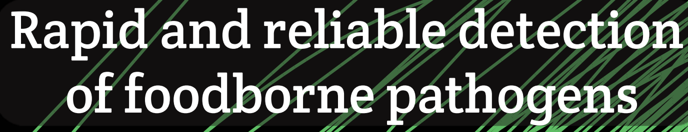

Rapid and reliable detection of foodborne pathogens

Exploring *Campylobacter* ecology in enrichments to improve detection

Maren I. Lanzl

Propositions

1. Uncertainty in *Campylobacter* detection outcomes is reduced by simplified protocols. (This thesis)

2. A combination of traditional and rapid microbiological methods results in more reliable detection of *Campylobacter* in foods. (This thesis)

3. Informal interdisciplinary knowledge exchange improves the quality of research.

4. Trust in science will increase if scientists adapt their communication style to their audience.

5. Lessons learned during the COVID-19 pandemic are forgotten quickly.

6. Balancing mental welfare and workload is only possible through a combination of awareness, open communication, and realistic expectations.

7. Toxic positivity on social media is a danger to emotional balance, especially of impressionable individuals.

Propositions belonging to the thesis, entitled

Rapid and reliable detection of foodborne pathogens – Exploring *Campylobacter* ecology in enrichments to improve detection

Maren I. Lanzl

Wageningen, 16 May 2023

Rapid and reliable detection of foodborne pathogens

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Maren Ina Lanzl

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1

General introduction and outline of the thesis

Within the genus of *Campylobacter*, some species are recognized as pathogens capable of causing human bacterial gastroenteritis called campylobacteriosis (Friedman et al., 2000). The two species most associated with disease are *Campylobacter jejuni* and *C. coli* (Kaakoush et al., 2015), which often live asymptomatically in the intestinal tract of birds and other farm animals (Newell et al., 2011; Revez et al., 2014). *Campylobacter* cells can disperse in the environment through shedding of faeces into environmental waters, fertilization of crops with manure and cross-contamination during slaughtering (Ahmed et al., 2013; Van Gerwe et al., 2010). As a result, the pathogenic cells can be found in a variety of food products, such as broiler meats, raw red meats, untreated water and milk, fruits and vegetables (Mohammadpour et al., 2018; Zbrun et al., 2020). *C. jejuni* and *C. coli* are the leading cause of foodborne gastroenteritis in the European Union (EFSA & ECDC, 2021), therefore it is important not only to test food products for the presence of *Campylobacter* but also to regularly monitor their prevalence to allow timely interventions to food-processing operations in case of rising *Campylobacter* concentrations in certain food products. Above that, pathogenic *Campylobacter* have a low dose-response relation (Teunis et al., 2018) and therefore, it is crucial to also be able to detect low amounts of *Campylobacter* in food products. A common approach is the use of an enrichment step to support the resuscitation of damaged cells and increase cell concentrations to the required levels needed for subsequent detection (Baylis et al., 2000; Park et al., 1983). For the detection of low levels of *Campylobacter* spp. from food products in the European Union (EU), the protocols described by the International Organization for Standardization (ISO) 10272-1:2017 are frequently applied. Although these protocols have a very low detection limit, their execution is laborious and confirmed results are only available after several days. Furthermore, the reliability of the selectivity of the enrichment media can currently not be assured due to the presence of competitive microorganisms (Hazeleger et al., 2016). Even though *Campylobacter* spp. have been studied extensively, relatively little is known concerning the behaviour of *Campylobacter* cells during enrichment. In the following sections, the *Campylobacter* physiology, their disease burden and prevalence in different food product groups are introduced as well as the current methods and challenges which arise regarding the detection of foodborne *Campylobacter*. Finally, an outline will be given of the research questions addressed in the different chapters, including a general discussion that addresses the

novel insights, remaining uncertainties and perspectives for future research when it comes to the detection of *Campylobacter* spp. from foods.

Campylobacter, the microorganism

The physician and microbiologist Theodor Escherich was first to observe the characteristic spiral-shaped cells while studying the colon of children suffering from enteric disease in 1886 (Escherich, 1886). Over almost the next century, several scientists recognized the, by then called, *Vibrio* and '*Vibrio* related' cells in human and veterinary medicine (King, 1957; Levy, 1946; Middelkamp and Wolf, 1961; White, 1967). In 1963, the organism was renamed as *Campylobacter* (meaning 'curved bacteria') by French scientists, Sebald and Véron (Sebald and Véron, 1963). Until the late 1960s, reports on *Campylobacter* cells were based exclusively on microscopic observations as cultural isolation of the cells proved to be difficult but in 1968, *Campylobacter* cells were for the first time successfully isolated from human faeces (Dekeyser et al., 1972).

Campylobacter species are Gram-negative, non-spore-forming bacteria with a single polar flagellum, bipolar flagella or no flagellum, depending on the species (Man, 2011; Vandamme, 2000). Healthy cells equipped with flagella are highly motile and move in a corkscrew-like motion. The cells are small and slender, approximately 0.5-5 μm long and 0.2-0.8 μm wide (Vandamme, 2000).

<u>Growth</u>

Generally, *Campylobacter* spp. can grow in a temperature range between 30-45 °C (Doyle and Roman, 1981; Hazeleger et al., 1998; Park et al., 1991) but the species most relevant for food safety belong to the group of thermotolerant campylobacters grow optimally at a temperature of 41.5 °C (Butzler and Skirrow, 1979; Forsythe, 2010). This narrow temperature range for growth is remarkable compared to other foodborne pathogens; some *Salmonella* strains have shown growth already at temperatures as low as 5 °C (D'Aoust, 1989) and growth of *Listeria monocytogenes* has been noted even at -1.5 °C (Lado and Yousef, 2007). The ability of pathogenic species to grow at low temperatures has been associated with the cellular up-regulation of cold shock proteins, which counteract some adverse effects of low temperatures, thereby aiding low temperature adaptation (Graumann and Marahiel, 1996; Hébraud and Potier,

1999; Phadtare, 2004). Thermotolerant campylobacters lack those cold shock proteins (Hazeleger et al., 1998; Park, 2002). And while the growth rate of other microorganisms gradually decreases near the minimal growth temperature, studies have shown that the growth rate of *Campylobacter* decreases from the maximum to zero very abruptly when the minimum and maximum growth temperatures are encountered (Hazeleger et al., 1998; Park, 2002).

Campylobacter spp. is considered a strictly microaerophilic bacterium, meaning that, on the one hand, it needs oxygen for growth to use as a terminal electron acceptor, but on the other hand, cannot grow under fully aerobic conditions (Krieg and Hoffman, 1986). It requires an oxygen concentration of 3-15% for growth as well as elevated levels of carbon dioxide and grows best in an atmosphere of 5% O_2, 10% CO_2 and 85% N_2 (Altekruse et al., 1999; Vandamme, 2000). Studies on *C. jejuni* strain 11168 showed that *C. jejuni* possesses two terminal oxidases, a *cb*-type cytochrome c oxidase and a *bd*-like quinol oxidase (Parkhill et al., 2000). Interestingly, the study also revealed the presence of alternative electron acceptors instead of oxygen, including fumarate, nitrate, nitrite, and N- or S-oxides (Kelly, 2001; Parkhill et al., 2000) which were initially thought to allow respiration and thus, growth in anaerobic conditions (Mendz et al., 1997). However, Sellars et al. (2002) proved that *C. jejuni* was unable to grow under strictly anaerobic conditions in the presence of any of the alternative electron acceptors. The same study also showed that these alternative respiratory pathways did contribute to growth under microaerobic conditions when the medium was supplemented with these alternative electron acceptors.

In order to maintain homeostasis and proliferation capacity, bacterial cells acquire different nutrients from their environment. Most other microorganisms primarily utilize glucose and a variety of other carbohydrates for energy production, but studies have shown that most thermotolerant campylobacters are unable to metabolize carbohydrates due to a lack of necessary transporters and enzymes (Gripp et al., 2011; Kelly et al., 2001; Line et al., 2010; Stahl et al., 2011; Velayudhan and Kelly, 2002). Instead, campylobacters make use of the citrate (TCA) cycle and depend heavily on its intermediates (Hofreuter, 2014; Stahl et al., 2012), some organic acids (Gripp et al., 2011; Guccione et al., 2008; Wright et al., 2009) free amino acids and peptides (Gao et

al., 2017; Guccione et al., 2008; Gundogdu et al., 2016; Hofreuter et al., 2008, 2006; Wright et al., 2009).

Species and subspecies

The genus *Campylobacter* is currently classified into 57 species and 16 subspecies ("lpsn.dsmz.de," 1997). Most of the strains were initially isolated either from humans displaying disease or from the food chain and agricultural environment (Sheppard and Maiden, 2015). However, studies have shown that wild animals are also a recurrent source of *Campylobacter* and that novel genotypes are frequently found and analysed especially in wild birds (Carter et al., 2009; French et al., 2009). At least 19 of the 57 species have been associated with infections in humans and/or animals in the past (Costa and Iraola, 2019). Recently, so called 'emerging species' such as *C. upsaliensis*, *C. lari*, *C. concisus* and *C. ureolyticus* are getting more attention as they are likely to contribute negatively to human health as well (Man, 2011; Tam et al., 2012). However, most reported *Campylobacter* infections in humans are caused by the thermotolerant *C. jejuni* and *C. coli* (Costa and Iraola, 2019; Facciolà et al., 2017; Man, 2011), they are therefore considered the two most important species within the *Campylobacter* genus. This is supported by the findings in a recent study by the European Food Safety Authority (EFSA) and the European Centre for Disease Prevention and Control (ECDC) which reported that 88.1% and 10.6% of all confirmed reported cases of *Campylobacter* infection in the EU were caused by *C. jejuni* and *C. coli*, respectively, while the remaining 1.3% of cases were caused by *C. fetus* (0.16%), *C. upsaliensis* (0.11%), *C. lari* (0.09%) and other species (0.94%) (EFSA & ECDC, 2021). Next to classification by species, campylobacters are also genetically differentiated by multilocus sequence typing (MLST) (Dingle et al., 2001). MLST makes use of genetic variation in seven housekeeping genes (*spaA, atpA, glnA, gltA, glyA, pgm* and *tkt*) (Miller et al., 2005). Based on sequence information of each housekeeping gene, allele numbers are assigned and each unique allelic profile (the combination of seven allele numbers) is assigned a sequence type (ST). As there are hundreds of different STs, closely related STs, sharing at least five out of seven allele numbers with another ST, can be clustered into clonal complexes (CCs) (Wieczorek et al., 2020). This way, molecular relationships between isolates can be established, which can be used to attribute isolates from human disease to potential sources (Colles and Maiden, 2012). The most common CCs associated with human disease are ST-21, ST-45 and ST-828

(Dearlove et al., 2016) but also the CCs ST-257 and ST-48 contained a noticeable number of isolates associated with human disease as well as animals (Colles and Maiden, 2012).

The impact of *Campylobacter* on human health

Campylobacteriosis

Infection with *C. jejuni* and *C. coli* usually leads to campylobacteriosis and is the most reported foodborne gastroenteric infection in humans in the EU and has been so since 2005 (as depicted in **figure 1**) (EFSA & ECDC, 2021).

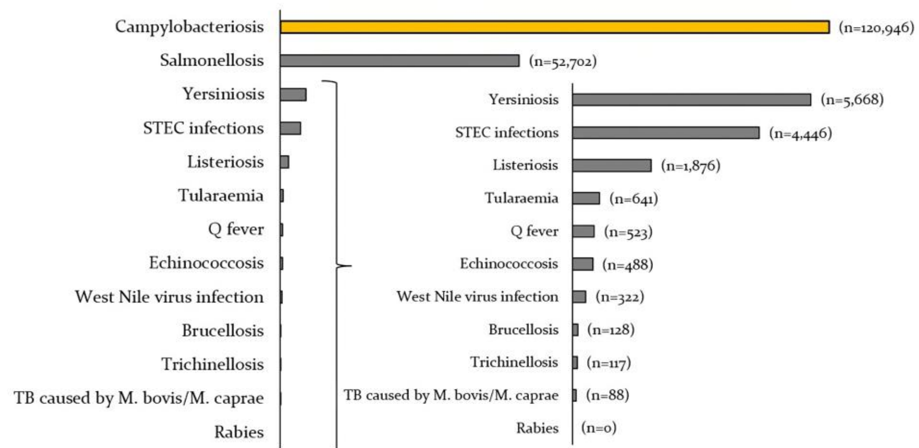

Figure 1: Reported number of cases of confirmed human zoonoses in the EU, 2020 (modified from EFSA & ECDC, 2022).

After an incubation period of 2-5 days (World Health Organization, 2020) gastrointestinal disease consisting of diarrhoea (ranging from mild to severe and even bloody), abdominal pain, nausea, fever, headache and vomiting sets in (Butzler, 2004; Butzler and Skirrow, 1979; Man, 2011). Generally, symptoms persist for 3-7 days, however, the severity of the symptoms varies from complete absence to severe, long-lasting illness. The extremity of the symptom manifestation is dependent on several factors such as the pathogenic potential of the strain and the number of cells ingested (dose) (Hara-Kudo and Takatori, 2011; Medema et al., 1996; Robinson, 1981a). Several studies assessed the pathogenic potential of different *C. jejuni* isolates by determining

the presence of different virulence factors and found that strains differed in their capacity to harbour the different virulence genes, which could influence their pathogenic potential (Abd El-Hamid et al., 2019; Frazão et al., 2017; Rawat et al., 2018). In 1988, Black et al. conducted a trial, in which they administered adult volunteers with food artificially contaminated with different concentrations of two *C. jejuni* strains to establish a dose-response relation for *Campylobacter* infections from food (Black et al., 1988). They found not only that one of the strains was more likely to cause illness and that illnesses were more severe, but they also concluded that already 800 cells were sufficient to cause campylobacteriosis (Black et al., 1988). Later, mathematical models and microbiological risk assessments were used to estimate a dose-response relation. Medema et al. (1996) suggested that the ingested dose resulting in illness was $9 \cdot 10^4$ cells but also mentioned that the risk differed between *C. jejuni* isolates. It is well established that *C. jejuni* is responsible for most foodborne *Campylobacter* infections, but *C. coli* has been recognized to be responsible for a fourth of all campylobacteriosis cases (Gürtler et al., 2005; Inglis et al., 2011) and symptoms are similar to those induced by *C. jejuni* (Kaakoush et al., 2015). It is important to mention that, although it has been suggested that the infectious dose of *C. jejuni* is much lower than other foodborne pathogens (Kothary and Babu, 2001), no clear dose-response relation can be established as it is not only dependent on the pathogenicity of the strain but on other factors like the health state and demographics of the individuals ingesting the *Campylobacter* cells. Although everyone can be infected by *Campylobacter*, infections are predominantly common in certain age groups; in industrialized countries, the incidence rate is generally higher in young children until the age of four (Butzler, 2004; Levesque et al., 2013; Tenkate and Stafford, 2001), young adults between the age of 15-24 (Ang et al., 2011; Nielsen et al., 2013) and the elderly population (>80 years) (Levesque et al., 2013; Nielsen et al., 2010). Interestingly, independent of age, males appear to have a higher risk of infection compared to females (Green et al., 2020; Levesque et al., 2013; Moffatt et al., 2017). Green et al. (2020) hypothesized that this might be due to physiological or genetic differences rather than behavioural factors. As is true for several pathogenic microorganisms, pregnant women and immunocompromised individuals are also at higher risk of *Campylobacter* infections (Janssen et al., 2008; Mizuno et al., 2022). Fortunately, the disease is usually self-limiting, and the mortality rate is low compared to other foodborne pathogens. The latest One Health Report by EFSA & ECDC

documented a mortality rate of 0.04% in the EU in 2020 while this was (much) higher for *Salmonella* spp. (0.1%) and *Listeria monocytogenes* (8.9%) (EFSA & ECDC, 2021). However, in some cases, an acute infection can develop into serious long-term illness like Guillain-Barré or Miller-Fisher syndrome, which both are severe neurological dysfunctions (Scallan et al., 2011; Skarp et al., 2016; World Health Organization, 2020), reactive arthritis (Pope et al., 2007) and irritable bowel syndrome (Berumen et al., 2021). The risk of contracting those sequelae are small (<2%) (Esan et al., 2020), but both, campylobacteriosis and its sequelae are a health and economic burden to the human population (Connerton and Connerton, 2017). For public health measures, the disease burden of *Campylobacter*-associated illness can be expressed in Disability Adjusted Life Years (DALYs) (World Bank, 1993). The estimation of DALYs is basically a sum of a population's years of life lost (YLL) to premature death and the years lived with any acute or chronic illness caused by, in this case, an infection with *Campylobacter* (YLD), weighed with a factor for the severity of illness (Chen et al., 2015). The estimated disease burden of *Campylobacter* in the Netherlands in 2019 was 19 DALYs per 100,000 citizens, which is much higher than for other bacterial pathogens (*Salmonella* spp.: 6.4; *L. monocytogenes*: 1.1; STEC O157: 0.9) (Lagerweij et al., 2019). In addition to the negative impact on human health, *Campylobacter*-associated illness also is costly as the cost of illness (COI) per case was calculated at 920 € in the Netherlands in 2019 which sums up to a total COI of 67 million euros for all reported *Campylobacter* infections (Lagerweij et al., 2019). It has been recognized that most *Campylobacter* infections are not reported to authorities and that the number of reported infections are just the tip of the iceberg. Since in most cases, illness manifests itself as self-limiting diarrhoea and general malaise, most people affected wait for symptoms to subside rather than seek medical help (Borgdorff and Motarjemi, 1997). Havelaar et al. (2013) estimated that, in 2009, only 1/47 cases of campylobacteriosis were reported in the EU. Oftentimes, it is unclear where *Campylobacter* infection were contracted as campylobacters can be found in a variety of reservoirs.

Campylobacter reservoirs and transmission routes

Person-to-person contact, direct contact with farm animals, pets, environmental sources and food were all identified as significant risk factors for campylobacteriosis (as depicted in **figure 2**) (Domingues et al., 2012).

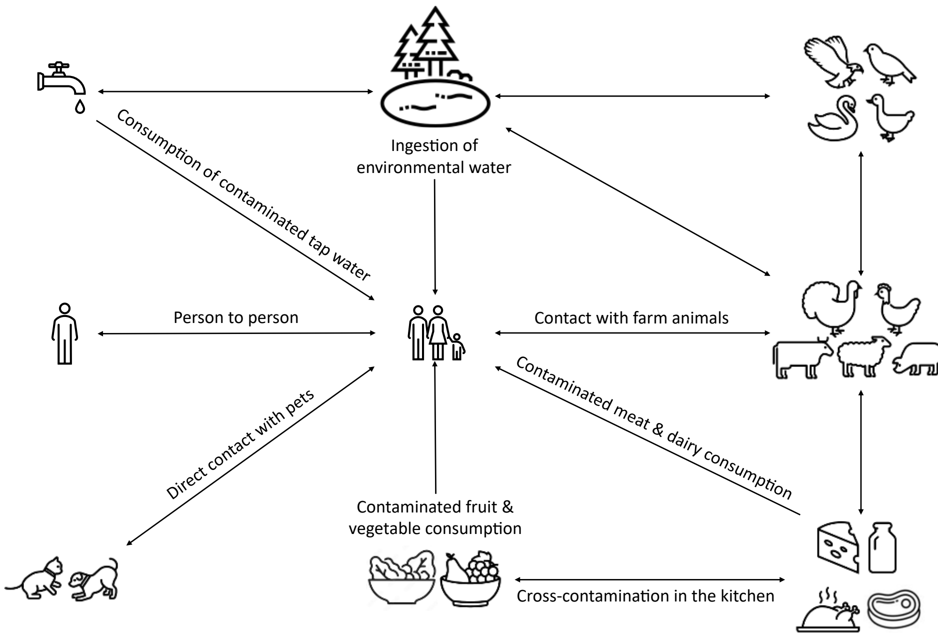

Figure 2: Reservoirs and transmission routes associated with *Campylobacter*.

International travel, especially to Asia was also often correlated with *Campylobacter* infection, probably due to a combination of the above-mentioned transmission routes (Ekdahl and Andersson, 2004; Mughini-Gras et al., 2014). Although person-to-person contact has been identified as a possible transmission route, *Campylobacter* is not usually spread directly from one person to another, but can happen via the faecal-oral route (Robinson, 1981b). The gastrointestinal tract of warm-blooded animals, including chicken, poultry, cattle, pigs and ruminants is a natural habitat for *Campylobacter* spp. (Hald et al., 2016; Kaine, 2003) and it has been shown that direct contact with these domesticated animals and their surroundings (e.g., transport crates) can lead to transmission of *Campylobacter* spp. (EFSA & ECDC, 2019; Frosth et

al., 2020; Levallois et al., 2014). But also, pets can carry *Campylobacter*. Examining the faeces of pets revealed that the prevalence of campylobacters, predominantly *C. jejuni* was high especially in (young) dogs and cats (Bojanić et al., 2017; Chaban et al., 2010; Thépault et al., 2020). The risk of *Campylobacter* transmission through wild animals has also been assessed but small rodents, deer and boars were found to have low levels or be free of *Campylobacter* spp. (Kaakoush et al., 2015; Meerburg et al., 2006; Morita et al., 2022; Tomino et al., 2020). On the other hand, wild birds have been found to be significant risk factors (Hald et al., 2016; Jamali et al., 2015). Hald et al. (2016) investigated the *Campylobacter* carriage in 52 wild bird species surrounding animal production farms in Denmark and found that just above 60% and 20% of thrushes and sparrows carried *Campylobacter* in their faeces. Jamali et al. (2015) found *Campylobacter* spp. in the intestinal contents of almost 40% and little over 25% of all examined ducks and geese, and isolated *C. jejuni* in more than 85% of *Campylobacter*-positive samples. *Campylobacter* cells carried in the intestinal tracts of wild birds are shed into environmental water sources like lakes and streams, which, in turn, can become a risk for contracting campylobacteriosis. Mughini-Gras et al.(2016) investigated the origin of *Campylobacter* strains in Dutch surface waters and found that most of the isolates were attributed to poultry (51.7%) and wild birds (37.3%) and contaminated the waters through animal faeces, sewage affluent and agricultural run-off. The consumption of untreated or contaminated water regularly is responsible for *Campylobacter* outbreaks in humans (Gilpin et al., 2020; Hyllestad et al., 2020; Pedati et al., 2019). Water has therefore been identified as a transmission route as well, not only to humans but to production animals as well. Frosth et al. (2020) demonstrated that, amongst other sources, contaminated drinking water and water ponds were potential reservoirs that can play a role in the transmission routes of *Campylobacter* to chickens. It has been demonstrated that colonization of production animals occurs at farm level (Signorini et al., 2013) and, in case of chickens, once *Campylobacter* is introduced into the flock, it spreads rapidly and colonizes the intestinal tracts of most chickens within a week where cells stay until slaughter (Horrocks et al., 2009; Newell et al., 2011; Van Gerwe et al., 2010). For a long time, *Campylobacter* was believed to be a commensal intestinal inhabitant, however, it has been suggested that the human pathogen might also have adverse effects on chicken welfare (Humphrey et al., 2014). In addition to all the mentioned risk factors, the highest risk for contracting campylobacteriosis has been attributed to the consumption and handling of

contaminated foods (EFSA & ECDC, 2019; Fravalo et al., 2021; Kaakoush et al., 2015; Mohammadpour et al., 2018).

Campylobacter in food

Raw or undercooked poultry and red meat, raw milk, dairy products, fruits and vegetables are common vehicles for the transmission of *Campylobacter* species.

Poultry

Of all foods, poultry is recognized as the primary source of food-related transmission of *Campylobacter* to humans (Taylor et al., 2013) and (small) *Campylobacter* outbreaks regularly occur (Baker et al., 2020; Greig and Ravel, 2009; Lanier et al., 2018; Llarena and Kivistö, 2020). Poultry products are mainly contaminated with *Campylobacter* during industrial-sized slaughter and processing (Althaus et al., 2017; Boysen and Rosenquist, 2009; Melero et al., 2012; Zbrun et al., 2013). Campylobacters can be found as commensals in the intestinal tract of poultry at relatively high numbers (up to 10^8 colony forming units [cfu]/g of caecal samples) (Hermans et al., 2011; Hue et al., 2011; Newell et al., 2011) and subsequently can also be found in the faeces in similar or even higher numbers (up to 10^{10} cfu/g of faeces) (Dhillon et al., 2006; Sahin et al., 2002). Since cross-contamination starts from either faecal or caecal contents that are leaked during slaughter, cells can be found mainly on the surface of poultry meat and in the offal (Hansson et al., 2015; Stella et al., 2017; Walker et al., 2019). During slaughter, large numbers of carcasses, often from several farms combined, are handled in a relatively short period of time which inevitably results in cross-contamination from the skin/surface of *Campylobacter*-positive to *Campylobacter*-free carcasses throughout the different processing stages (Gruntar et al., 2015; Newell et al., 2011; Stella et al., 2021; Zbrun et al., 2013). Stella et al. (2017) investigated the prevalence and contamination level of *Campylobacter* on different poultry meat sections and found that parts containing skin had the highest prevalence (86.8%), followed by whole carcasses and offal (58.3% and 48.7%, respectively), whereas skinless parts showed a lower prevalence (32.7%). The same study also revealed that cell concentrations were highest in offal (>3 $\log_{10}$ cfu/g in 20% of samples), followed by skin-containing parts (>1-2 $\log_{10}$ cfu/g in almost 60% of samples), whereas the majority (>80%) of skinless samples contained only low levels (<1 $\log_{10}$ cfu/g) of *Campylobacter*. Hansson et al.

(2015) conducted similar research and found that the prevalence was higher in skin samples compared to samples containing muscle tissue and when cell concentrations were sufficiently high for direct enumeration, cell numbers were also higher in skin-containing samples. The concentration of campylobacters in poultry meat samples was also examined in three recent studies. Habib et al. (2022) and Di Giannatale et al. (2019) examined poultry meat samples from the United Arab Emirates and Italy and found that approximately 71% and 52% of *Campylobacter*-containing samples were contaminated with < 1 log_{10} cfu/g . Slightly higher *Campylobacter* concentrations were reported by Tedersoo et al. (2022) who examined fresh chicken meat from three Northern European countries and found that *Campylobacter* concentrations were < 2 log_{10} cfu/g in approximately 44% of positive samples. However, they also found that initial cell concentrations were > 3 log_{10} cfu/g in around 20% of samples. Habib et al. (2022) also reported that for around 7% of *Campylobacter*-positive samples, cell concentrations were above 3 log_{10} cfu/g.

Red meat

As is the case for poultry, red meat products are also mainly contaminated during slaughter and processing, since cells are commonly isolated from the intestinal tracts of cattle, pigs and sheep (Quintana-Hayashi and Thakur, 2012; Scott et al., 2012; Thépault et al., 2018). However, studies conducted on the prevalence of *Campylobacter* spp. on beef, pork, lamb and goat meat revealed that the prevalence of *Campylobacter* on red meat generally is lower than for poultry, with the highest prevalence (approximately 20%) measured for beef meat (Aydin et al., 2020; Biasi et al., 2011; Premarathne et al., 2017; Rahimi et al., 2010). As could be seen for poultry, offal samples of beef, pork and lamb showed a higher prevalence than the meats (Walker et al., 2019).

Raw milk and dairy products

Raw milk is primarily contaminated through faeces, mostly indirectly via the udder but occasionally also directly because of mastitis (Orr et al., 1995). Although *Campylobacter* spp. were detected in up to 25% of raw milk samples recently (Del Collo et al., 2017; El-Zamkan and Hameed, 2016), in general, the prevalence is quite low (<5%). Outcomes of two meta-analysis studies estimated the prevalence of *Campylobacter* spp. to vary between 1.0-1.4% in Europe (Christidis et al., 2016;

Taghizadeh et al., 2022) which is supported by data reported to EFSA which showed an overall prevalence of *Campylobacter* spp. in milk of 0.6% (Van Den Brom et al., 2020). It is strongly advised to consume milk only after pasteurization; however, some consumers prefer raw milk and outbreaks involving unprocessed milk sporadically occur (EFSA & ECDC, 2021; Kenyon et al., 2020; Longenberger et al., 2013).

<u>Fruits and vegetables</u>

(Cross-) contamination of raw fruits and vegetables most likely occurs during cultivation by manure or contaminated irrigation water, faeces of wild or domestic animals and/or unhygienic handling practices (Danis et al., 2009; Pintar et al., 2017). In general, the prevalence of *Campylobacter* spp. in fresh vegetables and fruits was low (0.23-0.53%) and the highest prevalence was associated with sprout crops (Mohammadpour et al., 2018; Verhoeff-Bakkenes et al., 2011).

Impact of food processing on *Campylobacter* in foods

It is important to mention that is often difficult to compare the prevalence and contamination levels in different food sources since the results are highly dependent on the applied sampling method and stage during production at which the samples were taken. Additionally, it does not mean that the consumption of food products contaminated with *Campylobacter* will inevitably lead to food-borne infection. Whether they constitute a risk at the point of consumption is dependent on several factors such as the contamination level, environmental factors (e.g., storage temperatures and gaseous atmosphere), food processing steps and food handling practices. Cardoso et al. (2021) observed the behaviour of consumers in their own kitchen when handling raw poultry and found that two-third of the consumers washed the chicken meat under running tap water, which may contaminate surrounding kitchen surfaces. Similarly, Bremer et al. (2005) conducted a survey in private households and reported less than half of the participants washing their hands after handling raw poultry meat. Equally important, cutting boards were not at all or improperly cleaned after handling raw poultry meat by just below half of the participants which has been identified as a potential vehicle for the transfer of campylobacters from raw to ready-to-eat products (Tang et al., 2011). All these improper kitchen practices can contribute to cross-contamination of *Campylobacter*

spp. in domestic kitchens as was shown by Gorman et al. (2002) who examined hands, different kitchen surfaces and cutting boards for the presence of campylobacters after raw chicken was handled and recovered campylobacters of around one fifth of the sampling sites.

