1998).

In some studies nutrient limitation leads to prolonged detection of culturable *C. jejuni* (Boucher et al. 1994; Hazeleger et al. 1995; Thomas et al. 1999). This is illustrated by the longer survival-times, especially at low temperatures (~4°C), in aqueous environments and nutrient poor potassium phosphate buffer containing 0.85% (wt/vol) NaCl (PBS), when compared to milk, meat or the nutrient rich brain heart infusion (BHI). At ~4°C cells remained culturable for over 50 days and for over 7 days in aqueous environments and chicken meat respectively (Table 1.3). On the contrary, other authors (Cappelier et al. 1999b; Mihaljevic et al. 2007; Klancnik et al. 2009) showed that starvation caused significant loss of culturability. This inconsistency found in literature regarding the effect of nutrient limitation might be the result of differences in media used, as medium was shown to have a direct effect on stress-survival of *C. jejuni* (Murphy et al. 2005).

The survival is also influenced by pH and a_w. At 4°C in Brucella broth *C. jejuni* was no longer detectable after 1 day at pH 4, whereas at pH 5 it remained culturable for over 13 days (Doyle and Roman 1981). On dry stainless steel surfaces inoculated with approximately 10^7 CFU/100 cm^2, and on Formica surfaces inoculated with 10^2-10^4 CFU/100 cm^2, *C. jejuni* levels decreased below the detection limit within 4 h at room temperature (Humphrey et al. 1995; Kusumaningrum et al. 2003).

Table 1.3. Time during which *Campylobacter* spp. was reported to remain culturable in different environments at ~4°C, ~12°C, ~25°C

Environment, Temperature	Survival (days)	Reference
Brucella broth + 1% agar pH 4, 4°C	<1	Doyle & Roman 1981
Brucella broth + 1% agar pH 5, 4°C	>13	Doyle & Roman 1981
PBS, 4°C	70	Hazeleger et al. 1995
BHI, 4°C	44	Hazeleger et al. 1995
Stream water, 4°C	>120	Rollins & Colwell 1986
Sterile river water, 5°C	>60	Thomas et al. 1999
Sterile drinking water, 4°C	>64	Cools et al. 2003
Ground water, 4°C	50-84	Cook & Bolster 2007
Unpasteurized milk, 4°C	6-14	Doyle & Roman 1982b
Chicken rinse, 4°C	10->14	Chan et al. 2001
Chicken wings, 5°C	>24	Zhao et al. 2003
Raw chicken breast, 2°C	24	Curtis et al. 1995
Chicken, minced meat, 4°C	>7	Svedhem et al. 1981
Chicken mince, micro-aerobic, 5°C	>33	Chynoweth et al. 1998
Sterile chicken mince, aerobic, 5°C	<26	Chynoweth et al. 1998
Autoclaved chicken meat, 4°C	>18	Blankenship & Craven 1982
Cooked minced beef, 2°C	49	Curtis et al. 1995
Fresh beef, 4°C	4 - >25	Hänninen et al. 1984
PBS, BHI, 12°C	15-20	Hazeleger et al. 1995
Sterile river water, 15°C	40-45	Thomas et al. 1999
Raw chicken breast, 10°C	13	Curtis et al. 1995
Cooked minced beef, 10°C	23	Curtis et al. 1995
Brucella broth + 1% agar pH 4, 25°C	<1	Doyle & Roman 1981
Brucella broth + 1% agar pH 7, 25°C	>4	Doyle & Roman 1981
PBS, BHI, 25°C	6-8	Hazeleger et al. 1995
Stream water, 25°C	28	Rollins & Colwell 1986
Sterile river water, 25°C	<5	Thomas et al. 1999
Skim milk, 25°C	>1	Purdy et al. 1999
Chicken skin, 25°C	>2	Purdy et al. 1999
Raw chicken breast, 20°C	6	Curtis et al. 1995
Chicken, minced meat, 20°C	3	Svedhem et al. 1981
Raw or sterile chicken mince, 25°C	>8	Chynoweth et al. 1998
Autoclaved chicken meat, 23°C	14 - >18	Blankenship & Craven 1982
Cooked minced beef, 20°C	7	Curtis et al. 1995
Fresh beef, 20°C	1 - >4	Hänninen et al. 1984

Campylobacter is relatively sensitive to freezing (Humphrey and Cruickshank 1985). Whilst several factors, including ice nucleation and dehydration, have been implicated in the freeze-induced injury of bacterial cells, oxidative stress plays a key-role in freeze-thaw induced killing of *Campylobacter* (Stead and Park 2000). Two to three log reductions in *Campylobacter* counts on poultry were shown within days or a few weeks at -20ºC. However, part of the cells survived and could be isolated from the surface of poultry after several weeks of frozen storage (Simmons and Gibbs 1979; Solow et al. 2003; Bhaduri and Cottrell 2004; Sandberg et al. 2005; Ritz et al. 2007).

Viable nonculturable state

As reviewed above, the effect of environmental conditions on the survival of *C. jejuni* has been studied extensively. However, there is a gap in the knowledge about the infective ability of the surviving cells and the influence of environmental conditions on the infectivity. Yet, the exposure is often measured as the number of culturable *C. jejuni* cells in a product. This is only a good measure if there is a correlation between the number of culturable *C. jejuni* cells and the infectivity. There is still much debate on the existence and importance of a viable nonculturable (VNC) state of *Campylobacter*, but transformation into a more resistant, potentially pathogenic VNC state could be a survival strategy (Rollins and Colwell 1986). A bacterium in the VNC state is defined as a cell which is metabolically active (viable), while being incapable of undergoing the cellular division (nonculturable) required for growth in or on a medium normally supporting growth of that cell (Oliver 1993). The VNC state was firstly reported by Xu et al. in 1982 for *Escherichia coli* and *V. cholerae*. Rollins and Colwell (1986) were the first to report a VNC form of *C. jejuni*. A sub-population of cells was reported, which do not grow in usual culture media, and cannot be resuscitated by traditional techniques, but remain physiologically active for several functions such as cellular elongation (Rollins and Colwell 1986), respiratory chain activity (Cappelier et al. 1997), and incorporation of radio-labelled substrates (Rollins and Colwell, 1986). However, these measurements of metabolic activity as indicator for viability are questioned, whilst some enzymatic activity might persist when cells die in a gradual manner (Dodd et al. 1997; Bogosian et al. 1998). To be of public health significance, VNC cells have to be able to recover to the metabolically active state, or have to remain virulent in the VNC state. It is not clear whether VNC cells can revert back to the culturable form. On the one hand, it is suggested that the VNC state of *Campylobacter* is a 'dormant'-like survival state for adverse environmental conditions and that VNC cells are capable of growth once circumstances improve. Indeed, recovery or reversion of the VNC form of *C. jejuni* was reported after passage through mice, chickens or embryonated eggs (Jones et al. 1991; Saha et al. 1991; Pearson et al. 1993: Stern et al. 1994; Cappelier et al. 1999a, b;

Chaveerach et al. 2003). On the other hand, the formation of *Campylobacter* VNC cells is suggested to be a passive degeneration process, which is supported by studies in which protein synthesis was shown to be not essential for the formation of VNC cells (Boucher et al. 1994; Hazeleger et al. 1995; Hudock et al. 2005) and studies which failed to recover VNC cells of *C. jejuni* after passage through humans, mice, rabbits, and chickens (Beumer et al. 1992; Medema et al. 1992; Van de Giessen et al. 1996; Hald et al. 2001). Many conflicting articles have also been published on the virulence properties of *Campylobacter* VNC cells. Some authors showed that the VNC cells were colonizing chickens and mice (Jones et al. 1991; Stern et al. 1994; Cappelier et al. 1999a), and Lazaro et al. (1999) showed VNC *C. jejuni* cells with intact DNA content which implies that they are still virulent. Klancnik et al. (2009) showed a reduced virulence activity and a decreased infection capacity *in vitro* (Caco-2 cells) and *in vivo* (mouse model) for VNC cells. Others showed that VNC cells were not infective in chickens and mice (Medema et al. 1992; Stern et al. 1994; Fearnley et al. 1996; Van de Giessen et al. 1996) and did not result in an antibody response in humans, mice and rabbits (Beumer et al. 1992).

The many conflicting articles published on the recovery and virulence properties of VNC cells might be explained by differences in animal models, *Campylobacter* strains and conditions under which the VNC cells were formed. Strain differences were shown in various studies and only part of the strains tested were shown to form VNC cells (Medema et al. 1992; Federighi et al. 1998; Chaveerach et al. 2003). Temperature has a marked effect on the culturability of *C. jejuni*; at higher storage temperature *C. jejuni* shows a faster transformation to nonculturability (Rollins and Collwell 1986; Medema et al. 1992; Boucher et al. 1994; Hazeleger et al. 1995; Federighi et al. 1998; Lazaro et al. 1999). Furthermore, nonculturable cells that were formed at 4ºC, showed a marked difference in membrane fatty acid composition, ATP levels and protein expression, in comparison with those formed at 20 to 25ºC (Hazeleger et al. 1995; Höller et al. 1998). The presence of oxygen and the nutrient concentration do also influence the culturability. Aerated formation of *C. jejuni* VNC cells is much faster (3 days), than non-aerated (10 days) (Rollins and Colwell 1986; Boucher et al. 1994), and cells can remain culturable for longer periods in nutrient-poor environments when compared to nutrient-rich (Hazeleger et al. 1995). Another factor in the formation of VNC cells is time. The VNC state might be composed of two phases. At first the loss of culturability with maintenance of cellular integrity and intact RNA and DNA (and, hence, possibly viability), and thereafter the degradation of RNA and DNA resulting in the loss of viability (Hudock et al. 2005).

Besides differences in animal models, strains and environmental conditions, it is difficult to be sure that no culturable cells are inoculated and regrowth of a limited number of cells which never lost culturability might be mistaken for recovery (Kell et al. 1998). Therefore the culture method used is of importance; recovery of stress-damaged bacteria is improved

when these organisms are plated on media with additional growth supplements against oxidative damage (Mizunoe et al. 1999), while recovery is reduced by selective agents which implicate an extra stress for the sublethally injured cells (Corry et al. 1995).

C. jejuni in food microbiology

Cross-contamination

Food-related *Campylobacter* infections can originate from the consumption of food products that were contaminated already before purchase. In addition, during preparation *Campylobacter* can spread from these contaminated food products to other food products that were originally not contaminated with this pathogen, a process referred to as cross-contamination. *Campylobacter* was shown in laboratory studies to be easily transferred during food preparation (De Wit et al. 1979; Dawkins et al. 1984; De Boer and Hahné 1990; Cogan et al. 1999; Cogan et al. 2002; Gorman et al. 2002). However *C. jejuni* is susceptible to air-drying on surfaces at room temperature, which results in a rapid decrease in cell count and even on highly contaminated surfaces (~10^8 CFU/cm^2) cell numbers will be decreased to below the detection limit in 4 h at dry surfaces. Few quantitative studies estimated the cross-contamination as result of improper food preparation by measuring the transfer of indicator bacteria or *Campylobacter* from foods to other foods via hands, cutting board or knife during different preparation steps (Zhao et al. 1998; Chen et al. 2001; Montville et al. 2001; Kusumaningrum et al. 2004; Luber et al. 2006). For *Enterobacter aerogenes,* a non-pathogenic indicator organism with attachment characteristics similar to *Salmonella* on chicken, the measured transfer from chicken to lettuce or cucumber via cutting board or hands ranged from 0.07% to 1% (Zhao et al. 1998; Chen et al. 2001; Montville et al. 2001). For *Campylobacter* the measured transfer from chicken fillets to bread via hands, and from chicken fillets to cucumber via cutting board and knife was 0.11% (Luber et al. 2006), whereas the mean transfer from chicken to salad via surfaces was 1% (Kusumaningrum et al. 2004). However it is difficult to compare results of these different transfer studies as result of the different food components, kitchen utensils, and preparation schemes used.

Consumers do not always wash their hands properly (Altekruse et al. 1996; Worsfold and Griffith 1997; Yang et al. 1998; Jay et al. 1999a, b; Angelillo et al. 2001), and/or adequately clean their cutting boards or kitchen utensils (Klontz et al. 1995; Altekruse et al. 1996; Worsfold and Griffith 1997; Yang et al. 1998; Jay et al. 1999a, b; Li-Cohen and Bruhn 2002; Mitakakis et al. 2004) after handling chicken or raw meat to prevent cross-contamination. Furthermore, cleaning procedures with detergent will not always effectively remove all *Campylobacter* (Coates et al. 1987; Scott and Bloomfield 1990, 1993; Cogan et

al. 1999, 2002, Mattick et al. 2003). Based on this knowledge, cross-contamination is believed to be an important contributory factor in the consumer exposure to *Campylobacter*. This is in accordance with the results of case-control studies which indicated the handling and consumption of chicken meat as the most important risk factor for campylobacteriosis (Humphrey et al. 2001; Evans et al. 2003; Kapperud et al. 2003; Anonymous 2005; Mylius et al. 2007). As thorough heating will kill *C. jejuni* (Doyle and Roman 1981; Sorqvist 1989; Park 2002), cross-contamination will be an important infection route for high contaminated food products and several outbreaks as result of cross-contamination were described (Brown et al. 1988; Anonymous 1998; Roels et al. 1998; Gent et al. 1999; Mazick et al. 2006).

Heating

In general *Campylobacter* is characterized as sensitive to heating, but considerable variation in heat resistance has been observed between strains (Blankenship and Craven 1982; Christopher et al. 1982; Waterman 1982; Sorqvist 1989; Juneja et al. 2001).

Table 1.4. Decimal reduction time (*D*) for *Campylobacter* spp. in different media at different temperatures

Food product	Temperature (ºC)	Decimal reduction time *D* (minutes)	Reference
Physiological saline	56	0.71-0.78	Sorqvist 1989
	58	0.24-0.28	
	60	0.12-0.14	
Milk	55	~1	Waterman 1982
	48	7.2-12.8	Doyle and Roman 1981
	55	0.74-1.0	
Ground meat	50	5.9-6.3	Koidis and Doyle 1983
	60	<1	
Ground chicken meat	49	20.5	Blankenship and Craven 1982
	53	4.85	
	59	0.79	
Whole chicken fillets	~127ºC	1.95	Bergsma et al. 2007
Diced chicken fillets	~109ºC	0.59	Bergsma et al. 2007

The overview of decimal reduction times (*D*) in several media at various temperatures in Table 1.4 shows that in liquid media (physiological saline and milk) the *D*-values are lower than in solid media (ground meat), which can be explained by the fact that in liquid media

the heat can easier reach the pathogens. Moreover, *Campylobacter* was shown to be present in the deep muscle tissues of chickens and in feather follicles (Altmeyer et al. 1985; Humphrey 1991; Berndtson et al. 1992; Scherer et al. 2006; Humphrey at al. 2007; Luber and Bartelt 2007), where it is even more protected to heat than in ground meat, and the attachment of cells to biological and other materials even more increases its heat tolerance (Blankenship and Craven 1982; Humphrey et al. 2007). Bergsma et al. (2007) indeed showed remarkably higher survival rates during stir frying in a kitchen environment when compared with extrapolated literature data of laboratory studies. So, undercooked chicken can be seen as risk factor for *Campylobacter*, as also implicated by various case-control studies (Corry and Atabay 2001; Padungton and Kaneen 2003; Butzler 2004).

Food products associated with C. jejuni

Food is a common and important vehicle for transmission of *C. jejuni*. Especially products of animal origin are often contaminated by *Campylobacter*, but fresh produce might also become contaminated during cultivation. Whether these products will constitute a risk at consumption or not, depends on the influence of environmental factors and food handling processes along the food chain on the number of *Campylobacter*. As result of its strict growth requirements, it is generally assumed that *Campylobacter* cannot grow in food during either processing or storage. However, *Campylobacter* is able to survive, especially at refrigerator temperatures, for times long enough to cover the time from purchase until the moment of consumption. Proper heating will eliminate *Campylobacter*, which also eliminates the risk on campylobacteriosis. But the risk of infection increases markedly if cross-contamination to ready-to-eat products occurs.

Poultry

Chickens used for meat production are referred to as broilers. The intestinal tract of warm-blooded birds and animals serves as natural habitat for *C. jejuni*. Especially broilers, whose body temperature is 41-42ºC, are highly contaminated with this organism and can be considered as primary reservoir for *C. jejuni* (Jørgensen et al. 2002; Park 2002). Inadequate disinfection between broiler placements, flock thinning, the use of multi-unit sites, the proximity of other livestock and/or pet animals, the presence of vermin (rodents, litter beetles, house flies, wild birds), contaminated drinking water, and lapses in biosecurity have been suggested as significant risk factors for horizontal transmission of *C. jejuni* to broiler flocks at pre-harvest level (Chuma et al. 1993; Corry and Atabay 2001; Bouwknegt et al. 2004; Humphrey et al. 2007). Vertical transmission cannot yet be ruled out as an occasional route, but the fact that broiler chicks generally do not become infected until around 3 weeks of age (Corry and Atabay 2001; Gibbens et al. 2001) makes it a route of

less importance (Humphrey et al. 2007). When a broiler flock first becomes infected with *Campylobacter*, the organisms usually spread so rapidly that close to 100% of birds are reported to become colonized within a few days (Jacobs-Reitsma et al. 1995; Corry and Atabay 2001). Once colonized, broilers normally remain asymptomatic carriers (Gibbens et al. 2001), with high levels in the large intestine, the caecum and cloaca (Welkos 1984; Achen et al. 1998; Corry and Atabay 2001). The concentration in faeces at farm level in 2004 in the Netherlands was 5.4-7.9 log CFU g^{-1} (Nauta et al. 2005). Many efforts have been made to reduce the number of *Campylobacter* during rearing, with the emphasis on on-farm biosecurity measures (hygiene procedures, exclusion of vermin, a clean water supply) and cleaning and disinfection. These strict measures unfortunately did not guarantee a decrease in *Campylobacter* positive flocks (Jacobs-Reitsma 2000; Corry and Atabay 2001; Humphrey et al. 2007). In 2008 the average prevalence in broiler poultry flocks was 71.2% in the EU, and 24.4% in the Netherlands and levels of contamination varied between less than 1 log CFU g^{-1} to higher than 4 log CFU g^{-1} of neck skin together with breast skin (EFSA 2010).

The slaughter and processing of large numbers of carcasses of different sources, in combination with the high prevalence of *Campylobacter* positive flocks, and the high concentration of *Campylobacter* per carcass when contaminated, makes cross-contamination from *Campylobacter*-containing to *Campylobacter*-free carcasses unavoidable (Mead et al. 1995; Humphrey et al. 2007). Poultry meat can be contaminated either directly from the intestinal contents and faeces or indirectly via the equipment. The contamination is usually present on the surface, and as the skin is normally not removed from carcasses, large numbers of *Campylobacter* can remain in and on the skin. Measures to diminish the contamination during slaughter and processing, like improved disinfection of transport crates, slaughter of uninfected flocks prior to infected flocks, careful attention to major points of cross-contamination on the line (Corry and Atabay 2001), have not proven effective. This results in a high *C. jejuni* contamination rate of poultry meat products as shown in Table 1.5.

Two review articles on *Campylobacter* contamination in retail poultry meats in the world showed a *Campylobacter* prevalence of 50% or more for retail poultry meats in most countries (Suzuki and Yamamoto 2009) and an average prevalence of 60% for retail chicken meat (Humphrey et al. 2007). This is consistent with the mean prevalence in chicken products at retail level of 49.9% as calculated from the literature data given in Table 1.5. In the Netherlands the prevalence was much lower than the average, 15.4%, for *Campylobacter* in fresh chicken products at retail in 2006 (VWA 2009). However, it is difficult to compare prevalence data as result of differences in sampling methods (skin- or meat-samples, carcass-rinse method), type of samples (carcass, fillet, cuts with skin, etc.),

Table 1.5. Presence and concentration of *Campylobacter* in chicken products at retail level

Sample	Positive/Total	(%)	Quantity	Country	Reference
Carcass: total skin	34/133	25.6	n.d.	Belgium	Uyttendaele et al. 1999
Carcass: breast, neck skin	70/331	21	n.d.	Belgium	Ghafir et al. 2007
Carcass: neck skin	642/1127	57	n.d.	N. Ireland	Wilson 2002
	136/171	80	4.1 log CFU carcass^{-1}	England	Jørgensen et al. 2002
	59/63	94	n.d.	UK	Moore et al. 2002
Carcass: rinse	28/144	19.4	<2 - >4 log CFU g^{-1}	Netherlands	VWA 2009
	130/184	70.7	n.d.	USA	Zhao et al. 2001
	73/95	77	n.d.	UK	Harrison et al. 2001
	142/171	83	4.9- 5.1 log CFU carcass^{-1}	England	Jørgensen et al. 2002
	38/45	84	<1 - >5 log MPN carcass^{-1}	Netherlands	Dufrenne et al. 2001
	43/50	86	2 log CFU ml^{-1}	Korea	Hong et al. 2007
	74/84	88	~1 log CFU/cm^2	Australia	Pointon et al. 2008
Carcass: neck skin & rinse	1203/1675	71.8	n.d.	UK	Meldrum et al. 2006
	526/727	72.4	n.d.	Wales, Ireland	Meldrum & Wilson 2007
Fillet: rinse	87/100	87	2.7 log CFU fillet^{-1}	Germany	Luber & Bartelt 2007
Fillet: 25 g	43/356	12	n.d.	Belgium	Ghafir et al. 2007
	22/45	48.9	n.d.	Bulgaria	Stoyanchev et al. 2007
Breast: 25 g	62/519	11.9	<2- 3 log CFU g^{-1}	Netherlands	VWA 2009
	68/95	72	n.d.	UK	Harrison et al. 2001
Breast: 10 g	6/10	60	n.d.	Italy	Denis et al. 2001
Legs: rinse	92/140	66	2.4 log CFU g^{-1} skin	Germany	Scherer et al. 2006
Legs: 25 g	100/602	16.6	<2 - 4 log CFU g^{-1}	Netherlands	VWA 2009
Legs: 10 g	10/12	83	n.d.	Italy	Denis et al. 2001

Table 1.5 - *Continued*

Sample	Positive/Total	(%)	Quantity	Country	Reference
Wings: rinse	18/19	95	n.d.	Spain	Mateo et al. 2005
Wings: 100 g	4/4	100	n.d.	UK	Sails et al. 2003
Wings: 25 g	18/83	21.9	<2 - >4 log CFU g^{-1}	Netherlands	VWA 2009
	41/45	91.1	n.d.	Bulgaria	Stoyanchev et al. 2007
Thigh: 100 g	10/14	71	n.d.	UK	Sails et al. 2003
Thigh: 25 g	40/45	88.9	n.d.	Bulgaria	Stoyanchev et al. 2007
Drumsticks: rinse	24/27	89	n.d.	Spain	Mateo et al. 2005
Drumsticks: 100 g	15/19	79	n.d.	UK	Sails et al. 2003
Drumsticks: 10 g	12/12	100	n.d.	Italy	Denis et al. 2001
Chicken meat	216/1401	15.4	<2 - >4 log CFU g^{-1}	Netherlands	VWA 2009
	98/198	49.5	n.d.	Spain	Dominguez et al. 2002
	444/890	49.9	n.d.	Ireland	Whyte et al. 2004
	126/155	81.3	n.d.	Italy	Pezzotti et al. 2003
	205/230	89.1	<0.3 - 110 MPN g^{-1}	New Zealand	Wong et al. 2007
Total fresh carcass	3198/5000	64.0			
Total without skin	152/501	30.3			
Total with skin	520/1646	31.6			
Total	5133/10294	49.9			

n.d. - not determined

and time of year when sampled. Furthermore the quantities analysed varied as did the microbiological media. From the data no clear difference between the different sampling methods was observed, which is in agreement with Jørgensen et al. (2002) who did show no statistical difference between the neck skin and carcass rinse method. When comparing the different type of samples, the prevalence on carcasses is twice as high (64%) when compared to chicken cuts with skin (31.6%) and without skin (30.3%). No difference in prevalence between fresh and frozen carcasses was observed, however the counts on frozen carcasses are reported to be lower (Dufrenne et al. 2001). As shown before (Denis et al. 2001; Pointon et al. 2008) the presence or absence of skin does not influence the prevalence in poultry cuts, which contrasts with Uyttendaele et al. (1999) who showed significantly higher contamination rates of poultry cuts with skin compared to poultry cuts without skin in Belgium. Next to the high prevalence found, the contamination levels of retail chicken meat were high, in the Netherlands levels of contamination varied between 10^2 to 10^4 CFU g^{-1} chicken product (VWA 2009). Although reductions in numbers of *C. jejuni* occur during chilled and frozen storage of poultry meat products, *C. jejuni* remained culturable for more than three weeks in chicken products stored at refrigerator temperatures (4ºC) (Blankenship and Craven 1982; Curtis et al. 1995; Chynoweth et al. 1998; Zhao et al. 2003) and during freezing at -20ºC (Sandberg et al. 2005; Ritz et al. 2007). Because of the high prevalence and high numbers of *Campylobacter* in retail poultry meat, consumers and food handlers play an important role in reducing the incidence of *Campylobacter* infection by proper heating and preventing cross-contamination in kitchens or food preparation areas (Humphrey 2001).

Studies in the United States, Europe, and Australia attributed 50-70% of all *Campylobacter* infections to the consumption of chicken (Harris et al. 1986; Deming et al. 1987; Adak et al. 1995). Epidemiological studies demonstrated a significant correlation between the handling and consumption of poultry and the occurrence of *Campylobacter* enteritis. As risk factors were identified the consumption of poultry in general, of poultry and poultry liver, of raw or undercooked chicken, of barbecued chicken, and of chicken prepared by or eaten in a commercial food establishment (Ikram et al. 1994; Schorr et al. 1994; Adak et al. 1995; Eberhart-Phillips et al. 1997; Neal and Slack 1997; Friedman et al. 2000; Studahl and Andersson 2000; Rodrigues et al. 2001; Evans et al. 2003; Neimann et al. 2003; Friedman et al. 2004; Michaud et al. 2004). Furthermore a direct relation between outbreaks of campylobacteriosis and consumption of poultry was found (Tauxe 1992; Pebody et al. 1997; Evans et al. 1998; Pearson et al. 2000). The role of poultry in the epidemiology of human campylobacteriosis was also clearly demonstrated in Belgium during the dioxin crisis in 1999, where the withdrawal of poultry and eggs from the market was accompanied by 40% reduction in human *Campylobacter* cases (Vellinga and van Loock 2002).

Red meat (pork, beef, lamb)

Pigs, cattle and sheep are important reservoirs for *Campylobacter*, commonly isolated from their intestinal contents. However, in spite of the frequent contamination of pigs, cattle and sheep, e.g. 0-80% (Stern 1981; Lammerding et al. 1988; Atabay and Corry 1998; Nielsen 2002; Payot et al. 2004), the prevalence of *Campylobacter* in red meat (pork, beef, lamb) after chilling is low, e.g. 0-2% (Stern et al. 1985; Madden et al. 1998). The low contamination rate of red meat is explained by its low surface humidity after chilling, as *Campylobacter* is sensitive to dehydration (Oosterom et al. 1983; EFSA 2005). Red meat offal, on the other hand, stays moist and is much more frequently contaminated, e.g. 47-99% (Fricker and Park 1989; Kramer et al. 2000).

Case-control studies showed that infections from red meat animals can be acquired from the meat (Oosterom et al. 1984; Deming et al. 1987; Kapperud and Aasen 1992; Adak et al. 1995; Neimann et al. 2003; Schonberg-Norio et al. 2004; Carrique-Mas et al. 2005), but also via direct contact with farm animals (Kapperud and Aasen 1992; Eberhart-Phillips et al. 1997; Studahl and Andersson 2000; Evans et al. 2003; Kapperud et al. 2003; Neimann et al. 2003; Friedman et al. 2004), or indirectly via contamination of surface water by faeces or farm effluent.

Raw milk

All growth requirements are met in the gastrointestinal tract of cows and as a result the faeces of cows are often contaminated with *Campylobacter*. The faeces are the principal contamination source of milk, either directly or indirectly via the udder, and the most common route is through faecal contamination on the external surfaces of the udder and teat (Beumer et al. 1988; Atabay and Corry 1998; Stanley and Jones 2003). Direct contamination of milk as a consequence of mastitis also sometimes occurs (Orr et al. 1995). Although proper hygiene at milking can reduce both the incidence and level of contamination, and udders should be washed and dried prior to milking, this is not a completely effective control measure (Humphrey et al. 2007). Milk from all animals is collected in one tank and if one cow or milk sample was contaminated, this will result in contamination of the whole batch. The prevalence of *Campylobacter* in raw milk varied from 0.5 to 10.2% as shown in Table 1.6. As result of the antibacterial action of the lactoperoxidase system (LPS) in milk, *C. jejuni* (when present) will rapidly decrease in viable numbers (Beumer et al. 1985). This probably explains the higher prevalence found by Beumer et al. (1988), who inactivated the LPS system by raising the pH to 7.5, and by Humphrey and Hart (1988), who examined the milk within 1 h of collection, in contrary the other studies reported in Table 1.6 where samples were examined within 24 or 36 h after collection. The concentration of *Campylobacter* in raw milk is generally considered to be low; Humphrey and Beckett (1987) showed an MPN of 16 ± 30 CFU per 100 ml for raw

milk sampled within 1h, but sufficient to cause disease. The only way to ensure that people are protected from infection is pasteurization of the milk. Nevertheless a small group of consumers still prefers to drink raw milk. Raw milk is a well-documented vehicle in outbreaks (Harris et al. 1986; Kalman et al. 2000; Frost et al. 2002; Peterson 2003) and has also been implicated as a risk factor for sporadic infections (Hopkins et al. 1984; Saeed et al. 1993; Schorr et al. 1994; Adak et al. 1995; Eberhart-Phillips et al. 1997; Studahl and Andersson 2000; Neimann et al. 2003; Friedman et al. 2004; Michaud et al. 2004).

Table 1.6. Presence of *Campylobacter* in raw milk

Positive/Total	% positive	Country	Reference
8/1720	0.5	USA, Canada	Steele et al. 1997
1/69	1.4	France	Desmarus et al. 1997
2/130	1.5	Poland	Gomolka and Uradzinski 1996
1/62	1.6	Ireland	Whyte et al. 2004
5/248	2.2	USA	Jayarao et al. 2006
41/904	4.5	Netherlands	Beumer et al. 1988
67/1138	5.9	UK	Humphrey and Hart 1988
13/127	10.2	Pakistan	Hussain et al. 2007
138/4398	3.1		Total

Fruits and vegetables

Fruits and vegetables may be a risk factor for *Campylobacter* infection. Contamination of fruits and vegetables with *Campylobacter* is most likely to occur during cultivation, by soil, irrigation water, manure, faeces, air, wild or domestic animals, and/or unhygienic human handling. During harvest, especially in less developed countries, the hygiene of workers in the field is not always sufficient, which might result in contamination. Furthermore harvesting equipment and transport containers might be a source of (cross-) contamination. During storage, (cross-) contamination should be prevented and products should be protected against water, insects, rodents or other pests. During washing and processing cross-contamination between contaminated and not contaminated produce might take place, but proper washing with water might result also in a slight reduction in microorganisms by 10- to 100-fold at best (Adams et al. 1989; Beuchat 1996; Little and Gillespie 2008). After packaging, packaged products are better protected against cross-contamination than unpackaged products. At retail level improper packaging and cross contamination are important risk factors for *Campylobacter* contamination (Beuchat 1996). *Campylobacter* was detected in vegetables and fruits at retail level as shown in Table 1.7.

In general the prevalence was low (0 - 3.6%), with a mean prevalence of 0.42% for the total of studies. The much higher prevalence found by Phillips (1998) might be the result of the

more convenient atmospherical conditions for *Campylobacter* in a modified atmosphere packaging (MAP), whereas the far higher prevalence shown by Chai et al. (2007) and the relative high prevalence in the study of Kumar et al. (2001), might be caused by cross-contamination as result of less hygienic food handling conditions in Malaysia and India.
As large quantities of vegetables and fruits are served raw, even low prevalences of *Campylobacter* in these products might be a risk factor. Indeed several outbreaks were associated with the consumption of salads (Beuchat 1996; Anonymous 1998; Jacobs-Reitsma 2000; Little and Gillespie 2008) and also a case control study attributed the consumption of salad vegetables as important risk factor for *Campylobacter* infection (Evans et al. 2003). Outbreaks implicating fresh produce may be the result of contamination of the crop in the field or during processing, but may also be due to cross-contamination of the product from other contaminated raw foodstuffs during preparation.

Table 1.7. Prevalence of *Campylobacter* on vegetables and fruits reported in literature, detected by plating after enrichment

Product type	Positive/ Total	% positive	Country	Reference
RTE vegetables	0/3852	0/3852	UK	Sagoo et al. 2003b
Organic vegetables	0/3200	0/3200	UK	Sagoo et al. 2001
Unpacked salad vegetables	0/2950	0/2950	UK	Sagoo et al. 2003a
Vegetables supermarket	0/1031	0/1031	Canada	Park & Sanders 1992
RTE vegetables	0/361	0/361	Canada	Odumeru et al. 1997
Vegetables and fruit	0/300	0/300	Spain	Abadias et al. 2008
Whole lettuce	0/151	0/151	UK	Little & Gillespie 2008
Organic vegetables	0/86	0/86	Ireland	McMahon & Wilson 2001
RTE Vegetables	2/400	0.50	France	Federighi et al. 1999
Vegetables	2/279	0.72	Ireland	Whyte et al. 2004
Mushrooms	3/200	1.5	USA	Doyle & Schoeni 1986
Vegetables farmers' outdoor	9/533	1.7	Canada	Park & Sanders 1992
Vegetables	2/56	3.6	India	Kumar et al. 2001
Vegetables	28/309	9.1	Malaysia	Chai et al. 2007
MAP vegetables	20/90	22	UK	Phillips 1998
Total	79/19138	0.42		

Outline of this thesis

Campylobacter jejuni, a small, curved or spirally shaped highly motile microorganism, is identified as a major cause of bacterial gastroenteritis throughout the world. Serious complications such as the Guillain-Barré syndrome and reactive arthritis might follow infection. In this thesis data were generated in the fields of hazard characterization and exposure assessment, which can be used to identify risk factors for *Campylobacter*.

C. jejuni is regarded as generally sensitive; it does not multiply below 30ºC, is susceptible to atmospheric oxygen, is sensitive to low pH and organic acid, to drying and osmotic stress, to heating and freezing, and to disinfectants. The many cases of campylobacteriosis conflict with the strict growth requirements and sensitivity to stress of this pathogen. In **Chapter 2** the ability to grow in the presence of high oxygen concentrations, with or without pyruvate, was examined. The influence of different dissolved oxygen tensions on the growth yield and the activity of the enzymes catalase and superoxide dismutase, important in the oxidative defense system of *Campylobacter*, were studied.

C. jejuni can, and does, survive effectively for extended periods of time under non-growing conditions. While the effect of environmental conditions on the survival of *C. jejuni* has been studied extensively, the knowledge of the effect of environmental factors on the infectivity is scarce. Therefore, in **Chapter 3** the effect of environmental conditions (temperature, medium and atmosphere) on both the survival of *C. jejuni* and the infectivity of the surviving cells was investigated. The data were used to determine whether the number of culturable cells is related to the ability to interact with host cells, and the effect of strain differences was tested.

In practice the exposure is often measured as the number of culturable *C. jejuni* in a product and it is relevant to evaluate whether the culture method is effective to detect all infective *Campylobacter* cells. The formation of VNC *C. jejuni* cells as survival strategy is discussed in literature and the question rises whether nonculturable *C. jejuni* cells remain viable and cause infection in man. If VNC cells are still virulent, the risk for *C. jejuni* infection is largely underestimated. In **Chapter 4** a new approach was used to study the effect of nonculturable *C. jejuni* cells on the virulence in Int-407 cells. Instead of measuring the effect of nonculturable *C. jejuni* on their own, as done previously with the risk of few misleading culturable cells remaining, the effect of adding nonculturable *C. jejuni* on the infectivity in INT-407 cells was studied.

The high prevalence and concentration of *C. jejuni* on chicken meat and the high infection probability indicate that cross-contamination in the kitchen from raw foods, such as chicken, to other ready-to-eat foods will be an important risk for infection. To identify the

most critical transfer route for *Campylobacter*, the transfer rate for each individual step during consumer preparation of a chicken salad was estimated in **Chapter 5**.

So far poultry is seen as a major reservoir for *C. jejuni*, however not all cases of campylobacteriosis can be related to poultry. The question rises whether other not yet identified reservoirs or sources of *C. jejuni* do exist. Vegetables and fruits may be contaminated with *C. jejuni* by manure or irrigation water, and especially vegetables and fruits which are eaten raw may present a risk. To gain insight into the occurrence of *C. jejuni* on vegetables and fruits more data have to be collected. **Chapter 6** reviews the qualitative and quantitative occurrence of *C. jejuni* on vegetables and fruit.

Finally, in **Chapter 7** this thesis is concluded with a general discussion including a risk assessment of three food groups and closing remarks.

2

Pyruvate relieves the necessity of high induction levels of catalase and enables *Campylobacter jejuni* to grow under fully aerobic conditions

Abstract

The many cases of campylobacteriosis reported worldwide seemingly conflict with the strict growth requirements and sensitivity to environmental stress of *Campylobacter jejuni*. In this study, the need for a micro-aerobic environment [dissolved oxygen tension (DOT): 0.1-90%; 100% = air saturation] and the adaptive responses to oxygen stress were studied.
The growth of *C. jejuni* in continuous culture was assessed under different DOT in the presence or absence of pyruvate. In medium without pyruvate, continuous cultures of *C. jejuni* showed typically micro-aerobic behaviour and cells were unable to grow fully aerobically. However in the presence of pyruvate (25 mmol l^{-1}), continuous cultures of *C. jejuni* were able to grow in a broad DOT range, varying from 0.1 to at least 90%, and the catalase activity was decreased. In conclusion, adding pyruvate decreases the concentration of hydrogen peroxide, which enables *C. jejuni* to grow aerobically.

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Introduction

Campylobacter jejuni is the leading cause of bacterial foodborne gastroenteritis throughout the world (Friedman et al. 2000; Oberhelman and Taylor 2000). Several cases of campylobacteriosis reported worldwide seemingly conflict with the strict growth requirements and the sensitivity to environmental stress of *C. jejuni* as described in literature (Butzler and Oosterom 1991; Altekruse et al. 1999; Murphy et al. 2006).

Currently *C. jejuni* is seen as an obligate micro-aerophile, which requires oxygen concentrations of 3-15% and carbon dioxide (CO_2) concentrations of 3-5% for satisfactory growth, using amino acids as carbon and energy sources (Hoffman et al. 1979a; Smibert 1984; Kelly 2001; Park 2002; Sellars et al. 2002). However, it has been suggested that *Campylobacter* spp. can adapt to aerobic growth (Jones et al. 1993; Chynoweth et al. 1998) or can grow aerobically in 10% CO_2 in moist air (Fraser et al. 1992). *C. jejuni* has a respiratory metabolism with a branched electron transport chain based on the use of oxygen as a terminal electron acceptor; but alternative terminal electron acceptors can also be used (Mendz et al. 1997; Sellars et al. 2002). The partial reduction of oxygen to water during microbial respiration results in toxic reactive oxygen intermediates (ROI). ROI, such as the superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2) are implicated in lethal damage to nucleic acids, proteins and membranes (Fridovich 1978; Imlay and Linn 1988).

For the defense against the damaging effects of oxidative stress *C. jejuni* can make use of a single iron co-factored superoxide dismutase (SOD) (Pesci et al. 1994; Purdy and Park 1994; Purdy et al. 1999), and of catalase (KatA) (Grant and Park 1995). SOD catalyses the conversion of superoxide to H_2O_2 and oxygen, and the accumulation of H_2O_2 is prevented by the action of KatA. The sensitivity of *C. jejuni* to high oxygen concentrations might be caused by high oxidative stress, whereby the capacity of the defense systems is exceeded.

Next to protective enzymes, the addition of protective components, such as blood, charcoal, $FeSO_4$, metabisulfite and pyruvate can protect *C. jejuni* against oxygen stress and even enable *C. jejuni* to grow fully aerobically (George et al. 1978; Hoffman et al. 1979b; Bolton et al. 1984; Fraser et al. 1992; Hodge and Krieg 1994). These compounds are thought to act as quenchers of ROI, but the exact mechanism is not yet clear.

The first aim of this study was to investigate the effect of different oxygen tensions on growth yield and KatA and SOD activities of *C. jejuni*. More insight into these activities might help to further elucidate the toxic effect of oxygen on *C. jejuni* and determine why *C. jejuni* cannot grow in fully aerobic environments without protective components. The second aim of this study was to examine the effect of adding pyruvate on growth yield, and KatA and SOD activities of *C. jejuni* grown under different oxygen tensions. Mendz et al. (1997) showed that *C. jejuni* can use pyruvate as electron acceptor. It was examined

whether pyruvate acts as a quencher of ROI as suggested in literature (Hodge and Krieg 1994), or that the protective effect of pyruvate results from its use as an alternative electron acceptor for fermentation, as a switch in the metabolism from respiration to fermentation would subsequently result in the formation of less ROI.
In most studies on the effect of oxygen, the cultivation conditions were described as the percentage oxygen in the gaseous phase. However, the dissolved oxygen tension (DOT) better reflects the availability of oxygen and the conditions at which the cells are exposed, as the DOT is not only dependent on the percentage of oxygen in the input gas, but also on the geometry of the system, the biomass concentration and its specific rate of oxygen consumption (Alexeeva et al. 2002). In this study, continuous cultures were used in which the DOT was controlled.

Materials and methods

Growth conditions

C. jejuni NCTC 11168 was obtained from the National Collection of Type Cultures (Colindale, UK). Laboratory stocks of cells were stored at -70°C in a mixture of 80% brain heart infusion broth (BHI; Difco, Franklin Lakes, New Jersey, USA) and 20% glycerol. Inocula were prepared by thawing one cryovial of which 0.5 ml was used to inoculate 100 ml of BHI in a wide-necked Erlenmeyer flask. After 24 h of culturing at 37°C in a micro-aerobic atmosphere (10% O_2, 5% CO_2, 85% N_2) while shaking (100 rev min^{-1}), 5 ml was used to inoculate a Bioflo fermentor (New Brunswick Scientific, Edison, New Jersey, USA; culture volume is 1 l) or a 1-l vessel controlled by the ADI 1030 controller (Applikon, Schiedam, The Netherlands). Cultures were grown at a constant dilution rate (growth rate) of 0.1 h^{-1} (medium supply per vessel volume) in BHI without glucose (20 g l^{-1}; Difco) with or without 25 mmol l^{-1} of pyruvate (Acros Organics, Geel, Belgium). Pyruvate was sterilized by filtration (0.2 μm) and added aseptically to a medium which had been sterilized for 15 min at 121°C. A pH of 7.0 was maintained automatically using 1 mol l^{-1} of HCl and the temperature was maintained at 37ºC. The DOT (100% = air saturation) was measured using an Ingold O_2 sensor (Mettler Toledo, Tiel, The Netherlands) and regulated by adjusting the agitation speed (200-600 rev min^{-1}) and the in-going percentage of oxygen in the gas supply. The in-going gas consisted of compressed air, nitrogen, and CO_2 (5%, except at high DOT conditions). Baffles were installed in the fermentor vessels to improve the oxygen transfer from the gaseous to the liquid phase. Anti-foam A (0.1 g l^{-1}, Sigma-Aldrich, Zwijndrecht, The Netherlands) was added to the culture to prevent excessive foaming.

Sampling

C. jejuni was grown in continuous culture under different DOT (0.1, 1, 10, 25, 50, 75 and 90%; 90% is maximal reachable DOT during aeration with pure compressed air as part of the oxygen is consumed by the growing bacteria) in the presence or absence of pyruvate. To reach steady-state conditions, cells were sampled 2 days after setting the DOT. The growth yield, activities of oxygen stress-protective enzymes (KatA and SOD), activities of fermentation enzymes (lactate dehydrogenase and alcohol dehydrogenase) and the metabolites used and formed were determined for all the conditions. All conditions were sampled independently in triplicate.

Purity and growth yield

The purity of samples was verified microscopically and by streak plating, using Columbia agar plates supplemented with 5% lysed horse blood (CAB; Oxoid, Basingstoke, UK), incubated for 48 h at 37ºC under micro-aerobic conditions (10% O_2, 5% CO_2, 85% N_2).
The growth yield was monitored by measuring the dry weight. A tube was dried for 48 h at 105ºC and weighed. In the tube 15 ml of the culture was centrifuged (12 min, 4500 ***g***, 4ºC); the supernatant was then discarded and the pellet was resuspended in water, which was repeated twice. Following the final wash the tube with the pellet was dried for 48 h at 105ºC and re-weighed. The dry weight (of 15-ml culture) was calculated as the weight of the tube with the pellet minus the weight of the tube at the start.

Preparation of cell-free cell extracts (CFE)

Approximately 40 ml of the culture was harvested by centrifugation (12 min, 4500 ***g***, 4ºC). The pellet was washed with 70 mmol l^{-1} potassium phosphate-buffered saline (PBS; 9 g l^{-1} NaCl), pH 7.2 and centrifuged. Thereafter, the pellet was resuspended in 4 ml of PBS. The cells were disrupted by sonication (6 min, 50% duty cycle, 100 W; Branson 450 sonifier, Branson, Danbury, Connecticut, USA). The sonified suspensions were centrifuged for 10 min at 10 000 ***g*** at 4°C and the supernatant was used as CFE. KatA activity was measured directly; the remainder of the CFE was stored at -20°C for later use in other determinations.

KatA activity

The KatA activity was measured by monitoring the enzymatic breakdown of H_2O_2 at 240 nm as described by Beers and Sizer (1952).

SOD activity

The SOD activity of CFE was determined with an SOD Assay Kit - WST (Dojindo, Maryland, USA) as described in the manual. Technically the highly water-soluble tetrazolium salt WST-1, which produces a water-soluble formazan dye upon reduction with an O_2^-, was used as the indicating scavenger for the superoxide generated by the xanthine oxidase reaction. The rate of the reduction with superoxide is linearly related to the xanthine oxidase activity and is inhibited by SOD. One unit of SOD activity was defined as the amount of enzyme that provides 50% of inhibition. The inhibition activity was determined by a colorimetric method.

Lactate dehydrogenase (LDH) activity

The LDH activity of CFE was measured by determining the oxidation of NADH (0.15 mmol l^{-1}) in phosphate buffer (pH = 7.2) using pyruvate (1 mol l^{-1}) as the electron acceptor at 340 nm, as described by Streekstra et al. (1987).

Alcohol dehydrogenase (ADH) activity

The ADH activity of CFE was measured by determining the reduction of NAD (0.15 mmol l^{-1}) in sodium pyrophosphate buffer (12 mmol l^{-1}) using ethanol (350 mmol l^{-1}) as the electron donor at 340 nm, according to Clark and Cronan (1980).

Cellular protein determination

Cellular protein content of CFE was determined according to Lowry et al. (1951). Bovine serum albumin (Sigma) was used as a standard.

Determination of metabolites used and formed

High-performance liquid chromatography (HPLC) analysis of supernatants was performed exactly as described by Alexeeva et al. (2003).

Statistical analysis

The KatA activity, SOD activity, and dry weight, measured at different DOT, and in the presence or the absence of pyruvate, were compared by the Student's *t*-test for statistically significant differences.

Results

Growth in BHI without protecting components

In the complete DOT range of 1-75% the KatA activity increased significantly. In contrast, the SOD activity only increased significantly in the initial DOT range from 1% to 25%; when exposed to higher DOT no statistically significant differences in SOD activity were measured (Fig. 2.1a, b). The maximum observed KatA activity of 58 mmol H_2O_2 min^{-1} mg^{-1} protein was reached at a DOT of 75% (Fig. 2.1a). At a DOT of 25% the maximum observed SOD activity of 0.1 U mg^{-1} protein was reached (Fig. 2.1b). The growth yield of *C. jejuni* remained constant when the DOT was 25% or lower; when exposed to higher DOT in the range of 25-50% the growth yield diminished significantly, and at a DOT above 75% the growth rate did not match the dilution rate and consequently *C. jejuni* was washed out (Fig. 2.1c).

Growth in BHI with pyruvate added

In the presence of pyruvate, only at a DOT of 75% and higher the KatA activity was above 10 mmol H_2O_2 min^{-1} mg^{-1} protein, an activity already reached at a DOT of 10% when grown without pyruvate. The highest observed activity in cells grown at 90% DOT (16 mmol H_2O_2 min^{-1} mg^{-1} protein) was much lower than the highest observed KatA activity observed when grown without pyruvate (58 mmol H_2O_2 min^{-1} mg^{-1} protein) (Fig. 2.1a). In contrast to the large effect on KatA, pyruvate did not significantly affect the SOD activity (Fig. 2.1b).