Due to its strict growth requirements, it is generally assumed that thermotolerant *Campylobacter* spp. cannot grow on food during processing and storage. However, cells are often able to survive on/in food until the moment of consumption (Ahmed et al., 2013; Kärenlampi and Hänninen, 2004; Kocic et al., 2012). *Campylobacter* is sensitive to several food processing-related stresses, which often take place at the same time, among others but not exclusively temperature, gaseous atmosphere and desiccation.

Temperature

Heat treatments of food products is generally recognized as the most effective method to inactivate campylobacters. A lot of research has been conducted on the heat inactivation of campylobacters in different media and food products and results are difficult to compare, however, they all concluded that *Campylobacter* can be characterized as sensitive to heating. For example, Sakkaf and Jones modelled the heat resistance of *C. jejuni* in liquids at different temperatures and determined that at 56.5 °C, 90% of cells could be inactivated already after 9.8 s for some cases, and at 60 °C, the same proportion of cells was in inactivated in less than 2 s (Al-Sakkaf and Jones, 2012). Logically, pasteurization of milk showed to successfully kill campylobacters (Gill et al., 1981; Waterman, 1982) if performed correctly; a recent sporadic *Campylobacter* outbreak was linked to a partial failure in the pasteurization process of raw milk (Fernandes et al., 2015). Heat inactivation times generally increase in solid food matrices. Sampers et al. (2010) studied the survival of *Campylobacter* spp. on chicken burgers subjected to heat stress and results showed that after two minutes of pan-frying cell numbers started to decrease and after four minutes, when internal temperatures reached 57.5 °C, a reduction in *Campylobacter* concentrations was achieved beyond the detection limit of conventional plating methods.

Transportation and storage of perishable foods such as raw meats occurs at low temperatures. For example, cattle carcasses which are shipped long distance from South America to Europe are transported frozen (-18 to -25 °C) (BMT, n.d.). Studies

on the effect of frozen storage on the viability of campylobacters showed that viability decreased significantly at the beginning of frozen storage and remained relatively stable for several weeks. For the biggest part, this can be attributed to ice nucleation and dehydration during the initial stage of freezing (Bhaduri and Cottrell, 2004; Georgsson et al., 2006; Maziero et al., 2010). Other perishable meats shipped short distance are often transported chilled (-1.5to 5 °C) (BMT, n.d.) or stored in the refrigerator in stores and at the consumer until consumption or further processing. Sampers et al. (2010) showed that *Campylobacter* concentrations remained stable during 14 days of storage at refrigeration temperatures and a study by Bhaduri and Cottrell (2004) revealed that concentrations decreased slightly ($<1 \log_{10}$) during 7 days of refrigerated storage. Interestingly, the culturability of campylobacters is most impaired at ambient temperatures, which has been linked to upregulated metabolic processes (Buswell et al., 1998; Hazeleger et al., 1995; Rollins and Colwell, 1986) and a more severe decrease in *Campylobacter* concentrations could be observed during storage of inoculated minced chicken at ambient temperatures compared to refrigerated storage (Blankenship and Craven, 1982).

Gaseous atmosphere

Oftentimes, these inactivation and survival studies are conducted under aerobic conditions, which can also influence the survival due to the microaerobic growth requirements of *Campylobacter*. Byrd et al. (2011) evaluated the effect of different oxygen concentrations (ranging from 100-0% O_2) on the survival of *Campylobacter* during chilled storage and concluded that viability decreased the most under high oxygen concentrations. Similar results were also obtained by Boysen et al. (2007) who found that *C. jejuni* died significantly faster in a high oxygen-containing gas mixture compared to gas mixtures in which oxygen was replaced by nitrogen.

Desiccation

The rapid chilling of perishable products such as pig carcasses is achieved through ventilation of cooling rooms, which has shown to reduce *Campylobacter* spp. from the carcass surface (Oosterom et al., 1983). *Campylobacter* is described as sensitive to desiccation compared to other foodborne pathogens (Fernández et al., 1985) and the minimum water activity (a_w) for growing has been defined at 0.987 with an optimum at 0.997 (Park, 2002; Silva et al., 2011). Oosterom et al. (1983) investigated the effect of

water activity on the survival of campylobacters on the surface of tiles and found that viable cells could only be detected on visibly wet surfaces. This is especially relevant regarding the survival of *Campylobacter* cells on surfaces as they can play a role in cross-contamination.

All these factors and others can have an influence on the cell concentration, viability and culturability of foodborne pathogens such as *Campylobacter* (Wesche et al., 2009). Considering its high potential to cause infection, it is important to be able to verify control measures of food products by assessing if *Campylobacter* is present and/or in which concentrations.

Methods for the detection of *Campylobacter* spp. from foods

Depending on the contamination level and state of the cells, a qualitative or quantitative approach needs to be taken and over the years, different detection and quantification methods have been developed. In general, there are two types of principles, namely cultural and molecular detection methods and both principles have their advantages and disadvantages.

Cultural methods

For the cultural detection and enumeration of *Campylobacter* spp. from foods, protocols have been developed by the International Organization for Standardization (ISO). The current ISO 10272 document consists of two sections; 10272-1 describes procedures for the detection of *Campylobacter* while 10272-2 focusses on the enumeration. In the previous version from 2006, there used to be only one protocol to detect *Campylobacter* from food, however, ISO regularly evaluates methods and new research findings are considered in order to ensure the reliability of the protocols as best as possible. Currently, ISO 10272-1:2017 describes three protocols for the detection of campylobacters from food (International Organization for Standardization, 2017). An overview of the protocols is depicted in **figure 3**. While procedure C should be followed for products with expected high numbers of campylobacters such as poultry caecal contents or raw poultry meat, procedures A and B are meant to be followed when the expected number of campylobacters on the food products is low. Oftentimes it is complicated for users to estimate how many

campylobacters to expect on a sample and therefore procedures A or B are sometimes run in parallel with procedure C. Procedures A and B both contain a selective enrichment step.

In theory, the purpose of an enrichment is to provide (stressed) cells with an environment designed to optimally support the damage repair and growth of the cells by supplying them with favourable temperatures, microaerobic atmosphere and nutrients. However, selective agents can also be added to a nutritious base to simultaneously suppress the growth of competing microorganisms as is done for

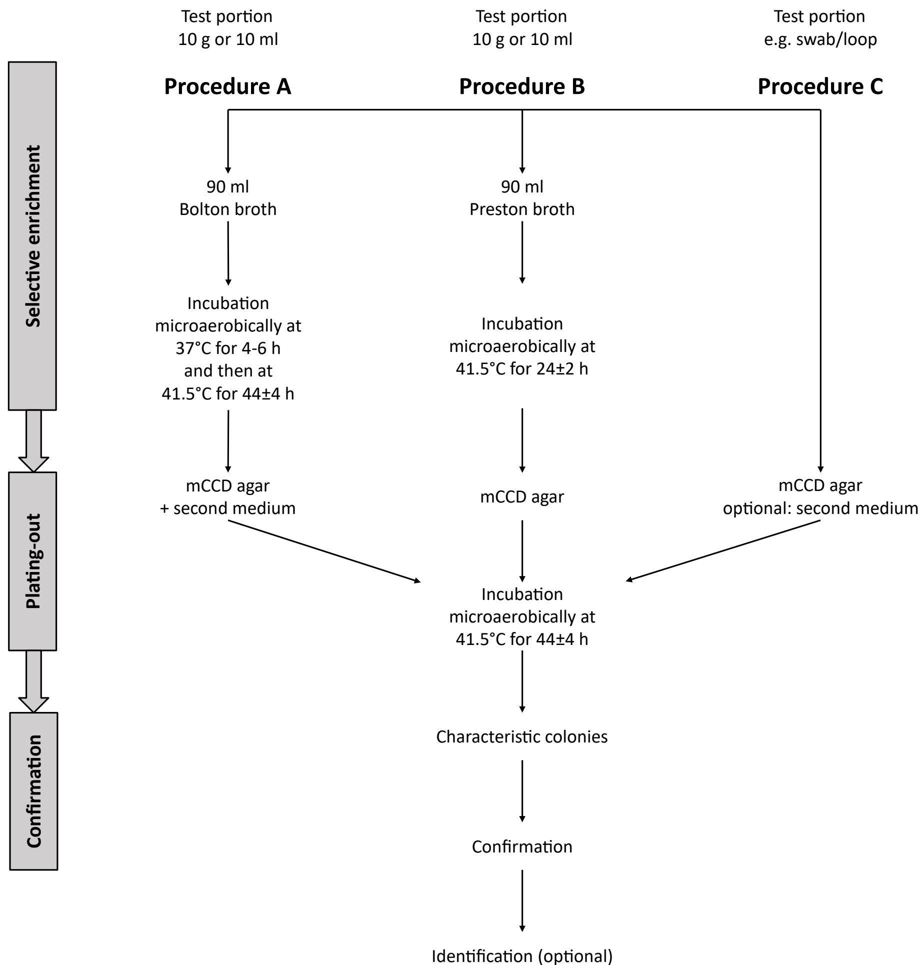

Figure 3: Overview of detection protocols described in ISO 10272-1:2017.

Listeria monocytogenes for example, but also for *Campylobacter* enrichments. In case of ISO 10272-1, the decision as to which procedure should be used is dependent on whether the user expects the amount of background microbiota on the sample to be low or high and whether they expect *Campylobacter* cells to be stressed/injured. In case of low amount of background microbiota, procedure A should be used but on the other hand, procedure B should be followed when the sample is expected to contain high levels of background microbiota. The biggest difference between procedures A and B lies in the selective enrichment step as either a 48 h-enrichment in Bolton broth (BB; procedure A) or a 24 h-enrichment in Preston broth (PB; procedure B) is executed. Both media contain a mixture of nutrients but differ from each other mainly in the composition of antibiotics. BB contains cefoperazone, vancomycin, trimethoprim and amphotericin B while, in PB, the former two antibiotics are replaced by polymyxin B and rifampicin to more severely inhibit the growth of background microbiota during enrichment (Chon et al., 2017; Hazeleger et al., 2016). Although *Campylobacter* are naturally resistant to a range of antibiotics (Luangtongkum et al., 2009) and normally should be able to grow in the presence of all of these antibiotics, it has been shown that, in some cases, PB inhibited the growth of *Campylobacter* which was attributed to polymyxin B (Baylis et al., 2000; Hazeleger et al., 2016; Paulsen et al., 2005). This is the main reason why procedure A should be used if campylobacters are expected to be damaged as a result of earlier food processing steps, such as by freezing or heating. After enrichment, campylobacters are expected to have reached high concentrations (~10^8-10^9 cfu/g) and cells are isolated from the enrichment by streaking 10 µl of the enrichment fluids onto selective agar plates. After incubation, a maximum of five characteristic colonies are first streaked onto unselective blood agar plates which are again incubated. The sample is considered *Campylobacter*-positive if one of the five colonies can be confirmed by investigating the morphology and motility, the absence of growth at 25 °C and the presence of oxidase activity.

The cultural detection protocols consisting of an enrichment step followed by isolation and confirmation are quite easy to follow and are relatively inexpensive, only detect living cells and at the end, *Campylobacter* isolates are obtained. The biggest advantage is their high sensitivity, as, in theory, with enrichment-based procedures

one single living cell could be detected in 10 g of sample. This can be converted to a lower detection limit in enrichments of -2 log_{10} cfu/ml.

However, the methods also have their drawbacks as they are quite laborious and there is not one protocol which seems to work well for all food samples, thereby affecting the reliability of the outcomes. Furthermore, in recent years, another challenge has arisen, namely the emergence of other antibiotic resistant *Enterobacteriaceae* which can grow on/in the used culturing media and consequently negatively affect their selectivity. Although efforts have been made to confront this issue, no protocol has been developed yet which restores selectivity without forfeiting sensitivity. Another big disadvantage is the expenditure of time of the enrichment protocols. While negative results can be obtained after 3-4 days (3 days for procedure B; 4 days for procedure A), confirmed positive results can only be obtained after approximately one week (6-7 days for procedure B; 7-8 days for procedure A). This is a challenge especially for products with a short shelf life such as raw refrigerated meats and ready-to-eat products. At the moment a positive detection result is confirmed, the products often have either already been consumed or at least are at the consumer's home which makes recalls problematic.

Molecular detection methods

Over the past two decades, a lot of rapid, culture-independent detection methods have been developed for *Campylobacter* spp.. They are based on two different methodologies, namely immunological and nucleic acid-based methods (Ricke et al., 2019).

The former uses the affinity of antibodies for *Campylobacter* species-specific surface antigens and the most common examples for these techniques are enzyme-linked immunosorbent assays (ELISAs) and flow cytometry (Heo et al., 2009; Kawatsu et al., 2008; Qian et al., 2008; Ricke et al., 2019; Steele et al., 2002). Although immunological methods have shown to be more specific than cultural methods regarding the detection of pathogens on a genus level (Granato et al., 2010), they also have their limitations as cross-reactivity with other *Campylobacter* species (next to *C. jejuni* and *C. coli*) may occur. With regards to the detection of campylobacters from food, it might not be of high importance which *Campylobacter* species is detected, however, if species differentiation is aimed for, immunological methods might not be optimal

since this lack of specificity can give false-positive detection outcomes (Couturier et al., 2013; Gharst et al., 2013; Myers and Jackson, 2011) and it has been suggested that quantitative polymerase chain reaction (qPCR) assays might be a more reliable option for the detection of *Campylobacter* in chilled and frozen poultry samples (Reis et al., 2018).

Nucleic acid-based methods make use of highly specific DNA or RNA fragments (so-called primers) which can be designed to either only detect *Campylobacter* spp. or also differentiate between species. The primer design depends on whether a singleplex or multiplex PCR assay is required, so whether only one or several targets should be detected. Likewise, the design is also dependent on the PCR machine and intended PCR variation For campylobacters, protocols have been designed for different PCR variations such as conventional (or endpoint), multiplex and real-time quantitative (q-)PCR. The most general approach is end-point PCR with primers for 16S rRNA which allows for the detection of *Campylobacter* species. After amplification, the PCR product is loaded onto an agarose-gel and after gel-electrophoresis, the specific PCR product is visible in the form of a band of a certain (known) molecular weight. This way, it can be assessed whether *Campylobacter* spp. were present in the sample. A more advanced variation is multiplex PCR which can also be conducted quantitatively in real-time. The multiplex setup uses different primer sets unique for different species and a fluorescent dye (so-called probe) for each of the primer sets and thereby allows for species differentiation. An overview of frequently used primer sets for the differentiation of *Campylobacter* spp. was recently given in a review article by Ricke et al. (2019). Real-time qPCR can be used qualitatively but, when suitable standard curves are included, can also give quantitative information about the contamination level of a sample. While outcomes of conventional PCR are visible only after gel-electrophoresis (~4 h), qPCR results can be tracked live as PCR amplification cycles occur and results are generally obtained within 2 h. However, these molecular methods also have their drawbacks as they tend to be more expensive, require highly specific primers, are difficult to optimize and, as with ISO protocols, must be re-evaluated regularly to ensure their sensitivity and selectivity considering emerging *Campylobacter* species and food matrix components which could interfere with detection. As a fact, it has been shown that detection outcomes are sometimes unreliable in complex matrices as DNA extraction methods are compromised and

inhibitors disrupt PCR amplification (Park et al., 2014; Pontiroli et al., 2011). Moreover, since generally, no prior culturing step is included in the protocols, no differentiation can be made between live and dead *Campylobacter* cells (Gharst et al., 2013; Kralik and Ricchi, 2017; Oyarzabal and Battie, 2012; Ricke et al., 2019). One of the biggest drawbacks of molecular methods lies in the relatively high amount of starting material needed in order to be able to conduct the protocols reliably. Generally, a minimal cell concentration between 10^3-10^5 cfu/ml is required for reliable results using PCR, and slightly lower concentrations are required for real-time PCR (Wang and Salazar, 2016).

Whether a cultural or molecular detection method should be applied to detect campylobacters in food products is dependent on several factors, such as the availability of equipment and trained personnel in the laboratories, the number of samples to be tested and the sample type. The biggest deciding factor is the amount of *Campylobacter* cells to be expected in the food product. If cell concentrations are beneath the detection limit of molecular methods, an approach utilizing an enrichment step is unavoidable to be able to also detect low amounts of *Campylobacter* in a food product. This is one of the reasons while ISO 10272-1 is still viewed as the gold standard for the detection of *Campylobacter* spp. in foods (Porte et al., 2016; Stingl et al., 2021). Since campylobacters are inevitably suspected to unfavourable conditions during food processing, transport and storage cells are likely to have obtained sub-lethal damage and therefore, ISO 10272-1 procedure A (enrichment in BB) is a logical choice.

Current regulations concerning the legal limits of *Campylobacter* in food

For the Netherlands, there are currently two regulations concerning the allowed limits of *Campylobacter* in different food product groups. Regulation (EC) No. 2073/2005 describes microbiological criteria for foodstuffs and sets limits regarding *Campylobacter* in broiler carcases. This regulation sets a process hygiene criterion for broiler carcases after chilling in form of a two-class sampling plan as depicted in **table 1**. 50 sampling units (n) must be examined using the enumeration protocol of ISO 10272. As of this moment, 15 of these sampling units (c) are allowed to contain *Campylobacter* up to the microbiological limit (m) of 1000 cfu/g. The sampling plan

increases in stringency as the maximum number of samples allowed to be marginally defective (c) previously was 20 and decreases to 10 samples by January 1, 2025. In case a lot is rejected, actions must be taken to identify the source of the high contamination level and take appropriate control measures (Commission of the European Communities, 2005; Dahms, 2004; European Commission, 2017).

Table 1: **2-class sampling plan for broiler *carcasses* after chilling according to regulation (EU) No. 2073/2005**

Sampling plan		Limits	Method
n	**c**	**m**	
50	c=20 (from 23.08.2017) c=15 (from 01.01.2020) c=10 (from 01.01.2025)	1000 cfu/g	ISO 10272-2

n: number of sampling units to be examined
c: maximum number of sampling units allowed to be marginally defective
m & M: microbiological limits (allowed to exceed the limit m but not exceed M)

Next to this, the Dutch regulation named 'Warenwetbesluit Bereiding en behandeling van levensmiddelen' (WBBL) specifies that *Campylobacter* needs to be absent in 25 g or ml of processed food products which underwent a microorganism-reducing treatment and are not heated before consumption, also known as ready-to-eat foods (Overheid.nl, 2021). While the two-class sampling plan only relies on direct plating of the (diluted) sample onto selective solid media, the testing of ready-to-eat foods requires an enrichment step to be able to detect one *Campylobacter* cell in the food sample.

Detection challenges

Currently, one could think of a *Campylobacter*-enrichment as a black box as information regarding the growth kinetics of *Campylobacter* spp. during enrichment is scarce. Research outcomes have shown that damaged cells recover better in BB than in PB (Baylis et al., 2000; Hazeleger et al., 2016) and that enrichment usually starts with low amounts of campylobacters and ends with significantly higher amounts. During enrichments, bacterial cells undergo four stages of growth, as schematically depicted in **figure 4**.

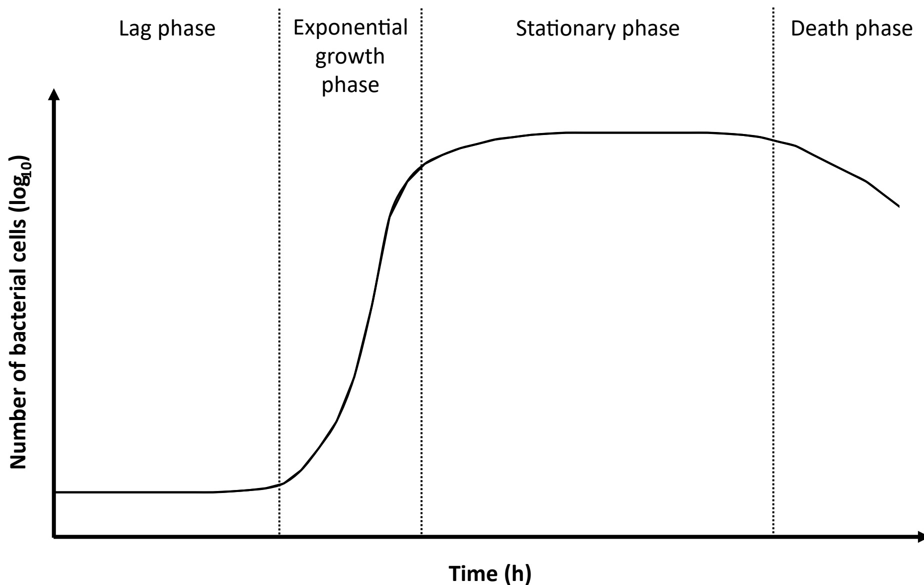

Figure 4: Schematic overview of the four stages of bacterial growth.

During the first stage, the so-called lag phase, no growth can be observed. Cells have shown to be metabolically active (Bertrand, 2019), but it is assumed that cellular processes taking place during lag phase are necessary for adaptation to the new environmental conditions, damage repair and synthesis of cellular components needed to initiate growth (Bertrand, 2019; Rolfe et al., 2012). A lot of research has been conducted on the effect of different food-related stresses on the survival and viability of *Campylobacter* (Bhaduri and Cottrell, 2004; Georgsson et al., 2006; Maziero et al., 2010; Park, 2002; Sopwith et al., 2008; Wilson et al., 2008) but the impact of stresses

on the lag phase of *Campylobacter* during enrichment has not yet been assessed. Quantifying the lag-duration of different *Campylobacter* strains could provide information which could help to assess current enrichment durations. Increased lag phase could potentially negatively impact outcomes if cells need a substantial amount of the enrichment duration to initiate growth. This, in combination with a low growth rate and growth inhibition due to competitors present could lead to false-negative detection outcomes. On the other hand, if the lag phase of tested strains is generally short, cells start growth faster and, if growth is not hindered by other factors, reach detectable concentrations well before the end of the current enrichment duration (48 h), This, in turn, could lead to faster detection outcomes if the enrichment duration could be shortened. Quantifying the growth kinetics of different isolates of *C. jejuni* and *C. coli* could also have added value concerning variability during enrichment. Variability is the expression of biological heterogeneity like genetic and phenotypic differences between strains (inter-species variability), or heterogeneity within a population due to the presence of sensitive and robust sub-populations (intra-species variability). Furthermore, it is yet unknown which cellular processes take place during this crucial phase and analysis of proteomic changes could identify biomarkers which could be indicative for recovery and growth initiation.

During the second stage, the exponential growth phase, cells are assumed to have recovered fully and are growing rapidly in the enrichment medium. Again, a considerable amount of research has been conducted so far on the metabolic growth requirements of *Campylobacter*, especially *C. jejuni* (Gao et al., 2017; Gripp et al., 2011; Guccione et al., 2008; Gundogdu et al., 2016; Hofreuter, 2014; Kelly et al., 2001; Stahl et al., 2012; Wright et al., 2009) but no information is available on either the exact composition of BB or the substrate utilization during enrichment. Information on this topic could identify whether BB is sufficiently rich for the recovery and growth of campylobacters or if the medium is lacking crucial nutrients which could aid the faster (recovery and) growth of campylobacters.

In the third stage, the stationary phase, cell growth is slowed down, a balance between growing and dying cells can be observed. and for a prolonged time, cell concentrations remain high, but stable. In general, the entry of bacteria into this phase can be caused by the exhaustion of nutrients, unfavourable changes in pH, or accumulation of toxic

by-products (Jaishankar and Srivastava, 2017; Navarro Llorens et al., 2010). Data on the growth kinetics of campylobacters during enrichment is scarce, but Hazeleger et al. (2016) showed that, in pure cultures, campylobacters reached cell concentrations of around 8 log_{10} cfu/ml, which is approximately one log_{10} lower than other bacteria such as *Escherichia coli* (Hazeleger et al., 2016; Wang et al., 2015).

As mentioned earlier, it has been recognized that other antibiotic-resistant bacteria, mainly Extended-spectrum beta-lactamase (ESBL-) producing *Enterobacteriaceae* have shown to grow in culture media developed to be selective for *Campylobacter* and their growth impairs detection when ISO 10272-1 is followed (Habib et al., 2011; Hazeleger et al., 2016; Jasson et al., 2009; Seliwiorstow et al., 2016). ESBLs are enzymes which can hydrolyse penicillin's, aztreonam, and third generation cephalosporins (Bradford, 2001; Hawkey and Jones, 2009; Paterson and Bonomo, 2005; Pitout and Laupland, 2008; Shaikh et al., 2015) and the latter is a component of both, the selective supplement of BB and the selective solid medium modified Charcoal-Cefoperazone-deoxycholate agar (mCCDA) (ThermoFisher Scientific, n.d.). Efforts have been made to restore the selectivity of media by adjusting the antibiotic cocktail (Chon et al., 2017; Jo et al., 2017; Kim et al., 2016; Moran et al., 2011) in order to suppress the growth of ESBL-producers but unfortunately, these antibiotics sometimes also suppress the growth of campylobacters which can led to false-negative detection outcomes (Baylis et al., 2000; Goossens et al., 1986; Hazeleger et al., 2016; Paulsen et al., 2005). Hazeleger et al. (2016) quantified the growth of campylobacters when grown in the absence and presence of ESBL-producing *Escherichia coli* and found that growth of campylobacters in BB was significantly hindered in the presence of ESBL-producers. As a fact, they reported a difference in *Campylobacter* concentration of approximately 3 log_{10} cfu/ml in stationary phase when co-cultured with ESBL-producers. This observed growth suppression of campylobacters could negatively impact detection outcomes if cell concentrations remain below the detection limit of molecular methods or *Campylobacter* colonies are overgrown by colonies of competitors in culture-based detection methods. It has been assumed that competition for medium substrates is the reason for the premature growth arrest, but this has not yet been confirmed.

The duration of the lag phase (λ), maximum specific growth rate (μ_{max}) and maximum cell concentration reached in stationary phase (asymptote) can be estimated using

different mathematical models. In order to reliably estimate growth parameters, cell concentrations must be measured regularly throughout enrichment and growth curves must be constructed which then can be fitted with models such as the modified Gompertz model (Zwietering et al., 1990), the three-phase model (Buchanan et al., 1997) and the Baranyi-model (Baranyi and Roberts, 1994). By fitting these models, the duration of the lag phase can be estimated, and comparisons can be made between the recovery time of healthy cells and injured cells during enrichment. The same is true for μ_{max} and the asymptote. The last stage of bacterial growth is the death phase, which is marked by a decrease in viable cells after stationary phase.

In addition to the mentioned unclarity that still exists when it comes to enrichments, it is also still unclear how to apply molecular methods for the detection of *Campylobacter* from complex food matrices when cells are damaged and present in low numbers. Several studies have mentioned that, for these situations, a preceding enrichment cannot be avoided (He and Chen, 2010; Ivanova et al., 2014; Josefsen et al., 2004; Mayr et al., 2010; Rantsiou et al., 2010; Sails et al., 2003) and efforts have been made to develop protocols combining cultural methods with molecular detection. Ivanova et al. (2014) demonstrated that *C. jejuni* was detected through real-time PCR from colonies grown on mCCDA after enrichment in BB and Mayr et al. (2010) combined a 40-48 h enrichment in PB with multiplex real-time PCR to detect different *Campylobacter* species. Other studies applied modified versions of BB (Rantsiou et al., 2010; Sails et al., 2003) or a modified sample preparation step prior to enrichment in BB (He and Chen, 2010). So far, no protocol has been developed yet which combines the original enrichment step as described in ISO 10272-1 procedure A with a molecular detection protocol that allows also for species differentiation.

Optimally, a detection protocol for *Campylobacter* from food products should have a high accuracy as the method should be very sensitive to detect low amounts of campylobacters, allow resuscitation of damaged cells and should also support the proliferation of both, clinically relevant and emerging *Campylobacter* species. At the same time, it should be sufficiently selective to either completely or at least partially suppress the growth of competing microorganisms which could hinder the growth of campylobacters, and detection (and differentiation) should be specific to avoid false-positive outcomes. Furthermore, an optimal method should be able to deliver results

faster than the current cultural method, be relatively inexpensive and user-friendly and protocols should be as simple as possible to leave little room for error. Additionally, next to detection, an optimal method should also allow species differentiation and quantification and should be easily adjustable in case of emerging species.

Objectives and outline of the thesis

The objective of this thesis was to shed light into the black box that is enrichment in order to assess whether the current enrichment protocol (procedure A of ISO 10272-1) is adequate for the detection of *Campylobacter* spp. from foods or if possible changes to the procedure could be proposed to improve the enrichment-based detection procedure. An overview of the research topics addressed in this thesis is given in **figure 5**.

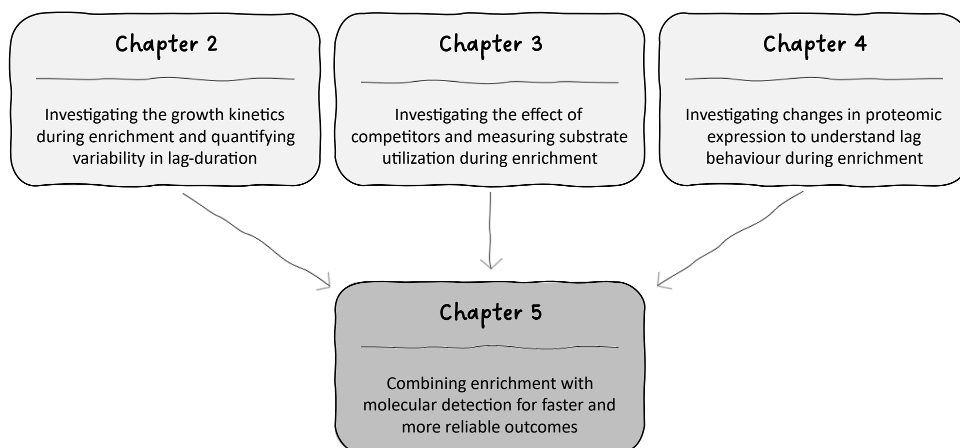

Figure 5: Overview of research topics addressed in each chapter of this thesis.