Adding pyruvate allowed growth over a wide DOT range from 0.1 to at least 90% (Fig. 2.1c). Neither LDH, nor ADH activity was found in CFE of *C. jejuni* (data not shown) and no fermentation products (succinate, lactate, acetate, formate, fumarate, and ethanol) were detected (data not shown) by HPLC analysis.

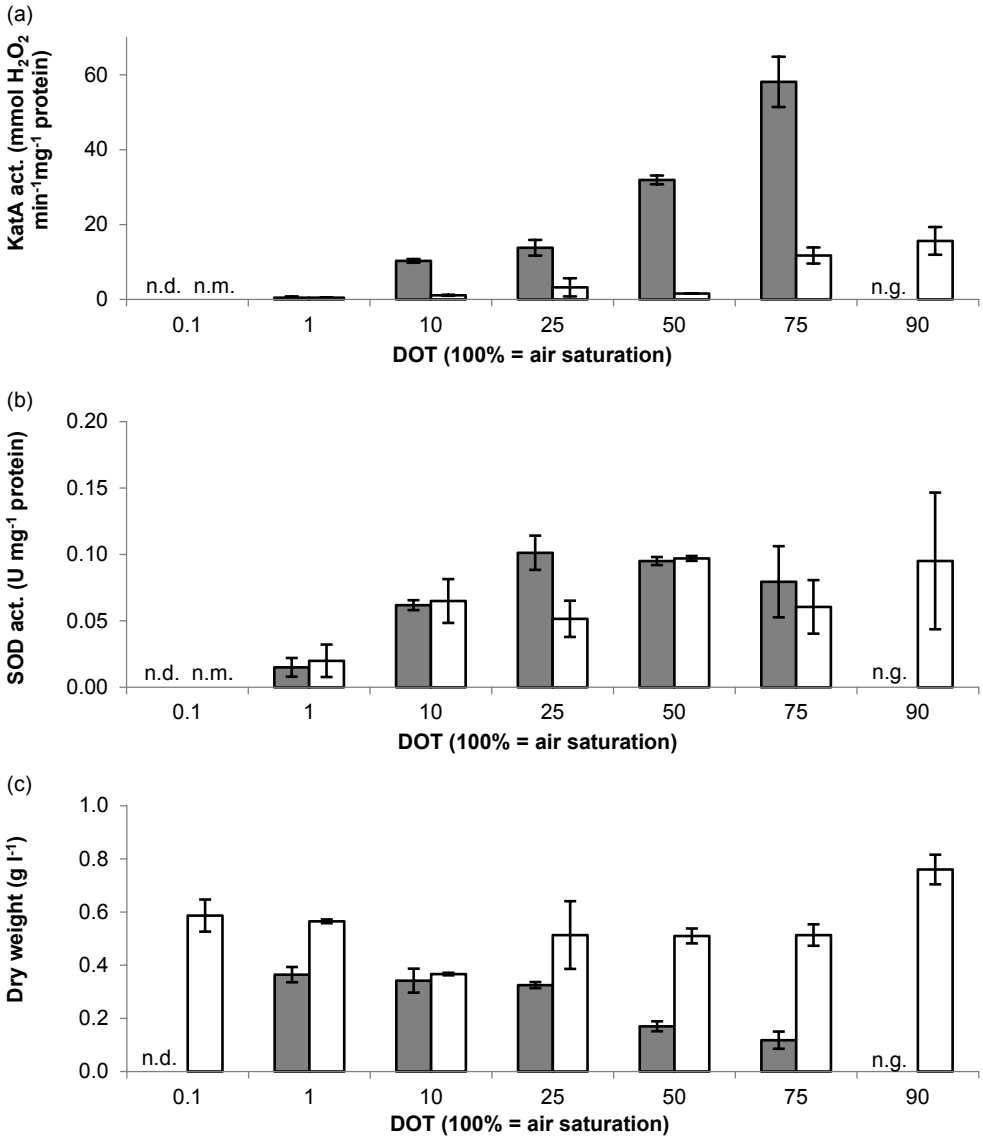

Figure 2.1. *C. jejuni* 11168 culture grown in brain heart infusion broth, in the absence (black) or presence of pyruvate (25 mmol l^{-1}; white), at dissolved oxygen tensions (DOT) varying from 0.1% to 90%: (a) the catalase (KatA) activity (mmol H_2O_2 min^{-1} mg^{-1} protein); (b) the superoxide dismutase (SOD) activity (U mg^{-1} protein); (c) the dry weight (g l^{-1}). All determinations were performed at least in triplicate and results are means ± standard deviation; n.d., not determined; n.m., not measurable; n.g., no sufficient growth.

Discussion

So far the effect of oxygen on the activities of the oxygen stress-protective enzymes KatA and SOD of *C. jejuni* has not been studied. We expected that an increase in DOT, leading to more oxygen stress and higher ROI concentrations, would result in elevated KatA and SOD activities, enzymes which are used to breakdown ROI. The measured KatA activity matched this assumption; the higher the DOT, from 0.1% to 90%, the higher the measured KatA activity. The rise in SOD activity in the DOT range from 1% to 25% also suggested SOD activity regulation as a function of the DOT. However at 25% a maximal induction of SOD was reached, where after the SOD activity stayed the same irrespective of the rise in DOT from 25% to 75%. In the absence of pyruvate, the KatA and SOD activities might not be sufficient at higher DOT to breakdown the ROI formed, resulting in the observed decrease in growth yield at DOT higher than 25% and a lesser growth rate to match the dilution rate at DOT higher than 75%. Besides the limited KatA and SOD activities, the presence of oxygen-sensitive enzymes might also account for the micro-aerobic behaviour of *C. jejuni.*

Next to the protective enzymes KatA and SOD, the presence of pyruvate was described to protect *C. jejuni* against the harmful effects of oxygen, enabling *C. jejuni* to grow aerobically (George et al. 1978; Bolton et al. 1984; Hodge and Krieg 1994). However, these studies were performed on plates or in flasks, in which the cultivation conditions were only given as the percentage of oxygen in the gaseous phase. In this study, the DOT was regulated in a continuous culture, which is a more appropriate measure for the availability of oxygen as it reflects better the DOT to which the bacteria are exposed, than the standard description of cultivation conditions given as percentage of oxygen in the gaseous phase (i.e. 3-21% O_2). Our measurements show that *C. jejuni* no longer acts micro-aerobically when pyruvate is added, but can grow in a broad DOT range from 0.1% to at least 90%, during which the growth yield remains high.

This protective effect of pyruvate might be attributed to a switch in metabolism from aerobic respiration to fermentation when pyruvate is added, as pyruvate is used during fermentation rather than oxygen and no toxic ROI are formed. *C. jejuni* is known to possess enzymes that are capable of fermenting pyruvate to various products like lactic acid and alcohol (Mendz et al. 1997). However, our HPLC results did not show the formation of any fermentation products and also the typical fermentation enzymes, LDH and ADH, could not be detected, which excludes a switch from respiration to fermentation as a possible explanation for the protective effect of pyruvate.

Instead, our results confirm that the protective effect of pyruvate might be based on the ability of pyruvate to act as a quencher of ROI, as assumed in literature (Bolton et al. 1984;

Hodge and Krieg 1994). The measured decreased KatA activity in the presence of pyruvate in our study supports the suggestion of Giandomenico et al. (1997) that chemical elimination of H_2O_2 by pyruvate can compete effectively with protective enzymes such as KatA. In other words, addition of pyruvate decreases the concentration of H_2O_2, which enables *C. jejuni* to grow aerobically.

In conclusion, while currently *C. jejuni* is seen as an obligate micro-aerophile, growing under conditions with 3-15% oxygen, this study showed that, in the presence of pyruvate, also aerobic growth of *C. jejuni* is possible. As amino acids can be converted to pyruvate, further research into environmental pyruvate concentrations is needed to determine if environments with correct pyruvate concentrations for growth of *C. jejuni* do exist. However, to serve as source of *C. jejuni* these environments also have to fulfil other strict growth requirements of *C. jejuni*, for instance a growth temperature above 30°C.

3

Campylobacter jejuni: a study on environmental conditions affecting culturability and *in vitro* adhesion/invasion

Abstract

Nongrowing cultures of *Campylobacter jejuni* lose their culturability. It is unclear whether this loss in culturability also affects their ability to interact with host cells. The purpose of this study was to determine the relevance of the number of culturable cells to the ability to adhere/invade in Caco-2 cells. For *C. jejuni* C356, culturability and adhesion/invasion were monitored in time (days) under different storage conditions (temperature, medium, atmosphere). Decrease rates of both culturability and adhesion/invasion were dependent on the conditions used, but the number of adhering/invading cells per culturable cell was not affected by the environmental conditions. Furthermore, five strains were monitored at one condition. The culturability and adhesion/invasion decrease rates did not significantly differ per strain; however the number of adhering/invading cells per culturable cell was strain dependent. In conclusion, culturability and adhesion/invasion of *C. jejuni* are linearly related. The number of adhering/invading cells per culturable *C. jejuni* cell is strain dependent, but is not affected by environmental conditions. So, it was shown that the number of culturable cells is a good measure for the *in vitro* adhesion/invasion of *C. jejuni.*

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Introduction

Campylobacter jejuni has been identified as the major cause of bacterial foodborne gastroenteritis throughout the world (Blaser 1997; Friedman et al. 2000; Oberhelman and Taylor 2000; De Wit et al. 2001b). *C. jejuni* is generally regarded as micro-aerophilic, growing in environments containing 3-5% CO_2 and 3-15% O_2, and thermophilic, growing in a small temperature range from 30°C to 45°C. Since usual food storage conditions do not match these growth conditions, a point of interest will be how long *C. jejuni* can survive and remain infective under various storage conditions.

Many studies have been conducted on the decrease in culturability of *C. jejuni* in different environments. Maximal survival was shown at low temperatures and an increase in temperature led to a faster decline in culturability (Doyle and Roman 1981; Svedhem et al. 1981; Blankenship and Craven 1982; Hänninen et al. 1984; Rollins and Colwell 1986; Reynolds and Draughton 1987; Phebus et al. 1991; Curtis et al. 1995; Hazeleger et al. 1995; Thomas and Mabey 1996; Buswell et al. 1998; Chynoweth et al. 1998; Thomas et al. 1999; Yoon et al. 2004). Nutrient limitation was shown to lead to prolonged detection of culturable *C. jejuni* (Boucher et al. 1994; Hazeleger et al. 1995; Thomas et al. 1999). At 4°C, the absence of oxygen was shown to diminish the decrease in culturability in time (Koidis and Doyle 1983; Phebus et al. 1991), but no difference was found at 21°C (Phebus et al. 1991).

In practice, exposure is often measured as the number of culturable *C. jejuni* present, which will only be a good indication of the risk of infection if culturability and infectivity are related. If nonculturable cells are able to cause infection in humans, culturability as a measure for the infection risk will result in large underestimations of the risk in case of large amounts of nonculturable cells being present. Many articles with contradictory results on the infectivity of nonculturable cells of *C. jejuni* have been published. Some authors showed that nonculturable cells were unable to infect chicks, mice and human volunteers (Beumer et al. 1992; Medema et al. 1992; Fearnley et al. 1996; Van de Giessen et al. 1996), while other authors demonstrated that nonculturable cells were infectious in chicks and mice (Jones et al. 1991; Stern et al. 1994; Cappelier et al. 1999a). The inconsistency found in literature regarding the infectivity of nonculturable cells might be the result of strain variations, differences in exposure challenge levels and routes, differences in methods used to determine nonculturability, or varying conditions under which nonculturable cells were formed.

Interestingly, in all studies in which nonculturable cells were shown to be infective, the nonculturable cells were formed at 4°C (Jones et al. 1991; Stern et al. 1994; Cappelier et al. 1999a) and in experiments using higher temperatures no infectivity was found (Beumer et

al. 1992; Medema et al. 1992). Furthermore Hazeleger et al. (1995) showed physiological differences between nonculturable cells being formed at various temperatures. Nonculturable cells formed at 4°C showed characteristics, including intracellular/extracellular ATP-ratio and membrane fatty acid composition, comparable to culturable cells. Whereas nonculturable cells formed at 25°C showed significant degeneration; intracellular/extracellular ATP-ratio and membrane fatty acid composition were clearly different to culturable cells.

Studying *Campylobacter* infection is complicated by the lack of simple animal models that mimic the disease seen in humans (Friis et al. 2005). In this study the ability of *C. jejuni* cells to adhere to and/or invade in Caco-2 cells, a well-known *in vitro* model for studying interactions between pathogens and human epithelial cells during infection, was used as measure for the infectivity.

The decrease in culturability and adhesion/invasion of *C. jejuni* C356 was determined in time (days), after storage at various temperatures (4, 12 and 25°C), in different types of media (nutrient rich, nutrient limited) and atmospheres (aerobic, micro-aerobic and anoxic). The main purpose of the experiment reported here was to clarify the relationship between the number of culturable cells and the adhesion/invasion, and to study the effect of environmental conditions on culturability and adhesion/invasion of *C. jejuni* C356. *C. jejuni* C356 was chosen based on its good adherence and invasion properties shown in preliminary studies (data not shown). Furthermore the effect of strain variability was studied for five strains at 12°C in nutrient limited medium under aerobic conditions.

Materials and methods

C. jejuni *strains*

For most experiments *C. jejuni* C356 (chicken faeces; ID-Lelystad B.V., Lelystad, The Netherlands) was used. For the experiments on strain variation *C. jejuni* 11828 (reference strain; National Collection of Type Cultures (NCTC), Colindale, UK), *C. jejuni* 11168 (reference strain; NCTC), LB99HU (human patient; RIVM, Bilthoven, The Netherlands) and *C. jejuni* B258 (chicken faeces; RIVM) were also used. *C. jejuni* strains were stored at -70ºC in brain heart infusion broth (BHI, Beckton Dickinson, Sparks, MD, USA) plus 20% (v/v) glycerol in vials. For culturing, the content of one vial was thawed and put in an Erlenmeyer flask containing 50 ml BHI. The Erlenmeyer flask was incubated while shaking (100 rev min^{-1}) in a custom-made incubator (NuAire, Plymouth, MN, USA) creating a micro-aerobic atmosphere (10% O_2, 5% CO_2, 85% N_2) at 37ºC. After 24 h, strains were subcultured in 100 ml fresh BHI and were grown under the same conditions for another 24 h to stationary phase as measured by OD growth curves (data not shown).

Storage experiments

Stationary phase cells were harvested by centrifugation at 3000 ***g*** and resuspended in either BHI or potassium phosphate-buffered saline solution (PBS; 50 mmol l^{-1} potassium phosphate, 8.5 g l^{-1} NaCl, pH 7.2). All cultures were stored in the dark. The culturability and infectivity were tested with different combinations of strain and storage conditions (medium, temperature, and atmosphere). For aerobic storage and micro-aerobic storage (10% O_2, 5% CO_2, 85% N_2) cultures were stored while shaking (100 rev min^{-1}) in wide-necked Erlenmeyer flasks covered with cotton wool. For studying survival in an anoxic atmosphere, cultures were stored stationary in tightly closed 15 ml tubes with no headspace left (Greiner).

Samples were taken after different storage time intervals, varying from 0 to 41 days, depending on the storage conditions. For each sampling point a different set of Erlenmeyer flasks or Greiner tubes was used, initially set up from the same batch of cells, so that at T0 all flasks had the same cell density. At least two independent data sets starting at different days were determined for all tested conditions. In figures and calculations all collected data points of different sets were combined.

Culturability assays

Plate counts were performed in duplicate by spread plating 0.1 ml of appropriate decimal dilutions of cell suspensions in sterile peptone (Difco, 1 g l^{-1}) saline (Merck, 9 g l^{-1} NaCl) solution on Colombia agar base with 5% (v/v) lysed defibrinated horse blood (CAB, Oxoid, Basingstoke, UK). The plates were incubated micro-aerobically in a jar using BBL Campypak (Becton Dickinson) at 37ºC for 72 h. Culturability is represented as log CFU ml^{-1} bacterial cell suspension.

Adhesion/invasion assays

Caco-2 cell line, growth media and conditions: Caco-2 cells, human colon adenocarcinoma cells isolated from a primary colonic tumor in a 72-year-old Caucasian male were obtained from the American Type Culture Collection (Caco-2, ATCC HTB-37). In the experiments passages 30 to 45 were used. Cells were maintained in Culture Medium (CM): Dulbecco's Modified Eagle Medium, 25 mmol l^{-1} HEPES, containing 4.5 g l^{-1} D-glucose (Gibco, Life Technologies Ltd, Paisley, Scotland, UK) supplemented with 10% heat inactivated (30 min at 60ºC) foetal calf serum (FCS, Integro b.v., Zaandam, The Netherlands), 0.1% MEM nonessential amino-acids (Gibco), 6 mmol l^{-1} L-glutamine (Gibco) and 50 µg ml^{-1} gentamycin (Gibco). Cells were stored at -135ºC in CM plus 10% (v/v) DMSO (Sigma Chemical Co., St Louis, MO, USA). After a quick thawing procedure in a water bath kept at 37°C, the content of one vial ($\pm 10^6$ cells) was put in a culture-flask with 10 ml CM. The

cells were grown routinely in flasks in a CO_2 5% (v/v) incubator at 37ºC. For experimental assays, confluent cultures were trypsinized and seeded in CM in 12-well tissue culture plates (Costar, Corning Costar Europe, Badhoevedorp, The Netherlands) at 160,000 cells ml^{-1}, 1 ml per well, and incubated in a CO_2 5% (v/v) incubator at 37ºC. Medium was changed three times a week. The 12-well plates were used 11 to 13 days after seeding.

Adhesion/Invasion assay: Prior to the experiment, the medium in the 12-well plates was replaced by 1 ml prewarmed CM without FCS and gentamycin. After 1 h the plates were inoculated with 40 µl bacterial suspension per well. The bacteria from the storage experiment were allowed to adhere to and/or invade in the cells for 3 h in a CO_2 5% (v/v) incubator at 37ºC. After incubation, the monolayers were rinsed three times with CM without FCS and gentamycin and the cells were lysed with 1 ml 1% (v/v) Triton-X100 (Merck, Amsterdam, The Netherlands) in distilled water. The amount of adhered and/or invaded bacteria was determined by plating 0.1 ml of serial dilutions of lysed Caco-2 cell suspensions on CAB and counting the resulting colony forming units (CFU) after 72 h incubation at 37ºC under micro-aerobic conditions. All assays were performed in triplicate. Adhesion/invasion is expressed as log CFU $well^{-1}$, corresponding with log CFU per 40 µl bacterial cell suspension.

Effect of storage conditions and strain variation on culturability and adhesion/invasion

Data on culturability and adhesion/invasion in time were fitted to a linear model in Microsoft Office Excel:

$$\log C = a_c - b_c t \quad (1)$$

$$\log I = a_i - b_i t \quad (2)$$

with log C as the culturability (log CFU ml^{-1} bacterial suspension) at time t (days), log I as the adhesion/invasion (log CFU $well^{-1}$) at time t (days), a_c as the initial culturability (log CFU ml^{-1} bacterial suspension) at t = 0 days, a_i as the initial adhesion/invasion (log CFU $well^{-1}$) at t = 0 days, b_c as the decrease rate of culturability in day^{-1}, and b_i as the decrease rate of adhesion/invasion in day^{-1}.

A two-sample t test was used to test the difference in decrease of culturability and adhesion/invasion between storage conditions (temperature, medium, and atmosphere) and strains. The significance level used was $\alpha = 0.05$.

Relation between culturability and adhesion/invasion

In the adhesion/invasion assay, plates were inoculated with 40 µl bacterial suspension per well. For reasons of comparability, in determining the relation between culturability and adhesion/invasion, the culturability is not expressed as log C (log CFU ml^{-1} bacterial cell suspension), but is converted to log C^* the number of culturable cells added to one well:

$$\log C^* = \log (0.04 * 10^{\log C}) \quad (3)$$

Firstly, a two-sample t test was used to test the difference between the decrease in the number of culturable cells in time and the decrease in the number of adhesive/invasive cells in time. The significance level used was $\alpha = 0.05$. Secondly, log I was plotted as function of log C^* and fitted to two linear regression equations (4) and (5):

$$\log I = r \log C^* + q \quad (4)$$

$$\log I = \log C^* + q \quad (5)$$

with log I as the adhesion/invasion at time t (log CFU $well^{-1}$), log C^* as the culturability at time t (log CFU $well^{-1}$), r as the regression coefficient, and q as the predicted x-axis intercept.

Results

The results of the decrease in culturability and adhesion/invasion in time for *C. jejuni* C356, suspended in PBS or BHI and stored at different temperatures (4°C, 12°C and 25°C) under different atmospheric conditions (aerobic, micro-aerobic and anoxic), are shown in Fig. 3.1 a-c. In Fig. 3.1d the decrease in culturability and adhesion/invasion in time for five strains (C356, 11828, 11168, LB99HU and B258) in PBS at 12°C under aerobic conditions is shown. In all tested situations, the data for both culturability and adhesion/invasion, fitted a linear model [log (N) = a - bt] with a high degree of goodness of fit (r^2 = 0.87-0.99; Table 3.1).

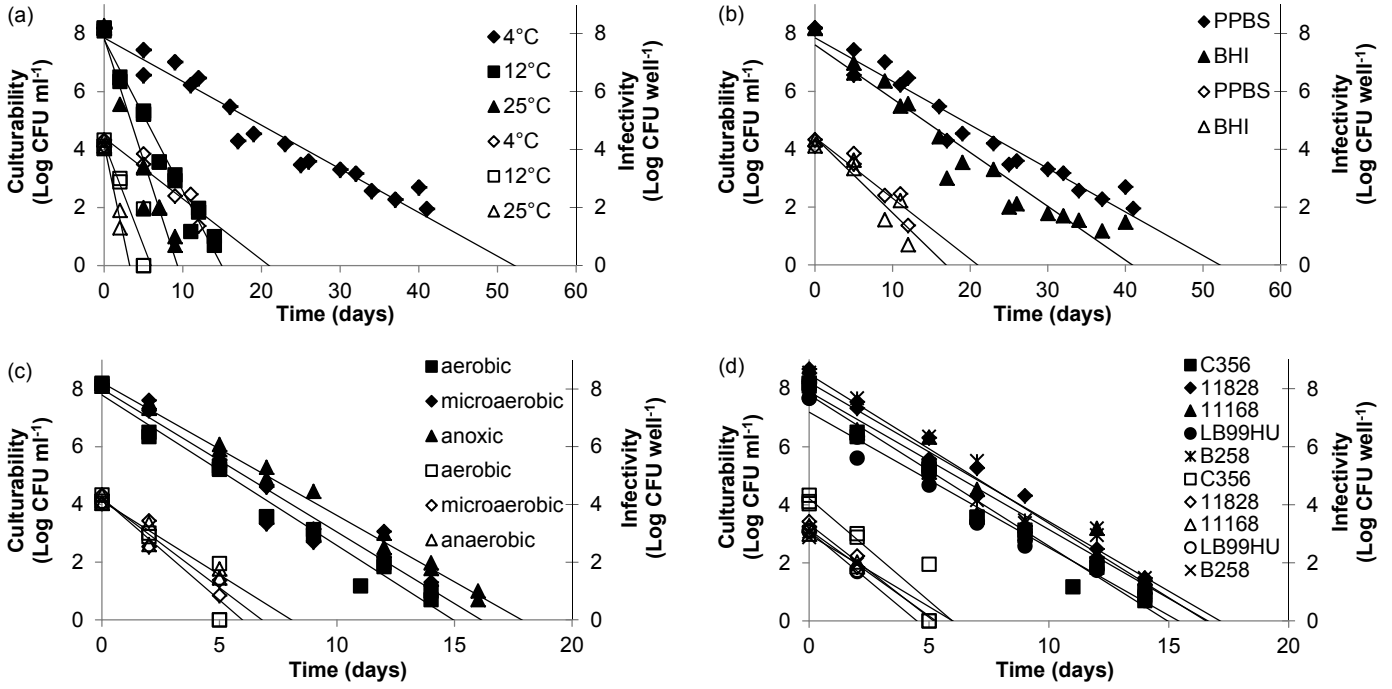

Figure 3.1. Linear regression curves for the decrease in culturability and adhesion/invasion in time. Closed symbols: Culturability (log CFU ml^{-1} bacterial suspension; detection limit: 0.70 log CFU ml^{-1}), Open symbols: Caco-2 adhesion and/or invasion (log CFU $well^{-1}$, containing 160,000 Caco-2 cells at the time of seeding; detection limit: 0.22 log CFU $well^{-1}$; data points at the x-axis are below the detection limit, but are represented if at specific conditions at least one of the experiments is above the detection limit). (a-c) under different storage conditions for *Campylobacter jejuni* C356: (a) effect of temperature (4°C, 12°C and 25°C) in PBS under aerobic conditions; (b) effect of medium (BHI and PBS) at 4°C under aerobic conditions; (c) effect of atmosphere (aerobic, micro-aerobic and anoxic) in PBS at 12°C, and (d) for different strains (C356, 11828, 11168, LB99HU, B258) in PBS at 12°C under aerobic conditions.