Chapter 1 provides a general introduction to *Campylobacter* physiology, its impact on human health, reservoirs and transmission routes, available detection methods and current challenges concerning the detection of *Campylobacter* spp. in food products.

Chapter 2 describes the variability in lag-duration of a set of *C. jejuni* and *C. coli* strains during enrichment in BB with and without prior stress treatment. By subjecting a selection of strains to different food-related stresses, we gained inside not only into the effect these stresses had on the viability of the strains but also on the recovery duration during enrichment. This way we could estimate the variability in lag-duration which allowed us to predict whether strain variability and stress-induced heterogeneity in outgrowth affected the risk for false-negative detection outcomes.

Chapter 3 focusses on the composition of selective BB and the utilization of the media compounds by *Campylobacter* spp. and ESBL-producing *Escherichia coli* throughout enrichment. By utilizing (Ultra-) High-performance liquid chromatography, we were able to identify a range of media compounds and quantify changes in their extracellular concentration when *C. jejuni, C. coli* and *E. coli* were enriched in pure or co-culture. This way we could assess whether campylobacters and *E. coli* compete for the same media components and whether adjustments to the enrichment settings (medium and gaseous atmosphere) could improve the growth kinetics of campylobacters in the presence of ESBL-producing *E. coli*.

Chapter 4 concentrates on the proteomic changes *C. jejuni* undergoes at the start of enrichment. By using liquid chromatography-mass spectrometry, we measured changes in the proteomic expression during the first hours of enrichment to generate insight into the intracellular processes that take place during the lag phase of reference and refrigeration-stressed *C. jejuni* cells.

Chapter 5 describes the development of a *Campylobacter* detection protocol which combines enrichment in BB with detection by means of a multiplex real-time PCR. We used the knowledge gained in chapters 2 to 4 to shorten the duration of enrichment and designed a qPCR protocol which, in combination with enrichment in BB, allows for the detection and differentiation of *C. jejuni* and *C. coli* in naturally contaminated food samples.

Chapter 6 provides a general discussion in which I will discuss all the results obtained during this research, their contribution to the food safety of *Campylobacter* and the lessons learned. I will also discuss topics related to the detection of *Campylobacter* and what I believe has yet to be investigated to further improve detection.

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Variability in lag-duration of *Campylobacter* spp. during enrichment after cold and oxidative stress and its impact on growth kinetics and reliable detection

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Abstract

Campylobacter jejuni and *Campylobacter coli* continue to be the leading cause of zoonotic gastroenteritis in the European Union, making reliable detection in food important. Low storage temperatures and atmospheric oxygen concentrations during food production can cause sub-lethal damage or transient non-culturability which is why ISO 10272-1:2017 includes an enrichment step to repair cell damage and increase cell concentrations, thereby supporting detection of campylobacters from foods. The aim of this study was to assess the variability in lag-duration of *C. jejuni* and *C. coli* during enrichment after different food-relevant stress treatments and evaluate its impact on growth kinetics and reliability of detection outcomes. Therefore, 13 *C. jejuni* and 10 *C. coli* strains were subjected to cold stress during refrigerated and frozen storage. Refrigerated storage did not significantly reduce culturability, but frozen storage reduced cell concentrations by 1.6±0.1 log_{10} cfu/ml for both species. Subsequently, cells were enriched following ISO 10272-1:2017-A and cell concentrations were determined over time and lag-duration and growth rate were determined by fitting the Baranyi-model. Without prior stress treatment, mean lag-duration for *C. jejuni* and *C. coli* was 2.5±0.2 h and 2.2±0.3 h, respectively. Refrigerated storage increased lag-duration for *C. jejuni* to 4.6±0.4 h and for *C. coli* to 5.0±0.4 h and frozen storage increased lag-duration to 5.0±0.3 h and 6.1±0.4 h for *C. jejuni* and *C. coli*, respectively. Comparison of strain- and biological variability showed that differences in recovery after cold stress can be attributed mainly to strain variability since strain variability after refrigeration and freeze stress increased respectively 3-fold and 4-fold while biological variability remained constant. A subset of strains was also subjected to oxidative stress that reduced cell concentrations by 0.7±0.2 log_{10} cfu/ml and comparison of recovery patterns after oxidative and freeze stress indicated that recovery behaviour was also dependent on the stress applied. A scenario analysis was conducted to evaluate the impact of heterogeneity in outgrowth kinetics of single cells on the reliability of detection outcomes following ISO protocol 10272-1:2017. This revealed that a ‘worst-case’-scenario for successful detection by a combination of the longest lag-duration of 7.6 h and lowest growth rate of 0.47 h^{-1} still resulted in positive detection outcomes since the detection limit was reached within 32.5 h. This suggests that other factors such as competitive microbiota can act as a causative factor in false-negative outcomes of tested food samples.

Introduction

Campylobacter spp. are Gram-negative, microaerophilic, spiral-shaped rods that live as commensals in the intestinal tract of predominantly birds, but also sheep, cattle and pigs, cats and dogs and they can survive in environmental waters and even sand (Jones, 2001; Ogden et al., 2009; Vandamme & De Ley, 1991). *Campylobacter* spp. continues to be the leading cause of registered zoonotic gastroenteritis in the European Union (EU) for more than 10 years, with approximately 84% and 10% of campylobacteriosis cases caused by *Campylobacter jejuni* and *Campylobacter coli*, respectively (European Food Safety Authority, 2019).

Thermotolerant campylobacters need at least a temperature as high as 30 °C to grow but grow optimally at a temperature range of 37-42 °C and require an atmosphere with reduced oxygen levels of 3-5% (Adams & Moss, 2014; Mead, 2004). This often leads to the conclusion that campylobacters seem to be fragile, also since they appear to be much more sensitive to environmental conditions compared to other foodborne pathogens (Jasson et al., 2007; Mihaljevic et al., 2007). Indeed, it has been shown that *Campylobacter* spp. are highly susceptible to desiccation, low pH and heat stress even below 60 °C (Chaveerach et al., 2003; Nguyen, et al., 2006; Oosterom et al., 1983). During food production, *Campylobacter* spp. can be exposed to increased atmospheric oxygen concentrations, which can lead to the production of reactive oxygen species (ROS), which can cause growth arrest and damage of the cell membrane and important proteins within the cell (Gundogdu et al., 2016; Imlay, 2003; A. Klančnik et al., 2009). Removal of ROS is mediated by several enzymes including superoxide dismutase (SOD), Catalase (KatA), cytochrome c peroxidase and alkyl hydroperoxide reductase (Ahp) (Van Vliet, Ketley, Park, & Penn, 2002). It has been shown that *Campylobacter* spp. cannot grow below 30 °C, partly due to a lack of cold shock proteins (Bhaduri & Cottrell, 2004; Hazeleger et al., 1998; Parkhill et al., 2000). Studies on the effect of freeze stress and survival of *Campylobacter* spp. during storage at temperatures of -20 °C and lower showed that decrease in cell concentration was highest at the begin of frozen storage and remained relatively stable for several weeks, which probably can be attributed to the formation of ice crystals during the initial stage of freezing (Bhaduri & Cottrell, 2004; Georgsson et al., 2006; Lee et al., 1998; Maziero & De Oliveira, 2010).

Although *Campylobacter* spp. are generally unable to multiply outside the animal host, it has been shown that cells are able to survive long-term under adverse environmental conditions (Lee et al., 1998; Park, 2002; Sopwith et al., 2008; Wilson et al., 2008). So, even though campylobacters must endure oxidative stress and unfavourably low temperatures during transport and storage of food products, cells have shown to be able to survive the hostile environment (Sampers et al., 2010) and possibly cause disease. This, together with the high potential to cause disease (Black et al., 1988) increases the importance of a sensitive, yet selective detection method to verify food safety measures.

Currently, the protocol of the International Organization for Standardization (ISO), ISO 10272-1:2017 is applied in the European Union for the detection of *Campylobacter* spp. from foods. It consists of three procedures, two of which contain selective enrichment to amplify cells to a detectable level (procedures A and B). Procedure A uses Bolton Broth and is applied when injured campylobacters are expected in the food product whereas procedure B uses Preston Broth and is applied when high amounts of background microflora are expected (International Organization for Standardization, 2017). Regardless of the procedure chosen, growth initiation might be preceded with a lag-phase (Zwietering et al., 1990). The lag-duration is not only dependent on the severity of stress a cell endured prior to enrichment, but can also be affected by strain-dependent differences in robustness and ability to recover from stress (Booth, 2002; Jasson et al., 2007). Strain variability has been previously described by (Whiting & Golden, 2002) as an inherent property of microorganisms which cannot be reduced when strains undergo identical treatments under the same conditions. Next to strain variability, also biological variability has to be considered. Aryani et al. (2015) defined biological or reproduction variability as the difference between independently reproduced experiments of the same strain performed on different experimental days from new pre-cultures and newly prepared media. Both strain variability and biological variability can have an impact on reliable detection of *Campylobacter* spp. when following ISO protocols, since increased recovery duration in enrichment might lead to false-negative outcomes.

In this study, the effect of different food-relevant stresses on the lag-duration of 23 *Campylobacter* isolates was assessed. Biological and strain variabilities in the obtained lag-duration λ were quantified and compared.

Furthermore, a scenario analysis and Monte Carlo simulations on outgrowth kinetics of sub-lethally injured *Campylobacter* spp. during enrichment were conducted to evaluate the impact of strain variability in recovery and growth kinetics on the reliability of detection outcomes following ISO protocol 10272-1:2017.

Materials and methods

Bacterial strains and preparation of stationary phase cultures

A selection of 13 *Campylobacter jejuni* and 10 *Campylobacter coli* strains of different origin (human, food and environmental isolates) and sequence type (ST) was collected (details can be found in **table S 1 of the supplementary materials**). Whenever possible, clonal complexes or STs frequently associated with disease were selected (Colles & Maiden, 2012; Dearlove et al., 2016). *Campylobacter* stock cultures were grown in Heart Infusion broth (Bacto HI, Becton, Dickinson and Company) for 24 h at 41.5 °C, then supplemented with 15% glycerol (Fluka) and stored at -80 °C. To obtain pre-cultures for stress and enrichment experiments, *C. jejuni* and *C. coli* were plated from the -80 °C vials onto Columbia agar base (CAB, Oxoid, supplemented with 5% (v/v) lysed horse blood (BioTrading Benelux B.V. Mijdrecht, Netherlands) and 0.5 % agar (Bacteriological agar No.1, Oxoid)) and grown microaerobically for 24 h at 41.5 °C. Subsequently, single colonies were resuspended in HI and cultured for 24 h at 41.5 °C to obtain stationary phase cultures. Afterwards, a 1:500 dilution was made in unselective Bolton broth (BB, Oxoid, supplemented with 5% (v/v) sterile lysed horse blood (BioTrading Benelux B.V., Mijdrecht, Netherlands without the addition of selective supplements) and cultured for 24 h at 41.5 °C to reach the stationary phase. For the application of oxidative stress, cultures were prepared by diluting the culture grown in HI-broth in a ratio of 1:500 in Bolton broth (BB, Oxoid) without supplementation of horse blood and antibiotics and this culture was subsequently grown for 24 h at 41.5 °C. Cell concentrations were determined by plating appropriate dilutions on CAB. All cells were cultured under microaerobic conditions (5 % O_2, 10 %

CO_2, 85 % N_2) in flushed jars (Anoxomat WS9000, Mart Microbiology, Drachten, Netherlands) unless stated otherwise.

Application of stress treatments

Refrigeration stress and freeze stress

All 23 strains cultured to the stationary phase in unselective Bolton broth with the addition of 5% sterile lysed horse blood were decimally diluted in peptone physiological salt solution (PPS, Tritium Microbiologie) to a cell concentration of approximately 10^4 cfu/ml for application of freeze stress and 10^3 cfu/ml for application of refrigeration stress (considering the difference in stress severity and consequent difference in reduction in cell viability, aiming at a cell concentration of approx. 10^3 cfu/ml after application of stress) and inoculated 1:6 in 5 ml of Bolton broth with addition of 5% (v/v) sterile lysed horse blood and without addition of selective supplements in 15 ml plastic tubes (Greiner centrifuge tubes, Merck). Tubes were placed standing upright at -20 °C or 4 °C for 64 ± 1 h for frozen and refrigerated storage, respectively. Afterwards, refrigeration-stressed cultures were transferred to room temperature and allowed to warm up for 10 min. Freeze-stressed cultures were transferred to room temperature and allowed to stand until defrosted. Cell concentrations after both stresses were determined by plating appropriate dilutions on CAB and stress-induced reduction in cell numbers was calculated.

Combined mild refrigeration and atmospheric oxygen stress

A selection of strains, namely *C. jejuni* strains WDCM 00005 and 81-176 and *C. coli* strains Ca 2800 and WDCM 00004 were exposed to atmospheric oxygen. Cultures grown to the stationary phase in unselective Bolton broth (without the addition of sterile lysed horse blood) were diluted to 10^4 cfu/ml and inoculated 1:10 in 27 ml of unselective Bolton broth without supplementation of sterile lysed horse blood in sterile 250 ml Erlenmeyer flasks with cotton stopper. Samples were incubated at 12 °C for 64 ± 1 h at 160 rpm shaking conditions to induce atmospheric oxygen and mild refrigeration stress. Cell concentrations after atmospheric oxygen stress were determined by plating appropriate dilutions on CAB and stress-induced reduction in cell numbers was calculated.

Quantification of growth parameters during enrichment

Infusion bottles were filled with 42 ml of Bolton Broth, closed with a rubber stopper and aluminium cap and sterilized. Subsequently, bottles were supplemented with 5 % sterile horse blood and 450 µl of the selective enrichment supplement (Oxoid SR0208E). Bottles were filled with 5 ml of either reference or stressed cultures, resulting in a starting cell concentration in the enrichment broth of approximately 2 log_{10} cfu/ml. Additions of fluids to sterilized infusion bottles was achieved using syringes to puncture the rubber stopper of the bottles. The head space of infusion bottles was flushed for 2 min with a gas-mixture of 5 % O_2, 10 % CO_2 and 85 % N_2 by a home-made gas flushing device using syringes to puncture the rubber stopper. Inoculated infusion bottles were incubated in water baths at 37 °C for the first 5 h and subsequently transferred to 41.5 °C for the remaining 43 h following ISO 10272-1:2017. At regular time intervals, 1-2 ml samples were taken from the bottles using a syringe and after each second sample, bottles were flushed again with the appropriate gas mixture. Samples were immediately decimally diluted in PPS, plated onto CAB and incubated for 48 h at 41.5 °C. Two biologically independent reproductions per strain and stress treatment were performed on different days.

Model fitting to estimate growth parameters during enrichment

Plate counts were transformed to log_{10} cfu/ml and growth curves were constructed using Microsoft Excel 2010. Growth curves were fitted with the modified Gompertz model (Zwieteringet al., 1990), the three-phase model (Buchanan, Whiting, & Damert, 1997) and Baranyi-model (Baranyi & Roberts, 1994) using the Solver add-in of Excel. The models were ranked based on the mean square error of the model (MSE_{model}) previously described by (Den Besten et al., 2006).

<u>Baranyi-model</u>:

$$\log_{10} N(t) = \log_{10} N(0) + \frac{\mu}{\ln(10)} \cdot A(t) - \frac{1}{\ln(10)} \cdot \ln\left[1 + \frac{exp[\mu \cdot A(t)] - 1}{10^{[\log_{10} N(max) - \log_{10} N(0)]}}\right] \quad [1]$$

$$A(t) = t + \frac{1}{\mu} \cdot \ln\left[exp(-\mu \cdot t) + exp\left(-\mu \cdot t_{lag}\right) - exp\,(-\mu \cdot t - \mu \cdot t_{lag})\right] \quad [2]$$

t = elapsed time during enrichment (h)
$\log_{10} N(t)$ = population at time t ($\log_{10}$ cfu/ml)
$\log_{10} N(0)$ = initial cell population ($\log_{10}$ cfu/ml)
μ = maximum specific growth rate (h^{-1})
$\log_{10} N(max)$ = final population ($\log_{10}$ cfu/ml)
t_{lag} = lag-duration of the growth curve (h)

Modified Gompertz model:

$$\log_{10} N(t) = \log_{10} N(0) + (\log_{10} N_{max} - \log_{10} N_0) * \exp\left\{-\exp\left[\frac{\frac{\mu}{\ln(10)} * exp(1)}{\log_{10} N(max) - \log_{10} N(0)} * (\lambda - t) + 1\right]\right\} \quad [3]$$

t = elapsed time during enrichment (h)
$\log_{10} N(t)$ = population at time t ($\log_{10}$ cfu/ml)
$\log_{10} N(0)$ = initial cell population ($\log_{10}$ cfu/ml)
μ = maximum specific growth rate (h^{-1})
$\log_{10} N(max)$ = final population ($\log_{10}$ cfu/ml)

Three-phase linear model:

Lag Phase: For $t \leq t_{lag}$, $\log_{10} N_t = \log_{10} N_0$ [4]

Exp. Growth Phase: For $t_{lag} < t < t_{max}$, $\log_{10} N_t = \log_{10} N_0 + \frac{\mu}{\ln(10)} (t - t_{lag})$ [5]

Stationary Phase: For $t \geq t_{max}$, $\log_{10} N_t = \log_{10} N_{max}$ [6]

t = elapsed time during enrichment (h)
t_{lag} = time when lag-duration ends (h)
t_{max} = time until maximum population density is reached (h)
$\log_{10} N_t$ = population at time t ($\log_{10}$ cfu/ml)
$\log_{10} N_0$ = initial cell population ($\log_{10}$ cfu/ml)
μ = maximum specific growth rate (h^{-1})
N_{max} = final population ($\log_{10}$ cfu/ml)

The two-tailed t-test was used to evaluate the statistical significance of differences in the reduction after stress treatments between species as well as differences in lag-duration of cultures during enrichment at different conditions ($p<0.05$).

To evaluate the increase in lag-duration due to stress pre-treatment, the mean lag-duration derived from enrichments of stressed cells was subtracted from the mean lag-durations derived from enrichments of the reference condition following equation 7.

$$\Delta_{lag} = \left(\frac{\lambda_{stress\ rep.1} + \lambda_{stress\ rep.2}}{2}\right) - \left(\frac{\lambda_{ref.condition\ rep.1} + \lambda_{ref.condition\ rep.2}}{2}\right) \quad [7]$$

$\lambda_{\text{stress rep.1}}$ = lag-duration (h) after stress of reproduction 1
$\lambda_{\text{stress rep.2}}$ = lag-duration (h) after stress of reproduction 2
$\lambda_{\text{ref.condition rep.1}}$ = lag-duration (h) in reference condition of reproduction 1
$\lambda_{\text{ref.condition rep.2}}$ = lag-duration (h) in reference condition of reproduction 2

Quantifying biological and strain variability

To quantify variability, methods previously described by Aryani et al. (2015b) were applied. Biological and strain variability were calculated for all strains and histories according to equations 8 and 9.

Biological variability: $$MSE_{Biological} = \frac{RSS}{df} = \frac{\sum_{S=1}^{23}\sum_{R=1}^{2}(\lambda_{RS} - \lambda_S)^2}{n-p} \quad [8]$$

MSE = mean square error
λ_{RS} = lag-duration (h) of each biological reproduction "*R*" and strain "*S*"
λ_S = average lag-duration (h) of λ_{RS} from two biological reproductions for strain "*S*"
df = no. of data points (n=2*23) minus the number of parameters (p=1*23)

Strain variability: $$MSE_{Strain} = \frac{RSS}{df} = \frac{\sum_{S=1}^{23}(\lambda_S - \bar{\lambda})^2}{n-p} \quad [9]$$

λ_S = average lag-duration (h) of λ_{RS} from two biological reproductions for strain "*S*"

$\bar{\lambda}$ = average lag-duration (h) of all 23 strains
df = no. of data points (n=23) minus the number of parameters (p=1)

The F-test was used to compare biological and strain variability in lag-duration during enrichment of the reference condition, as well as variability in lag-duration during enrichment after refrigeration stress and freeze stress. Data was considered significantly different at a p-value of 0.05 or lower.

Predictive modelling for scenario analysis

A three-phase linear model (Buchanan et al., 1997) was used to predict the bacterial growth curve during enrichment and to identify factors which could lead to false-negative detection outcomes. Equations 4 until 6 were used.

By varying different biological parameters such as the initial cell concentration ($\log_{10} N_0$), lag-duration (λ) and bacterial growth rate (μ) a scenario analysis was conducted to assess when/if the detection level set to 3 $\log_{10}$ cfu/ml was reached. The initial cell concentration was set to -2 $\log_{10}$ cfu/ml at the beginning of enrichment to mimic a scenario wherein 10 g of food product containing 1 cell is mixed with 90 ml of enrichment broth. The maximum cell concentration ($\log_{10} N_{max}$) was set to 9 $\log_{10}$ cfu/ml. By varying the duration of recovery λ (h) and growth rate μ (h^{-1}), predictions on growth after freeze stress and worst-case scenario analyses were conducted. Variations in lag duration λ were simulated by using the mean lag-duration derived after model fitting of growth kinetics during enrichment after freeze-stress of 23 strains (5.4 h) as well as the mean value with subtraction and addition of two standard deviations (±2.2 h). For variation of growth rate μ, the mean values (0.93 h^{-1}) was used as well as the mean value with subtraction and addition of two standard deviations (±0.47 h^{-1}).

The analysis was conducted using Microsoft Excel 2010 using the Solver add-in. Furthermore, the risk of false-negative detection outcomes after enrichment following ISO 10272-1:2017 procedure A was determined by means of a Monte Carlo simulation using the @RISK version 7.5 (Palisade Corporation) add-in in Microsoft Excel 2010. The impact of changes in μ and λ on the ability to reach the detection limit of 3 $\log_{10}$ cfu/ml was assessed. The parameters μ and λ were estimated to be normal-distributed

with the calculated standard deviations from all reproductions for refrigerated and freeze-stressed strains. To determine the risk of false-negative detection outcomes of freeze-stressed cells after 48 h of enrichment in Bolton broth, simulations were done with 100.000 iterations using Latin Hypercube sampling in combination with a Mersenne twister random number generator.

Results

Reduction in cell concentration of *C. jejuni* and *C. coli* after cold stress

The reduction in cell concentrations of 13 *C. jejuni* and 10 *C. coli* strains after 3 days of refrigerated and frozen storage at +4 °C and -20 °C, respectively, were determined and are presented in **Figure 1**. The reductions of the *C. jejuni* strains after refrigerated storage ranged from none to 0.2 log_{10} cfu/ml with an average for the 13 strains of 0.1±0.02 log_{10} cfu/ml. *C. coli* showed a similar reduction ranging from none to 0.3 log_{10} cfu/ml with an average of the 10 strains of 0.1±0.03 log_{10} cfu/ml. A two-tailed *t*-test showed no significant differences in reduction between the two species after refrigeration (p=0.61).

Storage at -20 °C resulted in a mean reduction of 1.5±0.05 log_{10} cfu/ml with reduction ranging from 0.9 to 1.7 log_{10} cfu/ml for *C. jejuni*. For *C. coli* reductions ranged from 1.1 to 2.1 log_{10} cfu/ml with an average of 1.7±0.09 log_{10} cfu/ml. A two-tailed *t*-test showed a rather similar, but just significantly different reduction after frozen storage for both species (p=0.04).

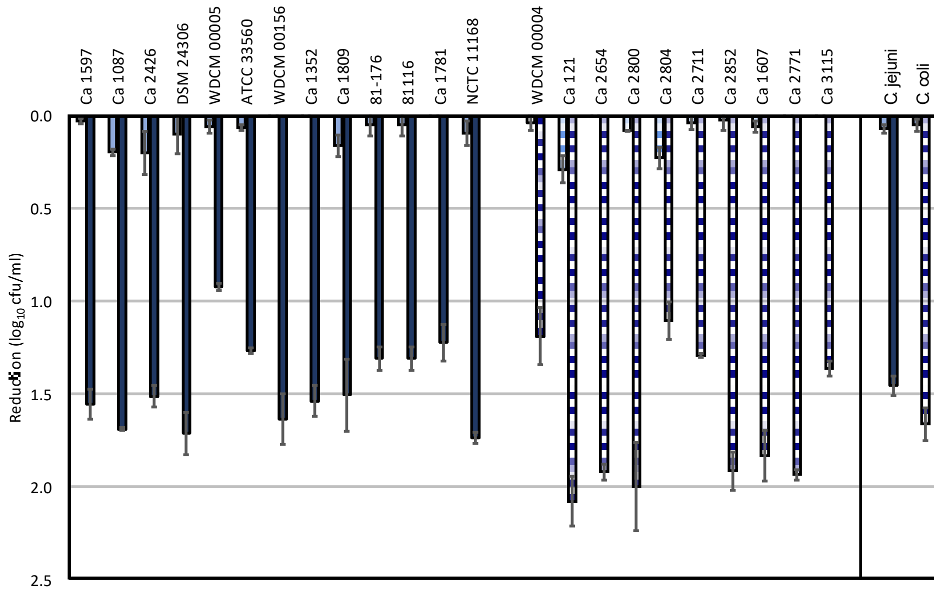

Figure 1: Reduction in cell concentration of *C. jejuni* (filled bars) and *C. coli* (striped bars) after refrigerated storage (light blue colored bars) and frozen storage (dark blue colored bars). Error bars depict the standard error of the biological reproductions (n=2).

Lag-duration of *C. jejuni* and *C. coli* during enrichment after different treatments

Lag-duration during enrichment of 13 *C. jejuni* and 10 *C. coli* strains without prior stress treatment as well as after refrigerated and frozen storage were determined by fitting the growth curves with the Baranyi-model. Initially, data were fitted with three different growth models; the Baranyi-model (Baranyi & Roberts, 1994), three-phase model (Buchanan et al., 1997) and modified Gompertz model (Zwietering et al., 1990). The reason for this was to assess and select the model which showed the best fit overall for the experimental data collected. Overall, the three-phase model showed the worst fit (highest MSE for 55% of the fittings), followed by the modified Gompertz model (highest MSE_{model} for 25% of the fittings), and the Baranyi-model (highest MSE_{model} for 20% of the fittings). Because the fitting performances of the Baranyi and the modified Gompertz models were rather comparable, all data sets were fitted with both models and lag-duration estimates were compared. Outcomes of this analysis revealed that

the model choice did not have a significant influence on the estimated lag-duration of any group (p=0.06 for lag of reference cells, p=0.36 for lag of refrigeration-stressed cells and p=0.13 for lag of freeze-stressed cells). Since the Baranyi model gave the best fit overall, this model was chosen for data representation. Mean lag-duration for each strain and after each history is depicted in **figure 2**. Without prior stress treatment, mean lag-duration for *C. jejuni* and *C. coli* was 2.5±0.2 h and 2.2±0.3 h, respectively. Refrigerated storage increased mean lag-durations for *C. jejuni* to 4.6±0.4 h and for *C. coli* to 5.0±0.4 h. Frozen storage led to a longer lag-duration for both species, with a mean lag-duration of 5.0±0.3 h and 6.1±0.4 h for *C. jejuni* and *C. coli*, respectively. A trend for interspecies differences in lag-duration could only be seen after frozen storage (p=0.02).

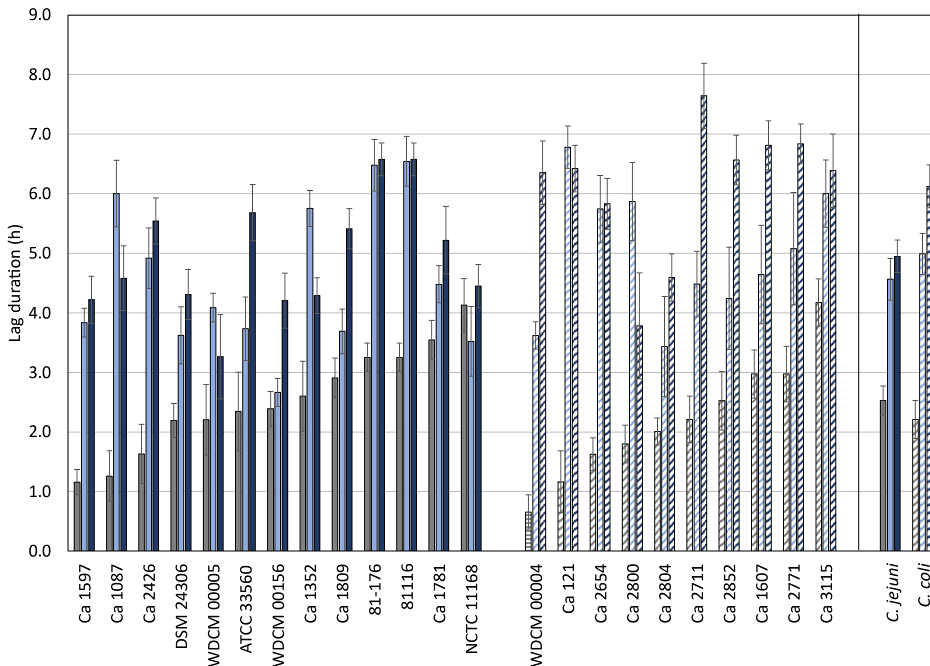

Figure 2: Recovery duration of *C. jejuni* (filled bars) and *C. coli* (striped bars) with(out) prior stress treatment. Mean lag-duration without prior stress treatment (grey colored bars), after refrigerated storage (light blue colored bars) and frozen storage (dark blue colored bars). Error bars depict the standard error of the biological reproductions (n=2).

Quantifying variability

Figure 3 shows the calculated biological and strain variability using the lag-duration determined for 23 strains and two reproductions per strain after three different experimental treatments: without prior stress (reference condition), after refrigeration stress and after freeze stress. Comparison of biological variability between the reference condition and refrigeration stress (just significant, $p=0.03$), reference condition and freeze stress ($p=0.11$) as well as comparison between the biological variability of both stress treatments ($p=0.76$) indicated that biological variability remained relatively constant for all three treatments.