Table 3.1. The culturability and adhesion/invasion in time, depending on storage conditions and strain variation[1]

C. jejuni	Storage conditions			log $C = a_c$-$b_c t$			log $I = a_i$-$b_i t$		
strain	Medium	Temperature	Atmosphere	a_c	b_c	r^2	a_i	b_i	r^2
C356	BHI	4°C	Aerobic	7.61	0.19	0.93	4.41	0.26	0.87
C356	BHI	12°C	Aerobic	7.86	0.57	0.98	4.18	0.72	0.91
C356	BHI	25°C	Aerobic	7.90	0.98	0.95	4.16	1.35	0.98
C356	PBS	4°C	Aerobic	7.86	0.15	0.95	4.41	0.21	0.09
C356	PBS	12°C	Aerobic	7.79	0.52	0.97	4.22	0.71	0.88
C356	PBS	25°C	Aerobic	7.84	0.84	0.95	4.16	1.28	0.97
C356	PBS	12°C	Micro-aerobic	8.01	0.50	0.95	4.20	0.62	0.94
C356	PBS	12°C	Anoxic	8.22	0.46	0.98	4.12	0.51	0.94
11828	PBS	12°C	Aerobic	8.50	0.51	0.98	3.33	0.64	0.95
11168	PBS	12°C	Aerobic	7.90	0.47	0.94	3.31	0.59	0.96
LB99HU	PBS	12°C	Aerobic	7.19	0.47	0.97	3.10	0.69	0.99
B258	PBS	12°C	Aerobic	8.23	0.48	0.95	2.97	0.50	0.98

[1] log C, culturability at time t (log CFU ml^{-1} bacterial suspension); log I, adhesion/invasion at time t (log CFU well^{-1}); a_c, initial culturability (log CFU ml^{-1} bacterial suspension) at $t = 0$; a_i, initial adhesion/invasion (log CFU well^{-1}) at $t = 0$; b_c, decrease rate of culturability (day^{-1}); b_i, decrease rate of adhesion/invasion (day^{-1}); t, time (days).

Effect of temperature

The *C. jejuni* C356 suspensions incubated at 4°C remained culturable for over 40 days and retained their ability to adhere and/or invade Caco-2 cells for at least 12 days, while for the *C. jejuni* C356 suspensions stored at 12°C and 25°C culturability was no longer measured after respectively 16 and 12 days and adhesion and/or invasion was not detected after respectively 7 and 5 days (Fig. 3.1a).

Effect of medium

At 4°C, the *C. jejuni* C356 suspensions incubated in PBS showed a higher number of culturable and infective cells than the cells stored in BHI at all time points (Fig. 3.1b). Furthermore the two-sample t test showed that the decrease rates of culturability and adhesion/invasion in PBS and BHI are statistically different at 4°C ($p = 0.02$). At 12°C and 25°C, a higher culturability and adhesion/invasion were also found in PBS compared to

BHI (data not shown), but the difference between the decrease rates of culturability and adhesion/invasion in both media at these temperatures was not statistically significant (respectively $p = 0.12$ and $p = 0.18$).

Effect of atmosphere

For *C. jejuni* C356 suspensions incubated at 12°C, the number of culturable and adhesive/invasive cells decreased faster with increasing percentages of oxygen in the atmosphere. Under anoxic conditions cells remained culturable for at least 16 days and retained their ability to adhere and/or invade Caco-2 cells for at least 5 days, while under aerobic conditions culturability was no longer measured after 12 days and adhesion/ invasion were not detected in two out of three cases after 5 days (Fig. 3.1c). Statistical analysis showed a significant difference between aerobic and anoxic storage ($p = 0.04$), but the differences between micro-aerobic and aerobic or anoxic storage were not significant (respectively $p = 0.49$ and $p = 0.38$).

Culturability versus adhesion/invasion

Under all tested combinations of storage conditions and strains, no significant differences between the decrease rates of culturability and adhesion/invasion were shown.
For all tested storage conditions of *C. jejuni* C356, log I was plotted as function of log C^* (Fig. 3.2) and fitted to equations (4) and (5), resulting in:

$\log I = 1.07 \log C^* - 3.10$ (r^2: 0.83)

$\log I = \log C^* - 2.70$ (r^2: 0.83)

For strains 11828, 11168, LB99HU and B258 (PBS, 12°C, aerobic) the curves fitted to equation (5) are shown in Fig. 3.2.

Strain variation

In PBS under aerobic conditions at 12°C strain variation did not significantly influence the decrease in survival and adhesion/invasion ($p > 0.05$; Fig. 3.1d). However, the number of adhesive/invasive cells per culturable cell differed per strain, from approximately one infective cell in 500 culturable cells for strain C356 to around one in 8000 for strains 11828 and B258 (Fig. 3.2).

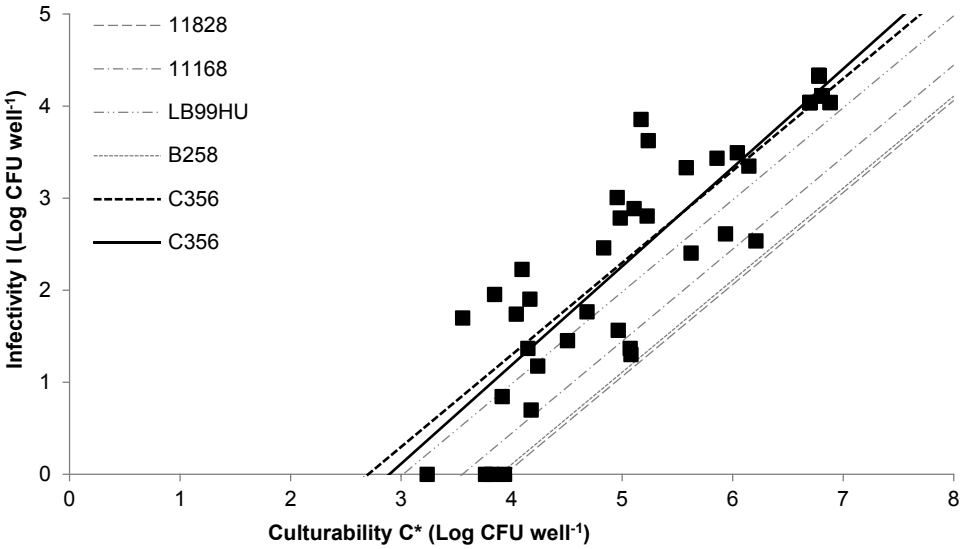

Figure 3.2. Relation between the adhesion/invasion I (log CFU well^{-1}) and the culturability C^* (log CFU well^{-1}); one well containing 160,000 Caco-2 cells at the time of seeding. For *C. jejuni* C356 all data points measured under various conditions (temperature, medium, and atmosphere) were incorporated (data points at the x-axis are below the detection limit: <0.22 log CFU well^{-1}, but are represented if at specific conditions at least one of the experiments is above the detection limit). Straight line: fit according to $\log I = r \log C^* + q$, and interrupted line: fit according to $\log I = \log C^* + q$. For strains 11828, 11168, LB99HU and B258 (PBS, 12°C, aerobic conditions) no data points, but only the curves fitted according to $\log I = \log C^* + q$ are shown.

Discussion

While the effect of environmental conditions on the survival of *C. jejuni* has been studied extensively, the knowledge of the effect of environmental factors on the infectivity is scarce. Our results with respect to the culturability of *C. jejuni* have confirmed the reported behaviour of *C. jejuni* as affected by medium, temperature, atmosphere over a period of days (Doyle and Roman 1981; Svedhem et al. 1981; Blankenship and Craven 1982; Koidis and Doyle 1983; Hänninen et al. 1984; Rollins and Colwell 1986; Reynolds and Draughton 1987; Phebus et al. 1991; Boucher et al. 1994; Curtis et al. 1995; Hazeleger et al. 1995; Thomas and Mabey 1996; Buswell et al. 1998; Chynoweth et al. 1998; Thomas et al. 1999; Yoon et al. 2004). However as the extent of survival is affected by the model medium, it is difficult to compare the survival times in our study to those found in literature in which other media were used. Only Hazeleger et al. (1995) studied survival in PBS and BHI, and their survival curves are in accordance with the results as shown in this article.

For *C. jejuni* C356 the decrease in culturability and adhesion/invasion in time was strongly influenced by the temperature, and slightly by the medium and atmosphere used.
For example, the decrease rates for culturability and adhesion/invasion kept in PBS under aerobic conditions at 4°C are respectively 0.15 and 0.21 day^{-1}, raising the temperature to 12°C leads to higher decrease rates of 0.52 and 0.71 day^{-1} and changing the medium to BHI leads to higher decrease rates of 0.19 and 0.26 day^{-1}. Changing the atmosphere to anoxic (12°C) leads to lower decrease rates of 0.46 and 0.51 day^{-1}.
In conclusion, prolonged survival and *in vitro* adhesion/invasion at low temperatures, in PBS and/or under anoxic conditions were found when compared to respectively higher temperatures, BHI and/or under aerobic conditions. This can be explained by a lower metabolism, leading to a delayed exhaustion of substrates and a reduced formation of toxic reactive oxygen intermediates leading to less oxidative cell damage (Hazeleger et al. 1995).
In Fig. 3.1 the culturability and adhesion/invasion in time seemed to decrease in parallel with all various tested conditions. Furthermore, no significant differences between the decrease rates of culturability and adhesion/invasion were shown under all tested combinations of storage conditions and strains. These findings indicate that culturability and adhesion/invasion are linearly related. Consequently for *C. jejuni* C356 all data points measured under various conditions (temperature, medium, and atmosphere) were fitted to equation (4) resulting in $\log I = 1.07 \log C^* - 3.10$ (r^2: 0.83); and equation (5) giving $\log I = \log C^* - 2.70$ (r^2: 0.83). As equations (4) and (5) fit the data equally well, there seems no need to incorporate the regression coefficient r in the formula, which is consistent with the finding that 1 was situated in the 95% confidence level of r. Equation (5) can be rewritten as $I = 10^{-2.70} * C^* = 0.002\ C^*$. The number of culturable cells is indeed linearly related to the number of adhering and/or invading cells, independent of the environmental conditions used. This means that 0.2% of the culturable *C. jejuni* C356 cells (so one in 500 cells) adhere to and/or invade in Caco-2 cells.
Given that the linear relation between culturability and adhesion/invasion in our study was not depending on the types of temperature, medium or atmosphere used, the contradictory data as reported in the articles on the infectivity of nonculturable cells (Jones et al. 1991; Beumer et al. 1992; Medema et al. 1992; Stern et al. 1994; Fearnley et al. 1996; Van de Giessen et al. 1996; Cappelier et al. 1999a) are probably not the result of the different environmental conditions under which nonculturable cells were formed but have to be the result of other differences in experimental set-up, like differences in methods and strains used. Our study illustrated that, during aerobic storage in PBS at 12ºC, the number of adhering/invading cells per culturable cell, is strain dependent as reported previously (Newell et al. 1985b; Bukholm and Kapperud 1987; Everest et al. 1992; Oelschlaeger et al. 1993; Tay et al. 1996; Harvey et al. 1999; Biswas et al. 2000). The curves for adhesion/invasion versus culturability in our study are comparable to data reported by Hu

and Kopecko (1999) and Friis et al. (2005) who also found a linear relation between the culturability and the adhesion/invasion in the dose range from log 3 to log 7 culturable *C. jejuni* cells per well, after which at a certain dose a maximum value of adhesive/invasive cells per culturable cell was reached. The number of adhering/invading cells per culturable cell in our study was shown to be strain dependent, varying from one in 500 to one in 8000, which is in the range of values measured in the linear part of the curves found in literature, from around one in 20 (Hu and Kopecko 1999; Friis et al. 2005) to one in 10 000 000 (Friis et al. 2005) depending on the strain used. Given that decrease rates of both survival and adhesion/invasion do not differ significantly per strain, but that the number of adhering/invading cells per culturable cell does, highly adhering/invading strains will not only give a higher risk on infection but will also present a risk over a longer period of time.
In conclusion, considering the Caco-2 model to be a good model for infectivity *in vivo*, the results of our study show that culturability gives a good indication of the risk of infection as culturability and adhesion/invasion are linearly related. Therefore, the absence of culturable *C. jejuni* cells is an indication that a product can be regarded as representing a very low risk with respect to campylobacteriosis.

4

Lack of response of INT-407 cells to the presence of nonculturable *Campylobacter jejuni*

Abstract

Many contradictory articles on the infectivity of nonculturable *Campylobacter jejuni* can be found. We studied the effect of nonculturable *C. jejuni* in an *in vitro* assay. To prevent the potential effect of a few culturable bacteria in the nonculturable suspension, INT-407 cells, which mimic the outer cell layer in the small intestines, were exposed to culturable *C. jejuni* suspensions with or without nonculturable *C. jejuni*. The number of bacteria adhering to and/or invading INT-407 cells and the IL-8 secretion were measured. No differences were found between bacterial suspensions with or without nonculturable *C. jejuni* added. These findings show that nonculturable *C. jejuni* do not adhere to or invade INT-407 cells and do not induce an immune response. As previous studies showed a correlation between the used *in vitro* assays and the effect *in vivo*, our study strongly suggests that culturability is a good indicator of the risk for *C. jejuni* infection.

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Introduction

Campylobacter jejuni is the leading cause of bacterial foodborne gastroenteritis throughout the world (Blaser 1997; Friedman et al. 2000; Oberhelman and Taylor 2000; De Wit et al. 2001b). It is a Gram-negative, motile microorganism, which is primarily micro-aerophilic. Remarkably, this pathogen grows within a short temperature range, being unable to multiply at temperatures above 45°C or below 30°C. At conditions where *C. jejuni* cannot grow, such as low temperature or in spent medium, it loses its culturability. It is crucial to know whether nonculturable *C. jejuni* can cause an infection, as in practice the exposure is often measured as the number of culturable *C. jejuni* in a product.

In the literature many contradictory articles on the infectivity of nonculturable *C. jejuni* can be found. Some authors showed that nonculturable *C. jejuni* were not infective in chicks, mice and human volunteers (Beumer et al. 1992; Medema et al. 1992; Fearnley et al. 1996; Van de Giessen et al. 1996) while others demonstrated that nonculturable *C. jejuni* were colonizing chicks and mice (Jones et al. 1991; Stern et al. 1994; Cappelier et al. 1999a). The inconsistency in the literature about the infectivity of nonculturable *C. jejuni* might be the result of differences in methods, conditions and strains used. A marked difference between studies is the temperature at which nonculturable *C. jejuni* were formed. While some authors did not find any infectivity of nonculturable *C. jejuni* formed at 4°C (Medema et al. 1992; Fearnley et al. 1996; Van de Giessen et al. 1996), in all studies reporting on nonculturable *C. jejuni* causing infection, the nonculturable *C. jejuni* were formed at 4ºC (Jones et al. 1991; Stern et al. 1994; Cappelier et al. 1999a), whereas in all experiments using higher temperatures no infectivity was found (Beumer et al. 1992; Medema et al. 1992). Interestingly Hazeleger et al. (1995) showed that nonculturable *C. jejuni* formed at 4^{o}C showed characteristics, including intracellular/extracellular ATP-ratio and membrane fatty acid composition, comparable to culturable *C. jejuni*, whereas nonculturable *C. jejuni* formed at 25^{o}C were clearly different. The aim of this study was to further elucidate if nonculturable *C. jejuni* formed at 4°C can be infective.

The nonculturability of suspensions, in studies in which nonculturable *C. jejuni* were found to be infective, is often discussed. If only one or a few undetected culturable bacteria remain present, the observed infectivity might be caused by these undetected culturable bacteria instead of by the nonculturable bacteria. To avoid the misleading effect of a few culturable bacteria, in our study the infectivity of culturable *C. jejuni* suspensions and the infectivity of the same culturable *C. jejuni* suspensions supplemented with a high number of nonculturable *C. jejuni*, formed at 4°C, were measured. The comparison was made for different doses of culturable *C. jejuni* in the absence and presence of nonculturable *C.*

jejuni, as the infectivity might be dose dependent (Hu and Kopecko 1999; Mooney et al. 2003; Friis et al. 2005).

For our study, INT-407 cells were chosen, based on various studies showing that the human cell lines Caco-2 and INT-407 mimic best the outer cell layer in the small intestines (De Melo and Pechère 1990; Konkel et al. 1992). In preliminary work (data not shown) adhesion and invasion were measurable in both Caco-2 and INT-407 cells, but IL-8 secretion after exposure with *C. jejuni* was only detected in INT-407 cells. The INT-407 cells were used for two *in vitro* infectivity tests. First, the adhesion and invasion assay, which is based on the binding to and entry in host cells of *C. jejuni*, an important factor in the pathogenesis of *C. jejuni* (Wooldridge and Ketley 1997). Second, the IL-8 assay in which the *C. jejuni*-stimulated secretion of the cytokine IL-8, an early signal for the mucosal inflammatory response (Eckmann et al. 1993; Jung et al. 1995), is determined as a measure for the immune response. A disadvantage of the adhesion and invasion assay is that the outcome is measured by plate counting. If nonculturable *C. jejuni* do not recover their culturability during the assay, but do adhere or invade, the infectivity of nonculturable *C. jejuni* is underestimated. As the IL-8 assay is based on measuring an immune response, even if nonculturable *C. jejuni* do not recover, their effect on the infectivity is likely to be measured by the IL-8 assay. As *in vitro* studies have established that the invasive and adhesive ability of *C. jejuni* strains differ (Newell et al. 1985b; Bukholm and Kapperud 1987; Everest et al. 1992; Oelschlaeger et al. 1993; Tay et al. 1996; Harvey et al. 1999; Biswas et al. 2000) and, furthermore, that the *C. jejuni*-stimulated IL-8 secretion is strain dependent (Hickey et al. 1999, 2000) four *C. jejuni* strains were selected. *C. jejuni* 70.2 and BF were chosen as for these strains the formation of infective nonculturable *C. jejuni* has been described by Cappelier et al. (1998, 1999a). C356 and 82/69 were selected for their good adherence and invasion properties found in preliminary research (data not shown).

Materials and methods

Culturing C. jejuni *strains*

Strains were stored at -70°C in brain heart infusion broth (BHI, Difco, Sparks, MD, USA) plus 20% (v/v) glycerol in cryovials. For culturing *C. jejuni* strains 70.2 and BF (INRA, Nantes, France; both isolated from human faeces), C356 (ID-Lelystad B.V., Lelystad, The Netherlands; isolated from chicken faeces) and 82/69 (ID-Lelystad; isolated from chicken faeces, same serotype also found in human faeces), the content of one vial (0.5 ml) was thawed and put in a wide-necked Erlenmeyer flask with 50 ml BHI. The flask was incubated while shaking at 100 rev min^{-1} in a custom-made incubator (NuAire, Plymouth, MN, USA) with a micro-aerobic atmosphere (10% O_2, 5% CO_2, 85% N_2) at 37ºC. After

±24 h, 0.5 ml was subcultured in 100 ml fresh BHI and incubated under the same conditions for ±16 h. These suspensions were used in the cell line assays.

Culturability

Plate counts were performed by spread plating 0.1 ml of appropriate decimal dilutions of bacterial suspensions in sterile peptone (Difco, 1 g l^{-1}) saline (Merck, Amsterdam, The Netherlands; 9 g l^{-1} NaCl) solution on Colombia agar base with 5% (v/v) defibrinated horse blood (CAB, Oxoid, Basingstoke, UK). The plates were incubated micro-aerobically at 37ºC in a jar with BBL® Campypak (Becton Dickinson, Sparks, MD, USA) for 72 h.

Nonculturable C. jejuni *suspensions*

To obtain nonculturable *C. jejuni*, strains were cultured as described above. After culturing, the bacterial suspensions (±10^9 *C. jejuni* ml^{-1}) were stored aerobically without shaking at 4°C in wide-necked Erlenmeyer flasks covered with cotton wool and kitchen foil to prevent dehydration. When plate counts were below the minimal detection level (=10 CFU ml^{-1}), usually after 30 days, suspensions were considered to be nonculturable. For the infection assays, in which 40 µl suspension was used, this corresponds to 4×10^7 nonculturable *C. jejuni* $well^{-1}$ and ≤ 0.4 culturable *C. jejuni* $well^{-1}$.

INT-407 cell line, growth media and conditions

Human embryonic intestinal cells (INT-407) obtained from the American Type Culture Collection were maintained in minimal essential medium with Earle's salts and without glutamine (EMEM, Gibco, Life Technologies ltd, Paisley, Scotland) supplemented with 10% heat inactivated (30 minutes at 60ºC) foetal bovine serum (FBS, Integro b.v., Zaandam, The Netherlands), 6 mM L-glutamine (Gibco) and 50 µg ml^{-1} gentamycin (Gibco). Cells were grown routinely in 10 ml culture medium in a 75-cm^2 flask (Corning Costar Europe, Badhoevedorp, The Netherlands) in a CO_2 5% (v/v) incubator at 37ºC. Confluent stock cultures were washed and released with 0.05% trypsin-EDTA and new stock cultures were seeded with 10^5 cells ml^{-1}. For the adhesion/invasion and IL-8 assays, 12-well tissue culture plates (Corning Costar Europe) were seeded with 160,000 INT-407 cells ml^{-1} per well. The plates were incubated in a CO_2 5% (v/v) incubator at 37ºC; the medium was changed three times a week. The plates were used 8 days after seeding.

Infectivity assays

Prior to the experiment, the medium overlaying the 8-day-old monolayers in the 12-well plates was replaced by pre-warmed EMEM, supplemented with 6 mM L-glutamine. After 1

h the cultures were inoculated with 10^3-10^{10} culturable *C. jejuni* well^{-1} without or with 4×10^7 nonculturable *C. jejuni* of the same strain per well. Bacteria were allowed to adhere to and invade INT-407 cells for 2 h in a CO_2 5% (v/v) incubator at 37ºC. After this incubation the bacteria were removed by rinsing the monolayers three times with EMEM.
To study adhesion and invasion, the INT-407 cells were lysed with 1 ml 1% (v/v) Triton-X100 (Merck) in distilled water. The number of bacteria adhering to and/or invading INT-407 cells well^{-1} was determined by plating serial dilutions of the suspensions on CAB and counting the resulting CFU, after 72 h incubation at 37ºC under micro-aerobic conditions. Adhesion and invasion assays were performed in triplicate.
To study IL-8 secretion, 1 ml EMEM with 50 μg ml^{-1} gentamycin was added to the cells, followed by incubation for 24 h in a CO_2 5% (v/v) incubator at 37ºC. Subsequently, supernatants were collected and stored at -70ºC to be analysed later. INT-407 cells without addition of bacteria were used as control. IL-8 concentrations were determined in triplicate using an IL-8 ELISA according to Garssen et al. (1999).

Results

Adhesion and invasion

The number of bacteria adhering to and/or invading INT-407 cells well^{-1} after infection with increasing numbers of bacteria, in a range of 10^3-10^{10} bacteria well^{-1}, was determined for four different strains, C356, BF, 70.2, and 82/69, in the absence or presence of $\pm4\times10^7$ nonculturable *C. jejuni* of the same strain per well (Fig. 4.1).
A similar trend was observed for all four strains: the number of bacteria adhering to and/or invading INT-407 cells well^{-1} increased as the number of *C. jejuni* increased until a maximum was reached; however, the curves differed per strain. The minimal needed number of culturable *C. jejuni* well^{-1} at which adhesion and invasion in the assay was measured, varied from $\pm5\times10^2$ for *C. jejuni* BF to $\pm1\times10^6$ for *C. jejuni* 70.2. The dose at which the maximal adhesion and invasion was reached, differed from $\pm5\times10^6$ *C. jejuni* BF well^{-1} to $\pm1\times10^9$ *C. jejuni* 70.2 well^{-1}. The maximum number of bacteria adhering to and/or invading INT-407 cells, varied from $\pm8\times10^4$ *C. jejuni* well^{-1} for strains C356, BF and 82/69 to $\pm2\times10^3$ *C. jejuni* well^{-1} for strain 70.2. No difference was seen between measurements in the absence or presence of nonculturable *C. jejuni*.

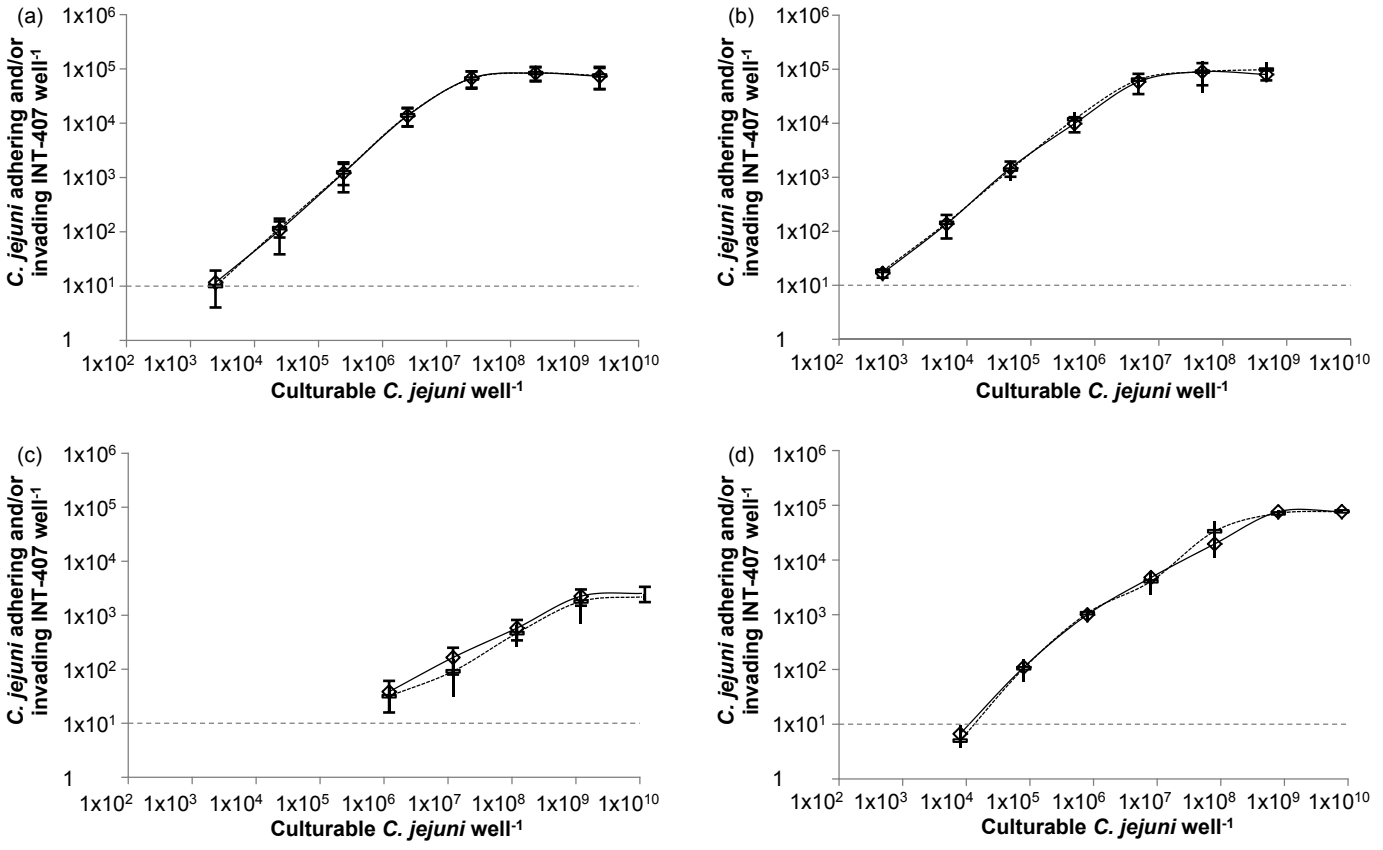

Figure 4.1. Bacteria adhering to and/or invading INT-407 cells per well after incubation with increasing numbers of culturable *C. jejuni* in the absence (-) or presence (◊) of ± $4x10^7$ nonculturable *C. jejuni* $well^{-1}$: (a) *C. jejuni* C356; (b) *C. jejuni* BF; (c) *C. jejuni* 70.2; (d) *C. jejuni* 82/69. The detection limit of the assay was 10 bacteria adhering to and/or invading INT-407 cells $well^{-1}$.

IL-8

The IL-8 secretion by INT-407 cells after infection with the four strains in the absence or presence of nonculturable *C. jejuni* of the same strain was measured (Table 4.1).
The induced IL-8 secretion varied significantly per strain, with the lowest IL-8 secretion induced by *C. jejuni* 70.2 and the highest IL-8 secretion measured after infection with *C. jejuni* BF. For all strains, no difference was found between measurements in the absence or presence of nonculturable *C. jejuni*.

Table 4.1. IL-8 secretion by INT-407 cells, after incubation with culturable *C. jejuni* in the absence or presence of ± $4x10^7$ nonculturable *C. jejuni* well^{-1}

C. jejuni strain	Bacteria well^{-1} *	IL-8 secretion (pg well^{-1})**	
		In the absence of nonculturable *C. jejuni*	In the presence of nonculturable *C. jejuni*
C356	8.2×10^6	153 ± 27	137 ± 20
BF	7.6×10^6	270 ± 31	284 ± 50
70.2	8.0×10^7	73 ± 22	79 ± 26
82/69	9.2×10^7	123 ± 35	106 ± 12
None		8 ± 2	-
None		-	8 ± 5***

*As undiluted overnight cultures are used, the number of bacteria added per well differs.
**All IL-8 determinations were performed in triplicate, results are means ± standard deviation.
*** Cocktail (1:1:1:1) of nonculturable *C. jejuni* of the four strains was used.

Discussion

Many conflicting articles have been written on the existence and importance of nonculturable *C. jejuni* (Beumer et al. 1992; Jones et al. 1991; Medema et al. 1992; Stern et al. 1994; Fearnley et al. 1996; Van de Giessen et al. 1996; Cappelier et al. 1999a). To further elucidate if nonculturable *C. jejuni* can be infective, the effect of adding nonculturable *C. jejuni* on the infectivity in INT-407 cells was studied instead of measuring the effect of nonculturable *C. jejuni* on their own.
No differences in adhesion and invasion were found when nonculturable *C. jejuni* were added, which implies that nonculturable *C. jejuni* do not adhere or invade *in vitro*. However, to measure adhesion and invasion, nonculturable bacteria have to recover their culturability. If nonculturable *C. jejuni* can adhere or invade but cannot recover their culturability, measuring the adhesion and invasion would result in an underestimation of the

infectivity, although the curves in Fig. 4.1 would be affected. The curves all show an increase in the number of bacteria adhering to and/or invading INT-407 cells well^{-1} until a maximal is reached, as previously described by Biswas and colleagues (2000, 2003, 2004). If nonculturable *C. jejuni* were able to adhere or invade, but not to recover their culturability, a competition with the culturable *C. jejuni* would be expected. This competition between culturable and nonculturable *C. jejuni* would result in a decrease in the measured number of bacteria adhering to and/or invading INT-407 cells well^{-1}, which would have affected the slope of the curves in Fig. 4.1. The number of bacteria adhering to and/or invading INT-407 cells dose^{-1} and the slope of the curve were not affected; again indicating that nonculturable *C. jejuni* cannot adhere or invade. Furthermore, the results of the IL-8 assay which are based on measuring an immune response, also strongly support the assumption that nonculturable *C. jejuni* are not infective, since the addition of nonculturable *C. jejuni* did not affect IL-8 secretion.

Our findings strongly indicate that nonculturable *C. jejuni* formed at 4°C are not infective *in vitro*, and conflict with literature in which nonculturable *C. jejuni* formed at 4°C were found to be infective (Jones et al. 1991; Stern et al. 1994; Cappelier et al. 1999a). Cappelier et al. (1999a) even reported that nonculturable *C. jejuni* of strains, BF and 70.2, both used in this study, were infective in two animal models. The infectivity of nonculturable *C. jejuni* in these former studies might be addressed by the presence of few culturable *C. jejuni*. In two studies (Stern et al. 1994; Cappelier et al. 1999a) culturability was determined by selective enrichment. As sublethally injured *C. jejuni* are sensitive to selective agents, selective enrichment will negatively influence the culturability (Humphrey 1986) and the presence of a few culturable *C. jejuni* might be not detected. Another explanation might be the use of *in vivo* instead of *in vitro* models, although previous studies showed a correlation between the adhesion and invasion properties and IL-8 values *in vitro* to the infectivity *in vivo* (Newell and Pearson 1984; Fauchere et al. 1986; Bacon et al. 2000).

Our results confirm that the adhesion and invasion and the IL-8 secretion are strain dependent. The more adhesive and invasive strains, appeared to be the ones which also induced the highest levels of IL-8 in INT-407 cells, as previously shown by Hickey et al. (1999, 2000). Next to strain-dependency, the level of adhesion and invasion was also found to be dose dependent as previously shown (Hu and Kopecko 1999; Mooney et al. 2003; Friis et al. 2005). Our data illustrate the importance of measuring the invasion and adhesion at different doses when comparing strains. For example at a high dose (> 8×10^8 *C. jejuni* well^{-1}) the number of bacteria adhering to and/or invading INT-407 cells was comparable for *C. jejuni* BF and 82/69, while at a low dose (8×10^3 *C. jejuni* well^{-1}) the number of bacteria adhering to and/or invading INT-407 cells varied a factor 20. *C. jejuni* 70.2 is in all aspects the least infective strain: the minimal dose to measure adhesion and invasion is the highest compared to the other strains, and the maximal number of bacteria adhering to

and/or invading INT-407 cells is by far the lowest. The cause of the differences in infectivity between strains has not yet been elucidated.

In conclusion, our findings indicate that nonculturable *C. jejuni* do not adhere or invade INT-407 cells and do not induce IL-8 secretion. Therefore, assuming that the INT-407 model is comparable to the effect *in vivo*, the number of culturable *C. jejuni* in a product is a good measure for the infection risk of a product.

5

Quantification of *Campylobacter jejuni* cross-contamination via hands, cutlery, and cutting board during preparation of a chicken fruit salad

Abstract

Using artificially contaminated chicken, the quantitative overall effect of *Campylobacter jejuni* cross-contamination, either via cutlery, cutting board, or hands, on the microbiological quality of a chicken salad was tested to identify the most critical transfer route. The end contamination level of salads prepared according to different scenarios, with or without cross-contamination, was compared. It was shown that the mean transfer rate calculated for all salads prepared allowing cross-contamination was 0.12% of the initial number of *C. jejuni* on the chicken fillet (8.8 ± 0.2 log CFU). The difference in calculated transfer rates for the tested cross-contamination routes was not significantly different ($p > 0.05$). The prevention of cross-contamination by replacing cutlery and cutting board after handling raw chicken and the prevention of hand contact resulted in considerably reduced end contamination levels (< 2.4 log CFU) or noncontaminated end products. The results of this study emphasize the importance of preventing cross-contamination during food handling in reducing the risks on foodborne infections, and they provide useful data for quantitative microbiological risk assessment.

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Introduction

It is expected that 40 to 60% of the cases of foodborne illness originate from meals prepared in private kitchens (Bryan 1988; Fein et al. 1995; Scott 1996; Humphrey et al. 2001; Cogan et al. 2002; Duff et al. 2003). Consumer knowledge of food handling practices and consumer behaviour in private kitchens were examined (Klontz et al. 1995; Altekruse et al. 1996; Worsfold and Griffith 1997; Jay et al. 1999a, b; Mitakakis et al. 2004), and furthermore, the presence of bacteria in kitchens and their survival and/or transfer during food handling were investigated (De Wit et al. 1979; ; Scott and Bloomfield 1990; Zhao et al. 1998; Chen et al. 2001; Cogan et al. 2002; Kusumaningrum et al. 2004; Luber et al. 2006). The results of these studies raise important concerns about food handling practices and unhygienic behaviour in consumer kitchens, and confirm that improper consumer food handling increases the risk on foodborne infections.

The leading cause of bacterial foodborne gastroenteritis, responsible for 50% of confirmed cases in Western Europe and the United States, is *Campylobacter jejuni* (Blaser 1997; Lawson et al. 1999; Centers for Disease Control and Prevention 2000; Friedman et al. 2000; Oberhelman and Taylor 2000; De Wit et al. 2001b; WHO 2001). This organism is micro-aerophilic, can only grow at temperatures above 30°C (Butzler and Oosterom 1991; Altekruse et al. 1999; Park 2002), and is inactivated by thorough heating (Doyle and Roman 1981; Sorqvist 1989; Park 2002). However, *C. jejuni* can survive under kitchen conditions (Butzler and Oosterom 1991; Altekruse et al. 1999; Park 2002) and is easily transferred from raw food products to hands and food contact surfaces (De Wit et al. 1979; Dawkins et al. 1984; De Boer and Hahné 1990; Cogan et al. 1999; Kusumaningrum et al. 2003, 2004; Luber et al. 2006). Based on these two characteristics and the low number of *Campylobacter* already leading to infection (Robinson 1981; Black et al. 1988), cross-contamination during food preparation was determined as major risk factor for campylobacteriosis in the home (Kapperud et al. 2003; Anonymous 2005; Mylius et al. 2007).

Few quantitative microbiological studies on cross-contamination have been conducted, focusing on the transfer of indicator bacteria (Zhao et al. 1998; Chen et al. 2001; Montville et al. 2001), on individual steps in the preparation process (Kusumaningrum et al. 2003), or on the transfer from different chicken parts to different ready-to-eat foods (Kusumaningrum et al. 2004, Luber et al. 2006). However, data from these studies are not sufficient to identify the most critical transfer route for *Campylobacter*. In this study, a chicken fruit salad was prepared to collect quantitative data on *C. jejuni* cross-contamination. Artificially inoculated chicken breast fillets were chosen as contamination source, as epidemiological studies identified poultry as a major risk factor for campylobacteriosis (Studahl and

Andersson 2000; WHO 2001; Kapperud et al. 2003; Neiman et al. 2003; Friedman et al. 2004). Salads were prepared according to different scenarios. First, a best-case scenario preparation scheme was designed in which cross-contamination was prevented. Subsequently, cross-contamination routes via hands, cutlery, and cutting board were introduced one route at the time, and then the combination of all three routes. The overall effects on the contamination level of the finally prepared chicken meal were determined and compared to identify the most critical transfer route.

Materials and methods

Preparation of C. jejuni *strain cocktail*

Five *C. jejuni* strains were used in this study: NCTC 11828 and NCTC 11168 (National Collection of Type Cultures, Colindale, UK; both human isolates), LB99HU (RIVM, Bilthoven, The Netherlands; human isolate), B258 (Wageningen University and Research Centre, Wageningen, The Netherlands; chicken isolate), and 82-69 (ID-Lelystad B.V., Lelystad, The Netherlands; chicken isolate). Strains were preserved at -70°C in cryovials containing brain heart infusion broth (BHI, Oxoid, Basingstoke, UK) supplemented with 20% (v/v) glycerol. For culturing, one cryovial was thawed, and 0.1 ml was used to inoculate 100 ml BHI in a wide-neck Erlenmeyer flask. The flasks were incubated while shaken at 100 rev min^{-1} in a custom-made incubator (NuAire, Plymouth, MN, USA) in a micro-aerobic atmosphere (10% O_2, 5% CO_2, 85% N_2) for 24 h at 37ºC. Subsequently, 0.1 ml of these cultures was transferred to 100 ml fresh BHI and incubated overnight under conditions as described above. A cocktail containing all five *C. jejuni* strains was prepared by combining equal volumes of the *C. jejuni* strains in a sterile test tube.

Chicken breast fillets

Fresh, chilled chicken breast fillets (100-150 g) were purchased in different batches at a local supermarket, For practical reasons, chicken fillets were stored frozen and thawed before use, under the assumption that *C. jejuni* sticks as well to defrosted chicken as it does to fresh chicken. Excessive moisture, resulting from the freeze thaw process, was removed with paper towel to more closely imitate the fresh chicken surface. Both sides of the fillets were inoculated with 0.5 ml (total 1 ml) of the *C. jejuni* strain cocktail, which was evenly spread using a plastic, sterile spreader. An inoculation level of 10^{8-9} CFU $fillet^{-1}$ was chosen to be able to measure distinct differences between various scenarios. Before use, the fillets were stored separately in plastic bags overnight at ±4°C to mimic retail and/or household storage conditions (no loss in bacterial counts was observed; data not shown).

Preparation of chicken fruit salads

Ingredients were purchased from a local supermarket: fresh chicken fillet (100-150 g), pine-apple (canned, 140 g), apple (one), orange (one), sterilized sour cream (200 ml), curry, and salt. All salads were prepared in a consumer kitchen with the utensils in-house. At the start of each salad preparation, the kitchen-bench and utensils were cleaned with detergent and hot water, and dried with paper towel. Hands were washed with warm water and soap, and dried with paper towel.

The salads were prepared according to the following method. A pan was filled with a stock cube and water, and the water was brought to a boil. The foil containing the chicken was opened, and the chicken was transferred to the cutting board. The chicken was cut into two pieces and placed into the boiling water for 10 min. The fruit was cut into pieces. After boiling, the chicken was transferred to the cutting board and allowed to cool for 10 min before the chicken was cut into pieces. The ingredients were then all mixed.

Modifications in salad preparation tools were made according to the different scenarios (all scenarios were prepared at least in triplicate):

-Best-case scenario: cutlery and cutting board contaminated during handling raw chicken; hands washed and both cutlery and cutting board replaced; clean cutlery used to cut fruit and cooked chicken on clean cutting board

-Hands: cutlery, cutting board, and hands contaminated during handling raw chicken; both cutlery and cutting board were replaced, and hands were not washed; clean cutlery and unwashed hands used to cut fruit and cooked chicken on clean cutting board

-Cutlery: cutlery and cutting board contaminated during handling raw chicken; hands washed and cutting board replaced, but cutlery not replaced; contaminated cutlery used to cut fruit and cooked chicken on clean cutting board

-Cutting board: cutlery and cutting board contaminated during handling raw chicken; hands washed and cutlery replaced, but cutting board not replaced; clean cutlery used to cut fruit and cooked chicken on contaminated cutting board

-Full cross-contamination: cutlery, cutting board, and hands contaminated during handling raw chicken; hands not washed and both cutlery and cutting board not replaced; contaminated cutlery and unwashed hands used to cut fruit and cooked chicken on contaminated cutting board

Microbiological analysis of chicken fruit salads

End contamination levels of salads after preparation were determined by use of the most probable number (MPN) method (De Man 1983) in combination with spread plating a range of decimal dilutions on agar plates. As plate counts are more precise and give smaller confidence intervals than do MPN counts, in general data from the plate count method were

used. However when plate counts were below the detection limit, MPN count results were applied.

Salads were analysed either on the same day or after overnight storage at 4°C (no loss in bacterial counts in prepared salads was observed during overnight storage at 4°C; data not shown). Part of the salad was mixed in a 750-ml glass blender (with foam arrester; Waring, Torrington, Conn., USA) for 1 min, resulting in salad homogenate. Furthermore, an additional homogenate (salad mix) was prepared by mixing approximately 70 g (exact weight noted) of salad with 200 ml sterile peptone physiological saline (PPS; 1 g l^{-1} peptone and 9 g l^{-1} NaCl; NVI, Bilthoven, The Netherlands) for 1 min using a 300-ml stainless steel blender (Waring).

MPN and plate count

The inoculation solutions (in triplicate) were as follows: 10 g of salad homogenate in 100 ml of Preston broth (Oxoid), 1 g of salad homogenate in 10 ml of Preston broth, 1 g of salad mix in 10 ml of Preston broth, and 1 ml of 10^{-1} and 10^{-2} dilutions of salad mix in 10 ml of Preston broth, respectively. After incubation, jars and tubes were checked for growth of *C. jejuni* by streak plating loopfuls on Karmali agar plates (Oxoid). Suspected colonies were confirmed by phase-contrast microscopy. The MPN of organisms present in the chicken salad was calculated, taking into account the exact weighed amount of salad used for enumeration, using a Microsoft Office Excel spreadsheet, based on the MPN method described by De Man (1983).

For the plate counts, appropriate dilutions of salad mix in PPS (10^{-1} - 10^{-4}) were spread plated (100 µl) on Karmali agar plates. Incubation conditions were as follows. All tubes, jars, and plates were micro-aerobically incubated at 37°C (broth: 48 ± 4h, agar: 72 ± 4h) either by use of jars with BBL Campypak (Becton Dickinson, Sparks, MD, USA) or in the three-gas incubator (no differences in bacterial counts were observed between incubation in jars or in the three-gas incubator; data not shown).

Transfer rates

Out of the determined end contamination levels of prepared salads and the initial inoculation level of the chicken breast fillets (i.e., the number of *C. jejuni* in 1 ml of the inoculation cocktail) the transfer rates from chicken to salad for the different scenarios were calculated as: transfer rate (%) = (CFU per prepared salad/CFU per chicken fillet) * 100. According to Chen et al. (2001), the log transfer rates are normally distributed. Therefore, the average transfer rates were determined by averaging the log transfer rates, where after the average log values were antilog transformed.

Statistical analysis

The two-sample *t* test was used to test the difference between the transfer rates for the various preparation scenarios. The significance level used was $\alpha = 0.05$.

Results

Using artificially inoculated chicken breast fillets (8.8 ± 0.2 log CFU chicken fillet^{-1}), salads were prepared according to different cross-contamination scenarios: full cross-contamination (all three routes), via hands, via cutlery, via cutting board, and no cross-contamination (best-case scenario). The average end contamination level of salads prepared according to scenarios allowing cross-contamination was 5.8 ± 0.6 log CFU salad^{-1}, while the prevention of cross-contamination (best-case scenario) led to considerably reduced end contamination levels (<2.4 log CFU salad^{-1}) or noncontaminated salads.

For each prepared salad, the transfer rate was calculated out of the initial contamination of the chicken breast fillets and the end contamination level (Table 5.1). Mean transfer rates for the different cross-contamination scenarios are given in Table 5.2. The calculated transfer rates for the various cross-contamination routes are not significantly different ($p > 0.05$), and the mean transfer rate calculated for all salads prepared allowing cross-contamination was 0.12%.

Table 5.1. Inoculation levels of chicken breast fillets, end contamination levels in prepared salads, and the calculated transfer rates for the different scenarios

Scenario	Measurement	Inoculation level (log CFU fillet^{-1})		End contamination* (log CFU salad^{-1})		Transfer rate (%)
		mean	95% CI	mean	95% CI	
Full	1	8.5	8.4 - 8.6	6.2	5.9 - 6.4	0.44
	2	9.0	8.9 - 9.0	6.0	5.6 - 6.3	0.11
	3	9.0	8.9 - 9.0	6.1	5.8 - 6.4	0.15
Hands	1	8.5	8.4 - 8.6	*5.8*	*5.1 - 6.3*	0.17
	2	9.0	8.9 - 9.0	5.8	5.2 - 6.1	0.07
	3	8.6	8.4 - 8.6	6.1	5.8 - 6.4	0.38
	4	9.0	8.9 - 9.0	6.1	5.8 - 6.4	0.15
	5	9.0	8.9 - 9.0	6.1	5.8 - 6.4	0.14
Cutlery	1	8.8	8.7 - 8.8	6.0	5.7 - 6.3	0.18
	2	8.5	8.4 - 8.6	*4.3*	*3.6 - 5.0*	0.01
	3	9.0	8.9 - 9.0	6.1	5.8 - 6.4	0.14
	4	8.6	8.4 - 8.6	6.1	5.8 - 6.3	0.32
	5	9.0	8.9 - 9.0	6.2	5.9 - 6.4	0.18
Cutting board	1	8.8	8.7 - 8.8	6.0	5.6 - 6.3	0.18
	2	8.5	8.4 - 8.6	5.2	3.6 - 5.8	0.05
	3	9.0	8.9 - 9.0	6.1	5.7 - 6.4	0.13
	4	8.2	8.2 - 8.2	*4.3*	*3.5 - 4.9*	0.01
	5	9.0	8.9 - 9.0	6.2	5.9 - 6.4	0.17
BC	1	8.8	8.7 - 8.8	*1.8*	*1.0 - 2.3*	1.0×10^{-5}
	2	8.4	8.4 - 8.4	n.d.		
	3	9.0	8.9 - 9.0	n.d.		
	4	8.2	8.2 - 8.2	n.d.		
	5	8.6	8.5 - 8.7	n.d.		
	6	8.6	8.5 - 8.7	n.d.		
	7	8.6	8.5 - 8.7	n.d.		
	8	8.6	8.5 - 8.7	*2.4*	*1.7 - 2.9*	5.5×10^{-5}

*In general, data from plate counts were used. However when plate counts were below the detection limit, MPN count results were applied. MPN count values are given in italics.

n.d. - not detected. Both plate counts and MPN counts below detection limit (± 1.4 log CFU salad^{-1}).