Strain variability increased as the severity of stress increased, namely approximately three times higher compared to the reference condition after refrigerated storage and approximately four times higher after frozen storage. Comparison of strain variability between the reference condition and stress treatments showed significant differences ($p<<0.001$, for both, refrigeration and freeze stress). Biological and strain variability were almost equal during enrichment without prior stress ($p=0.03$). After refrigeration and freeze stress, strain variability was significantly higher than reproduction variability ($p<<0.001$, in both cases). Notably, similar conclusions could be made when the lag durations were estimated using the modified Gompertz model (see **figure S 1 of the supplementary materials**).

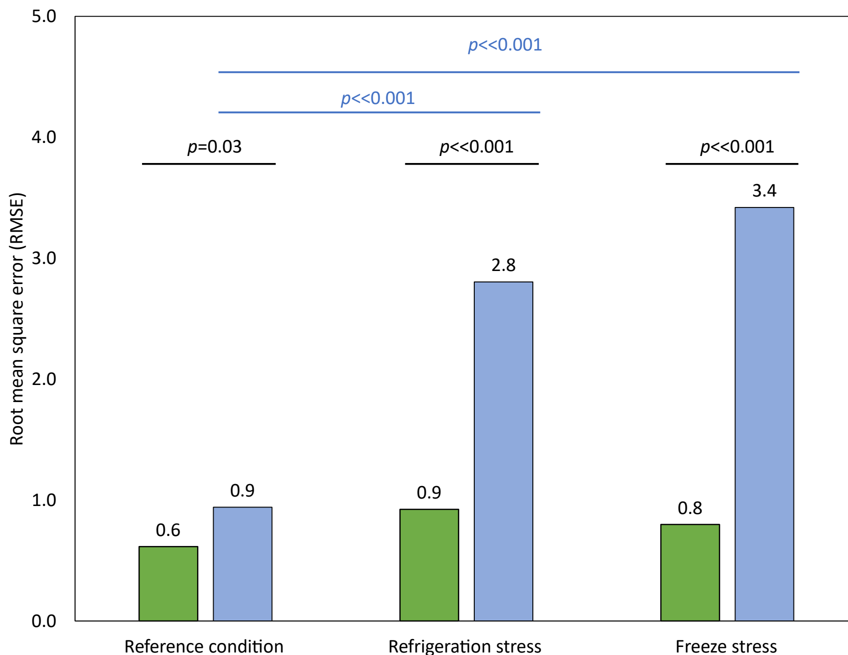

Figure 3: Comparison of biological variability (green-coloured bars) and strain variability (blue-coloured bars) of the lag-duration after three experimental treatments. Significance testing showed significant differences between biological and strain variability in growth experiments conducted after all three treatments. Comparison of biological variability between the sets of experiments showed no significant difference, except between reference condition (RC) and refrigeration stress (RS) ($p=0.03$). Comparison of strain variability between RC and RS as well as RC and FS showed significant differences ($p<0.05$ in both cases). No significant difference could be seen after comparing biological variability in growth experiments conducted after both stress treatments ($p=0.05$).

Effect of stress history on recovery during enrichment

After determination of lag-duration after freeze stress, one fast- and one slow-recovering strain of each species were selected to investigate whether trends in lag-duration are similar after application of combined mild refrigeration and atmospheric oxygen stress. Strain selection was made after comparing the lag-duration of reference cells with the lag-duration of freeze-stressed cells. For *C. jejuni*, strain WDCM 00005 was chosen as a fast-recovering strain and strain 81-176 was selected as a slow-recovering strain. For *C. coli*, strain Ca 2800 showed the fastest recovery after freeze-

stress, while strain WDCM 00004 was chosen as a slow-recovering strain. **Figure 4** depicts the described difference in lag-durations in enrichment of the four selected strains after oxidative and freeze stress. Oxidative stress reduced cell concentrations by 0.7±0.2 log_{10} cfu/ml on average, while freezing resulted in a mean decrease of 1.4±0.5 log_{10} cfu/ml (corresponding data is displayed in **figure S 3 of the supplementary materials**). Visualization of lag-duration after the different stresses allowed comparison of stress-dependent recovery behaviour of each strain. *C. jejuni* isolate WDCM 00005 and *C. coli* isolate Ca 2800 recovered relatively fast from freeze stress (Δ_{lag} of 1.1 h and 2.0 h, respectively). Exposure to atmospheric oxygen concentrations led to an increase in Δ_{lag} for strain WDCM 00005 by approximately a factor 3 (2.8x) and a factor 2.0 for strain Ca 2800. Recovery of *C. jejuni* strain 81-176 was comparable after both stresses (Δ_{lag} of 3.3 h and 2.9 h after freeze and oxidative stress, respectively). and lag-duration of *C. coli* strain WDCM 00004 was similar after both stresses as well. These stress effects indicate that after similar reduction, recovery behaviour during enrichment following ISO 10272-1:2017 was not only strain-dependent but also affected by the type of stress the population encountered prior to enrichment.

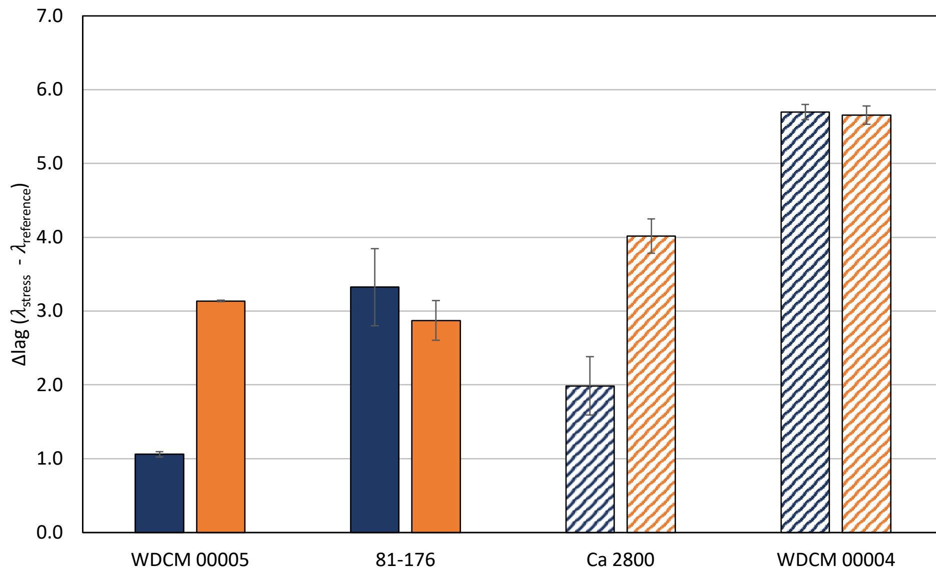

Figure 4: Comparison of absolute lag-durations of four strains after freeze-stress (dark blue-coloured bars) and oxidative stress (orange-coloured bars). Two fast- and slow-recovering strains after freeze stress of *C. jejuni* (filled bars) and *C. coli* (striped bars) were subjected to oxidative stress and subsequently enriched following ISO 10272-1:2017, procedure A. The bars depict the increase in lag-durations to compare relative values for lag-duration. Error bars depict the standard error of the biological reproduction (n=2).

Scenario analysis

The data obtained for the 23 *Campylobacter* isolates from enrichments conducted after cold stress treatments was used to predict growth kinetics during enrichment following ISO 10272-1:2017, procedure A. According to this procedure, one *Campylobacter* cell present on/in 10 g or ml of food sample is enriched in 90 ml of Bolton Broth for 48 h, resulting in an initial concentration of -2 $\log_{10}$ cfu/ml. Subsequently, 10 µl of the enriched broth is streaked onto selective solid media and *Campylobacter*-typical colonies are confirmed. Therefore, cell concentrations after enrichment must be at least 2 $\log_{10}$ cfu/ml in order to transfer on average one cell onto selective solid media. In this study the detection limit was set to 3 $\log_{10}$ cfu/ml to minimize the risk of false-negative outcomes after enrichment. **Figure 5** shows the outcomes of the scenario analysis. To simulate variations in lag-duration λ, initially

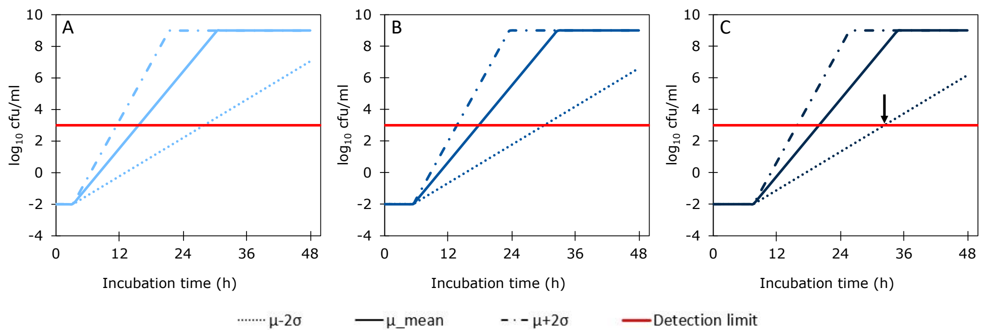

Figure 5: Scenario analysis of growth kinetics of freeze-stressed *Campylobacter* spp. in monoculture during enrichment in Bolton Broth following ISO 10272-1:2017. Initial inoculation level ($\log_{10} N_0$) was set to one *Campylobacter* cell/100 ml of Bolton broth. Simulation of differences in lag-duration were achieved by plotting (A) the mean lag-duration derived from all enrichments conducted after freeze stress treatments -2 standard deviations (3.2 h), (B) mean lag-duration (5.4 h) and (C) mean lag-duration +2 standard deviations (7.6 h). For all three scenarios, growth rates were estimated by plotting the mean growth rate (0.93 h^{-1}) as well as low (0.47 h^{-1}) and high (1.39 h^{-1}) growth rate. Low and high growth rates were estimated by plotting mean growth rate ±2 standard deviations. Worst case scenario is depicted in the dotted line of graph (C). The black arrow indicates the time point during enrichment, when the detection limit of 3 $\log_{10}$ cfu/ml is reached (t=32.5 h).

the mean lag derived after model fitting of the growth kinetics during enrichment of the 23 freeze-stressed isolates was used (5.4 h) (figure 5, B). Particularly short but especially long recovery durations were also simulated by usage of the mean value with subtraction (figure 5, A) and addition (figure 5, C) of two standard deviations (±2.2 h). For variation of growth rate μ, the mean values (0.93 h^{-1}) was used as well as the mean value with subtraction (dotted line) and addition (striped line) of two standard deviations (±0.47 h^{-1}). A worst-case scenario analysis was conducted to simulate the growth kinetics of a single cell in 100 ml Bolton broth with a maximum lag duration of 7.6 h and a minimal growth rate of 0.47 h^{-1}. Results show that the detection limit was reached within 32.5 h (marked by a black arrow in figure 5, C). This indicates that false-negative detection outcomes are probably not due to slow growth and long lag-duration. The outcomes of the scenario analysis can be underpinned by the Monte Carlo simulation that was conducted to determine the risk of false-negative detection outcomes of freeze-stressed cells after 48 h of enrichment

in Bolton broth. For that 100.000 iterations were used and for most simulations (94.3%) the detection limit of 3 $\log_{10}$ cfu/ml was reached already within 24 h while the probability of reaching the detection limit at the end of enrichment (after 48 h) was 100%. Outcomes of the Monte Carlo simulation are displayed in **figure S 4 of the supplementary materials.**

Discussion

Reduction in cell concentration of *C. jejuni* and *C. coli* after cold stress

In this study, cold stress treatments were applied for 64 h to cells suspended in Bolton broth base supplemented with 5 % sterile lysed horse blood. Trials were done in a food-based fluid (chicken rinse) following methods described by Birk et al. (2004) to assess reduction over time after refrigeration and freeze stress and results indicated that cells of the same inoculum showed comparable reduction in both media (data not shown). Also, reduction in cell concentration over time during short-term refrigerated and frozen storage was monitored in trial experiments to set the cold storage time. Results showed, that freezing at -20 °C resulted in an initial sharp decrease in cell concentration which is probably due to the formation of ice crystals during freezing but remained relatively constant until the end of the measurement (5 days, data not shown). Refrigerated storage at +4 °C in BB base resulted in minor reduction of cell concentrations. This might be attributed to decrease in metabolic rate of cells cultured at refrigeration temperatures in BHI previously described by Hazeleger et al. (1995). Overall, after refrigerated and frozen storage in BB, a reduction of less than 0.5 $\log_{10}$ cfu/ml and approximately 1.5 $\log_{10}$ cfu/ml was reached in the current study, respectively. Those results are in line with other studies conducted on the survival after exposure to cold temperatures (Bhaduri & Cottrell, 2004; Georgsson et al., 2006; Haddad et al., 2009; Klančnik et al., 2008; Maziero & De Oliveira, 2010; Sampers et al., 2010). Throughout this study, the unselective solid medium CAB was used for the determination of cell concentrations after the application of stress in order to increase the chance to detect sub-lethally injured cells, however it cannot be excluded that the tested stresses might trigger the viable but nonculturable (VBNC) state (Portner et al., 2007; Rollins, 1986).

Lag-duration of *C. jejuni* and *C. coli* during enrichment after cold stress

Results of this study showed, that refrigerated storage did not result in a major decrease in cell concentration (approximately 0.1 log_{10} cfu/ml for both species). Nonetheless, lag-duration after refrigerated storage increased significantly by a factor of approximately 2.0 compared to the lag-duration of strains without prior stress treatment. Although refrigeration stress resulted in a relatively low reduction in culturability compared to freeze stress, strain variability in subsequent lag-duration after both stresses was comparable (p=0.18) and significantly higher than for reference cells. Interestingly, no correlation could be found for both cold-stress treatments between reduction and subsequent lag-duration for the tested *C. jejuni* and *C. coli* strains (corresponding data can be found in **figure S 2 of the supplementary materials**). It has been shown that *Campylobacter* spp. do not express cold-shock proteins, which are often associated with cell division at low temperatures (Hazeleger et al., 1998; Phadtare et al., 1999). However, cells have demonstrated to be able to show respiration, chemotaxis and protein synthesis at temperatures as low as 4 °C, although at rates much lower than at 37 °C (Hazeleger et al., 1998). Metabolism appears to be affected by low temperatures, as the production of succinate, an amino acid which is secreted during growth was decreased at low temperatures (Hofreuter, 2014; Höller et al., 1998). It might be possible, that even though cells did not incur severe damage to their cell membrane during refrigeration, cells still need to adapt to the rapid change of temperature from refrigeration to enrichment temperatures of 37 °C. It has been shown, that changes in temperature even within the range of growth lead to transcriptional changes over time with gene up- and downregulation lasting for at least 50 minutes (Stintzi, 2003).

The impact on cell viability of storage at temperatures comparable to those of conventional household freezers has been studied in detail (Georgsson et al., 2006; Jasson et al., 2007; Maziero & De Oliveira, 2010; Sampers et al., 2010). However, relatively little is known about the recovery behaviour of freeze-stressed *Campylobacter* cells during enrichment. In this study, frozen storage significantly increased the lag-duration of both *Campylobacter* species compared to reference cells by a factor of approximately 2.3. It has been recognized that freezing mainly results in damage to the membrane resulting in cell leakage as well as to DNA or DNA synthesis

due to ice crystal formation during the freezing process (Wesche et al., 2009). Humphrey and Cruickshank described that *Campylobacter* cells showed to be more sensitive to antibiotic agents after exposure to freeze stress (Humphrey & Cruickshank, 1985). The observed increase in lag-duration might be attributed to a combination of these factors. Next to a general period which is needed to adapt to the enrichment environment, cells need to initiate and carry out repair processes to deal with structural damages. This might be further impeded by the presence of the cocktail of antibiotic compounds in Bolton broth (International Organization for Standardization, 2017).

Effect of oxidative stress history on recovery during enrichment

In this study, four strains were selected to be subjected to oxidative stress based on their recovery behaviour during enrichment after freeze-stress. The strains selected were two fast-recovering and two slow-recovering strains of both, *C. jejuni* and *C. coli*. This was done to investigate, whether those strains showed a similar recovery pattern after exposure to oxidative stress. Results showed that the recovery trend was not consistent. This might be due to the lack of a general stress response system as it can be found in other bacteria and therefore differences in cellular stress response (Parkhill et al., 2000). On average, reduction after the oxidative stress treatment applied in this study was relatively limited (0.7±0.2 log_{10} cfu/ml). Under aerobic conditions, more reactive oxygen species accumulate potentially leading to damage of cellular components such as nucleic acids and proteins (Gundogdu et al., 2016; Kaakoush et al., 2009; Oh et al., 2015). Generally, *C. jejuni* has developed specific adaptation mechanisms for survival under atmospheric oxygen which deal with the removal of ROS from the cytoplasm (Kim et al., 2015). It has also been recognized, that *C. jejuni* is less susceptible to oxidative stress at low temperatures than at 42 °C, suggesting that temperature can affect oxidative stress resistance (Garénaux et al., 2008). Indeed, results of preliminary experiments showed that oxidative stress treatments conducted for 64 h in Bolton broth at 20 °C led to a significantly higher reduction than at 12 °C (data not shown). These experiments also showed that reduction was less when cells were suspended in Bolton broth base without the addition of sterile horse blood or selective supplements than when stress treatments were conducted in sterile water (data not shown). It has been suggested that pyruvate

itself and in a combination with sodium metabisulfite and ferrous sulphate can have a protective effect against oxidative stress in aerobic conditions (Chou et al., 1983; Verhoeff-Bakkenes et al., 2008). The former two can be found in Bolton broth base. Consequently, in this study, cells grown to stationary phase were transferred into Bolton broth base and subsequently exposed to atmospheric oxygen by shaking incubation at 12 °C for 64 h. It is possible, that the limited reduction observed might be attributed to the combination of low temperature and choice of Bolton broth as a medium with protective components for coping with oxidative stress.

Scenario analysis

In this study, the detection limit was set to 3 log_{10} cfu/ml, which equals on average to the transfer of 10 cells onto selective agar after enrichment, in order to reduce the risk of false-negative detection outcomes. Also, an initial contamination of 1 cell per 10 g or ml of food product was applied to mimic the growth kinetics of single cells during enrichment. In reality, contamination on broiler meat in Europe is often higher and in the range of 10^2-10^3 cfu/10g of food sample (Guyard-Nicodeme et al., 2015), but here the aim was to provide growth predictions based on the lowest initial cell concentration since this will decrease the chance of overestimating positive detection outcomes. In this study, the average μ_{max} was set to 0.93 h^{-1} as well as the 2.5 and 97.5 percentile (0.47 and 1.39 h^{-1}) to include strain specific slow and fast growth rate. Comparison with literature showed, that μ_{max} of *Campylobacter* in different growth media is often between 0.7 and 0.9 h^{-1}. Battersby et al. (2016) determined a mean μ_{max} of *Campylobacter* spp. in Bolton broth of 0.7 h^{-1} and Hazeleger et al. (1998) calculated the μ_{max} of *Campylobacter* spp. during growth at approximately 40 °C in Brain Heart Infusion broth to 0.7-0.9 h^{-1}. However, this scenario analysis is based on the outcomes of experiments conducted with isolates from various isolation sources in monoculture in culture media after undergoing a single cold-stress treatment. In reality, cells are often confronted with a multitude of processing steps which can induce sub-lethal damage (Keener et al., 2004). The combination of stressful factors could lead to increased lag-duration. A worst-scenario analysis showed that a single cell at the beginning of enrichment with a maximum growth specific rate as low as 0.47 h^{-1} could reach the detection limit still within 48 h even with lag duration as long as 23.5 h (data displayed in **figure S 5 of the supplementary materials**). However, with the stress

conditions applied in this study, no lag-duration above 8 h was found. The scenario of presence of competitive microbiota has not been considered in this study. In practice, meat, especially broilers can also be contaminated with Extended-spectrum beta-lactamase (ESBL-) producing Enterobacteriaceae. In the United Kingdom and Belgium, ESBL-producing bacteria were found on approximately 65% and 60% of tested broilers, respectively (Depoorter et al., 2012; Randall et al., 2017) and in a study from the Netherlands, 94% of all chicken breasts tested were positive for ESBL-producing bacteria (Stuart et al., 2012). Therefore, there is a possibility of co-culture scenarios during enrichment wherein the growth of *Campylobacter* cells is suppressed by a more dominant strain following the principle of the Jameson effect, that is often attributed to production of specific inhibitors of growth by one species against another (Hazeleger et al., 2016; Mellefont et al., 2008; Overdevest et al., 2011). ESBL-producing *Enterobacteriaceae* have been recognized as a challenge for reliable detection of campylobacters in food (Hazeleger et al., 2016). Further research will focus on the impact of competitive microbiota on the growth kinetics and detection outcomes of *Campylobacter* spp. during enrichment following ISO 10272-1:2017.

Conclusion

Refrigerated and frozen storage led to an increase in lag-duration of 13 *C. jejuni* and 10 *C. coli* strains in Bolton broth. Variability in lag-duration could be mainly attributed to strain variability, since biological variability was constant for all cold stress treatments and rather comparable to the reference condition. Exposure of cells to oxidative stress before enrichment showed that lag duration was not only strain dependent but also influenced by the type of stress applied. A scenario analysis on the growth kinetics of *Campylobacter* spp. during enrichment in monoculture highlighted that even in a worst-case scenario starting from one cell the limit for further successful detection was reached within 32.5 h and the probability of reaching the detection limit within 48 h was 100%. Based on these data, it seems that failures in reliable detection outcomes are not due to prolonged lag-duration and/or a reduction in specific maximum growth rate even to values as low as 0.47 h^{-1}. The outcomes of this research narrow down the reasons for false-negative detection outcomes as they regularly occur in practice. As competitive microbiota challenge the success of enrichment-based

detection, the effect of competitive microbiota on reliable detection will be subject for further research.

Acknowledgments

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Supplementary materials

Table S 1: Detailed description of the strain selection used in this study. N.a. indicates, that sequence type is unknown. Strains written in bold are reference strains used for performance testing of culture media in ISO 10272-1:2017

Species	Strain name	Isolation source	Sequence type (Clonal complex)
C. jejuni	NCTC 11168	UK, 1977, human stool	ST-21
C. jejuni	81116	UK, 1981, human stool	ST-45
C. jejuni	81-176	USA, 1985, human stool	ST-42
C. jejuni	**WDCM 00005**	no data, no data, human stool	n.a.
C. jejuni	**WDCM 00156**	USA, no data, human stool	n.a.
C. jejuni	ATCC 33560	no data, no data, bovine faeces	ST-403
C. jejuni	DSM 24306	NL, no data, chicken faeces	ST-21
C. jejuni	Ca 1809	NL, no data, chicken meat	ST-21
C. jejuni	Ca 1781	NL, no data, chicken meat	ST-45
C. jejuni	Ca 1597	NL, no data, manure	ST-45
C. jejuni	Ca 1352	NL, no data, chicken meat	ST-48
C. jejuni	Ca 2426	NL, no data, sheep manure	ST-48
C. jejuni	Ca 1087	NL, no data, chicken meat	ST-257
C. coli	**WDCM 00004**	no data, no data, marmoset faeces	n.a.
C. coli	Ca 121	NL, no data, pig manure	ST-828
C. coli	Ca 1607	NL, no data, chicken manure	ST-828
C. coli	Ca 2654	NL, 2017, turkey meat	n.a.
C. coli	Ca 2800	NL, 2017, chicken meat	n.a.
C. coli	Ca 2804	NL, 2017, lamb meat	n.a.
C. coli	Ca 2852	NL, 2017, chicken meat	n.a.
C. coli	Ca 2711	NL, 2017, bovine faeces	n.a.
C. coli	Ca 3115	NL, 2017, lamb meat	n.a.
C. coli	Ca 2771	NL, 2017, bovine faeces	n.a.

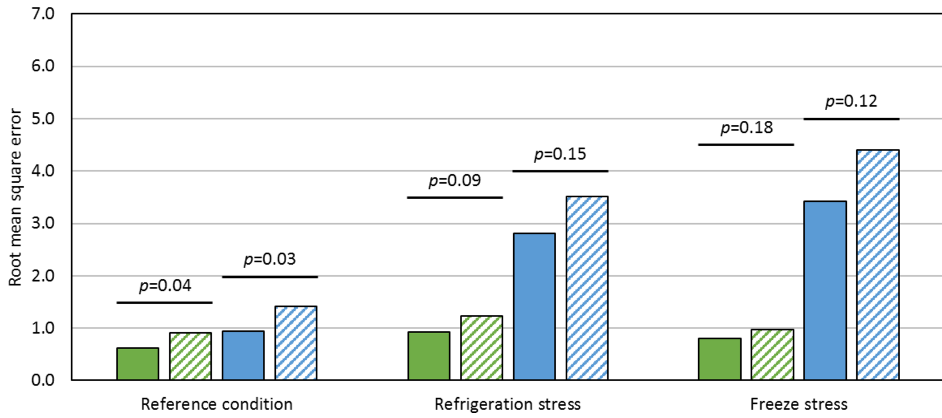

Figure S 1: Comparison of biological variability (green-coloured bars) and strain variability (blue-coloured bars) of the lag-duration after fitting the Baranyi-model (filled bars) and the modified Gompertz model (striped bars).

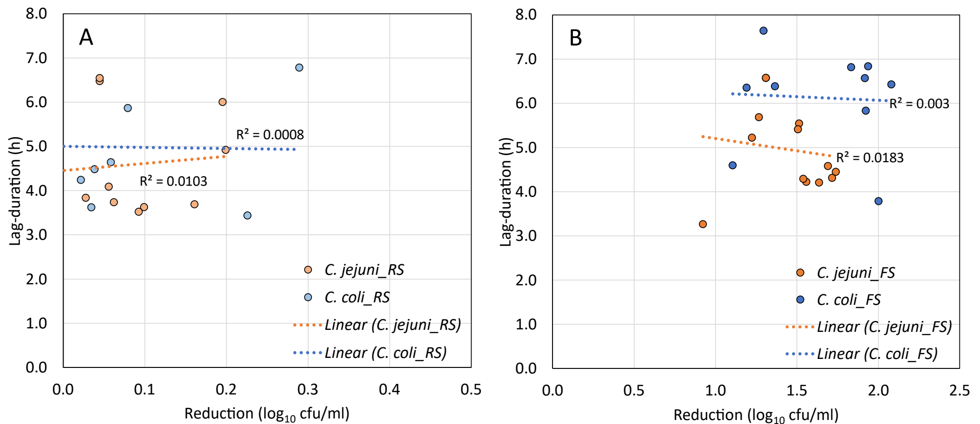

Figure S 2: Comparison of reduction in cell concentration and subsequent lag-duration of *C. jejuni* (orange-coloured dots) and *C. coli* (blue-coloured dots) after (A) refrigerated storage and (B) freeze stress.

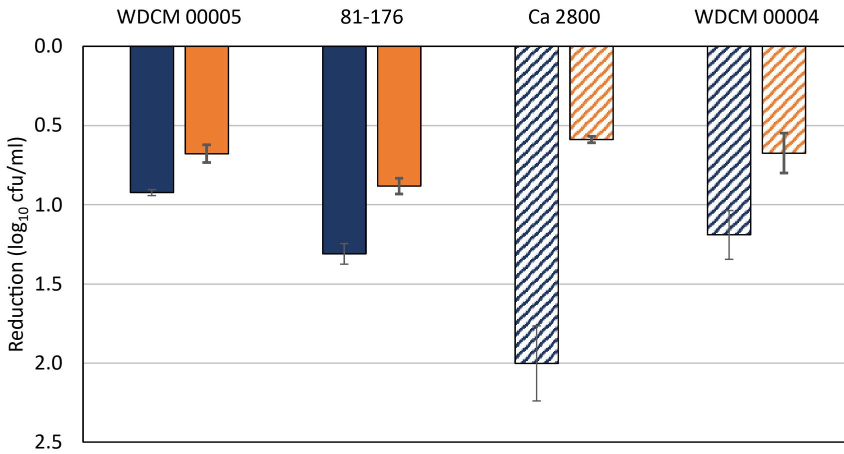

Figure S 3: Comparison of reduction in cell concentration after freeze stress (dark blue-coloured bars) and oxidative stress (orange-coloured bars). Average reduction of the four strains after freeze- and oxidative stress was 1.4±0.5 $\log_{10}$ cfu/ml and 0.7±0.2 $\log_{10}$ cfu/ml, respectively (n=4). Error bars depict the standard error of the biological reproductions (n=2).

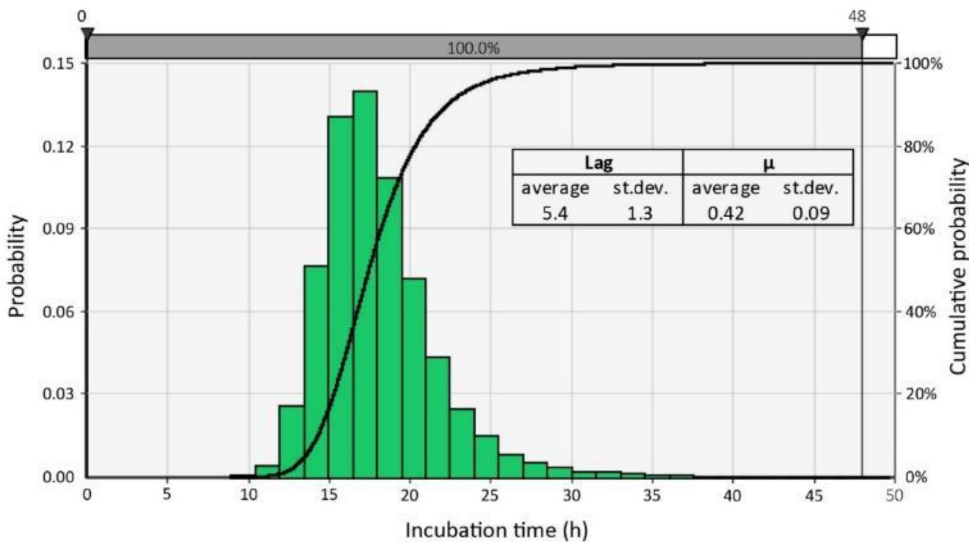

Figure S 4: Outcomes of Monte Carlo Simulation for freeze-stressed cells during enrichment in Bolton Broth. For 100.000 iterations, the time was determined for a single cell to reach the detection limit of 3 $\log_{10}$ cfu/ml after freeze-stress.