Table 5.2. Mean transfer rates for the different cross-contamination scenarios

Scenario	Log transfer rate (%)		Transfer rate (%)		
	mean	SD	mean	95% Cl	
Full	-0.71	0.31	0.19	0.05 -	0.80
Hands	-0.80	0.27	0.16	0.05 -	0.54
Cutlery	-1.01	0.67	0.10	0.00 -	2.04
Cutting board	-1.12	0.49	0.08	0.01 -	0.69
All*	-0.93	0.47	0.12	0.01 -	0.96

*The summation of all salads prepared allowing cross-contamination

Discussion

This study showed that, irrespective of the transfer route, 0.12% of *Campylobacter* initially present on chicken breast fillets was transferred to prepared salads. This high transfer emphasizes the significance of cross-contamination as a risk for campylobacteriosis, and it proved the importance of prevention of cross-contamination in preparing safe food.

The measured transfer rates for hands (0.16%), cutlery (0.10%), and cutting board (0.08%) in this study were of the same order as reported in literature: for *C. jejuni*, 0.11% from chicken fillets to bread via hands and 0.11% from chicken fillets to cucumber via cutting board and knife (Luber et al. 2006); for the indicator organism *Enterobacter aerogenes*, from chicken to lettuce via hands 0.07% to 0.74% and 0.1 to 1% via cutting board (Zhao et al. 1998; Chen et al. 2001; Montville et al. 2001). The various recipes used in these former studies made it difficult to compare the different transfer routes. Therefore, in our approach, the preparation scenarios were kept identical with exception of one cross-contamination route at a time, which facilitated the comparison of different cross-contamination routes to identify the most critical one. As the transfer rates for the various cross-contamination routes were not significantly different, it is concluded that the different cross-contamination routes tested, via hands, cutlery, and cutting board were equally important.

With the great impact of cross-contamination shown, results of former consumer studies on consumer behaviour are very alarming. After handling raw meat or poultry, 34 to 58% of the consumers do not wash their hands properly (Altekruse et al. 1996; Worsfold and Griffith 1997; Jay et al. 1999a; Angelillo et al. 2001; Li Cohen and Bruhn 2002), 25 to 39% of consumers do not clean their cutting board adequately (Klontz et al. 1995; Altekruse et al. 1996; Worsfold and Griffith 1997; Jay et al. 1999a; Li Cohen and Bruhn 2002; Mitakakis et al. 2004), and 29 to 45% do not clean their kitchen utensils sufficiently (Worsfold and Griffith 1997; Jay et al. 1999a). Moreover, Jay et al. (1999b) showed that self-reported behaviour often positively contrasted with actual observations, and as most of

these surveys were based on interviews (Klontz et al. 1995; Altekruse et al. 1996; Jay et al. 1999a; Angelillo et al. 2001; Li Cohen and Bruhn 2002; Mitakakis et al. 2004) the reality might be far worse.

Furthermore, even if consumers try to prevent cross-contamination by hand washing and the cleaning or replacement of cutlery and cutting board, small failures may result in contamination of the end product. This is clearly illustrated by our findings that, whilst beforehand best-case salads were expected to be *Campylobacter* negative, two of eight best-case salads showed positive MPN counts. The positive best-case salads might be explained by inefficient hand washing or cleaning procedures in between preparation of two salads, i.e. washing with detergent or soap and hot water and drying using paper towel. In accordance, Chen et al. (2001) did recover bacteria from hands after washing with soap and hot water and drying with a paper towel, and Cogan et al. (1999, 2002) reported that part of the bacterial contamination on hands and surfaces remained present after cleaning with detergent and hot water, followed by drying. In contrast various other authors showed the applied hand washing (Dawkins et al. 1984; Coates et al. 1987; Brown et al. 1988) or cleaning techniques (Dawkins et al. 1984; Ak et al. 1994; Mattick et al. 2003) to be sufficient to remove all bacteria from hands or surfaces. The difference in measured efficiency between studies might be the result of different prewash contamination levels used. Especially at high inoculation levels as used in this study, the maximal reduction by the used techniques might not be enough to remove all *Campylobacter*.

We used artificially contaminated chicken fillet with a high contamination level of 9 log CFU fillet^{-1} to be able to measure distinct differences between various scenarios. The *Campylobacter* contamination level of chicken breast fillets in The Netherlands varies from 2.9 to 4.0 log CFU fillet^{-1}, with mean 3.3 log CFU (Nauta et al. 2005); however these data are based on just a few flocks sampled in the summer of 2004. In practice contamination levels of chicken breast fillets might vary more widely, as seen for whole carcasses (Dufrenne et al. 2001). The determined mean transfer of *Campylobacter* of 0.12% during preparation of salads when allowing cross-contamination in combination with a mean contamination level of 3.3 log CFU fillet^{-1} would result in *Campylobacter* remaining in more than half of improperly prepared salads. This high level of contaminated products after unsafe food preparation, together with the high percentages of consumers reported to improperly prepare their food thereby enabling cross-contamination, indicates an important role for cross-contamination as a cause of campylobacteriosis during consumer preparation of chicken. Therefore, it is very important to take measures to prevent cross-contamination during consumer food handling practices, as long as the levels of *Campylobacter* on chicken products remain high.

6

Consumption of raw vegetables and fruits: a risk factor for *Campylobacter* infections

Abstract

The purpose of this study was to determine the prevalence of *Campylobacter* in fresh vegetables and fruits at retail level in The Netherlands, and to estimate its implications on the importance of vegetables and fruits as risk factor for campylobacteriosis.

Thirteen of the 5640 vegetable and fruit samples were *Campylobacter* positive, resulting in a prevalence of 0.23% (95% confidence interval (Cl): 0.12-0.39%). The prevalence of packaged products (0.36%, 95% Cl: 0.17-0.66) was significantly higher than of unpackaged products (0.07; 95% Cl: 0.01-0.27). No statistical differences were found between seasons.

Combining the mean prevalence found in this study with data on the consumption of vegetables and fruits, an exposure of 0.0048 campylobacters ingested per person per day in The Netherlands by transmission via vegetables and fruits, was calculated. This exposure, as input in a Beta-Poisson dose-response model, resulted in an estimated number of 4.6×10^5 cases of infection with *Campylobacter* per year for the whole Dutch population. This constitutes the consumption of raw vegetables and fruits, especially when packaged, to be a risk factor for *Campylobacter* infections.

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Introduction

Campylobacter jejuni is the leading cause of bacterial foodborne gastroenteritis throughout the world (Friedman et al. 2000; WHO 2001). The organism is micro-aerophilic and can only grow at temperatures above 30°C (Park 2002). The required growth conditions are met in the gastrointestinal tract of warm-blooded animals, including birds, the main reservoir for *C. jejuni*. Transmission to humans is considered to occur mainly via foods of animal origin, especially poultry and raw milk, and contaminated drinking water (Eberhart-Phillips et al. 1997; Studahl and Andersson 2000; Frost et al. 2002; Kapperud et al. 2003). However transmission of *C. jejuni* through contaminated fresh produce of non-animal origin, i.e. raw vegetables and fruit, might also be of significant importance. This is illustrated by several outbreaks of campylobacteriosis associated with raw vegetables and fruits (Bean and Griffin 1990; Centers for Disease Control 2000; Jacobs-Reitsma 2000; Harris et al. 2003; Mandrell and Brandl 2004; Sivapalasingam et al. 2004), and the epidemiologic study of Evans et al. (2003) showing salad vegetables to be the second highest risk factor for *Campylobacter* infection, after consumption and preparation of chicken.

As result of changes in food production and distribution practices, consumer preferences, social demographics, and enhanced epidemiological surveillance using improved methods to identify and track pathogens (Hurst and Schuler 1992; Beuchat and Ryu 1997; Tauxe et al. 1997; Sivapalasingam et al. 2004; Little and Gillespie 2008), the number of documented foodborne outbreaks associated with raw vegetables and fruits has increased during the last three decades (Beuchat 2002; European Commission 2002; Buck et al. 2003; Harris et al. 2003; Sivapalasingam et al. 2004). This implicates a growing importance for vegetables and fruits as risk factor for campylobacteriosis.

The common hypothesis is that the consumption of raw vegetables and fruits constitutes a risk on campylobacteriosis as result of cross-contamination during food preparation by another contaminated food, such as raw poultry. However, several studies reported the presence of *Campylobacter* on raw vegetables and fruits at retail level before food preparation (Doyle and Schoeni 1986; Phillips 1998; Federighi et al. 1999; Kumar et al. 2001; Whyte et al. 2004; Chai et al. 2007) and *C. jejuni* was shown to survive sufficiently long on vegetables and fruits to pose a risk to the consumer (Castillo and Escartin 1994; Kärenlampi and Hänninen 2004). Before consumer food preparation, vegetables and fruits can become contaminated with pathogenic microorganisms, during production, harvesting, processing, packaging, distribution, or at retail level. Pre-harvest contamination sources include faeces, presence and survival in soil, irrigation water, natural or inadequately composted manure, air (dust), wild or domestic animals, and/or human handling. Post-harvest factors which influence the contamination of vegetables and fruits are improper

hygiene of workers, harvesting equipment, transport containers, wild and domestic animals, air (dust), wash and rinse water, sorting-, packing-, cutting-, and further processing equipment, ice, transport vehicles, improper storage (temperature and physical environment), improper packaging, and cross-contamination at retail level (Beuchat 1996).
In most previous studies reporting on the presence of *Campylobacter* spp. on raw vegetables and fruits, the uncertainty was very large as result of the limited number of samples taken and the low prevalence found. The purpose of this study was to calculate with higher accuracy the prevalence of *Campylobacter* in fresh vegetables and fruits at retail level in The Netherlands out of a large set of monitoring data. Calculations on the prevalence were grouped per type of vegetable or fruit, per sampling date, and per type of packaging. Furthermore the human exposure to *Campylobacter* via vegetables and fruits was calculated, to assess the importance of vegetables and fruits as risk factor for *Campylobacter* infections.

Materials and methods

Sampling

The Food and Consumer Product Safety Authority (VWA) of The Netherlands is the official governmental authority for human and animal health protection. To safeguard public health and animal health and welfare different aspects on the quality of food and consumer products are monitored, including the microbiological quality of food at retail level. The microbiological monitoring outcomes are gathered in the ISI-database, for different pathogenic microorganisms in specific food, to facilitate statistical estimations on the occurrence of pathogens in food. With this knowledge, possible public health risks can be better estimated and strategies on reduction of these risks can be made.
Within the microbiological monitoring programs, the presence of *Campylobacter* spp. in fresh vegetables and fruits was monitored. Samples were taken randomly by VWA inspectors at the main retail points for vegetables and fruits, mainly supermarkets and fruit- and vegetable shops, throughout The Netherlands. Both vegetable and fruit samples were taken as a whole portion and minced and homogenized in a food processor. For the detection of *Campylobacter* spp. a method derived from the ISO 10272: 1995 (International Organization for Standardization 1995) was used. The method consisted of a selective enrichment of 25 g of the homogenized sample in charcoal cefoperazone deoxycholate broth (CCD) incubated under micro-aerobic conditions (5 % O_2, 10 % CO_2 and 85% N_2) for 24 h at 42°C. *Campylobacter* spp. were isolated by streaking a loopful on CCD-agar incubated under micro-aerobic conditions for 48 h at 42 °C. Typical colonies were confirmed using wet mount and latex agglutination test (Meretic^{tm}-Campy from Meridian

Biosciences, Cincinnati, USA). The theoretical detection limit for this method was 1 CFU/25 g. Large numbers of samples were taken to make statistical reliable estimations on the prevalence of *Campylobacter* spp. in vegetables and fruits.
From January 2000 until December 2005, 5640 vegetable- and fruit products at retail level were analysed for the presence of *Campylobacter* spp..
Of all samples, the kind of vegetable or fruit, the sampling date, and the type of packaging were documented. From October 2000 until April 2001 and from July 2001 until October 2001 no vegetable and fruit samples were taken within the monitoring program.
To enable calculations on exposure, microbiological monitoring data were classified into fresh produce groups (leafy vegetables, fruit crops, root crops, cabbage, mushrooms, onions/garlic, stem- and sprout crops, mixed salads/vegetables, fruit, and mixed fruits), as described in the Dutch National Consumption Survey 2003 (Hulshof et al. 2004).

Calculations

Out of the number of samples positive for *Campylobacter* spp. (N) and the total number of samples T, the prevalence P was calculated. Based on cumulative binomial distribution, the 95% confidence intervals for the prevalence were determined. Results were divided per vegetable and fruit group, per three months and for different packaging methods.

$$P = N / T \qquad (1)$$

The Dutch food consumption survey 2003 reported a mean daily consumption of 24.3 g raw vegetables m_v and 27.3 g fruits consumed with skin m_f per person (Hulshof et al. 2004). Together these values give the mean daily consumption of raw vegetables and fruits with skin m.

$$m = m_v + m_f \qquad (2)$$

As no concentration was measured but only if *Campylobacter* spp. were present in 25 g, the minimal *Campylobacter* concentration c in contaminated products was used for calculations, which is equal to the detection limit (1 CFU/25 g =0.04 CFU g^{-1}).
The mean dose d was calculated by multiplying the mean daily consumption of raw vegetables and fruits in gram per person per day in The Netherlands m; and the *Campylobacter* concentration c in CFU $gram^{-1}$ contaminated product.

$$d = m * c \qquad (3)$$

The estimated dose and the calculated prevalence P of contaminated products in retail in the Netherlands were used to estimate the probability of infection with *Campylobacter*, using a Beta-Poisson model as described by Nauta et al. (2005):

$$P_{inf,day} = [1 - (1 + d / \beta)^{-\alpha}] * P \quad (4)$$

with $P_{inf,day}$ the probability of infection per day given a consumed dose d and a prevalence P, with α and β parameters. Teunis and Havelaar (2000) give $\alpha = 0.145$, $\beta = 7.589$.
The probability of infection per person per year $P_{inf,year}$ equals:

$$P_{inf,year} = P_{inf,day} * 365 \quad (5)$$

For the Dutch population $P_{Nl,year}$ the average of the number of inhabitants at the start (15.9×10^6, January 2000) and at the end of the sampling period (16.3×10^6, December 2005) was taken, giving 16.1×10^6 inhabitants (CBS 2010). The expected number of cases of infection per year for the whole Dutch population $P_{Nl,year}$, then equals:

$$P_{Nl,year} = P_{inf,year} * 16.1\times10^6 \quad (6)$$

The overall equation composed out of the estimations above is:

$$P_{Netherlands,\ year} = [1- (1 + ((m_v + m_f) * c)/\beta)^{-\alpha}] * P * 365 * 16.1\times10^6 \quad (7)$$

Furthermore the mean daily dose *dd*, expressed as the mean number of *Campylobacter* ingested per person per day by transmission via vegetables and fruits at Dutch population level was calculated by multiplying the prevalence P, the mean daily consumption m and the *Campylobacter* concentration c.

$$dd = P * m * c \quad (8)$$

Results

The amount of samples; the prevalence of *C. jejuni* in different vegetable and fruit products with a 95% confidence level; and data on consumption of fruits and vegetables from the Dutch National Consumption Survey 2003 (Hulshof et al. 2004) are presented in Table 6.1.
Leafy vegetables (2 of 562; 1 endive and 1 watercress), fruitcrops (2 of 1157; 2 cucumbers), stem- and sproutcrops (1 of 50; 1 leek), mixed salads/vegetables (5 of 2549), vegetable and fruitsalads (1 of 159), and mixed fruits (2 of 779) were positive for

Campylobacter, whereas the other vegetables and fruits tested negative. The average prevalence of all the analysed vegetables and fruits is 0.23% (13 out of 5640), with 95% confidence interval: 0.12-0.39%.

Table 6.1. The occurrence of *Campylobacter* spp. (number of samples analysed, number of positive samples, prevalence, and 95% confidence interval) and the consumption of raw vegetables and fruits with skin (per Dutch person per day) presented per vegetable or fruit group

Source	Number of samples	Number of samples positive for *Campylobacter* spp.			Mean daily consumption[1] (g day^{-1})
		N	*P* (%)	95% Cl	
Leafy vegetables	562	2	0.36	0.04 - 1.28	6.98
Fruit crops	1157	2	0.17	0.02 - 0.62	12.56
Root crops	196	0	0	0 - 1.86	0.89
Cabbage	127	0	0	0 - 2.86	0.49
Mushrooms	8	0	0	0 - 36.94	0.03
Onions, Garlic	42	0	0	0 - 8.41	1.79
Stem- and sprout crops	50	1	2.0	0.05 - 10.65	0.10
Mixed salads/vegetables	2549	5	0.20	0.06 - 0.46	1.46
Vegetables-fruit mix	159	1	0.63	0.02 - 3.45	*
Fruit	11	0	0	0 - 28.49	26.46
Mixed fruit	779	2	0.26	0.03 - 0.92	0.82
Total	5640	13	0.23	0.12 - 0.39	51.55
Vegetables	4691	10	0.21	0.10 - 0.39	24.26
Vegetables-fruit mix	159	1	0.63	0.02 - 3.45	*
Fruit	790	2	0.25	0.03 - 0.91	27.29

[1] (Hulshof et al. 2004)

* there is no data available on the mean daily consumption of vegetable-fruit mix, as this category could not be classified into the fresh produce groups as described in the Dutch National Consumption Survey

To calculate the mean daily dose (*dd*), the average prevalence of *C. jejuni* in vegetables and fruits was multiplied with the mean daily consumption of raw vegetables and fruit with skin and the minimal *Campylobacter* concentration in contaminated products, using equation 8:

$dd = 0.231\% * (24.26+27.29) * 0.04 = 0.0048$ CFU person^{-1} day^{-1}

Thereafter, the expected number of cases of infection with *Campylobacter* per year for the whole Dutch population was estimated using equation 7:

$$P_{Netherlands,\ year} = [1- (1+((24.26 + 27.29) * 0.04)/7.589)^{-0.145}]*365*16.1\times10^{6}*0.231\%$$

giving an estimated number of 4.6×10^{5} cases of infection with *Campylobacter* per year in The Netherlands. About 1/3 of the number of cases of infection is expected to lead to disease (Havelaar et al. 2000).
Furthermore, the monitoring data were used to study the occurrence of *Campylobacter* spp. on vegetables and fruits during the year (Fig. 6.1). No apparent seasonal effect of *Campylobacter* occurrence on vegetables and fruits was observed. Two peaks were found, for October to December 2002 and April to June 2003, which were caused by two respectively three positive samples that were taken at one retail facility on one day. These samples were of different vegetable and fruit sources and were pre-packaged, which excludes cross-contamination at retail level.

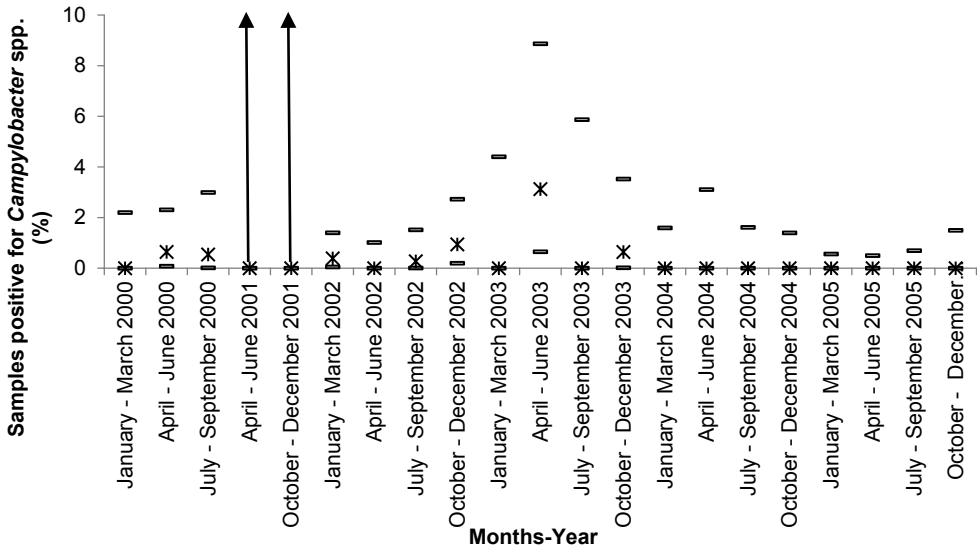

Figure 6.1. The occurrence of *Campylobacter* spp. (percentage positive samples and 95% confidence interval) given per three-month period from January 2000 till December 2005.

Finally the monitoring data were used to study the occurrence of *Campylobacter* spp. on vegetables and fruits for different packaging methods (packaged, modified atmosphere packaging (MAP), and unpackaged; Table 6.2).

Table 6.2. The occurrence of *Campylobacter* spp. (number of samples analysed, number of positive samples, percentage positive samples, and 95% confidence interval) per type of packaging (packaged, MAP-packaged and unpackaged)

Type of packaging	Number of samples	Number of samples positive for *Campylobacter* spp.		
		N	*P* (%)	95% Cl
Packaged	2792	10	0.36	0.17 - 0.66
MAP	161	1	0.62	0.02 - 3.41
Unpackaged	2687	2	0.07	0.01 - 0.27
Total	5640	13	0.23	0.12 - 0.39

A significant packaging effect was observed, the percentage of *Campylobacter* spp. positive samples was significantly higher (Fisher's exact test, p=0.023) in packaged products (0.36%, 95% Cl: 0.17-0.66) when compared with unpackaged products (0.07%, 95% Cl: 0.01-0.27). The same increase in *Campylobacter* occurrence was seen for MAP packaged products (0.62%, 95% Cl: 0.02-3.41), however the effect was not significant (Fisher's exact test, p=0.161) for these products and the confidence interval was very large, due to the low number of samples tested for MAP.

Discussion

The monitoring data presented in this paper give an overview of the prevalence of *Campylobacter* on vegetables and fruits in The Netherlands. Of all vegetables and fruits the 95% confidence intervals cover the mean prevalence of 0.23% as calculated for the total of all vegetables and fruits. Because of the large uncertainties per type of vegetable it was not possible to make distinctions between risks of specific types of vegetables.

The literature regarding the prevalence of *Campylobacter* on vegetables or fruits is summarized in Table 6.3. In many studies, small sample sizes (≤400) in combination with few or no positive samples resulted in large confidence intervals (Doyle and Schoeni 1986; Odumeru et al. 1997; Federighi et al. 1999; McMahon and Wilson 2001; Whyte et al. 2004; Little and Gillespie 2008). The prevalence found in our study fits in the large confidence limits of these studies. Only three other papers calculated the prevalence from a large set of monitoring data (>2500 samples). *Campylobacter* spp. were not detected in these UK studies (Sagoo et al. 2001, 2003a, b). In contrast, in another UK study *Campylobacter* spp. were shown to be present in 20 out of 90 MAP packaged vegetables (Phillips 1998).