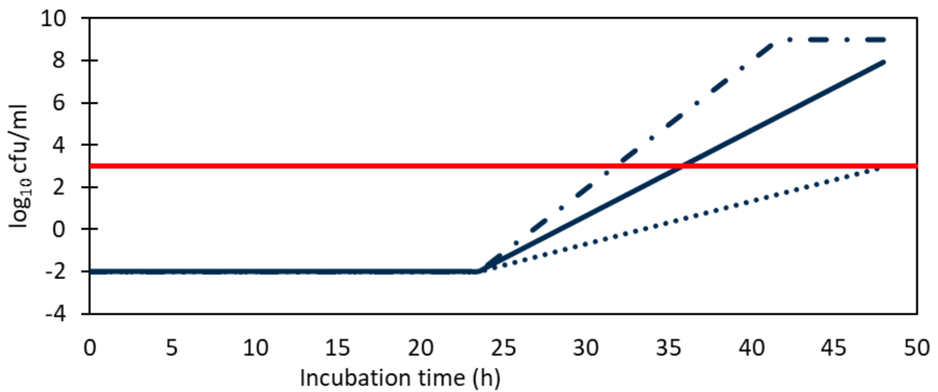

Figure S 5: Scenario analysis showing the effect of a lag-duration of 23.5 h and growth rate of 0.47 h^{-1} on reaching the detection limit within 48 h. Growth rates were estimated by plotting the mean growth rate (0.93 h^{-1}) as well as low (dotted line: 0.47 h^{-1}) and high (1.39 h^{-1}) growth rate. The red line depicts the detection limit of 3 log_{10} cfu/ml.

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3

Role of substrate availability in the growth of *Campylobacter* co-cultured with Extended-spectrum beta-lactamase-producing *Escherichia coli* in Bolton broth

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Abstract

It is well-established that Extended-spectrum beta-lactamase-producing (ESBL-) *Escherichia coli* challenge reliable detection of campylobacters during enrichment in Bolton broth (BB) following ISO 10272-1:2017. The overgrowth of *Campylobacter* by ESBL-*E. coli* in the enrichment medium BB can lead to false-negative detection outcomes, but the cause for the growth suppression is yet unknown. A plausible reason could be the competition-induced lack of certain growth substrates. Therefore, this study aimed to investigate whether campylobacters and ESBL-*E. coli* compete for the same medium components and whether this is the cause for the observed growth repression. The availability of possible growth substrates in BB was determined and changes in their extracellular concentration were measured over time during mono-culture enrichment of *C. jejuni, C. coli* or ESBL-*E. coli* as well as in co-culture enrichments of campylobacters and ESBL-*E. coli*. Comparative analysis showed lactate and fumarate utilization by *C. jejuni* and *C. coli* exclusively, whereas ESBL-*E. coli* rapidly consumed asparagine, glutamine/arginine, lysine, threonine, tryptophan, pyruvate, glycerol, cellobiose, and glucose. Both campylobacters and ESBL-*E. coli* utilized aspartate, serine, formate, a-ketoglutarate and malate. Trends in compound utilization were similar for *C. jejuni* and *C. coli* and trends in compound utilization were rather comparable during enrichment of reference and freeze-stressed campylobacters. Since final cell densities of *C. jejuni* and *C. coli* in co-cultures were not enhanced by the addition of surplus L-serine and final cell densities were similar in fresh and spent medium, growth suppression seems not to be caused by a lack of substrates or production of inhibitory compounds. We hypothesized that oxygen availability was limiting growth in co-cultures. Higher oxygen availability increased the competitive fitness of *C. jejuni* 81-176 in co-culture with ESBL-*E. coli* in duplicate experiments, as cell concentrations in stationary phase were similar to those without competition. This could indicate the critical role of oxygen availability during the growth of *Campylobacter* and offers potential for further improvement of *Campylobacter* spp. enrichment efficacy.

Introduction

For more than a decade, the foodborne pathogen *Campylobacter* is the leading cause for zoonotic gastroenteritis in the European Union (EU) and two species, in particular, are responsible for approximately 95% of human campylobacteriosis cases, namely *Campylobacter jejuni* (~84%) and *Campylobacter coli* (~10%) (European Food Safety Authority, 2019). Those thermotolerant species often live as commensals in the intestinal tract of especially avian species but are present in farm animals such as cattle, pigs, and sheep, too (Jones, 2001; Ogden et al., 2009; Vandamme and De Ley, 1991). Direct contact with animals and the consumption of undercooked foods have been identified as important sources for *Campylobacter* infections (Nauta et al., 2005). In the latter case, *Campylobacter* is introduced as a contaminant on the meat during slaughter (Shange et al., 2019) where they can survive for several weeks (Maziero and De Oliveira, 2010; Sampers et al., 2010). Although vegetables can be contaminated with *Campylobacter* cells as well (Mohammadpour et al., 2018), most human campylobacteriosis cases are associated with the consumption of raw or undercooked poultry and poultry products (European Food Safety Authority, 2019; Moore et al., 2006).

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Even though campylobacters can be present in relatively high cell concentrations (2-3 $\log_{10}$ cfu/g) on raw poultry meat (Guyard-Nicodeme et al., 2015), the detection of low amounts is crucial since *Campylobacter* cells can be present in low numbers on products that are consumed raw and campylobacters have shown to survive for a long time under adverse environmental conditions (Lee et al., 1998; Park, 2002; Sampers et al., 2010; Sopwith et al., 2008; Wilson et al., 2008) and conceivably still have a high potential to cause disease (Black et al., 1988). Hence, a sensitive yet selective method is necessary to detect *Campylobacter* in food and thereby verifying food safety control measures. In the European Union, the standard ISO 10272-1:2017 is widely applied for the detection of *Campylobacter* spp. from food products. Dependent on the expected amount of campylobacters and their history and the probable presence of competing background microbiota, different procedures are to be followed. If campylobacters are expected to be sub-lethally injured, enrichment is done in Bolton broth (BB; procedure A), while Preston broth is advised if the amount of competing background microbiota is expected to be high (procedure B) (International Organization for

Standardization, 2017). In reality, tested poultry products are often stored cold or frozen, which can induce sub-lethal damage to *Campylobacter* cells. It has been shown that the viability of *Campylobacter* spp. decreased by more than one log_{10} cfu/g when subjected to freezing (Bhaduri and Cottrell, 2004; Georgsson et al., 2006; Haddad et al., 2009; Klančnik et al., 2008; Maziero and De Oliveira, 2010; Sampers et al., 2010; Lanzl et al., 2020). On top of the viability-loss, recovery duration during enrichment also increased significantly after freeze stress compared to cells that had not been stressed before enrichment. Next to that, even though cell concentrations seem not to decline significantly after refrigerated storage, it has been shown that recovery duration during enrichment still increased significantly after refrigerated storage (Lanzl et al., 2020). At the same time contamination of food products with Extended-spectrum beta-lactamase (ESBL)-producing background microbiota cannot be excluded (Jasson et al., 2009). Enrichment in BB (procedure A) appears to be a logical choice for the recovery and growth of sub-lethally injured *Campylobacter* spp. from food products.

BB is assumed to be a nutrient-rich medium composed of enzymatic digest of animal tissues, lactalbumin hydrolysate, and yeast extract and is further supplemented with several organic compounds such as sodium pyruvate, alpha-ketoglutarate, and haemin to enhance the growth of *Campylobacter* spp. (International Organization for Standardization, 2017). However, the exact composition of the medium is not specified. Research has shown that most campylobacters are unable to metabolize carbohydrates due to interrupted Embden-Meyerhof and Pentose Phosphate pathways (Gripp et al., 2011; Kelly, 2001; Line et al., 2010; Stahl et al., 2011). Campylobacters do have a functioning set of enzymes for the citrate (TCA) cycle (Hofreuter, 2014; Stahl et al., 2012) and depend heavily on the TCA intermediates including alpha-ketoglutarate, succinate, fumarate, and malate for their energy needs. Likewise, it has been demonstrated, that *C. jejuni* can utilize the organic acids acetate, lactate, and pyruvate (Guccione et al., 2008; Thomas et al., 2011; Wright et al., 2009). *C. jejuni* can utilize free amino acids as well, with serine being the preferred amino acid followed by aspartate, asparagine, and glutamate (Hofreuter et al., 2008). However, also other amino acids such as proline and threonine can be metabolized (Guccione et al., 2008; Hofreuter et al., 2008; Wright et al., 2009) and it has been

suggested that peptides play an important role in amino acid catabolism of *C. jejuni* as well (Gao et al., 2017; Gundogdu et al., 2016; Hofreuter et al., 2006).

To suppress the growth of other background microbiota, BB is supplemented with a cocktail of antibiotic agents. However, it has been recognized that ESBL-producing *Enterobacteriaceae* hydrolyze the lactam ring of one of those antibiotics, namely, cefoperazone, thereby rendering the antibiotic inactive resulting in growth in BB (Chon et al., 2017; Hazeleger et al., 2016; Jasson et al., 2009). This explained the previously observed growth of ESBL-producing *Enterobacteriaceae* in BB, subsequently overgrowing *Campylobacter* spp. in BB and on mCCDA plates (Jasson et al., 2009; Moran et al., 2011). Determining the growth kinetics of *Campylobacter* spp. during enrichment in BB in the absence and presence of ESBL- *E. coli* (Hazeleger et al., 2016) showed that *Campylobacter* cell concentrations in the stationary phase were often lower in co-cultures which could lead to false-negative detection outcomes if ESBL-producers grow on insufficiently selective *Campylobacter* agar (such as mCCDA) thereby masking *Campylobacter* colonies

We hypothesized that compounds present in BB could be utilized by *Campylobacter* spp. as well as ESBL-*E. coli* during enrichment. Due to shorter lag-duration and higher growth rate of the latter, the medium might be depleted of shared compounds and this competition-induced lack of substrates might be the reason for growth suppression of campylobacters. Unfortunately, no information is available about the metabolic processes of *Campylobacter* spp. and ESBL-*E. coli* that take place during enrichment in BB when cells initiate growth and/or recover from stressful conditions (i.e., freeze stress). A thorough analysis and quantification of nutrients present in BB and an assessment of compound utilization by *Campylobacter* spp. and ESBL-*E. coli* during enrichment could aid the improvement of the enrichment medium to stimulate the growth of campylobacters to high concentrations when co-present with competitive microorganisms in the enrichment medium.

Materials and methods

Bacterial strains and preparation of working cultures

Two *C. jejuni* (WDCM 00005 and 81-176) and one *C. coli* isolate (Ca 2800) were selected for this study (details of all bacterial strains used in this study can be found in **table S 1 of the supplementary materials**). For the preparation of working cultures, *C. jejuni* and *C. coli* were plated from the -80 °C vials onto Columbia agar base (CAB, Oxoid, supplemented with 5% (v/v) lysed horse blood (BioTrading Benelux B.V. Mijdrecht, Netherlands) and 0.5% agar (Bacteriological agar No.1, Oxoid)) and grown microaerobically for 24 h at 41.5 °C. Subsequently, a single colony was resuspended in Heart Infusion broth (Bacto HI, Becton, Dickinson and Company) and cultured for 24 h at 41.5 °C to obtain stationary phase cultures. Afterward, working cultures were prepared by making a 1:500 dilution in unselective BB (Oxoid, supplemented with 5% (v/v) sterile lysed horse blood (BioTrading Benelux B.V., Mijdrecht, the Netherlands)) without the addition of selective supplements) and cultured for 24 h at 41.5 °C to reach the stationary phase.

For this study, initially, nine ESBL-producing *Enterobacteriaceae* strains (six *Escherichia coli*, one *Klebsiella pneumoniae*, one *Serratia fonticola,* and one *Enterobacter cloacae* isolate) were screened for their growth abilities in Brain Heart Infusion broth (Bacto BHI; Becton, Dickinson, and Company), unselective BB (BB-base + 5% sterile horse blood) and selective BB (BB-base + 5% sterile horse blood + selective supplement SR0208E), without and with pre-exposure to freeze stress for 3 days. Only three ESBL-*E. coli* isolates (RIVM 2, ESBL 3953, and ESBL 3874) were able to grow in selective BB after freeze stress (data not shown) and were therefore used in this study. Stock cultures of selected ESBL-*E. coli* strains were grown aerobically in BHI broth for 24 h at 37 °C, then supplemented with 15% glycerol (Fluka) and stored at -80 °C. To obtain working cultures, ESBL-*E. coli* were plated from the -80 °C vials onto BHI agar (Merck Millipore and 1.5% agar [Bacteriological agar No.1, Oxoid]) and grown aerobically for 24 h at 37 °C to obtain fresh colonies. One single colony was resuspended in BHI and cultured for 24 h at 37 °C to obtain stationary phase cultures. Afterward, a 1:500 dilution was made in unselective BB and cultured for 24 h at 37 °C to reach the stationary phase. Cell concentrations of the working cultures were determined by plating appropriate dilutions (made in peptone physiological salt

solution (PPS, Tritium Microbiologie)) on BHI agar plates and aerobic incubation for 24 h at 37 °C.

Application of stress treatments

For mono-culture enrichments, 1 ml of the working culture of each *Campylobacter* strain was inoculated in 5 ml of unselective BB in 15 ml plastic tubes (Greiner centrifuge tubes, Merck) to achieve a cell concentration of approx. 10^8 cfu/ml before the freeze stress treatment. To be able to observe possible growth suppression of *Campylobacter* spp., while still being able to quantify changes in extracellular metabolite quantities during co-culture enrichments, working cultures of *Campylobacter* and ESBL-*E. coli* were decimally diluted in unselective BB. Subsequently, 1 ml of the diluted ESBL-*E. coli* culture and 1 ml of the diluted *Campylobacter* culture were inoculated in 4 ml of unselective BB in plastic tubes.

For both, mono- and co-culture stress treatments, inoculated plastic tubes were placed standing upright at -20 °C for 64 ± 1 h for frozen storage. Afterward, freeze-stressed cultures were transferred to room temperature and allowed to stand until defrosted. The freeze-stress treatment resulted in a reduction of 1-1.5 $\log_{10}$ cfu/ml (data not shown).

Inoculation of infusion bottles, determination of growth kinetics, and sample preparation for HPLC and UPLC analysis

Infusion bottles were filled with 42 ml of BB, closed with a rubber stopper and aluminium cap, and sterilized. Subsequently, bottles were supplemented with 5% sterile horse blood and 450 µl of the selective supplement (Oxoid SR0208E). Bottles were filled with reference or freeze-stressed cultures using an inoculation level ($\log_{10}$ N_0) of 6-7 $\log_{10}$ cfu/ml to be able to observe outgrowth and possible growth suppression of *Campylobacter* spp. in co-culture while still being able to quantify changes in extracellular metabolite concentrations. In all other mono- and co-culture experiments (see sections 2.4 and 2.5) $\log_{10}$ N_0 was approx. 2 $\log_{10}$ cfu/ml. In all experiments, the addition of fluids to sterilized infusion bottles was achieved using syringes to puncture the rubber stopper of the bottles. The headspace of infusion bottles was flushed for 2 min with a gas mixture of 5% O_2, 10% CO_2, and 85% N_2 by a

homemade gas flushing device using needles to puncture the rubber stopper. Inoculated infusion bottles were incubated in water baths at 37 °C for the first 5 h and subsequently transferred to 41.5 °C for the remaining 43 h following ISO 10272-1:2017. After inoculation (t0), and after 2, 4, 6, 8, 24, and 48 h samples were taken from the bottles using a syringe and after 0, 4, 8, and 24 h bottles were flushed with the appropriate gas mixture.

Determination of growth kinetics

For mono-culture enrichments, samples were immediately decimally diluted in PPS (Tritium Microbiologie), plated onto CAB for *Campylobacter* spp. and incubated microaerobically for 48 h at 41.5 °C. For ESBL-*E. coli* monoculture experiments, samples were plated onto BHI-agar and incubated aerobically for 24 h at 37 °C. For co-culture enrichments, the plating was done onto RAPID *Campylobacter* agar (RCA; BIORAD) and Brilliance ESBL agar (BEA; OXOID) for campylobacters and ESBL-*E. coli*, respectively. All *Campylobacter* cells were cultured under microaerobic conditions (5% O_2, 10% CO_2, 85% N_2) in flushed jars (Anoxomat WS9000, Mart Microbiology, Drachten, Netherlands) unless stated otherwise. After incubation, cfu were counted and cell concentrations were calculated as log_{10} cfu/ml for each time point and sample. Three biologically independent reproductions per strain and stress treatment were performed on different days. In parallel to log_{10} counts determination, samples were taken for High-Performance Liquid Chromatography (HPLC) and Ultra-high Performance Liquid Chromatography (UPLC) analysis and transferred to two sterile Eppendorf tubes (2x0.5 ml), snap-frozen in liquid nitrogen, and stored at -20 °C until quantification of extracellular metabolites by HPLC and UPLC.

Sample preparation for HPLC and UPLC analysis

For the quantification of acetate, formate, lactate, propionate, pyruvate, citrate, fumarate, a-ketoglutarate, malate, succinate, ethanol, glycerol, cellobiose, and glucose, 500 µl of the defrosted sample was deproteinized by addition of 250 µl cold Carrez A (0.1 M potassium ferrocyanide trihydrate). After mixing, 250 µl cold Carrez B (0.2 M zinc sulfate heptahydrate) was added, followed by mixing and centrifugation at 17,000 x *g* for 10 minutes. 200 µl of the deproteinated sample was injected on an UltiMate 3000 HPLC (Dionex, Germany) equipped with an Aminex HPX-87H column

(300x7.8 mm) with guard-column (Biorad). As mobile phase, 5 mM H_2SO_4 was used at a flow rate of 0.6 ml/min. The column temperature was kept at 40 °C. Compounds were detected by a refractive index detector (RefractoMax 520). Amino acids (alanine, asparagine, aspartate, cysteine, glutamate, glutamine, arginine, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, valine) were quantified by UPLC. 40 µl of the defrosted sample was deproteinated by addition of 50 µl 0.1 M HCl, containing 250 µM norvaline as internal standard and 10 µl 30% sulfosalicylic acid (SSA). Subsequently, the solution was mixed and centrifuged at 17,000 x *g* for 10 minutes at 4 °C. Amino acids were derivatized using the AccQ•Tag Ultra derivatization kit (Waters, USA). 20 µl of the deproteinated supernatant or standard amino acids mixture was mixed with 60 µl AccQ•Tag Ultra borate buffer in glass vials. For deproteinated samples, 75 µl of 4 M NaOH was added to 5 ml borate buffer to neutralize the addition of SSA. Subsequently, 20 µl of AccQ•Tag reagent dissolved in 2 ml AccQ•Tag reagent diluent was added and immediately vortexed for 10 s. Then, the sample solution was heated at 55 °C in a heat block for 10 min. Amino acids were quantified by UPLC by injection of 1 µl sample on an UltiMate 3000 (Dionex, Germany) equipped with an AccQ•Tag Ultra BEH C18 column (150 mm x 2.1 mm x 1.7 µm) (Waters, USA) with BEH C18 guard column (5 mm x 2.1 mm x 1.7 µm) (Waters, USA). The column temperature was set at 55 °C and the mobile phase flow rate was maintained at 0.7 ml/min. Eluent A was 5% AccQ•Tag Ultra concentrate solvent A and Eluent B was 100% AccQ•Tag Ultra solvent B. The separation gradient was 0-0.04 min 99.9% A, 5.24 min 90.9% A, 7.24 min 78.8% A, 8.54 min 57.8% A, 8.55-10.14 min 10% A, 10.23-17 min 99.9% A. Compounds were detected by UV measurement at 260 nm. Glutamine and arginine could not be separated in the UPLC analysis due to overlapping peak areas.

Preparation of spent BB and enrichment in spent medium

After enrichments were performed as described in 2.3, 45 ml of spent media of ESBL-*E. coli* strain RIVM 2 in monoculture and co-culture with *C. jejuni* strain 81-176 were collected by transferring the (co-)culture to 50 ml Greiner tubes, and subsequent centrifugation at 17,000 x *g* for 5 min and filter-sterilization (0.2 µm filter, Sartorius Minisart™ Plus Syringe Filters, Thermo Fisher Scientific). The spent BB was then transferred into sterile 45 ml infusion bottles and used for enrichments in spent BB.

For mono-culture enrichments of *C. jejuni* strain 81-176 and *C. coli* strain Ca 2800 in spent medium, working cultures were serially diluted in PPS until approx. 10^3 cfu/ml and inoculated in spent medium to reach an inoculation concentration ($\log_{10} N_0$) of approx. 2 $\log_{10}$ cfu/ml, after which infusion bottles were flushed and incubated, and samples were taken after 0, 24, and 48 h.

Oxygen availability during enrichment

A co-culture enrichment with *C. jejuni* strain 81-176 and ESBL-*E. coli* strain RIVM 2 was performed in infusion bottles in four different atmospheric setups, starting with $\log_{10} N_0$ of approx. 2 log. In method 1, the bottle was flushed microaerobically at the start of enrichment and after 2, 4, 6, and 8 h. In method 2, the bottle was flushed microaerobically only at the start of enrichment, while in method 3, the bottle was flushed anaerobically (with N_2) only at the start of enrichment. In method 4, the bottle was not flushed, but two needles with attached 0.2 µm filters were introduced into the rubber stopper and the bottle was subsequently placed in a big jar (AJ9028) which was flushed microaerobically with the Anoxomat. All bottles were incubated at 41.5 °C for 24 h and cell concentrations were determined on RCA and BEA at the start of enrichment and after 24 h of incubation.

Data and statistical analysis

For all experiments conducted to assess the composition of BB as well as the utilization of compounds in BB during enrichment, three biological reproductions were taken and means, and standard errors were determined for each time point, strain, compound, and history. For the determination of cell concentrations ($\log_{10}$ cfu/ml), the mean and standard error of three biological reproductions were calculated. To determine whether differences in compound availability after 48 h (compared to t_0) were significant, Student's t-tests were performed, and significance was determined with *p*-values with Bonferroni-correction ($p=0.05/$(amount of t-tests performed)$= 0.05/132=0.00038$). To determine whether the increase in cell concentrations of *C. jejuni* and *C. coli* after 24 and 48 h of enrichment C-spent BB and C&E-spent BB (section 2.4) was significantly different compared to growth in fresh BB, Student's t-tests were performed using a significance value of $p=0.05$. Similarly, to determine whether differences in cell concentration of *C. jejuni* and *C. coli* after 24 h

of enrichment were significant in different atmospheric settings, Student's t-tests were performed using a significance value of p=0.05.

Results

Compound availability in selective BB

At the start of each monoculture enrichment the available amounts of the 20 essential amino acids, organic acids (acetate, formate, lactate, propionate, and pyruvate), TCA-cycle intermediates (citrate, fumarate, a-ketoglutarate, malate, and succinate), alcohols (ethanol and glycerol) and carbohydrates (glucose and cellobiose were measured and an overview is given in **figure 1 A**. Interestingly, BB contained all essential amino acids, of which the amounts were in most cases lower than 1 mM except for glycine (1.19±0.15 mM). Acetate, pyruvate, a-ketoglutarate, malate, ethanol, and the disaccharide cellobiose were present in relatively high amounts (between 1.11±0.21 mM for pyruvate and 2.47±0.67 mM acetate) and some other organic compounds were present in even higher amounts, as BB contains approximately 5 mM of lactate (5.09±0.3 mM) and more than 3 mM of succinate (3.1±0.35 mM). The concentration of each compound in BB can also be found in a table format **in table S 2 of the supplementary materials**.

Figures 1 B and C show the measured differences in the availability of each of the measured media components after 48 h of incubation (>24 h in the stationary phase) compared to the fresh medium. Graph B depicts the measured difference for each of the compounds in the enrichment medium for reference *Campylobacter* cells and freeze-stressed cells. Trends in compound utilization were similar for the two *C. jejuni* and one *C. coli* tested (detailed information on the utilization pattern of each strain can be found in the supplementary materials) and therefore changes in compound utilizations are represented as the overall mean of the genus *Campylobacter*.

Trends in compound utilization were comparable after 48 h of enrichment of reference and freeze-stressed campylobacters but observed changes (both positive and negative) were higher, yet insignificantly for reference cells compared to freeze-stressed cells during enrichment for all but one compound. The only exception was

lactate, which was consumed significantly ($p<0.001$) more by reference cells than freeze-stressed cells.

Likewise, a delay in compound utilization could be observed consistently for freeze-stressed cells throughout enrichment, which correlates with an increased lag duration of freeze-stressed campylobacters during enrichment (details on the kinetics of compound utilization in monoculture enrichments can be found in **figures S 1 to S 4 of the supplementary materials** and details on the growth kinetics of cells during enrichment can be found in **figure S 9 of the supplementary materials**). The same observations could be made for the trends in compound utilization of ESBL-*E. coli* as well, although differences were less obvious.

Acetate and succinate were secreted into the medium in high amounts, while other compounds (glycine, histidine, isoleucine, methionine, proline, valine, and glycerol) were secreted in lower, yet significant amounts after enrichment of reference and freeze-stressed *Campylobacter* cells. The amounts of serine, lactate, a-ketoglutarate, and malate decreased considerably after enrichment of reference and freeze-stressed *Campylobacter* cells, while a decrease in ethanol could be measured by reference cells as well. A lower, yet significant decrease in compound availability after 48 h of enrichment could too be observed for aspartate, citrate, formate, and fumarate.

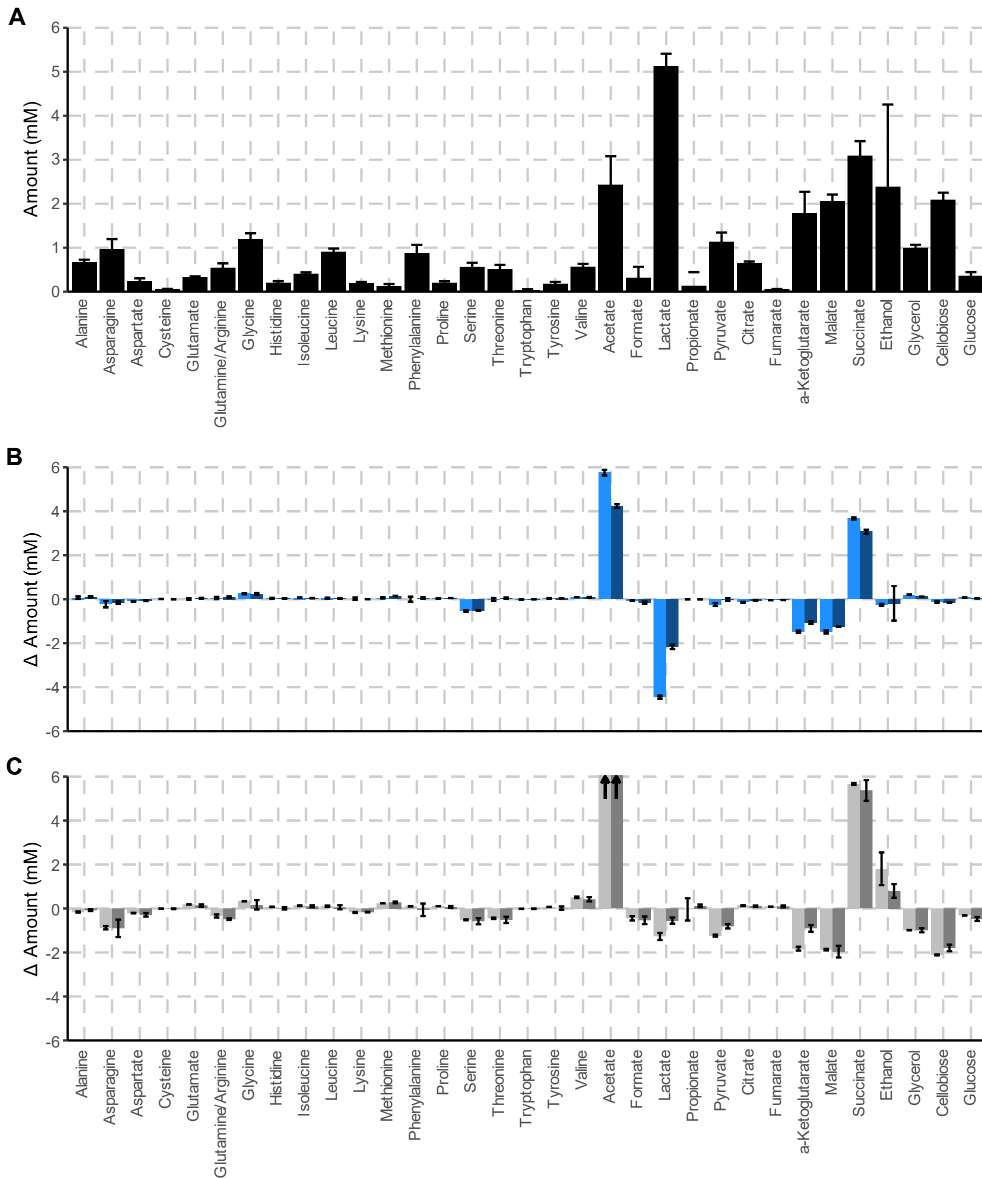

Figure 1: Composition of BB (A) and change of compound concentration after 48 h of enrichment of reference and freeze-stressed *Campylobacter* spp. (B) and ESBL-*E. coli* (C) cells. The composition of selective BB before enrichment is depicted in graph A. The amount of each compound (in mM) is indicated in black-colored bars. The error bars depict the standard deviation (n=36). *Figure text continued on next page.*

Figure 1 continued: Graph B shows the difference in compound amount after 48 h of mono-culture enrichment of reference (light blue) and freeze-stressed cells (dark blue) relative to the start of the enrichment of *Campylobacter* spp. (bars depict the average of three reproductions of two *C. jejuni* and one *C. coli* species). The error bars depict the standard deviation (n=3). Graph C shows the difference in compound amount after 48 h of mono-culture enrichment of reference (light grey) and freeze-stressed cells (dark grey) relative to the start of the enrichment of ESBL-*E. coli* (bars depict the average of three reproductions of 3 ESBL-*E. coli* strains). The error bars depict the standard deviation (n=9). In graph C, the arrows (for the compound acetate) indicate that the increase in amount was above 6 mM (17.2±0.6 mM and 15.3±1.7 mM for reference and freeze-stressed cells, respectively).