Striking is the high number of positive samples (49.5%) detected by Chai et al. (2007). This is the only study using a PCR based method for the recovery of *Campylobacter* in vegetables and fruits, which detects not only culturable but also nonculturable

Campylobacter. However former research indicated the number of culturable *C. jejuni* in a product to be a good measure for the infection risk of a product (Verhoeff-Bakkenes et al. 2009) and nonculturable *Campylobacter* were shown not to be infective *in vitro* (Verhoeff-Bakkenes et al. 2008). Only 28 of 153 samples positive for *Campylobacter* spp. by PCR were also positive by plating after enrichment, giving a prevalence of 9.1% (Cl: 6.1-12.8). Cross-contamination might have played a role in this (still) high contamination rate detected by plating and enrichment, as the hygienic condition and foodstuff handling methods in Malaysia may be poorer than in European countries and the USA (Chai et al. 2007), which might also explain the higher number of positive samples (3.6%) found in India (Kumar et al. 2001). The key role of hygienic handling of fresh produce is clearly illustrated by Park and Sanders (1992), who collected vegetable samples from farmers' outdoor markets as well as from supermarkets. From the vegetables produced and (or) stored under less sanitary conditions at farmers' outdoor market 9 of 533 were *Campylobacter* positive, while all 1031 more hygienic produced supermarket samples were negative for *Campylobacter*.

The exposure model was used to calculate the *Campylobacter* exposure by transmission via raw vegetables and fruits with skin. With a prevalence of 0.23%, a consumption of 51.55 g person^{-1} day^{-1}, a *Campylobacter* concentration of 0.04 CFU g^{-1}, the mean daily dose was estimated at 0.0048 CFU person^{-1} day^{-1}. The estimated number of cases of infection with *Campylobacter* as result of the consumption of raw vegetables and fruits per year for the whole Dutch population, based on a Beta-Poisson dose-response model and the calculated exposure, was 4.6×10^5 cases. A reasonable estimate is that the number of cases of disease is about equal to 1/3 of the number of cases of infection (Havelaar et al. 2000), i.e. 1.5×10^5. An epidemiological study estimated the number of gastroenteritis cases in The Netherlands per year by *Campylobacter* at 1.1×10^5 (De Wit et al. 2001b). Thus the risk assessment model might give an overestimation, or the epidemiological study might give an underestimation of the number of human cases of campylobacteriosis, although it must be kept in mind that the exposure model, the dose-response relationship and the epidemiological estimate are all uncertain. The epidemiological study focused on acute gastroenteritis, and all asymptomatic human cases were not considered, which might result in an underestimation. Furthermore, sample storage and culturing in medical microbiology may add to an underestimation of the real incidence of all symptomatic cases (Nauta et al. 2005). For the risk assessment model, the calculations on the exposure are based on various assumptions and are a simplified representation of reality. Important parameters like the prevalence, consumption and concentration are estimated with uncertainty, but are of great influence on the outcome of the exposure model. Firstly, as result of the small number of 13 positive samples, the mean prevalence of 0.23% for *Campylobacter* in fresh vegetables and

Table 6.3. The prevalence of *Campylobacter* on vegetables and fruits reported in literature, detected by plating after enrichment unless indicated otherwise

Source	Number of samples	Number of samples positive for *Campylobacter* spp.			Country	Reference
		N	*P* (%)	95% Cl		
Vegetables	309	153[1]	49.51	43.8 - 55.2	Malaysia	Chai et al. 2007
MAP vegetables	90	20	22.22	14.1 - 32.2	UK	Phillips 1998
Vegetables	309	28	9.06	6.1 - 12.8	Malaysia	Chai et al. 2007
Vegetables	56	2	3.57	0.44 - 12.3	India	Kumar et al. 2001
Mushrooms	200	3	1.50	0.31 - 4.3	USA	Doyle and Schoeni 1986
Vegetables supermarket	1031	0	0.00	0.00 - 0.36	Canada	Park and Sanders 1992
Vegetables farmers' outdoor	533	9	1.69	0.77 - 3.1	Canada	Park and Sanders 1992
Vegetables and fruits	5640	13	0.23	0.12 - 0.39	Netherlands	This study
Vegetables	279	2	0.72	0.09 - 2.6	Ireland	Whyte et al. 2004
RTE Vegetables	400	2	0.50	0.06 - 1.8	France	Federighi et al. 1999
Organic vegetables	86	0	0.00	0.00 - 4.2	N. Ireland	McMahon and Wilson 2001
Whole lettuce	151	0	0.00	0.00 - 2.4	UK	Little and Gillespie 2008
RTE vegetables	361	0	0.00	0.00 - 1.0	Canada	Odumeru et al. 1997
Unpacked salad vegetables	2950	0	0.00	0.00 - 0.12	UK	Sagoo et al. 2003a
Organic vegetables	3200	0	0.00	0.00 - 0.12	UK	Sagoo et al. 2001
RTE vegetables	3852	0	0.00	0.00 - 0.10	UK	Sagoo et al. 2003b
Total[2]	19138	79	0.42	0.33 - 0.52		

[1] detected by PCR

[2] only the plating after enrichment results from the study of Chai et al. (2007) are incorporated

fruits as calculated from the monitoring data and used for the exposure calculations in this study has a large 95% confidence interval of 0.12-0.39%. Consequently, the exposure can be almost twice as low or twice as high as calculated with the mean prevalence. Secondly, the mean daily consumption per person was used, which assumes that every single person in the Dutch population will consume fruits and vegetables every day with a constant amount of consumption. In reality, the amount of consumption will vary per person per day and is dependent on age, gender, region, education and other factors. Although the data will vary at an individual level, the exposure calculations are at population level and given the big uncertainty of the other input parameters the assumption of a mean daily consumption will not influence the exposure outcomes at a large extent. Thirdly, the minimal *Campylobacter* concentration, equal to the detection limit, is used for calculations, which might result in underestimation of the risk. Positive products are assumed to contain 1 CFU per 25 gram, which in reality is probably higher. Moreover below the detection limit products are assumed to contain no *Campylobacter*, but might still contain *Campylobacter* at a lower concentration (less than 0.04 CFU g^{-1}) which is not detected. Furthermore the exposure model might give an overestimation as result of clustering of exposure, and immunity which both are not incorporated in the model. To estimate the probability of infection with *Campylobacter* the Beta-Poisson dose-response model was used. This model does not include differences in pathogenicity of strains, differences in effects of food-matrix on *Campylobacter*, and genetic host differences, which might result also in an overestimation. Although models are a simplified version of reality, they are useful tools to give insight in the importance of the different parameters used.

It has been widely reported that poultry and raw milk are main transmission vectors of *Campylobacter*. A Dutch risk assessment study calculated 20 to 40% of the campylobacteriosis cases to be poultry related (Havelaar et al. 2005) and withdrawal of poultry from the market in Belgium was reported to lead to a 40% decline in the number of *Campylobacter* infections (Vellinga and van Loock 2002). To assess the importance of vegetables and fruits as risk factor for campylobacteriosis, the exposure data for vegetables and fruits were compared to the exposure data for chicken and raw milk reported earlier by Evers et al. (2004). The importance of the different parameters used was assessed with the exposure model. In spite of the low consumption of raw chicken (chicken liver; 0.03 g $person^{-1}$ day^{-1}) and raw milk (2.32 g $person^{-1}$ day^{-1}), exposure is highest via these transmission vectors (0.0173 CFU $person^{-1}$ day^{-1} for raw chicken and 0.0167 CFU $person^{-1}$ day^{-1} for raw milk) as a result of the high prevalence (33% for raw chicken and 4.5% for raw milk) and high concentration (1.75 CFU g^{-1} raw chicken and 0.16 CFU g^{-1} raw milk) of *Campylobacter* in these products. Evers et al. (2004) also estimated the exposure via cross-contamination during preparation of chicken. With a prevalence of 29% for chicken fillet and 33% for other chicken, a consumption of 7.41 g chicken fillet and 9.60 g other chicken

per person per day, a *Campylobacter* concentration of 1.75 CFU g^{-1}, and an estimated transfer and survival by cross-contamination of factor $10^{-3.6}$, they estimate the exposure at 0.0023 CFU $person^{-1}$ day^{-1}, which is in the same order as the exposure via vegetables and fruits as calculated in this study (0.0048 CFU $person^{-1}$ day^{-1}). The exposure via vegetables and fruits is highly influenced by the high consumption (51.55 g $person^{-1}$ day^{-1}), while the prevalence (0.23%) and concentration (0.04 CFU g^{-1}) of *Campylobacter* in vegetables and fruits are low in relation to raw chicken and raw milk. To summarize, the exposure via vegetables and fruits in this study (0.0048 CFU $person^{-1}$ day^{-1}) is about a quarter of the reported exposure via the main transmission vectors poultry (raw and prepared; 0.0197 CFU $person^{-1}$ day^{-1}) or via raw milk (0.0167 CFU $person^{-1}$ day^{-1}) (Evers et al. 2004).

With the confirmed importance of raw vegetables and fruits in the transmission of *Campylobacter*, a better insight in the hazards of vegetables and fruits is needed. Therefore the monitoring data were used to determine if the risk of vegetables and fruits depends on the season or the type of packaging. Former studies showed a seasonal peak during the summer for the numbers of human campylobacteriosis cases (Nylen et al. 2002; Sopwith et al. 2003; Miller et al. 2004; Little and Gillespie 2008). However no seasonal pattern in *Campylobacter* prevalence on vegetables and fruits could be observed in our study. The two observed peaks were caused by samples taken at the same retail facility at the same day and might be the effect of cross-contamination. The absence of seasonal peaks in *Campylobacter* prevalence on vegetables and fruits might be explained by the extensive import of vegetables and fruits from over the world from countries with opposite seasons. However seasonal peaks in the numbers of human campylobacteriosis cases might still be caused by vegetables and fruits, not as result of peaks in prevalence but caused by differences in preparation and consumption patterns during the year, e.g. more raw products consumed during the summer.

A significantly higher *Campylobacter* prevalence was observed in packaged products compared to unpackaged products. Many of the packaged products were ready-to-eat vegetables and fruits which had undergone (extensive) processing (chopping, mixing) during which contamination might have occurred. A higher prevalence of contamination has been shown before by Velani and Roberts (1991) for *Listeria monocytogenes* in ready-to-eat mixed salads. Processing might also have resulted in more favourable conditions, such as enhanced water activity or the release of nutrients. Moreover effective washing and decontamination of ready-to-eat products are difficult. The majority of these products are washed in water. Washing in water removes sand, soil and other debris efficiently, but gives a reduction in microorganisms of 100-fold at best (Adams et al. 1989; Beuchat 1996; Little and Gillespie 2008). Chlorine added to the washing water results in a further reduction in microorganisms, but not in total elimination (Adams et al. 1989; Beuchat and Ryu 1997). Furthermore the packaging atmosphere and the low temperature at which most

packaged vegetables and fruits are kept might have positively influenced the survival of *Campylobacter*, as survival is prolonged at low oxygen conditions (Koidis and Doyle 1983; Stern et al. 1986; Verhoeff-Bakkenes et al. 2009) and at low temperatures (Hazeleger et al. 1995; Verhoeff-Bakkenes et al. 2009). The atmosphere effect on survival was expected to be even larger for MAP packaged products, as shown before by Phillips (1998). Indeed the prevalence calculated for MAP products in our study was high. However the confidence interval was very large for MAP packaged products as result of the low number of MAP samples (n=161) and more samples have to be taken to determine if MAP packaging creates a significant risk for *Campylobacter* infections or not.

To conclude, the calculated *Campylobacter* prevalence on vegetables and fruits of 0.23% in combination with reported high consumption levels, indicate that the consumption of raw vegetables and fruits is a risk factor for *Campylobacter* infections. Especially packaged vegetables, for which a prevalence of 0.36% was calculated, constitute a higher risk and as consumption of pre-packaged ready-to-eat vegetables is still increasing, the number of *Campylobacter* infections caused by the consumption of these products might further increase.

7

General discussion

Introduction

Despite several decades of research, *Campylobacter* is still the leading cause of food-borne bacterial gastroenteritis in the world. In this thesis data were generated in the field of hazard characterization and exposure assessment. The outcomes can be used to identify risk factors for *Campylobacter* and to give better education on the safety and handling of food, leading to a decrease in the cases of campylobacteriosis.

Growth

C. jejuni is generally characterized as an obligate micro-aerophile that grows best at a partial oxygen tension of 3-15% and is generally regarded as unable to grow aerobically. However, in Chapter 2 of this thesis it was shown that the presence of pyruvate (25 mM = 275 mg/100 ml) enabled continuous cultures of *C. jejuni* to grow fully aerobically in BHI (20 g l^{-1} without glucose), whereas the same cultures showed typically micro-aerobic behaviour and were unable to grow fully aerobically in this medium without pyruvate. Adding pyruvate lead to a decreased catalase activity which supports the suggestion of Giandomenico et al. (1997) that chemical elimination of hydrogen peroxide by pyruvate can compete effectively with protective enzymes such as catalase. Foods which contain levels of pyruvate are for instance apple juice (50mg/100ml), red apples (~450mg/100g), red wine (~20mg/100ml), cheddar cheese (~48.6mg/100g), yoghurt (~32mg/100g), beer (~6.6 mg/100g), tomatoes (~0.19mg/100g), and onions (~0.7mg/100g) (Izco et al. 2002; Megazyme 2007; Souci et al. 2008). In products with low pyruvate concentrations, pyruvate can be formed out of the amino acids alanine, cysteine, glycine, serine, threonine and tryptophan, or during glycolysis out of phosphoenolpyruvate, as pyruvate is a key intermediate in the network of metabolic pathways, common in living organisms. High levels of amino acids can be found in protein rich foods, such as fish, meat, eggs, dairy products, beans, peas, and nuts. Furthermore other antioxidants in foods, such as vitamins (A, C, and E) and dietary polyphenols (flavonoids, phenolic acids and tannins) which are naturally abundant in fruits and vegetables might also have a protective effect against atmospheric oxygen (Kim and Lee 2004; Mattila et al. 2011). However, instead of stimulating growth as antioxidants, many polyphenols also possess bactericidal activities (Rauha et al. 2000; Wen et al. 2003; Cushnie and Lamb 2005; Boban et al. 2010) and will prevent growth of *Campylobacter*.

If pyruvate, its precursors or other non-bactericidal antioxidant levels in foods are sufficient to allow growth of *C. jejuni* under aerobic conditions, still all other growth requirements like temperature, pH and water activity have to be fulfilled for growth. For example, apple juice, red apples and red wine contain pyruvate above or around the concentration of 25

mM which was shown to allow aerobic growth in our study. Nevertheless as result of the low pH of apple juice, apples (3-4) and red wine (3.3-3.5) *Campylobacter* cannot grow in these products. Red wine was even described for its high bactericidal effects against *Campylobacter* as result of its low pH, in combination with the presence of polyphenol compounds, high alcohol content, and high organic acid content (Carneiro et al. 2008; Birk and Knøchel 2009; Ganan et al. 2009; Isohanni et al. 2010).

As *Campylobacter* is regarded as thermophilic, storage temperatures below 30ºC, such as refrigerator temperature or room temperature, also prevent *Campylobacter* to grow. However, Mattila and Frost (1988) described a 1 to 2 log increase, possibly through interaction with other species, on meat at 25ºC. Furthermore, Lee et al. (1998) also reported a 1 to 2 log increase of a clinically derived strain at 25ºC after inoculation on chicken skin packaged respectively under various packaging atmospheres. However these results were never confirmed and results published by other researchers indicate that populations of *C. jejuni* decline but can survive for different periods of time at these temperatures in different environments, which was also shown for storage at 4, 12 and 25ºC under different atmospheres in different media in Chapter 3 of this thesis.

Survival

C. jejuni survives for long periods in a broad range of environments. In Chapter 3 the effect of temperature, medium and atmosphere on the survival were examined. Temperature was the most influencing factor for survival. *C. jejuni* was shown to survive for over 40 days at 4ºC in BHI and PBS. Higher temperatures lead to shorter survival times; at respectively 12ºC and 25ºC *C. jejuni* was shown to survive for 16 and 12 days. This can be explained by the fact that lower temperatures result in a lower metabolism, leading to preservation of energy, delayed exhaustion of substrates, and reduced formation of toxic reactive oxygen intermediates leading to less oxidative cell damage (Hazeleger et al. 1995). The temperature of 4ºC is central in the preparation, storage and distribution of perishable foods that may be contaminated with *C. jejuni*. In practice this means that if foods are contaminated with *C. jejuni* during production or processing and thereafter stored in the refrigerator, the pathogen will probably remain present on the product until the moment of preparation by the consumer at numbers high enough to cause infection.

Survival was longest in PBS when compared to BHI, which is in accordance with literature describing that nutrient limitation increases the survival (Boucher et al. 1994; Hazeleger et al. 1995; Thomas et al. 1999). However these studies, including our study, were conducted in sterile liquid laboratory media and one may argue if the results will be the same for (solid) real foods. *C. jejuni* can survive for more than one week in milk and meat products

at 4ºC (Svedhem et al. 1981; Blankenship and Craven 1982; Doyle and Roman, 1982b; Hänninen et al. 1984; Curtis et al. 1995; Chynoweth et al. 1998).
Next to temperature and medium, in Chapter 3 the atmosphere was shown to slightly influence the survival. At 12ºC, under anaerobic conditions cells remained culturable for 16 days, while under aerobic conditions culturability was no longer measured after 12 days.
In many packaged products, the combination of low storage temperature and lower amounts of oxygen enables prolonged survival of *Campylobacter*. Indeed, in Chapter 6, a significantly higher *Campylobacter* prevalence was observed in packaged vegetable and fruit products (0.36%; Cl: 0.17-0.66) compared to unpackaged vegetable and fruit products (0.07%; Cl: 0.01-0.27). Many of the packaged products were ready-to-eat vegetables and fruits, which are processed and thereafter stored at refrigerator temperature. Next to the storage temperature and atmosphere in these products, processing might also influence the number of *Campylobacter*.

Viable nonculturable (VNC) cells

In literature there is a continuing discussion on the existence and importance of VNC *C. jejuni* cells. If VNC cells can still be virulent, culturability as a measure for the infection risk will result in large underestimations of the risk in case of large amounts of nonculturable cells being present. In Chapter 3 a linear relationship between the number of culturable cells and the capability to adhere to and to invade in Caco-2 cells was shown, which suggests that VNC cells are of no importance. During survival culturable cells will degrade to nonculturable, possibly viable, cells, but no effect of these cells was measured in this study. To further elucidate the role of VNC cells, in Chapter 4 of this thesis a new approach was used to study if VNC *C. jejuni* cells can cause infection in human cells. In former studies in which nonculturable *C. jejuni* were found to be infective, the absolute absence of culturable cells was often disputed. To avoid the misleading effect of few culturable cells, instead of only nonculturable cells a combination of nonculturable and culturable cell suspensions was used in our study. The suspensions were added at different doses and the resulting adhesion and invasion, and *C. jejuni* stimulated IL-8 secretion were measured. No significant differences in adhesion and invasion and no effect on the *C. jejuni* stimulated IL-8 secretion of culturable cell suspensions was observed when nonculturable cells were added. These findings once more indicate that nonculturable cells do not adhere or invade Int-407 cells and do not induce an immune response, and therefore are probably not a risk for *Campylobacter* infection. This conclusion stated in Chapter 4 in combination with the linear relationship between the number of culturable cells and the infectivity as shown in Chapter 3, leads us to the conclusion that VNC *C. jejuni* cells are not a plausible risk for campylobacteriosis, assuming that the INT-407 and Caco-2 models are comparable

to the effect *in vivo*. This indicates that the number of culturable cells in a product is a good measure for the infection risk.

Food handling

Although thermotolerant in nature, *Campylobacter* is readily inactivated by heat and does not survive pasteurization treatments or typical cooking procedures. Pasteurized food products and foods which are cooked properly are regarded to be not at risk for *Campylobacter* infection. However, communication on proper cooking procedures remains important as undercooked chicken has been shown as risk factor for *Campylobacter* in several case-control studies (Corry and Atabay 2001; Padungton and Kaneen 2003; Butzler 2004). On the opposite, preparation of heavily contaminated foods, can lead to the widespread dissemination of the pathogen around the food preparation area. Via direct or indirect contact, these foods can cross-contaminate cooked foods, ready-to-eat foods or other foods which will not be heated before consumption, which can lead to campylobacteriosis. Cross-contamination during food preparation is determined as major risk factor for campylobacteriosis at home (Kapperud et al. 2003; Anonymous 2005; Mylius et al. 2007). To prevent cross-contamination proper hygiene measures and cleaning of kitchen equipment and tools are essential, however high percentages of improper consumer behaviour enabling cross-contamination are reported (Klontz et al. 1995; Altekruse et al. 1996; Worsfold and Griffith 1997; Yang et al. 1998; Jay et al. 1999a, b; Angelillo et al. 2001; Li-Cohen and Bruhn 2002; Mitakakis et al. 2004). In Chapter 5, the quantitative overall effect of *Campylobacter jejuni* cross-contamination, either via cutlery, cutting board, or hands, was tested during preparation of a chicken fruit salad, using artificially contaminated chicken. It was shown that when cross-contamination was allowed, the mean transfer to the chicken salad was around 0.12% of the initial number of *Campylobacter* on the chicken fillet, which is in the same range as transfer rates found in literature (Zhao et al. 1998; Chen et al. 2001; Montville et al. 2001; Kusumaningrum et al. 2004; Luber et al. 2006). The transfer rates for the tested cross-contamination routes, cutlery, cutting board, or hands, were not significantly different from one and other. Replacing cutlery and cutting board after handling raw chicken, the prevention of hand contact, and good hand washing are evenly important hygiene measures, and if not properly executed they lead to an increased risk on *Campylobacter* infection.

Raw vegetables and fruit

Contamination of raw vegetables and fruits can appear along the food chain. Once contaminated, key determinant for the number of *Campylobacter* is survival, as most crops are cultivated at temperatures beneath 30ºC and have pH values beneath 4.9.

Artificially inoculated *C. jejuni* (10^8-10^9 CFU per leaf or soil) did survive and could be cultured from radish and spinach plants for at least 3 weeks at temperatures of 10 ºC and 16ºC, a temperature at which many cool-season crops are grown (Brandl et al. 2004). For crops which are grown at higher temperatures the survival is probably reduced, based on the findings in Chapter 3. After harvesting, most vegetables and fruits are kept at low temperature to prolong the shelf life, however this also prolongs the survival of *C. jejuni* as shown in this thesis. Especially in produce which is kept at chill temperature (around 4ºC) the probability is high that *C. jejuni* will survive until the moment of consumption. Furthermore, many of the chilled vegetable and fruit products which are kept at chill temperature at retail are pre-packaged (in vacuum or MAP). Low oxygen conditions in packaged products will even enlarge the survival of *C. jejuni* as shown in this thesis.
The finding in this thesis that pyruvate enabled *C. jejuni* to grow fully aerobically could have implications for the growth in certain vegetables and fruits with elevated levels of pyruvate, like red apples, or might prolong survival in these produce.
In this thesis *Campylobacter* prevalence (*P*) of 0.23% was estimated for raw vegetables and fruits at retail in the Netherlands. This prevalence fits the large confidence intervals of studies reported in literature, in which prevalences where reported from 0 to 22.2%, with a mean prevalence for all studies together of 0.42% as shown in Chapter 6.
No data on the *Campylobacter* concentration in fresh produce are available. Most studies tested only the presence of *Campylobacter* in 25 g product. Positive products will contain at least 1 CFU/25g, i.e. 0.04 CFU g^{-1}. This minimal *Campylobacter* concentration in contaminated products was used as concentration (*c)* for calculations.
Raw vegetables and fruits are consumed in large quantities. The Dutch food consumption survey 2003 reported a mean daily consumption of 24.26 g raw vegetables and 27.29 g fruits consumed with skin per person, giving a mean daily consumption (*m*) of 51.55 g raw vegetables and fruits consumed with skin (Hulshof et al. 2004).
The mean daily dose $dd_{raw\ vegetables\ and\ fruit}$, the mean number of *Campylobacter* ingested per person per day by transmission via vegetables at the Dutch population level, was calculated by multiplying the prevalence of contaminated products in the Netherlands (*P*); the mean daily consumption (*m*) of raw vegetables and fruits in gram per person per day in the Netherlands; and the *Campylobacter* concentration in CFU g^{-1} contaminated product (*c*) (Table 7.1), giving an exposure of 0.0048 CFU per person per day (= 1.7 CFU $person^{-1}$ $year^{-1}$).
The most frequently used dose-response relationship for *Campylobacter* is the Beta-Poisson model (Nauta et al. 2005) based on the data of a volunteer study of Black et al. (1988). The number of cases of infection per year for the whole Dutch population ($16.1x10^6$ individuals) $P_{Netherlands,\ year}$ estimated, based on the Beta-Poisson model, with two parameters: $\alpha = 0.145$, $\beta = 7.589$ (Teunis and Havelaar 2000) gives:

$P_{Netherlands,\ year} = [1 - (1 + (m * c) / \beta)^{-\alpha}] * P * 365 * 16.1x10^6$
$= [1- (1+(51.55 * 0.04)/7.589)^{-0.145}] *0.23\%*365*16.1x10^6$
$= 4.6x10^5$ infections per year in the Netherlands

Based on the study of Black et al. (1988) in which 29 people got ill out of 89 individuals that were infected, it is estimated that the number of cases of disease is about equal to 1/3 of the number of cases of infection (Havelaar et al. 2000; Nauta et al. 2005), i.e. $1.5x10^5$ cases of disease per year in the Netherlands as result of the consumption of raw vegetables and fruits.
Another risk during consumption of raw vegetables and fruits is cross-contamination via *Campylobacter* contaminated food products, such as chicken, which will be discussed in the following section on chicken meat.