The changes in compound availability after 48 h of enrichment of ESBL-*E. coli* are shown in graph C, with changes depicted in light and dark grey bars for reference cells and freeze-stressed cells, respectively. Results showed that amounts of acetate and succinate were considerably increased. In the case of acetate, an increase of 17.2±0.6 mM and 15.3±1.7 mM was measured for reference cells and freeze-stressed ESBL-*E. coli* cells, respectively. An increase in ethanol could also be observed which, however, was not significant due to large variation between reproductions. Next to that, the amino acids glutamate, isoleucine, methionine, proline, and valine were secreted in lower, yet significant amounts after enrichment of reference and freeze-stressed cells. The amounts of asparagine, glutamine/arginine, serine, threonine, pyruvate, a-ketoglutarate, malate, glycerol, cellobiose, and glucose decreased considerably after enrichment of reference and freeze-stressed ESBL-*E. coli* cells, while a yet insignificant decrease could be measured for lactate as well. A lower, yet significant decrease in compound availability after 48 h of enrichment could too be observed for aspartate, lysine, tryptophan, and formate.

Comparison of compound utilization of *Campylobacter* spp. and ESBL-*E. coli* during enrichment

The VENN diagram in **figure 2** gives an overview of the significant decrease of the measured compounds for reference and freeze-stressed cells of *Campylobacter* spp. (*C. jejuni* and *C. coli*) and ESBL-*E. coli*.

ESBL-*E. coli* was able to utilize a wider range of amino acids present in BB (namely asparagine, glutamine/arginine, lysine, threonine, and tryptophan). Next to that,

both, the target pathogens *C. jejuni* and *C. coli* and the competitors ESBL-*E. coli* were able to utilize aspartate, serine, formate, a-ketoglutarate, and malate present in BB during enrichment. Results showed that most compounds that were utilized by *Campylobacter* spp. were too, and more rapidly utilized, by ESBL-*E. coli.* Furthermore, the latter was able to utilize pyruvate, glycerol, and the two carbohydrates cellobiose and glucose during enrichment, while on the other hand, lactate, citrate, and fumarate decreased from the medium by *Campylobacter* spp., exclusively.

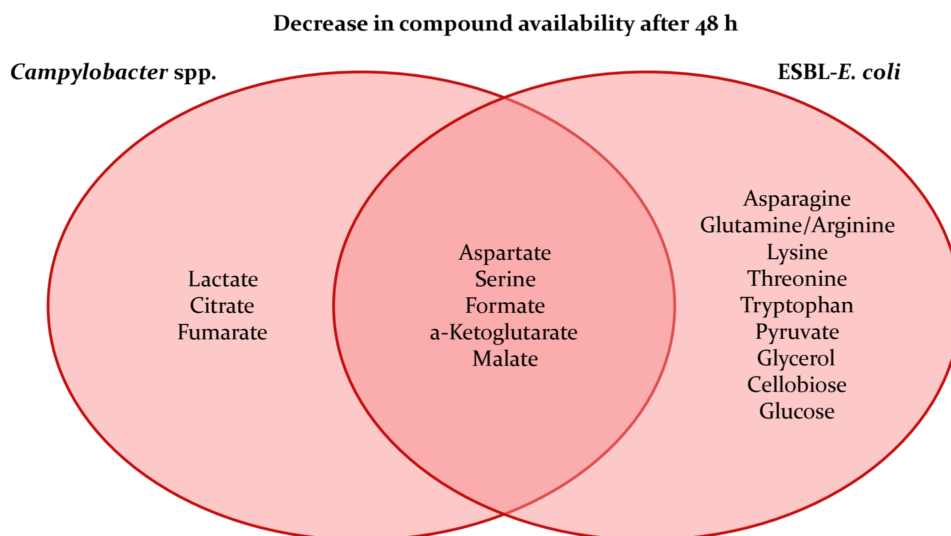

Figure 2: Significant decrease in compound availability after 48 h of enrichment in BB of *Campylobacter* spp. or ESBL-*E. coli.* The decrease of measured compounds is depicted in the red VENN diagram. Compounds depicted in the left and right circles are significantly taken up by *Campylobacter* spp. and ESBL-*E. coli*, respectively. Compounds utilized by both are depicted in the shared section. For all three sections, only compounds are depicted of which a significant decrease ($p<0.00013$) could be measured for reference and freeze-stressed cells.

Utilization of all compounds was also investigated during co-culture enrichments, whereby no differentiation could be made between utilization by campylobacters or ESBL-*E. coli* (an overview can be found in **figures S 5-8 of the supplementary materials**). Comparison of compound utilization patterns between mono- and co-culture enrichments revealed that trends in utilization of compounds during co-culture were more similar to those of ESBL-*E. coli* than campylobacters.

Serine utilization by *Campylobacter* spp. and ESBL-*E. coli* during mono- and co-culture enrichments

All compounds that were utilized by ESBL-*E. coli* were taken up almost to exhaustion within 48 h of enrichment with an initial inoculum of 6-7 log_{10} cfu/ml. While most compounds (aspartate, formate, a-ketoglutarate, lactate, and malate) were taken up from the medium by *Campylobacter* spp. to some extent, only serine, which is known to be the most preferred growth substrate for campylobacters, was taken up almost to exhaustion after 24 h by reference cells and after 48 h of enrichment by freeze-stressed *Campylobacter* cells. **Figure 3** depicts the changes in the amount of serine in the enrichment medium over time in monoculture and co-culture for reference and freeze-stressed cells. For campylobacters during mono-culture growth, initially, no significant change in extracellular serine could be observed. A significant uptake in serine could be measured after six h for reference cells and even later for freeze stressed cells, After 24 h of enrichment, approximately 93 and 81% of the available extracellular serine was taken up by reference and freeze-stressed cells of *Campylobacter* spp., respectively. After 48 h, the amount of extracellular serine was 0.038±0.002 mM and 0.034±0.001 mM for reference and freeze-stressed campylobacters, which constituted approximately 6-7% of serine initially available in BB. Notably, during the mono-culture growth of all three ESBL-*E. coli* strains, serine was taken up from the medium almost until exhaustion (~95%) already after 2 and 4 h of enrichment in BB of reference and freeze-stressed cells, respectively, and co-culture experiments showed similar serine utilization trends as ESBL-*E. coli* in mono-culture.

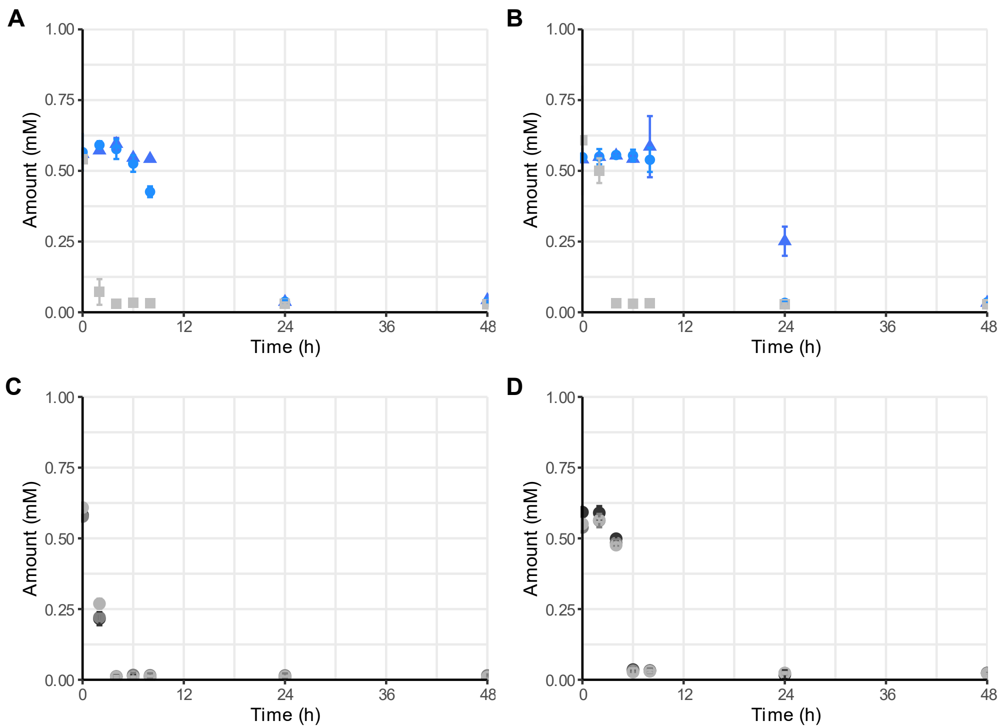

Figure 3: Utilization of serine by *C. jejuni*, *C. coli* and ESBL-*E. coli* during monoculture (panels A and B) and co-culture (panels C and D)-enrichment in BB. Serine utilization during mono-culture enrichments is depicted in graphs A for reference cells and B for freeze-stressed cells. The amount (mM) of serine at different time points and by different strains is depicted as blue dots and triangle for *C. jejuni* and *C. coli*, respectively and grey squares for ESBL-*E. coli*. Serine utilization during co-culture enrichments is depicted in graphs C for reference cells and D for freeze-stressed cells. During co-culture enrichment, no distinction could be made between serine uptake by campylobacters or ESBL-*E. coli*, therefore the serine amount (mM) is depicted in three shades of grey for the different co-culture combinations. The error bars depict the standard deviation of three reproductions (n=3).

Growth of *Campylobacter* spp. in L-serine-enriched and spent BB

With serine being a critical growth substrate for campylobacters, we hypothesized that a competition-induced lack of serine could be the reason for the faster transition of *Campylobacter* into stationary phase during co-culture enrichments resulting in a lower cell concentration (growth kinetics are depicted in **figure S 9 of the supplementary materials**). As a next step, BB was supplemented with 1 mM of L-serine and a mono-culture enrichment was performed with one *C. jejuni* and one *C. coli* strain. Observed differences in cell concentrations in BB and BB supplemented with 1 mM L-serine were less than 0.5 log_{10} cfu/ml after 24 and 48 h (data not shown). Since results showed that L-serine was rapidly utilized by ESBL-*E. coli* in mono-culture and that utilization trends during co-culture looked rather similar, a follow-up experiment was conducted to test whether supplementation of BB with 5 and 25 mM of L-serine could result in higher cell concentration of reference cells of *C. jejuni* 81-176 and *C. coli* Ca 2800 during co-culture enrichment with ESBL-*E. coli* RIVM 2. No obvious beneficial effect of supplementation with 5 or 25 mM of L-serine could be observed since the increase in cell concentration between the start of enrichment and the reach of the stationary phase (Δt_{24}-t_0) was less than 0.5 log_{10} cfu/ml for both *Campylobacter* strains compared to BB (**figure S 10 of the supplementary materials**). To assess whether the observed growth arrest of campylobacters during co-culture growth with ESBL-*E. coli* might be due to growth-induced lack of other medium compounds, mono-culture enrichments of *C. jejuni* and *C. coli* were conducted in spent BB that was obtained from 48 h-monoculture and co-culture incubations of *Campylobacter* and/or ESBL-*E. coli* (**figure 4**). The log-change in growth between stationary phase and initial cell concentration (Δt24-to) was determined to compare the growth ability of *C. jejuni* and *C. coli* in different spent BB compared to fresh BB. After 24 h of enrichment of strain 81-176 in C-spent and C&E-spent BB, an increase in cell numbers of 6.6±0.13 and 6.2±0.18 log_{10} cfu/ml, respectively, could be observed, which was comparable to the increase in cell concentrations of the same strain in fresh BB (6.5±0.18 log_{10} cfu/ml). Outcomes of Student's t-tests also confirmed that differences in log-increase after 24 h between both spent BB and fresh BB were insignificant (p=0.52 and p=0.25, respectively). The same trend could be observed after 48 h, as differences in log-increase remained similar (p=0.08 and p=0.06, respectively). For strain Ca 2800, after 24 h, a log-change

of 6.7±0.12 and 5.9±0.02 log_{10} cfu/ml could be observed in C-spent and C&E-spent BB, respectively, compared to 5.8±0.06 log_{10} cfu/ml in fresh BB. Correspondingly, significance testing showed, that growth was comparable in C&E-spent BB (p=0.28), but even better in C-spent BB (p=0.01). Comparably, log-changes after 48 h showed the same trends (p=0.05 and p=0.03, respectively). Since growth of both strains was similar in spent BB compared to fresh BB, this indicates not only that BB contains a surplus of essential substrates for the growth of campylobacters but also that the growth suppression during co-culture enrichment is not due to competition-induced lack of critical growth substrates or production of inhibitory compounds.

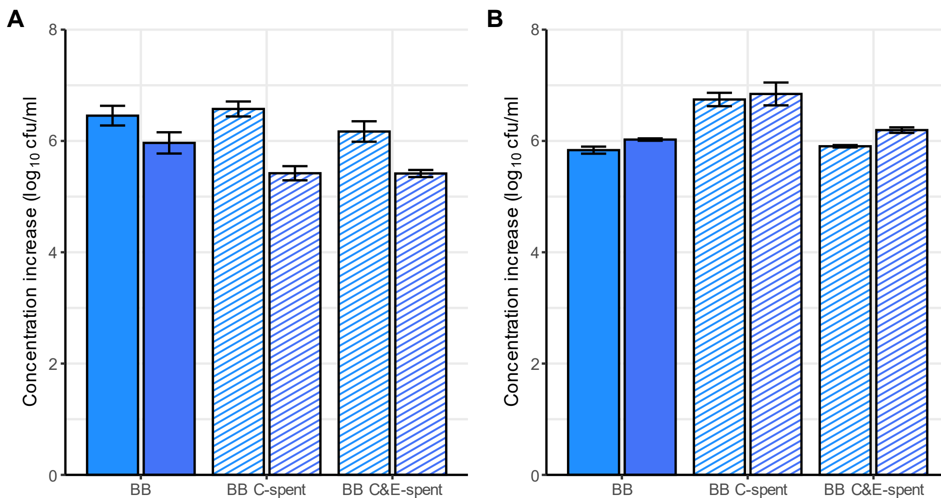

Figure 4: Increase in cell concentrations (after 24 and 48 h) of *C. jejuni* strain 81-176 (A) and *C. coli* strain Ca 2800 (B) during monoculture enrichment in fresh BB and spent BB. The increase in cell concentrations in BB and different spent media are depicted after 24 and 48 h of mono-culture enrichment in BB (medium and dark colored bars, respectively). Filled bars depict the increase in cell in cell concentrations of *C. jejuni* 81-176 (A) or *C. coli* Ca 2800 (B) in BB, while striped bars show the growth in two different spent BB (BB C-spent indicates the medium BB which was previously used for a 48-h enrichment of *C. jejuni*, while C&E-spent BB was used previously for a 48-h co-culture enrichment of *C. jejuni* and ESBL-*E. coli*). Error bars depict the standard deviation of 2 reproductions (n=2).

Growth of *C. jejuni* during co-culture enrichments in different atmospheric settings

Since metabolite production during mono- and co-culture enrichments indicated a potential lack of oxygen (e.g., no acetate switch and high production of succinate by both, and ethanol by ESBL-*E. coli*), we hypothesized that a lack of oxygen could be the reason for the lower stationary phase cell concentration of campylobacters during co-culture growth. Therefore, a duplicate experiment was conducted in four different atmospheric setups.

For all enrichments conducted in this study, small infusion bottles (volume of max. 100 ml) were used which were filled with 50 ml of enrichments fluids (45 ml selective BB + 5 ml diluted cells). The bottles were closed with rubber stoppers and the environment in the remaining headspace was altered in four different ways. For methods 1 to 3, headspaces were flushed only once or at different time points with gas mixtures either containing 5% O_2 or no oxygen at all, while method 4 allowed continuous gas exchange with a microaerobic environment throughout enrichment. To assess whether the different setups had an impact on the cell concentrations of *C. jejuni* strain 81-176 in stationary phase during co-culture enrichment with ESBL-*E. coli*, cell concentrations were determined at the start of enrichment and after 24 h (stationary phase). **Figure 5** shows that, while cell concentrations at the start were comparable for all four methods (~2 $\log_{10}$ cfu/ml), a comparison of cell concentrations in the stationary phase revealed, that *Campylobacter* strain 81-176 grew the least well in method 1 (7.2±0.08 $\log_{10}$ cfu/ml) and best in method 4 (8.4±0.11 $\log_{10}$ cfu/ml), with final CFUs reached similar to that in monoculture experiments. Further statistical analysis showed that differences in cell concentration after 24 h were only significant ($p=0.03$) for method 4 ($p=0.12$ for both method 2 and method 3) The growth of the facultatively anaerobic ESBL-*E. coli* strain was unaffected by the different methods as cell concentrations after 24 h were statistically insignificant ($p=0.96$, $p=0.39$ and $p=0.06$ for method 2, method 3 and method 4, respectively) for all four methods (between 8-8.5 $\log_{10}$ cfu/ml). Therefore, the outcomes of this experiment showed, that at least for *C. jejuni* strain 81-176, constant availability of microaerobic conditions lead to increased cell concentrations during co-culture enrichments with ESBL-*E. coli.*

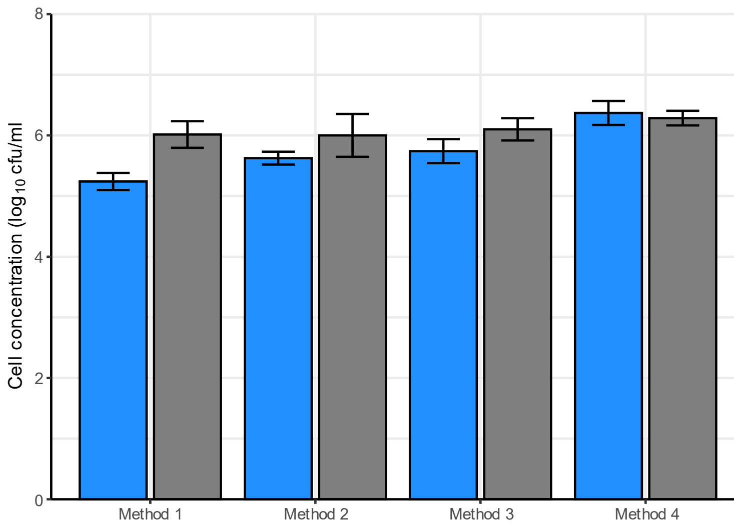

Figure 5: Increase in cell concentrations (Δ_{t24-t0}) of *C. jejuni* strain 81-176 (blue bars) and ESBL-*E. coli* strain RIVM 2 (grey bars) after 24 h of co-culture enrichment in four different atmospheric settings. Infusion bottles were flushed at the beginning of enrichment as well as after every two sampling points (method 1) or flushed only once at the beginning of enrichment either microaerobically (method 2) or anaerobically (method 3). For method 4, infusion bottles were not flushed but incubated in a microaerobic jar (AJ9028) with needles attached to the rubber stopper to allow constant gas exchange. Error bars depict the standard deviation of 2 reproductions (n=2).

Discussion

In this study, we measured the availability of extracellular amino acids, organic acids, TCA-cycle intermediates, carbohydrates, and alcohols in the *Campylobacter* enrichment medium BB as well as changes in their availability throughout mono- and co-culture enrichment of campylobacters and ESBL- *E. coli*. Some compounds like a-ketoglutarate and pyruvate are known to be added to the base to enhance the growth of campylobacters but a great proportion of the base consists of enzymatic digest of animal tissues, lactalbumin hydrolysate, and yeast extract, which together are rich sources of nitrogen, carbon, amino acids, peptides, vitamins, and carbohydrates. While most of these compounds are necessary and beneficial for the growth of other microorganisms, campylobacters rely on only a small selection of nutrients for their growth. It is fairly known, that, in contrast to other microorganisms, campylobacters

are generally unable to utilize some mono- and di-saccharides like glucose and cellobiose (Parkhill et al., 2000), which are both available in BB. In contrast, they rely on several available TCA-intermediates, organic acids, and amino acids for their growth (Mohammed et al., 2004; Stahl et al., 2012; Wright et al., 2009). In this study, the changes in compound availability were not only observed for reference but also for freeze-stressed campylobacters. Since results showed similar utilization patterns for reference and freeze-stressed *Campylobacter* and ESBL-*E. coli*, we could conclude that none of the examined compounds was utilized specifically for repair and recovery purposes. During mono-culture enrichments of *Campylobacter* spp., the concentration of extracellular serine, aspartate, lactate, a-ketoglutarate, formate, fumarate, and malate decreased significantly after 48 h of enrichment both for reference and freeze-stressed cells, which is in line with existing literature on the metabolic needs of *Campylobacter*. Membrane transporters have been identified for the uptake of most of these compounds (Stahl et al., 2012) and a periplasmic formate dehydrogenase has been described for the metabolism of formate (Kassem et al., 2017). It has been previously described that *C. jejuni* utilizes amino acids in sequential order with a high preference for serine followed by aspartate, while the uptake of other amino acids is dependent on the availability of other nutrients (Wright et al., 2009). In this study, serine was taken up from the medium (almost) to exhaustion after 48 h for both, reference and freeze-stressed cells, while at the same time, still almost half (46.1%) and approximately two-thirds (69.4%) of the initial aspartate was left in the medium. In this study, no significant uptake of asparagine, glutamate, and proline could be observed. It is possible, that the beforementioned amino acids were not utilized significantly within the 48 h-long enrichment, since there was still a high availability of the more preferred amino acid aspartate.

Likewise, glutamate, proline, pyruvate, acetate, and succinate have been identified as possible growth substrates for *C. jejuni* (Hofreuter, 2014; Stahl et al., 2012), and our study showed that the concentration of extracellular pyruvate decreased only for the *C. coli* strain tested, and this could be only observed for reference cells. It has been recognized that pyruvate plays an important role in the metabolism of *C. jejuni* (Velayudhan and Kelly, 2002), although no pyruvate transporters have been identified yet. However, it is known that pyruvate can be produced intracellularly through the metabolism of serine and lactate, and for some isolates also L-fucose (Stahl et al.,

2012). Previous studies have identified acetate and succinate as growth substrates for *C. jejuni* (Stahl et al., 2012). Wright et al. (2009) observed that acetate, which was previously secreted during the exponential phase, was taken up in late stationary phase (after 28 h) and used as a growth substrate by *C. jejuni* when grown in Brain-Heart-Infusion broth. They conjectured, that this 'acetate switch' was due to nutrient depletion. In this study, no 'acetate switch' could be observed, which is plausible since BB was still rich in growth substrates at the end of enrichment (after 48 h). To further substantiate this, a *C. jejuni* and a *C. coli* strain were grown in BB which was previously used for the enrichment of *C. jejuni* and both strains were still able to reach 8-8.5 log_{10} cfu/ml after 24 h of enrichment (**figure 4**), which further supports the conclusion that BB was a very rich medium, which more than sufficiently supports the growth of campylobacters throughout enrichment. Indeed, reported maximum specific growth rates in BB (Lanzl et al., 2020) were comparable to the reported maximum specific growth rates in unselective rich Brain heart infusion medium (Battersby et al., 2016; Hazeleger et al., 2016). Research on metabolism has been primary focussed on *C. jejuni* and considerably less on *C. coli*, which raised the question of whether compound utilization was similar for both species. A study by Wagley et al. (2014) compared the carbon source utilization of 13 *C. jejuni* and 2 *C. coli* isolates and concluded, that both species were generally able to utilize the same core carbon sources (serine, aspartate, asparagine, proline, pyruvate, malate, succinate, fumarate, lactate, and formate), while glutamate and glutamine were only utilized by certain *C. jejuni* strains and propionate only by the two *C. coli* strains tested. In this study, two isolates of *C. jejuni* and one *C. coli* isolate were tested and in general compound utilization patterns were similar for both species except for pyruvate, which was only utilized by the reference *C. coli* culture. It is possible, that trends for the utilization of certain compounds might be slightly different for other strains.

Although mono-culture enrichments of three ESBL-*E. coli* isolates showed that most of the compounds metabolized by campylobacters could be utilized by ESBL-*E. coli* as well, the current study demonstrates that the observed growth arrest of campylobacters during co-culture enrichments with ESBL-*E. coli* could not be explained by a lack of nutrients in BB, since campylobacters grew well in spent medium. One additional reason for conducting enrichments in C&E-spent BB (figure 4) was to investigate possible growth inhibition by inhibitory compounds produced

by *E. coli* (e.g., colicins) at high cell concentrations to outcompete *Campylobacter* during co-culture enrichments. This inhibitory behaviour was previously described for *E. coli* in co-competition with other microorganisms than *Campylobacter* (Cascales et al., 2007; Kerr et al., 2002; Lenski and Riley, 2002). The fact that *Campylobacter* growth in C&E-spent BB was comparable to that in fresh BB and that an agar plate diffusion assay did not show inhibition zones (data not shown) led to the conclusion that either no colicins were produced or at least not to an extent that would impede the growth of campylobacters. Note that throughout this study, cell concentrations were determined by applying the plating technique. Although it is a widely used method to determine differences in cell concentrations, this method has its limitations (e.g., plating error) and therefore, unless stated otherwise, all experiments were performed using (at least) two biologically independent reproductions. Next to inhibitory compounds, also an increase in acidification of BB was considered, especially concerning the high amounts of acetate and succinate produced by ESBL-*E. coli* during enrichment. However, pH measurements after 0, 24, and 48 h of enrichment in mono- and co-culture showed that acidification of the medium could be ruled out as well, as the pH of BB remained neutral (between 6.5-7.5) for all measurements throughout the study (data not shown), which is optimal for growth of campylobacters (Doyle and Roman, 1981). All results gathered until this point led to the conclusion, that BB sufficiently supports the growth of campylobacters during enrichment and that the observed early transition into stationary phase is not caused by lack of growth substrates or presence of inhibitory compounds in BB.

Based on the results of the duplicate experiments (**figure 5**) where a constant availability of a microaerobic environment lead to higher cell concentrations of *C. jejuni* strain 81-176 during co-culture enrichment, it is conceivable that oxygen availability could influence the final cell concentration of *Campylobacter* in co-culture with *E. coli*. Campylobacters are known to grow only under microaerobic conditions. Although they can get some energy from fermentation, low amounts of oxygen are required for the proper functioning of an enzyme (class I ribonucleotide reductase) responsible for DNA synthesis (Sellars et al., 2002). *E. coli* is widely known to be able to grow under both, aerobic and anaerobic conditions. Although energy yields are higher aerobically compared to anaerobically, at high growth rates, *E. coli* has been shown to exhibit 'overflow metabolism, where it switches from respiration to

fermentation to avoid a high density of respiratory proteins in the cell membrane (Szenk et al., 2017). We hypothesized that in microaerobic conditions, ESBL-*E. coli* consumes oxygen during growth depleting the medium and headspace of the infusion bottles of oxygen. As ESBL-*E. coli* are also able to grow anaerobically, their growth would be unaffected, while the growth of campylobacters, being obligate microaerobic, would be impeded.

Indeed, when oxygen exchange was made possible during the entire enrichment period, *C. jejuni* was able to grow to cell concentrations comparable to those of monoculture enrichments. While ISO 10272-1:2017 does not provide instructions or suggestions concerning the settings of an enrichment, our results indicate that facilitating the constant gas exchange with a microaerobic gas mixture might be used to optimize the enrichment of campylobacters in BB resulting in higher final cell concentrations. However, this experiment was conducted for one *C. jejuni* strain only and therefore, more experimental research should be conducted with also other *Campylobacter* strains to extend our findings.

Conclusion

This study aimed to investigate whether growth inhibition of *Campylobacter* spp. during co-culture enrichments with ESBL-*E. coli* in BB could be the result of a competition-induced lack of growth substrates. This study demonstrated that BB contains in surplus all essential substrates for the growth of *Campylobacter* in co-culture with competitive ESBL-*E. coli*. However, higher oxygen availability increases the competitive fitness of *Campylobacter* in co-culture with ESBL-*E. coli*. This opens avenues to optimize the enrichment method when competitive background flora is expected to be present.

Acknowledgments

The authors thank Dr. Marc Wösten and Dr. Nancy Bleumink-Pluym from the Department of Infectious Diseases & Immunology, Infection Biology of Utrecht University, and Dr. Greetje Castelijn and Dr. Menno Van Der Voort from Wageningen Food Safety Research for providing strains used in this research. Furthermore, the

authors thank all project partners (Biomérieux, DSM, Innosieve Diagnostics, Merck Darmstadt, NWO, and Wageningen Food Safety Research) for their financial and in-kind contributions. This work is part of the research program "Rapid and reliable enrichment-based detection of foodborne pathogens" with project number 15002, which is partly financed by the Dutch Research Council (NWO).

Supplementary materials

Table S 1: Detailed description of the strain selection used in this study. The strain written in bold is a reference strain used for performance testing of culture media in ISP 10272-1: 2017

Species	Strain name	Isolation source (country, year, source)
C. jejuni	81-176	USA, 1985, human stool
C. jejuni	**WDCM 00005**	no data, no data, human stool
C. coli	Ca 2800	NL, 2017, chicken meat
ESBL *E. coli*	RIVM 2	NL, 2011, chicken liver
ESBL *E. coli*	ESBL 3953	NL, no data, beef
ESBL *E. coli*	ESBL 3874	NL, no data, chicken manure

Table S 2: Availability of each of the tested compounds in BB. The average concentration and standard deviation (n=18) is given either in µM (for amino acids) or mM (for organic acids, TCA-cycle intermediates, alcohols and carbohydrates

Compound	Mean concentration ± SD
Alanine	670 ± 61 µM
Asparagine	954 ± 247 µM
Aspartate	237 ± 75 µM
Cysteine	49 ± 16 µM
Glutamate	322 ± 25 µM
Glutamine/Arginine	546 ± 105 µM
Histidine	199 ± 39 µM
Isoleucine	406 ± 35 µM
Leucine	910 ± 77 µM
Lysine	190 ± 31 µM
Methionine	120 ± 48 µM
Phenylalanine	868 ± 211 µM
Proline	203 ± 36 µM
Serine	564 ± 102 µM
Threonine	505 ± 111 µM
Tryptophan	34 ± 19 µM
Tyrosine	181 ± 44 µM
Valine	562 ± 76 µM
Acetate	2.47 ± 0.67 mM
Formate	0.33 ± 0.27 mM
Lactate	5.09 ± 0.30 mM
Propionate	0.16 ± 0.32 mM
Pyruvate	1.11 ± 0.21 mM
Citrate	0.64 ± 0.05 mM
Fumarate	0.05 ± 0.01 mM
a-Ketoglutarate	1.74 ± 0.50 mM
Malate	2.04 ± 0.16 mM
Succinate	3.10 ± 0.35 mM
Ethanol	2.56 ± 1.93 mM
Glycerol	0.99 ± 0.07 mM
Cellobiose	2.08 ± 0.16 mM
Glucose	0.36 ± 0.09 mM

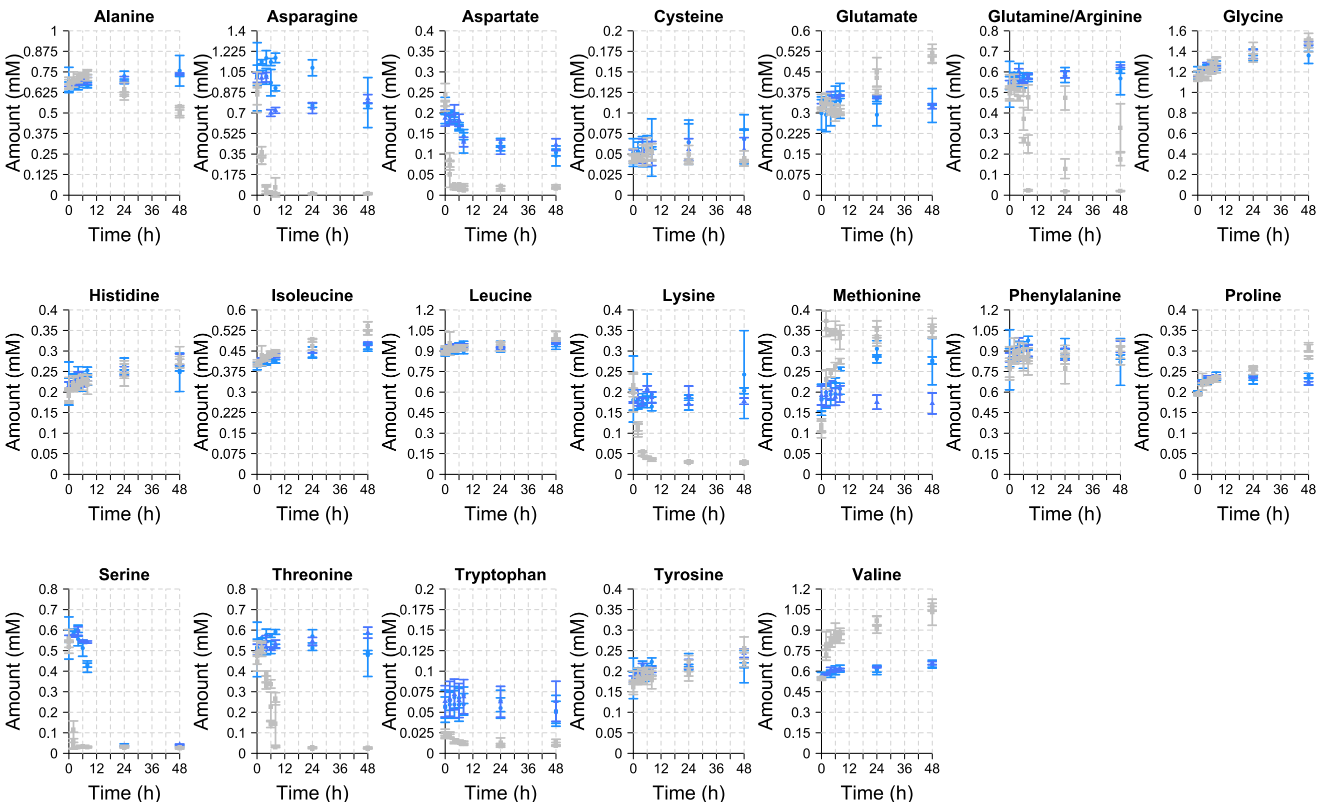

Figure S 1: Overview of trends in amino acid utilization of two *C. jejuni* (blue circles), one *C. coli* (blue triangle), and three ESBL-*E. coli* strains (grey squares) during monoculture enrichment of reference cells. Error bars depict the standard deviation of the concentrations at each time point of all strains per species and biological reproductions (n=2*3=6 for *C. jejuni*, n=1*3=3 for *C. coli*, and n=3*3=9 for ESBL-*E. coli*).

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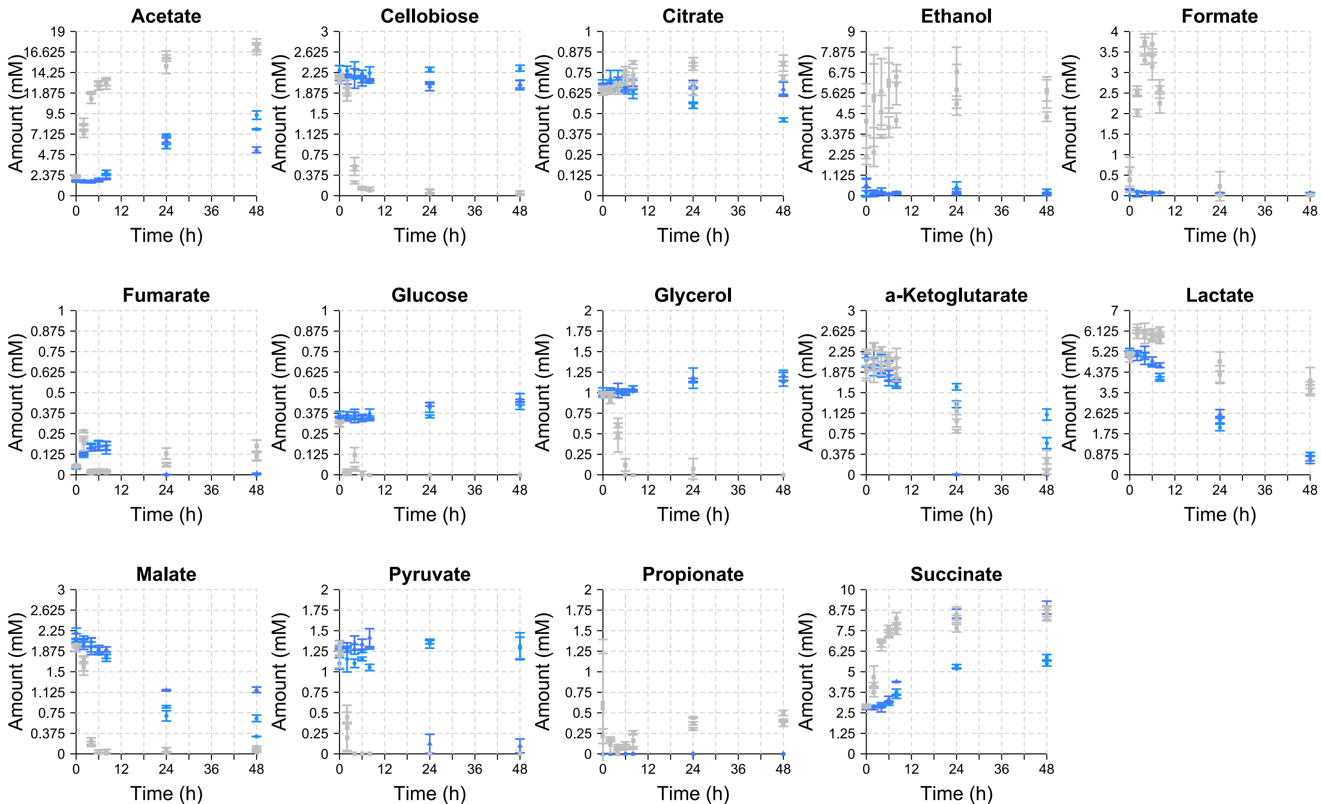

Figure S 2: Overview of trends in organic acid, TCA-intermediate, carbohydrate, and alcohol utilization of two *C. jejuni* (blue circles), one *C. coli* (blue triangle), and three ESBL-*E. coli* strains (grey squares) during monoculture enrichment of reference cells. Error bars depict the standard deviation of the concentrations at each time point of all strains per species and biological reproductions (n=2*3=6 for *C. jejuni*, n=1*3=3 for *C. coli,* and n=3*3=9 for ESBL-*E. coli*).

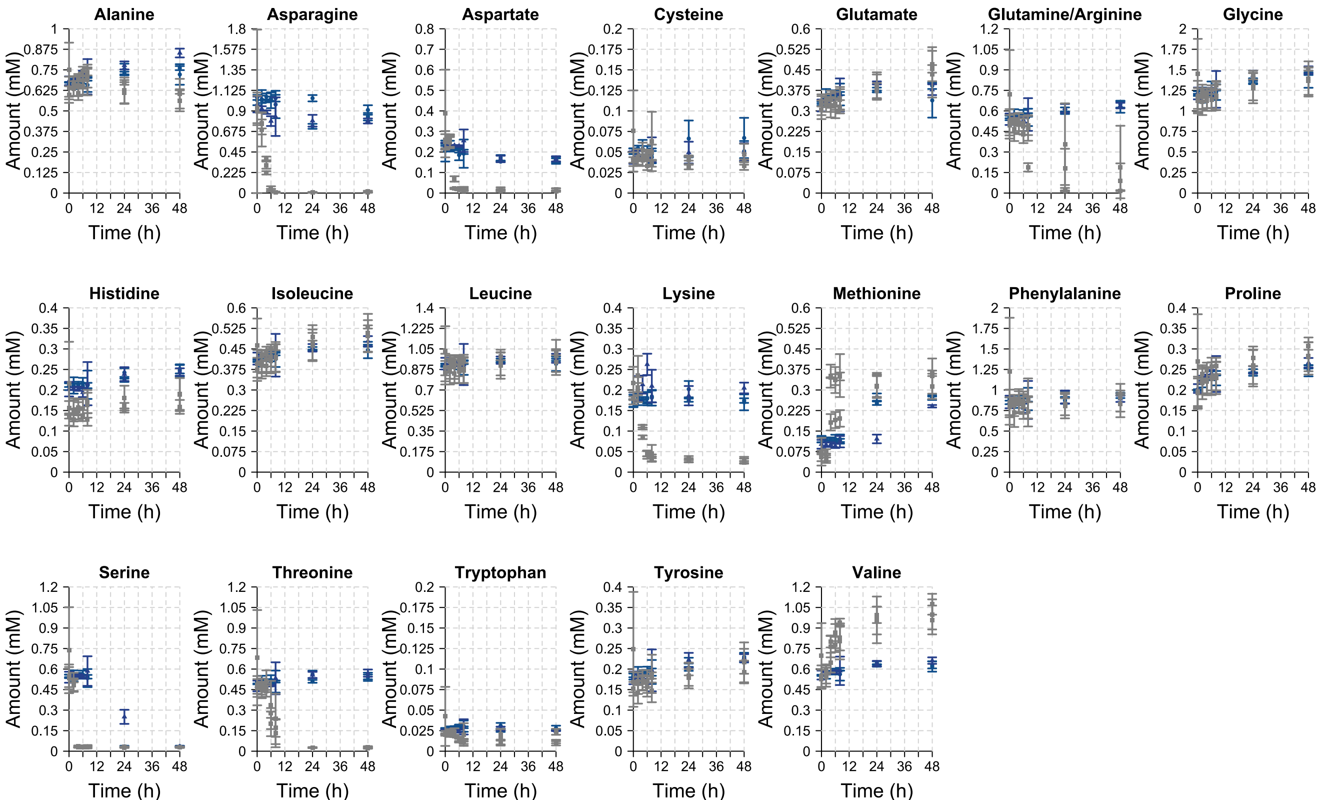

Figure S 3: Overview of trends in amino acid utilization of two *C. jejuni* (blue circles), one *C. coli* (blue triangle), and three ESBL-*E. coli* strains (grey squares) during monoculture enrichment of freeze stressed cells. Error bars depict the standard deviation of the concentrations at each time point of all strains per species and biological reproductions (n=2*3=6 for *C. jejuni*, n=1*3=3 for *C. coli,* and n=3*3=9 for ESBL-*E. coli*).

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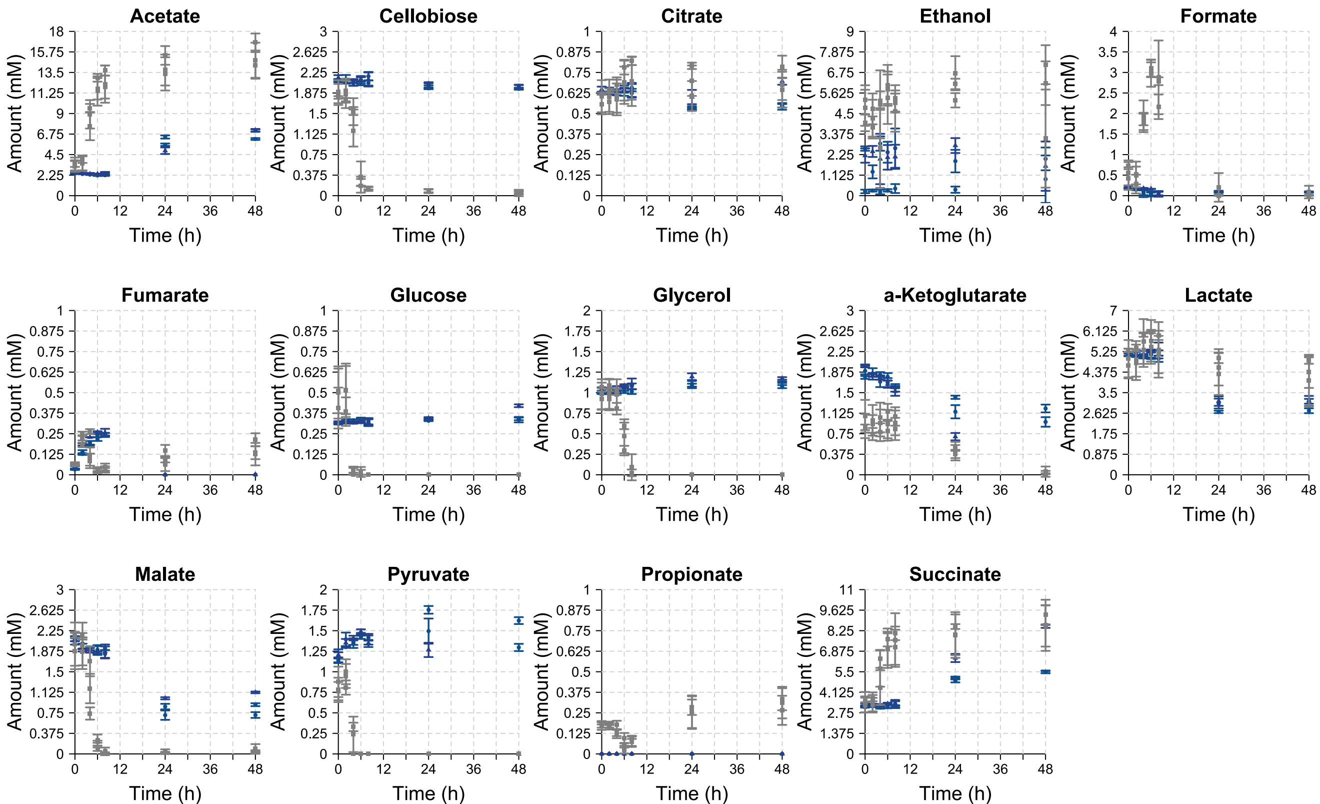

Figure S 4: Overview of trends in organic acid, TCA-intermediate, carbohydrate, and alcohol utilization of two *C. jejuni* (dark blue symbols), two *C. coli* (light blue symbols), and three ESBL-*E. coli* strains (red symbols) during monoculture enrichment of freeze-stressed cells. Error bars depict the standard deviation of the concentrations at each time point of all strains per species and biological reproductions (n=2*3=6 for *C. jejuni*, n=1*3=3 for *C. coli,* and n=3*3=9 for ESBL-*E. coli*).

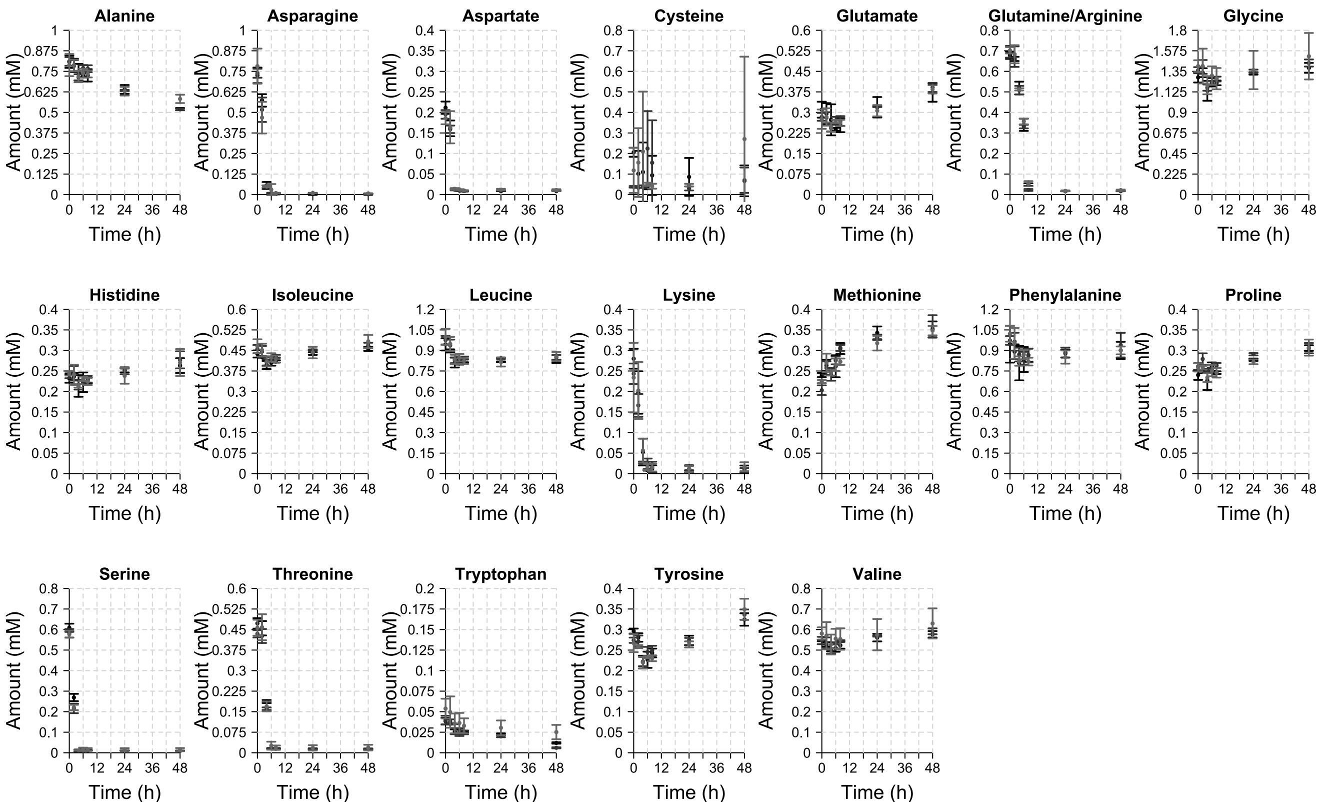

Figure S 5: Overview of trends in amino acid utilization of two *C. jejuni (Cj)* and one *C. coli (Cc)* in co-culture enrichments with ESBL-*E. coli* strain RIVM 2 in reference condition. Error bars depict the standard deviation of the concentrations at each time point of all strain-combinations and biological reproductions (n=3*3=9).

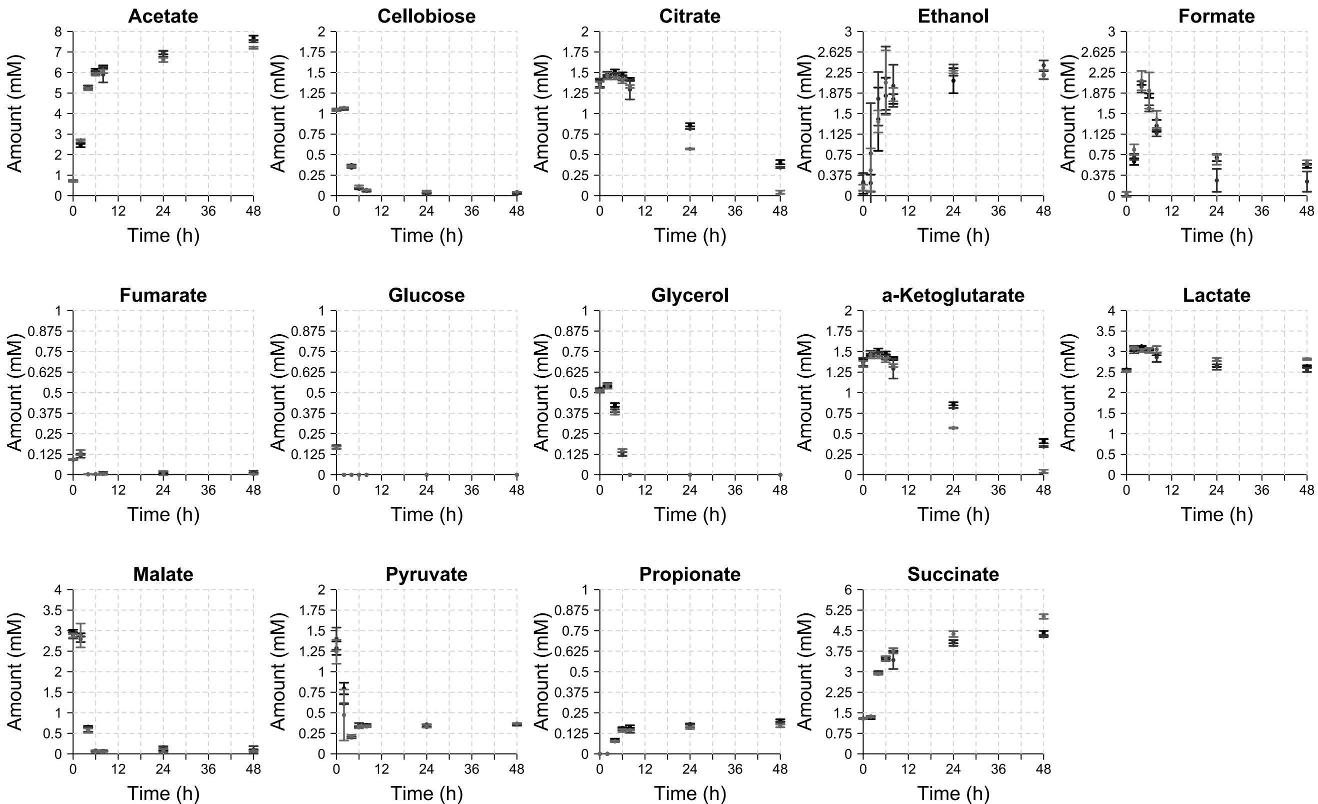

Figure S 6: Overview of trends in organic acid, TCA-intermediate, carbohydrate, and alcohol utilization of two *C. jejuni (Cj)* and one *C. coli (Cc)* in co-culture enrichments with ESBL-*E. coli* strain RIVM 2 in reference condition. Error bars depict the standard deviation of the concentrations at each time point of all strain-combinations and biological reproductions (n=3*3=9).

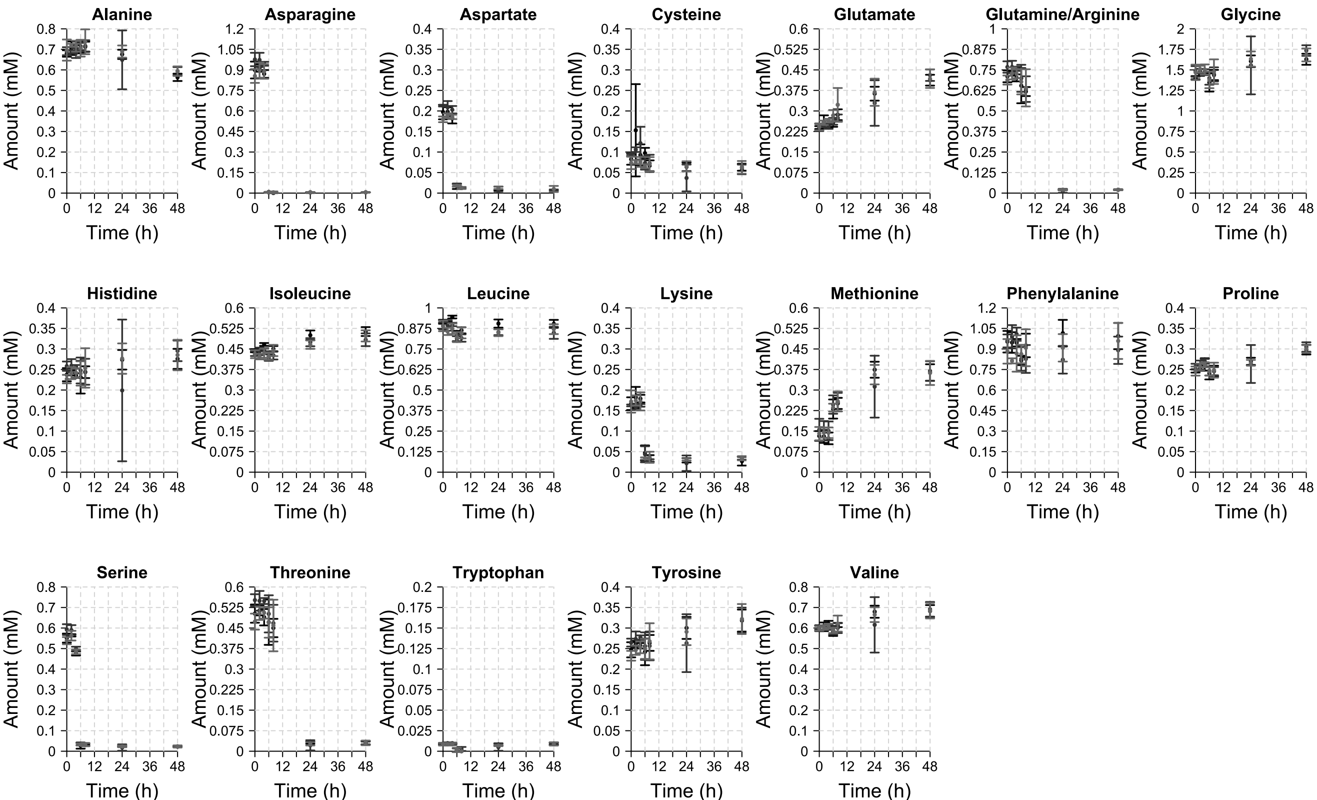

Figure S 7: Overview of trends in amino acid utilization of two *C. jejuni (Cj)* and one *C. coli (Cc)* in co-culture enrichments with ESBL-*E. coli* strain RIVM after freeze stress. Error bars depict the standard deviation of the concentrations at each time point of all strain-combinations and biological reproductions (n=3*3=9).

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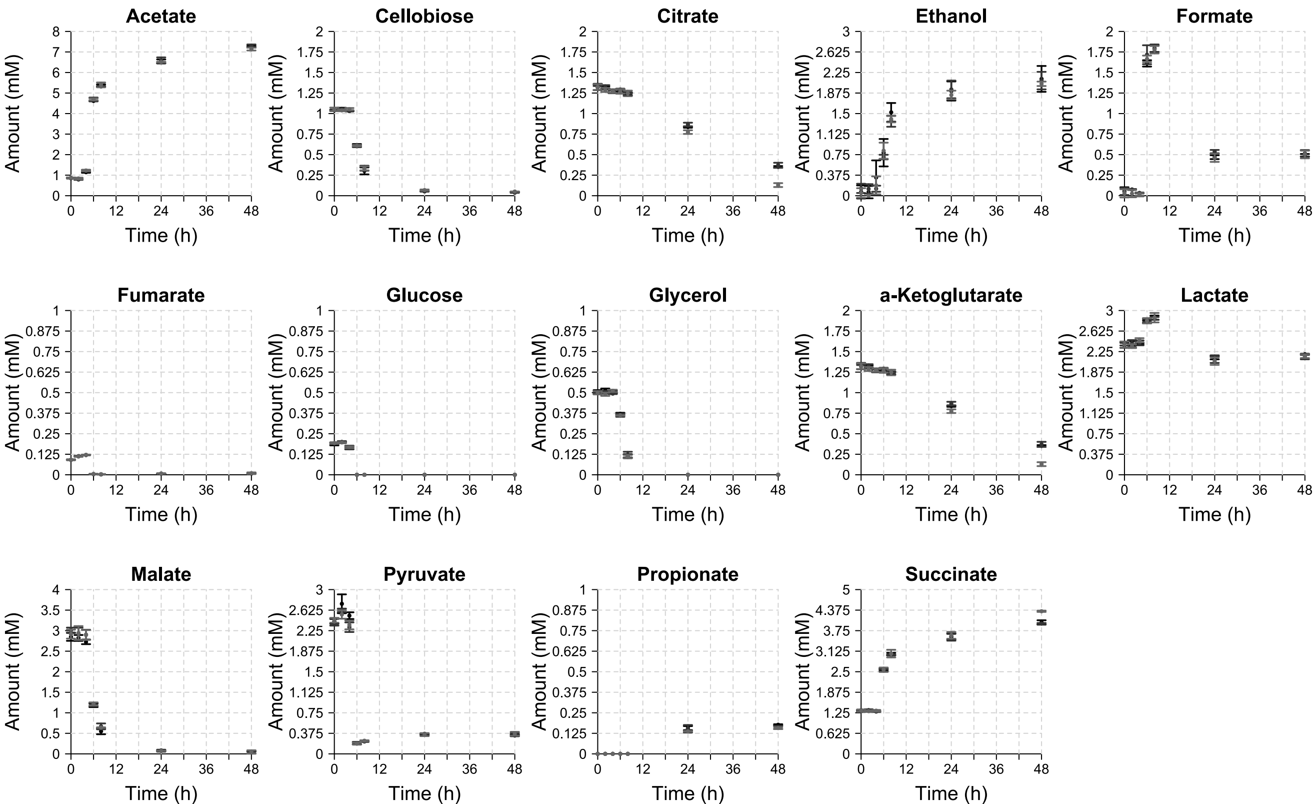

Figure S 8: Overview of trends in organic acid, TCA-intermediate, carbohydrate, and alcohol utilization of two *C. jejuni (Cj)* and one *C. coli (Cc)* in co-culture enrichments with ESBL-*E. coli* strain RIVM 2 after freeze stress. Error bars depict the standard deviation of the concentrations at each time point of all strain-combinations and biological reproductions (n=3*3=9).