Chicken meat

Data on the prevalence and concentration in chicken meat at retail is shown in Table 1.5. In the Netherlands in 2006 the prevalence was 15.4% for *Campylobacter* in fresh chicken products at retail (VWA 2009). Levels of contamination varied between 10^2 to 10^5 CFU per carcass (Dufrenne et al. 2001) and based on these data Evers et al. (2004) calculated the average contamination of chicken meat in the Netherlands at 1.75 CFU g^{-1} chicken meat.
Chicken meat is kept at chill temperatures and *Campylobacter* is no longer able to grow, as result of its minimum growth temperature of 30ºC. However, during normal, chilled storage of chicken meat *Campylobacter* will survive for more than three weeks, although a 1 to 2 log reduction in numbers might occur (Svedhem et al. 1981; Blankenship and Craven 1982; Curtis et al. 1995; Zhao et al. 2003). Frozen storage reduces the survival of *Campylobacter* on chicken meat, but *Campylobacter* cells were detected even after weeks of frozen storage (Simmons and Gibbs 1979; Solow et al. 2003; Bhaduri and Cottrell 2004; Sandberg et al. 2005; Ritz et al. 2007).
Most chicken meat is heated during preparation which will inactivate *Campylobacter*, however chicken livers are in half of the cases consumed (partly) raw (Evers et al. 2004). The Dutch food consumption survey reported a mean daily consumption of 0.06 g (partly) raw chicken liver, 7.41 g fillet (heated) and 9.60 g other chicken meat (heated) per person (Evers et al. 2004). The mean exposure by consumption of (partly) raw chicken livers $dd_{raw\ chicken}$ was calculated, with a prevalence (P) of 15.4%, a consumption (m) of 0.03 g per person per day and a *Campylobacter* concentration (c) of 1.75 CFU g^{-1}, giving 0.0081 CFU $person^{-1}$ day^{-1} (= 3.0 CFU $person^{-1}$ $year^{-1}$). The mean number of cases of infection per year for the whole Dutch population based on this exposure is estimated at $9.0x10^5$ infections per

year in the Netherlands, resulting in approximately 3.0 $x10^5$ cases of disease per year in the Netherlands.

Evers et al. (2004) also calculated the exposure as result of the consumption of raw chicken meat with the same parameter values for the consumption and the *Campylobacter* concentration, however a prevalence of 33% was used (Van der Zee et al. 2002), giving a twice as high exposure of 0.0173 CFU $person^{-1}$ day^{-1}.

Next to the exposure via raw chicken meat, there is the exposure as result of cross-contamination during preparation. In this thesis the importance of hand washing, replacing cutlery and cutting board after handling raw chicken and the prevention of hand contact were shown to be very important hygiene measures, resulting in not-contaminated end products. However, consumer surveys show that at least 34% of the consumers do not wash their hands properly and do not clean their kitchen utensils after handling raw chicken, and in reality this might be far worse. If one of these measures was not complied with, a transfer of around 0.12% of the initial number of *Campylobacter* on the chicken fillet to the end product (chicken salad) was shown in Chapter 5. Given these data, the mean exposure as result of cross-contamination during preparation $dd_{chicken\ by\ cross\ contamination}$ was calculated by multiplying the prevalence of contaminated products in the Netherlands (*P*); the mean daily consumption of chicken fillet and other chicken (heated) in gram per person per day in the Netherlands (*m*); the *Campylobacter* concentration in CFU g^{-1} contaminated product (*c*); the transfer rate from chicken to other end products; the percentage of people not behaving properly (Table 7.1), giving 0.0019 CFU $person^{-1}$ day^{-1} (= 0.68 CFU $person^{-1}$ $year^{-1}$). The mean number of cases of infection per year for the whole Dutch population based on this exposure is estimated at $7.6x10^4$ infections per year in the Netherlands, resulting in approximately 2.5 $x10^4$ cases of disease per year in the Netherlands.

This calculated exposure of 0.0019 CFU $person^{-1}$ day^{-1} in the Netherlands is consistent with the estimated exposure of 0.0023 CFU $person^{-1}$ day^{-1} in the Netherlands as calculated by Evers et al. 2004, who estimated the fraction of *Campylobacter* transferred by cross-contamination, based on data of two models described in a preliminary FAO/WHO report (Hartnett et al. 2001). Another study, based on a much more detailed risk assessment model for the transmission of *Campylobacter* from broiler meat, from entering the processing plant until consumption of a chicken breast meal, estimated the incidence as a consequence of the consumption of salad cross-contaminated after handling chicken breast fillet, at $1.23x10^4$ cases of campylobacteriosis per year in the Netherlands (Nauta et al. 2007), which is also in the same range as the incidence in this thesis.

Raw milk

Worldwide, the prevalence of *Campylobacter* in raw milk varies from 0 to 10.2 percent as shown in Table 1.6. In the Netherlands in 1988 the prevalence of *Campylobacter* in raw milk was 4.5% (Beumer et al. 1988); more recent data are not available. The concentration of *Campylobacter* in raw milk is generally considered to be low. The only report on the *Campylobacter* concentration in raw milk showed an MPN of 16 CFU/100 ml raw milk (Humphrey and Beckett 1987).

The milk will be cooled and stored chilled, which disables *Campylobacter* to grow, and moreover the antibacterial lactoperoxidase system in raw milk will result in a decrease in viable numbers (Beumer et al. 1985). However, *C. jejuni* was detected in artificially contaminated raw milk at 4ºC for over 6 days (Doyle and Roman 1982b).

Raw milk undergoes no reduction or elimination process and the risk on cross-contamination is small. Raw milk is consumed by a small part of the population. The Dutch food consumption survey 2003 reported a mean daily consumption of 2.32 g raw milk per person (Hulshof et al. 2004). The mean exposure via raw milk was calculated, based on a prevalence of 4.5%, a consumption of 2.32 g person^{-1} day^{-1} and a concentration of 0.16 CFU g^{-1} (Evers et al. 2004), giving 0.0167 CFU person^{-1} day^{-1} (= 6.1 CFU person^{-1} year^{-1}). The mean number of cases of infection per year for the whole Dutch population based on this exposure is estimated at 1.8x10^{6} infections per year in the Netherlands, resulting in approximately 6.1 x10^{5} cases of disease per year in the Netherlands.

Implications for public health

To quantify the risk on campylobacteriosis as result of the consumption of different foods (vegetables and fruits, (partly) raw chicken, chicken by cross-contamination and raw milk), the exposure and number of illnesses was estimated with a concise quantitative microbiological risk assessment (QMRA) model. In Table 7.1 the data and calculations from this thesis on vegetables and fruits, raw chicken, chicken by cross-contamination and raw milk have been summarized.

The exposure is highest via raw milk (0.0167 CFU person^{-1} day^{-1}) and chicken (0.0100 CFU person^{-1} day^{-1}; (partly) raw and by cross-contamination), as result of the high prevalence and concentration of these products. In contrast, the prevalence and concentration in vegetables and fruits are low, but as result of the very high consumption the exposure via raw vegetables and fruits (0.0048 CFU person^{-1} day^{-1}) is still about half the reported exposure via chicken.

Table 7.1. Prevalence, consumption, *Campylobacter* concentration, estimated *Campylobacter* exposure, and estimated number of cases of campylobacteriosis given for different foods

	Vegetables and fruits	(Partly) raw chicken	Chicken by cross-contamination	Raw milk
Prevalence[1]	0.23	15.4	15.4	4.5
Consumption[2]	51.55	0.03	Fillet: 7.41 Other: 9.60	2.32
Campylobacter concentration[3]	0.04	1.75	1.75	0.16
Transfer by cross-contamination[4]	-	-	0.12	-
People not behaving properly[5]	-	-	34	-
Estimated mean daily dose *dd*[6]	0.0048	0.0081	0.0019	0.017
Cases of infection $P_{Netherlands,year}$[7]	4.6×10^5	9.0×10^5	7.6×10^4	1.8×10^6
Cases of illness $N_{Netherlands,year}$[8]	1.5×10^5	3.0×10^5	2.5×10^4	6.1×10^5

[1] in %
[2] in g person^{-1} day^{-1}
[3] in CFU g^{-1}
[4] in %
[5] in %
[6] in CFU person^{-1} day^{-1}
[7] Number of cases of infection per year for the whole Dutch population
[8] Number of cases of illness per year for the whole Dutch population

Chicken is generally regarded as main transmission vector and based on limited Dutch data and extrapolation of international data, poultry was estimated to be responsible for 20-40% of all human cases of campylobacteriosis in the Netherlands (Havelaar 2002; Mangen et al. 2007; Vellinga and van Loock, 2002). Based on our data, next to chicken, also raw milk and raw vegetables and fruits can be seen as important transmission routes. However, the calculations in the QMRA model are a simplified representation of reality based on limited data, various assumptions and may lack aspects that might be relevant in reality. For instance, when regarding raw milk and (partly) raw chicken, in fact only a small subpopulation is highly exposed, resulting in the highest mean exposure for the whole Dutch population. This might be an overestimation, as immunity and clustering of exposure are both not incorporated in the model. Knowledge on immunity is scarce, and should be further elucidated. The effect of immunity will especially be of importance for people who are often exposed to *Campylobacter*, such as people who regularly consume high risk foods like raw milk or (partly) raw chicken, or abattoir-workers.

Also other important parameters are estimated with uncertainty, but are of great influence on the outcome. For the exposure model, data on prevalence and especially concentration in food are scarce, and to improve the outcomes of the model more detailed data are required. Detailed information on food consumption and preparation practices is essential as input in the model, in combination with quantitative information on pathogen behaviour under different environmental conditions in the food chain. Current food consumption surveys do provide very useful information on meal composition, but lack data of storage conditions, and preparation practices. Especially knowledge on preparation steps which influence the number of microorganisms, like heating of foods, peeling of fruits, or adding ingredients like salt or spices, and their effect on the number of *Campylobacter* is needed. Furthermore more knowledge on the quantitative occurrence of consumer handling practices which might result in cross-contamination is necessary (Havelaar et al. 2008).

The last important step in the model is the dose-response model for translating the exposure data into a risk on infection and illness. Data on dose-response processes is scarce. The used dose-response relation is based on a study on the effect of high doses of two laboratory adapted *Campylobacter* strains in a small group of healthy human volunteers (Black et al. 1988; Teunis and Havelaar 2000). Differences in the pathogenicity of *Campylobacter* strains are not incorporated in this model, nor the effect on different hosts or different food-matrices, which might lead to overestimation. Furthermore, data are lacking on the response at low-doses and as a consequence the used Beta-Poisson model might lead to gross overestimation of the risk at low doses (Teunis and Havelaar 2000). As low doses do occur at consumer level, a better insight in the effects of low doses would be desirable.

The concise QMRA model used to estimate the *Campylobacter* exposure can be used to compare the relative importance of these foods as shown above and it should be realized that the qualitative outcomes are more relevant than the precise numbers resulting from the quantitative analyses. Precise estimates of human cases cannot be given by QMRA, due to numerous sources of uncertainty along the food pathway and in the dose-response modelling (Havelaar et al. 2008; Mangen 2010, Nauta et al. 2005). However, it is tempting to compare the quantitative QMRA data with epidemiological estimates. Based on the calculations, vegetables and fruits, (partly) raw chicken, chicken by cross-contamination and raw milk together are estimated to be responsible for 1.1 $x10^6$ cases of campylobacteriosis per year in the Netherlands. This calculated total number of cases is much higher than the total average of approximately 8 $x10^4$ campylobacteriosis cases per year for the Netherlands, with a 90% Cl of 3 $x10^4$ and 1.6 $x10^5$ (Mangen et al. 2007; Havelaar et al. 2007) as estimated based on the SENSOR study, a Dutch prospective community-cohort study combined with a GP-cohort study that was conducted in 1999 (De Wit et al. 2001b, c). In general, the QMRA model estimates are much higher than expected

based on epidemiological estimates (Havelaar et al. 2008), but the uncertainty of both calculations is high. The QMRA model might give an overestimation as described above. Otherwise the epidemiological study might give an underestimation as it focused on acute gastroenteritis with loose stools or significant vomiting, and asymptomatic human cases were not considered, which might result in an underestimation (De Wit et al. 2001b). Sample storage and culturing in medical microbiology may also lead to an underestimation of the real incidence of all symptomatic cases (Nauta et al., 2005). Selection bias of the study group might also influence the outcomes; people who frequently suffer from gastroenteritis may be more interested in the topic and therefore be more likely to participate which might result in overestimation; whereas persons born abroad, which tended to have a higher incidence, were underrepresented in the Sensor study probably as result of language problems and cultural barriers which might also result in underestimation (De Wit et al. 2001b).

In conclusion

Despite its strict growth requirements and sensitivity to environmental stress, *Campylobacter* is the major cause of bacterial gastroenteritis in the world. The growth conditions might be less strict than generally assumed in literature. *C. jejuni* was shown to grow aerobically in the presence of pyruvate, and growth might also be possible in the presence of other antioxidants. However, as result of the strict minimal growth temperature (30ºC), *C. jejuni* is still assumed to be generally unable to grow in foods and therefore growth is not considered as a large risk for campylobacteriosis.

Notwithstanding the strict growth conditions, *C. jejuni* is able to survive for long periods of time in various environments. Temperature is the most influencing factor in survival and survival will be better at low temperatures (around 4ºC). As many foods are stored chilled, this will prolong the survival of *C. jejuni*. Furthermore survival is better at low oxygen conditions, as often present in packaged foods.

Culturability and infectivity are linearly related, and nonculturable cells were shown not to be infective (in cell cultures). Therefore, the absence of culturable *C. jejuni* cells is an indication that a product is representing a very low risk with respect to campylobacteriosis.

Warm-blooded animals can be highly contaminated with *Campylobacter* and this results in a wide spread in the environment, and food products of animal origin, like chicken meat and raw milk. But also products of non-animal origin, like vegetables and fruits can be contaminated. Once contaminated the risk is high that *Campylobacter* will survive on these food products until the moment of consumption, except if the product undergoes an elimination step during preparation, such as heating. Therefore, foods consumed rawly constitute a higher risk on *Campylobacter*, than foods which are heated before preparation.

However foods which are heated, such as chicken meat, can be highly contaminated with *Campylobacter* which creates a risk on cross-contamination during preparation. The high percentage of consumers who exhibit improper hygienic and cleaning behaviour, and the significant transfer of *Campylobacter* during improper food preparation, indicate cross-contamination as a high risk factor for *Campylobacter* infection.
Regarding specific foods, the prevalence and concentration are low on raw vegetables and fruit, but the quantity of raw vegetables and fruits consumed is very high. In contrast, raw chicken and raw milk show relative high prevalence and concentration levels, but the consumption is low. Despite the differences in parameters, the exposure calculated out of the combination of parameters indicates all these three foods as high risk factors for *Campylobacter*. So foods which are not heavily contaminated with *Campylobacter*, but are consumed in large quantities can constitute a similar high risk on *Campylobacter* infection as foods which are consumed in small quantities but are heavily contaminated with *Campylobacter.*

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Samenvatting

Campylobacter jejuni, een klein, gebogen of spiraalvormig beweeglijk micro-organisme, is wereldwijd geïdentificeerd als een van de belangrijkste bacteriële veroorzakers van gastro-enteritis. Infectie kan soms tot ernstige complicaties leiden zoals het Guillain-Barré syndroom en reactieve artritis. In het onderzoek omschreven in dit proefschrift zijn data gegenereerd op het terrein van gevarenkarakterisering en blootstellingschatting met als doel een beter inzicht te krijgen in de risicofactoren voor *Campylobacter*.

De invloed van omgevingsfactoren op groei, overleving en de vorming van niet-kweekbare cellen en het belang van deze niet-kweekbare cellen is onderzocht.

C. jejuni wordt gezien als een obligaat micro-aerofiel micro-organisme, dat alleen kan groeien onder micro-aerobe condities (3-15% O_2). In hoofdstuk 2 is echter beschreven dat in de aanwezigheid van pyruvaat volledig aerobe groei mogelijk is. Aerobe groei zou ook kunnen plaatsvinden in de aanwezigheid van andere antioxidanten. Echter eventuele groei in voedsel wordt sterk beperkt door de strikte minimum groeitemperatuur van 30ºC.

Het is bekend dat *C. jejuni* lange tijd kan overleven onder condities waar geen groei mogelijk is. In hoofdstuk 3 is de invloed van omgevingsfactoren (temperatuur, medium en atmosfeer) op de overleving getest. Temperatuur is de meest bepalende factor voor overleving. Overleving is beter bij lage temperatuur (rond 4ºC) en aangezien veel voedsel gekoeld wordt opgeslagen, zal dit de overleving in voedsel verlengen. Verder is de overleving beter bij verlaagde zuurstofspanning, zoals vaak aanwezig in voorverpakt voedsel. Deze bevindingen komen overeen met de literatuur. De kennis over de infectiviteit van de overlevende cellen is echter miniem. Daarom werd naast het effect van omgevingsfactoren op de kweekbaarheid, ook het effect op de infectiviteit onderzocht. Er is een lineair verband tussen de kweekbaarheid en infectiviteit aangetoond. Verder is in hoofdstuk 4 aangetoond dat het toevoegen van niet-kweekbare *C. jejuni* cellen aan kweekbare celsuspensies geen effect heeft op de infectiviteit (*in vitro*). De resultaten van deze twee studies leiden tot de conclusie dat de afwezigheid van kweekbare cellen in een product een goede indicatie is dat een product een zeer laag risico geeft op campylobacteriosis.

Voedsel kan besmet worden met *Campylobacter* gedurende de productie of naderhand bij de consument thuis. Eenmaal besmet is het risico hoog dat *Campylobacter* overleeft, behalve als het voedsel nog een bereidingsstap ondergaat waarbij *Campylobacter* wordt gedood, zoals verhitting.

Het effect van verschillende kruisbesmettingsroutes gedurende de bereiding van een kip-fruit-salade is bestudeerd in hoofdstuk 5. De gemiddelde overdracht door kruisbesmetting is 0.12% van het initiële aantal *C. jejuni* op kunstmatig besmette kipfilet. Er blijkt geen

belangrijkste besmettingsroute te zijn, de verschillend geteste routes, bestek, snijplank en handen, waren allemaal even belangrijk. De hoge prevalentie en concentratie van *C. jejuni* op kip, het hoge percentage consumenten dat onvoldoende hygiënisch handelt gedurende de voedselbereiding en de significante overdracht van *Campylobacter* gedurende dit onhygiënisch handelen, leidt tot een hoog risico op kruisbesmetting gedurende de bereiding van rauw hoog besmet voedsel, zoals kip, naar ander rauw te consumeren of bereid voedsel. Groenten en fruit kunnen worden besmet met *C. jejuni* gedurende teelt of verwerking. Vooral als groenten en fruit rauw en ongewassen geconsumeerd worden, zijn zij mogelijk een risico voor *Campylobacter*-infectie. In hoofdstuk 6 zijn Nederlandse data over het voorkomen van *Campylobacter* op groenten en fruit geanalyseerd. Dertien van de 5640 groenten- en fruitmonsters was *Campylobacter*-positief, overeenkomend met een prevalentie van 0.23% (95% betrouwbaarheidsinterval: 0.12-0.39%). De prevalentie in verpakte producten is significant hoger dan de prevalentie in onverpakte producten.
Tot slot zijn de nieuw verkregen data in combinatie met kwantitatieve literatuurdata over de prevalentie en concentratie van *Campylobacter* aan het begin van de consumentenfase samengevat voor drie voedselgroepen, groenten en fruit, kip, en rauwe melk. Met deze data als input in een kwantitatief risicoschattingsmodel, is de blootstelling en het resulterende aantal ziektegevallen berekend en onderling vergeleken om het relatieve belang van de verschillende risicofactoren te bepalen. De prevalentie en concentratie op groenten en fruit zijn laag, maar de hoeveelheid rauwe groenten en fruit die worden geconsumeerd is erg hoog. De prevalentie en concentratie op rauwe kip en in rauwe melk zijn hoog, maar de consumptiehoeveelheid is laag. De blootstelling berekend uit de combinatie van deze parameters is voor alle productgroepen hoog, wat al deze drie voedselgroepen aanmerkt als belangrijke risicofactoren voor *Campylobacter*. Dus laag besmet voedsel dat wordt geconsumeerd in hoge hoeveelheden, kan eenzelfde ricico op *Campylobacter* infectie geven als hoog besmet voedsel dat wordt geconsumeerd in kleine hoeveelheden.

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Curriculum vitae

Liesbeth Bakkenes werd op 1 december 1975 geboren te Ede. In 1994 behaalde zij het VWO diploma aan het Elzendaalcollege te Boxmeer. In datzelfde jaar startte zij haar studie Levensmiddelentechnologie aan de Wageningen Universiteit. Afstudeervakken werden afgelegd in twee richtingen: Levensmiddelenproceskunde, getiteld "De kwantificering van biofilms onder dynamische omstandigheden", en Levensmiddelenmicrobiologie, getiteld "De toxineproductie door *Staphylococcus aureus*". De studie werd afgerond met een stage bij TNO-Voeding, waarbij de microbiologische werking van pulsed electric field (PEF) en ultra hoge druk (UHD) conservering werd bestudeerd. In maart 2000 studeerde zij af.
Vanaf juli 2000 werkte zij aan haar promotieonderzoek bij het Microbiologisch Laboratorium voor Gezondheidsbescherming (MGB) van het Rijksinstituut voor Volksgezondheid en Milieu (RIVM) in samenwerking met de leerstoelgroep Levensmiddelenmicrobiologie van de Wageningen Universiteit. Ook zes maanden onderzoek in het Department of Biochemistry, Stellenbosch University, South Africa, maakten onderdeel uit van dit traject. De resultaten van dit promotieonderzoek staan beschreven in dit proefschrift.

List of completed training activities

Discipline specific activities

Courses

Physiology of food-associated microorganisms (2000), VLAG, Wageningen
Int. advanced course on food technology and food safety (2001), VLAG, Wageningen
Microbial physiology (2002), UVA/SILS, Amsterdam
Safety training in handling gas cylinders and equipment (2003), AFROX, Stellenbosch, SA

Meetings

SFAM Conference *Campylobacter*, *Helicobacter* and *Arcobacter* (2000), Glasgow, UK
PVE Conference day (2001), Lelystad
CHRO (2001), Freiburg, Germany (poster)
NVvM Conference day (2001, 2009, 2011), Vlaardingen (poster), Bilthoven Wageningen
Food Standard Agency Workshop on foodborne disease in the home (2002), London, UK
CARMA Conference day Campylobacteriosis in the Netherlands (2002, 2003), Bilthoven
SAFE Consortium Seminar Newly emerging pathogens (2003), Brussels, Belgium (poster)
EFFI/FMM Hygiene conference (2003), Ede

General courses

PhD week (2000), VLAG, Nijmegen
Procite course (2000), RIVM, Bilthoven
Mathematical modelling (2000), RIVM/VU, Bilthoven
Course competence profiles (2001), RIVM, Bilthoven
Scientific writing in English (2003), RIVM/Taalcentrum VU, Bilthoven
Course career perspectives (2005), WGS/Meijer&Meijaard BV, Wageningen

Optionals

Preparation of PhD research proposal (2000)
RIVM/MGB Research meetings (2000-2005)
Six months work-visit (2002-2003), Biochemistry Department, Stellenbosch University, Stellenbosch, South Africa

The research described in this thesis was conducted at the Microbiological Laboratory for Health Protection of the National Institute for Public Health and the Environment within the framework of project V251825, “Quantitative safety aspects of exposure to *Campylobacter jejuni*” by order and on the account of the Directory Board of the National Institute for Public Health and the Environment (RIVM).

Cover: Scanning electron microscopy of *Campylobacter jejuni*, photo by Marina Burger (RIVM)

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