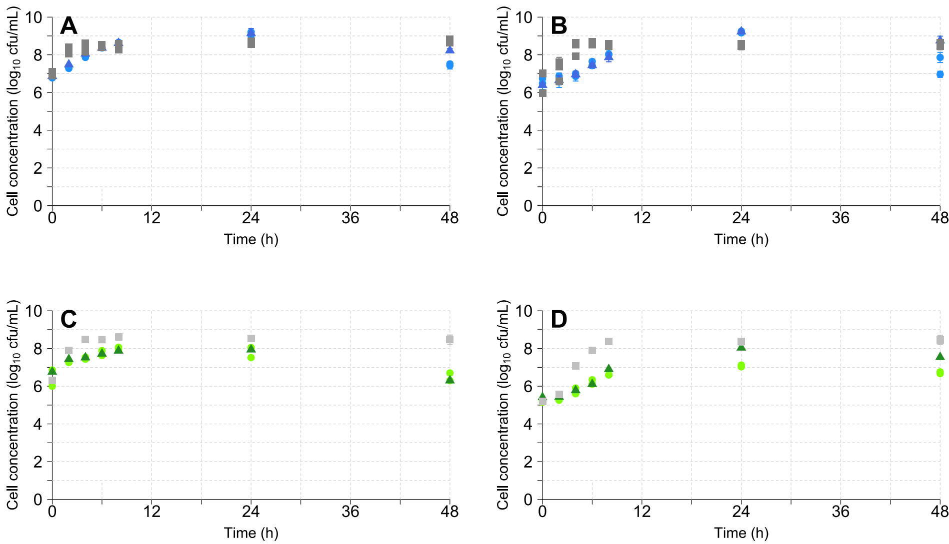

Figure S 9: Growth kinetics of *Campylobacter* spp. and ESBL-*E. coli* during enrichment when samples are taken for compound analyses. In monoculture (A&B), growth kinetics of two *C. jejuni* (blue circles), one *C. coli* (blue triangles), and three ESBL-*E. coli* strains (grey squares) are depicted during the enrichment of reference cells (A) and freeze-stressed cells (B). Error bars depict the standard deviation of the cell concentrations of the biological reproductions (n=3). In co-culture (C&D), growth kinetics of two *C. jejuni* (green circles) and one *C. coli* (green triangle) in co-culture with ESBL-*E. coli* strain RIVM 2 (grey squares) are depicted during in reference condition (C) and after freeze stress (D). Error bars depict the standard deviation of the cell concentrations of the biological reproductions (n=3 for campylobacters) and biological reproductions and strain combinations for ESBL-*E. coli* strain RIVM 2 (n=3*3=9).

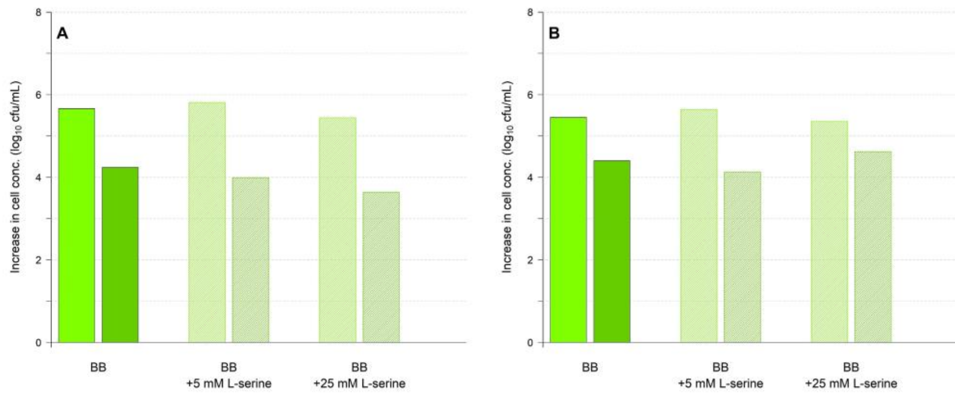

Figure S 10: Increase in cell concentrations after 24 and 48 h (Δt24-t0 and Δt48-t0, respectively) of *C. jejuni* strain 81-176 (A) and *C. coli* strain Ca 2800 (B) during co-culture enrichment with ESBL-*E. coli* strain RIVM 2 in BB, BB+5 mM L-serine and BB+25 mM L-serine. The increase in cell concentrations is depicted after 24 and 48 h of co-culture enrichment (medium and dark green colored bars, respectively). Filled bars depict the increase in co-culture growth of *C. jejuni* strain 81-176 (A) and *C. coli* strain Ca 2800 (B) in BB, while striped bars show the increase in co-culture growth in BB with an additional 5 or 25 mM of L-serine. For this experiment, one replicate was performed.

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The proteomic response of *Campylobacter jejuni* during lag phase in Bolton broth enrichment

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Abstract

The enrichment step is crucial for the reliable detection of *Campylobacter jejuni* from food products to support recovery of potentially stressed cells and to increase the concentration of cells to detectable levels. However, only little is known about the behaviour of *C. jejuni* during the initial lag phase in enrichments. Therefore, in this study, a proteomics approach was followed to gain insight into the recovery mechanisms during the lag phase of *C. jejuni* strain 81-176 when enriched in Bolton broth (BB). Cells were pre-cultured to stationary phase in BB-base and either directly transferred into selective BB or first exposed to refrigeration stress under aerobic conditions. Upon transfer of reference cells into selective BB, proteins associated with the maintenance of (membrane) protein quality were upregulated. In addition, redox enzymes and the anaerobic electron transfer protein complex MfrABC were upregulated during the lag phase of reference and refrigeration-stressed cells. The latter cells showed additional upregulation of proteins associated with several cell functions including purine metabolism, DNA and protein damage repair, iron acquisition, and additional electron acceptors, pointing to complementary factors involved in recovery of stressed cells. Outcomes of this study give insights into the lag phase of *C. jejuni* during enrichment in BB and demonstrated that protein quality and oxidative stress management are important factors in the recovery of reference and refrigeration-stressed *C. jejuni* cells.

Introduction

Campylobacter is an important food-borne pathogen as it is the leading cause of zoonotic gastroenteritis in the European Union (EFSA & ECDC, 2021). Most infections (~90%) are caused by the species *C. jejuni* (EFSA & ECDC, 2021) and therefore, it is important to be able to reliably detect campylobacters, but especially *C. jejuni* in food products. The current golden standard for the detection of *Campylobacter* spp. from foods are the protocols described in ISO 10272-1:2017 (International Organization for Standardization, 2017). It is widely known that campylobacters are susceptible to several food-processing conditions, such as chilling and freezing (Bhaduri and Cottrell, 2004; Georgsson et al., 2006; Lanzl et al., 2020; Maziero et al., 2010), heating (Sampers et al., 2010), and atmospheric oxygen (Boysen et al., 2007; Byrd et al., 2011) and these environments have shown to significantly reduce *Campylobacter* concentrations in food products. Cells can be expected to be (sub-lethally) injured and present only in low numbers after food processing and storage. It is therefore important to apply detection methods that include an enrichment step allowing the resuscitation and growth of campylobacters to reach cell concentrations sufficient for subsequent detection. ISO 10272-1 describes two enrichment-based protocols, but for the detection of sub-lethally injured cells an enrichment in selective Bolton broth (BB) is advised. Research has shown that BB supported the recovery of sub-lethally injured campylobacters better than the alternative Preston broth (PB) (Baylis et al., 2000; Hazeleger et al., 2016; Paulsen et al., 2005), and medium compound analysis during enrichment demonstrated that BB is a nutrient-rich medium (Lanzl et al., 2022) that does not lack critical growth substrates. However, while BB is a suitable medium to support recovery of stressed cells, the recovery duration (lag phase [λ]) of cold-stressed cells was significantly longer than for cells which did not undergo a preceding stress treatment (reference cells) (Lanzl et al., 2020). So far, the lag phase remains poorly understood (Bertrand, 2019; Hamill et al., 2020; Vermeersch et al., 2019) and currently, no scientific literature is available regarding the physiological processes that take place during the lag phase of *C. jejuni*. Therefore, the aim of this study was to generate insight into the intracellular processes that take place during the lag phase of reference and refrigeration-stressed cells of *C. jejuni* in selective BB with added supplement SR0208E containing antibiotics, using a proteomics approach.

Materials and methods

Bacterial strain, growth conditions, stress treatment and preparation of enrichment

Bacterial strain and growth conditions

For this study, *C. jejuni* strain 81-176 was used. Stock cultures were grown in Heart Infusion broth (HI, Bacto HI, Becton, Dickinson and Company) for 24 h at 41.5 °C, then supplemented with 15% glycerol (Fluka) and stored at –80 °C. Cells were plated from -80 °C vials onto Columbia agar base (CAB, Oxoid, supplemented with 5% (v/v) lysed horse blood (BioTrading Benelux B.V. Mijdrecht, Netherlands) and 0.5% agar (Bacteriological agar No.1, Oxoid)) and grown microaerobically for 24 h at 41.5 °C. Subsequently, a singly colony was resuspended in Heart Infusion broth (Bacto HI, Becton, Dickinson and Company) and cultured for 24 h at 41.5 °C to obtain stationary phase cultures. Afterwards, working cultures were prepared by making a 1:500 (v/v) dilution in Bolton broth (BB) base (Oxoid) without lysed horse blood and cultured for 24 h at 41.5 °C to reach the stationary phase.

Stress treatment

For the application of refrigeration stress, 6 ml of working culture were transferred into a 15 ml plastic tube (Greiner centrifuge tubes, Merck) and tubes were placed standing upright at 4 °C for 64±1 h under atmospheric oxygen conditions. Under these conditions, cell concentrations remained stable while lag-duration increased compared to reference cells (Lanzl et al., 2020).

Preparation of enrichment setup

For reference cells, working cultures were decimally diluted in BB base to a cell concentration of approximately 7.5 $\log_{10}$ cfu/ml and 5 ml were used to inoculate infusion bottles filled with 45 ml of selective BB (Bolton broth base with the addition of 450 µl of the selective supplement containing the antibiotics cefoperazone, trimethoprim, vancomycin and amphotericin B (Oxoid SR0208E)). The method for use of infusion bottles was previously described in detail (Lanzl et al., 2020). In short, additions of fluids to sterilized bottles (volume of 100 ml) was achieved using syringes

to puncture the rubber stopper of the bottles. Before addition of the working cultures, the headspace of the bottles was flushed for 2 minutes with a gas-mixture of 5% O_2, 10% CO_2 and 85% N_2 by a home-made gas flushing device. The same inoculation procedure was followed for refrigeration-stressed cells. Inoculated infusion bottles were incubated in a water bath at 37 °C for 3 and 4 h (for reference and refrigeration-stressed cells, respectively). Three biologically independent reproductions were performed.

Assessment of growth kinetics and determination of lag-duration

To determine the growth kinetics and estimate the lag-duration, samples were taken throughout enrichment (0, 2, 4, 6, 8, 10, 24 and 48 h), immediately diluted in peptone physiological salt solution (PPS, Tritium Microbiologie), plated onto CAB and incubated for 48 h at 41.5 °C to determine cell concentrations of reference and refrigeration-stressed cells during enrichment. Plate counts were transformed to log_{10} cfu/ml and growth curves were constructed using Microsoft Excel 2010. The Baranyi-model (Baranyi and Roberts, 1994) was fitted to the growth data using the Solver add-in of Excel to estimate the lag-duration of *C. jejuni* strain 81-176 in selective BB in reference condition and after refrigeration stress at a relatively high inoculum. Two biologically independent reproductions were performed.

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Sample preparation and proteomic analysis

Samples were taken at the start of enrichment as well as after 10 min, 30 min, 1 h, 2 h and 3 h for reference cells and after 4 h in case of refrigeration-stressed cells for proteomic analyses. For that, 45 ml of the culture was centrifuged at 10,000 *g* for two minutes, and the pellet was dissolved in 200 µl of 100 mM Tris (pH 8). Each sample was washed twice with 100 mM Tris (pH 8) to remove traces of selective BB. The pellet was then resuspended in 100 µl of 100 mM Tris (pH 8) and stored at -80 °C until further analysis. Each sample was allowed to thaw on ice and subsequently sonicated three times for 30 s on ice for cell lysis (MSE Soniprep 150). The protein content was measured using the Bradford assay (Bradford, 1976). Samples were prepared according to the filter assisted sample preparation protocol (FASP) (Wiśniewski et al., 2009) applying reduction with 15 mM dithiothreitol, alkylation with 20 mM acrylamide and digestion with sequencing grade trypsin overnight. Each prepared sample was

analysed by injecting 18 µl into a nanoLC-MS/MS (Thermo nLC1000 connected to an LTQ-Orbitrap XL) as described previously by (Liu et al., 2021). The quality of the nanoLC-MS/MS system was checked with PTXQC using the MaxQuant result files (Bielow et al., 2016). LCMS data with all MS/MS spectra were analysed with the MaxQuant quantitative proteomics software package (Cox et al., 2014) as described before (Smaczniak et al., 2012; Wendrich et al., 2017). A reference database containing all protein sequences of *C. jejuni* 81-176 (organism ID: 1436885, proteome ID: UP000018831) was downloaded from the UniProt database (Apweiler et al., 2004). MaxQuant ProteinGroups were filtered, and further bioinformatics and statistical analysis were performed with Perseus (Tyanova et al., 2016). Contaminants and reverse hits were filtered out and protein groups were filtered to contain minimally two peptides for protein identification of which at least one is unique and at least one is unmodified. All timepoints of each condition were compared against the first timepoint (0 h) of the same condition (reference cells and refrigeration-stressed cells). Proteins were considered to be significant differentially expressed when they showed an absolute fold change increase of 2 (equalling 1 log_2-fold increase) and a *p*-value lower than 0.05, and when they showed an absolute fold change increase of 8 (equalling 3 log_2-fold increase) and a *p*-value lower than 0.1. Proteomic analysis and visualisation were done in R version 4.1.0 (R Core Team, 2021).

Results and discussion

Lag-duration at high inoculum

In order to study the proteomic response of *C. jejuni* during the lag phase and onset of growth in selective BB, high inoculum concentrations had to be used to harvest sufficient number of cells and consequently enough protein for analysis. Therefore, the inoculum concentration of *C. jejuni* was 6.7±0.04 and 6.8±0.01 log_{10} cfu/ml, for reference and refrigeration-stressed cells, respectively. The cell concentration was measured until stationary phase was reached the lag-duration was estimated using the Baranyi growth model. **Figure S 1 of the supplementary materials** shows the cell concentrations during enrichment and the fitted growth model**.** The lag-duration of reference and refrigeration-stressed cells after transfer into selective BB was 0.9±0.04 h and 2.8±0.19 h, respectively. Therefore, samples were taken at the start of

enrichment (0 h) and after 10 min, 30 min, 1 h, 2 h and 3 h for reference and refrigeration-stressed cells, and an additional sample was taken after 4 h for refrigeration-stressed cells to cover the lag phase and the initial growth phase.

Protein expression during lag phase

When the protein expression data of reference and refrigeration-stressed cells were clustered (**figure 1 A)**, there was a clear cluster-distinction between the last two timepoints of reference and stressed cells (2h and 3h for reference cells and 3h and 4h for stressed cells) and the samples taken earlier during enrichment, which agrees with the estimation of the lag-duration and initiation of growth. As a result, the proteomic data was filtered, and a subset was selected including all timepoints of the first hour of reference cells and the first two h of refrigeration-stressed cells to focus further on the proteomic response during the lag phase. Furthermore, the clustering dendrogram also shows a clear separation of the proteome response between reference and stressed cells. The Venn-diagram in **figure 1 (B)** shows that 51 proteins were significantly differentially expressed in reference cells, of which 14 were also differentially expressed in refrigeration-stressed cells, and this overlap in expression could point to crucial cellular processes that take place during lag phase, regardless of whether cells received

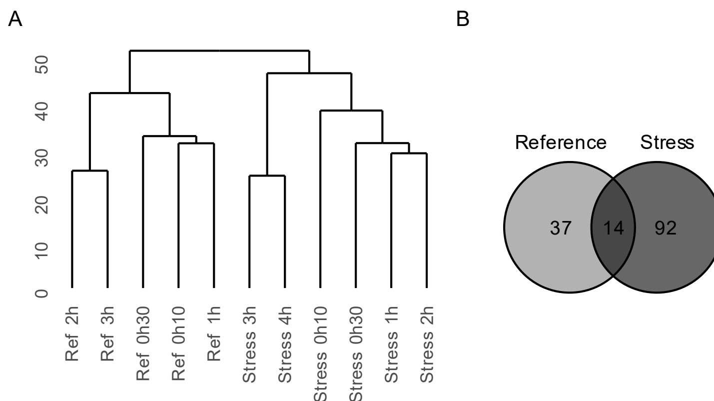

Figure 1: Clustering dendrogram (A) shows the hierarchical clustering of reference and refrigeration-stressed cells for all timepoints. The clustering was done with the complete-linkage clustering algorithm. **A Venn-diagram (B) shows the differentially expressed proteins during lag phase** (sampling points 0h10, 0h30, 1h of reference cells and 0h10, 0h30, 1h, 2h of stressed cells).

an additional stress treatment or not. An additional 92 proteins were uniquely expressed during the lag phase of refrigeration-stressed cells, which would suggest an additional adaptation or damage repair during enrichment of stressed *C. jejuni* cells.

Figure 2 shows in more detail the characterized proteins that were up- or downregulated in reference cells, of which 11 were also significantly differentially expressed in stressed cells. The representative proteins were divided into six relevant themes. A complete list of the log_2-fold change at all time points of all 51 proteins significantly differentially expressed during lag phase of reference cells can be found in **table S 1 in the supplementary materials**.

Adaptation and repair

Five proteins associated with adaptation to changing environments and repair were differentially expressed in reference cells. The exodeoxyribonuclease III (CJJ81176_0282) was highly downregulated in this study (log_2-fold change of -5.0). According to Gaasbeek et al. (2009), it is an orthologue to XthA in *Escherichia coli*, which removes damaged DNA at cytosines and guanines (Gaasbeek et al., 2009). As this protein was downregulated in this study it could imply that *C. jejuni* actively repaired DNA damages during late stationary phase but during lag phase of reference cells in fresh medium, DNA damage repair was less critical. A differential expression of two Clp-protease family proteins could be observed, namely the ATP-dependent Clp protease adapter protein ClpS (CJJ81176_1125) and the ATP-dependent chaperone protein ClpB (CJJ81176_0537). Both were upregulated after exposure to different stresses in previous research studies (Reid et al., 2008; Varsaki et al., 2015) and play a role in coping with damaged proteins. ClpS is important in degrading severely damaged proteins and was downregulated in this study. ClpB enables the refolding of mildly damaged proteins and was upregulated during lag-duration of reference cells. This could imply that cellular proteins only suffered minor damage during pre-culture growth to stationary phase and that the damaged proteins are repaired rather than degraded. An upregulation could also be observed for an ATPase (CJJ81176_0401), that is similar to FtsH, a membrane-anchored protease crucial for membrane protein quality control (Langklotz et al., 2012). An initial upregulation could be observed in the histidine kinase BumS (CJJ81176_1484). It has been recognized that this protein, together with the DNA-binding response regulator BumR is part of a two-component

regulatory system (Luethy et al., 2015). Goodman et al. (2020) recently suggested that BumS likely senses butyrate availability in the environment and controls the function of BumR, subsequently modulating the transcription of colonization factors. Since no significant differential expression of these factors was noted in our study, a possible role of BumSR and putative regulated genes in getting out of lag in *C. jejuni* remains to be elucidated.

Cell wall and ribosomes

During lag phase of reference cells, several cell wall/membrane related proteins were downregulated, including a putative membrane protein (CJJ81176_0335) and a Mur ligase family protein (CJJ81176_0816) but unfortunately, only little information is currently available regarding their precise function. Downregulation could also be observed for a peptidase (CJJ81176_1228) which has been shown to influence cell shape and be important in adherence, invasion and intracellular survival during *in vivo* experiments conducted by (Stahl et al., 2016). The transglycosylase RlpA (CJJ81176_0674) involved in cell wall organization was also downregulated. Interestingly, the only two cell wall synthesis associated proteins which were upregulated were transglycosylases (CJJ81176_0070 and CJJ81176_0554). Transglycosylases play a role in the polymerization of peptidoglycan as they form linear glycan chains, while transpeptidases connect those glycan chains through peptide cross-bridges. A downregulation could be observed for two penicillin-binding proteins (CJJ81176_0536 and CJJ81176_0680). The selective cocktail of BB contains the beta-lactam antibiotic cefoperazone that binds to specific penicillin-binding proteins (PBPs) located inside the bacterial cell wall, causing the inhibition of the third and last stage of bacterial cell wall synthesis (Reed et al., 2011). Although *C. jejuni* is resistant to beta-lactam antibiotics through either the presence of beta-lactamases or certain membrane processes (porins, efflux) (Iovine, 2013), the observed downregulation of the PBPs may further prevent inhibition of cell wall synthesis in the initial lag phase.

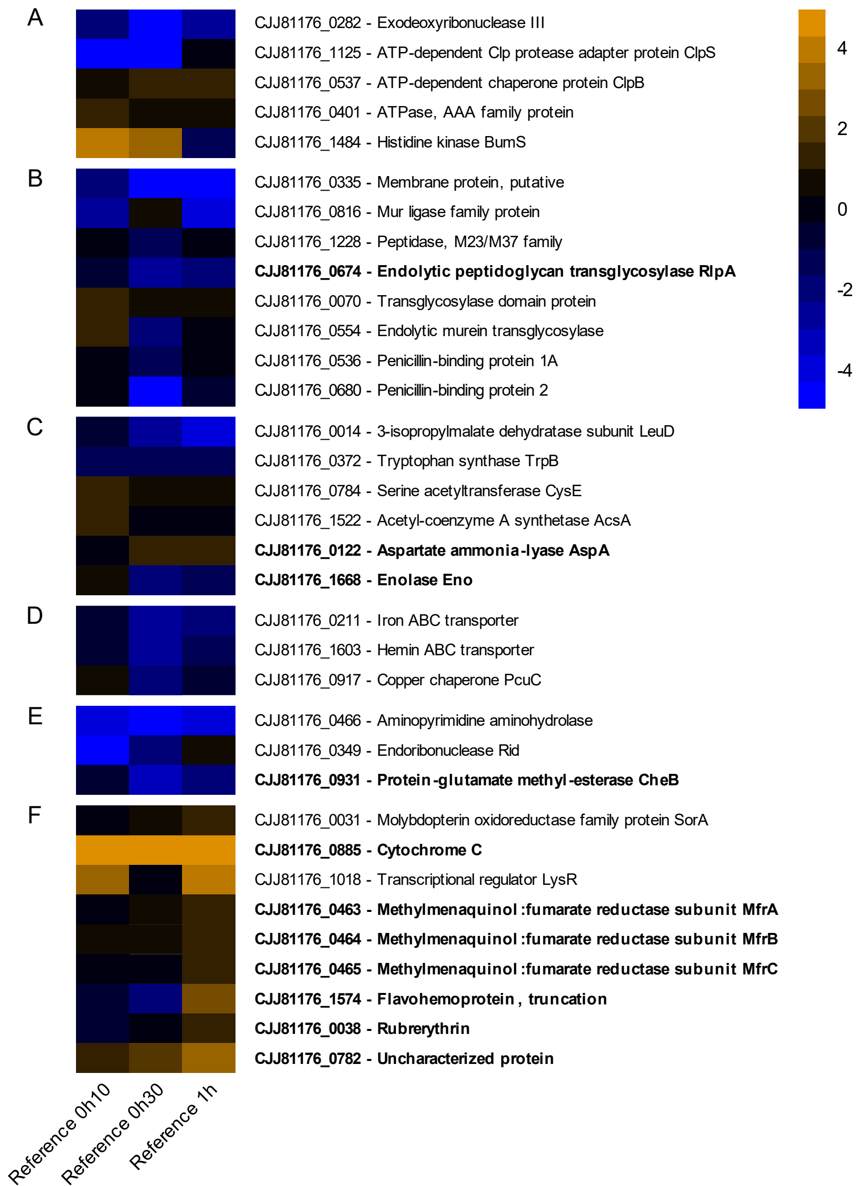

Figure 2: Heatmap of characterized proteins with significant differential expression during lag-duration of reference cells. The proteins marked in bold were also significantly differentially expressed during lag-duration of refrigeration-stressed cells. The heatmap is structured into six functional groups containing proteins associated with (**A**) adaptation and repair, (**B**) cell wall and ribosomes, (**C**) metabolism, (**D**) metal transport, (**E**) motility and (**F**) respiration and redox enzymes. The colours indicate the $\log_2$-fold change of the protein at different time points compared to the initial t0h sample.

Metabolism

When stationary phase reference cells were transferred to selective BB, a downregulation could be observed for subunits of two enzymes, 3-isopropylmalate dehydratase subunit LeuD (CJJ81176_0014) and tryptophan synthase TrpB (CJJ81176_0372), associated with amino acid biosynthesis. Interestingly, both proteins were upregulated in other studies after exposure to hyperosmotic stress (Cameron et al., 2012) and sub-inhibitory dose of erythromycin (Xia et al., 2013), respectively. This could imply that these proteins were increasingly expressed in unfavourable environments. Since *Campylobacter* cells in this study were transferred from late stationary phase into fresh medium, a downregulation of these proteins could indicate an adaptation to a more favourable nutrient-rich environment. In addition, upregulation of the serine acetyltransferase CysE (CJJ81176_0784) was observed. CysE catalyses the first step to synthesize the pathway intermediate O-acetylserine and the cysteine synthase CysK catalyses the second step using hydrogen sulfide to produce cysteine. However, in this study, no significant differential expression could be observed for CysK. Serine is *Campylobacter*'s most preferred amino acid (Wright et al., 2009) and utilization by *C. jejuni* during enrichment could also be observed in previous research wherein the same experimental setup regarding pre-culturing and enrichment was applied (Lanzl et al., 2022). In that study, *C. jejuni* was able to utilize serine faster in reference condition than after freeze-stress, in line with the upregulation of CysE observed during lag-duration of reference cells. The same study demonstrated that cysteine was available in selective BB, and a recent study by (Man et al., 2020) demonstrated that cysteine was taken up through the putative sodium:dicarboxylate transporter Cj0025c. However, no significant differential expression could be observed for this membrane protein (CJJ81176_0250) in this study. As cysteine plays a key role in a wide range of proteins and is crucial for the synthesis of many biomolecules important for growth (Hicks et al., 2022; Vorwerk et al., 2014), the exact mechanisms for cysteine acquisition remains to be elucidated. The acetyl-coenzyme A synthetase AcsA (CJJ81176_1522) was initially also upregulated. It catalyses the conversion of acetate into acetyl-CoA. It has been associated with the 'acetate switch', during which bacteria start breaking down acetate which they previously produced (Wolfe, 2005; Wright et al., 2009).

The aspartate ammonia-lyase AspA (CJJ81176_0122), which is part of the TCA-cycle converting aspartate to fumarate, was upregulated during the lag phase of reference and stressed cells. On the other hand, the enolase Eno (CJJ81176_1668) was downregulated, which is essential for carbohydrate degradation and catalyses the reversible conversion of 2-phosphoglycerate to phosphoenolpyruvate. The change in aspartate and fumarate concentration during enrichment was measured in a previous study (Lanzl et al., 2022) and results showed that *C. jejuni* indeed utilized aspartate already during the lag phase, while at the same time, fumarate concentrations increased. Later, during enrichment, a decrease in fumarate concentration in BB could be measured, which indicates that fumarate is metabolised during enrichment as well.

Metal transport

During lag phase of reference cells, a downregulation could be observed for three metal-associated transporter proteins; an iron ABC transporter (CJJ81176_0211), a hemin ABC transporter (CJJ81176_1603) and the copper chaperone PCuC (CJJ81176_0917), which probably is involved in copper trafficking (Garg et al., 2021). The same authors also suggested that copper trafficking and homeostasis were linked to the cbb_3-type cytochrome c oxidase which is involved in respiration of *C. jejuni*. Interestingly, Rolfe et al. (2012) focused on the transcriptomic changes during lag phase of *Salmonella* Typhimurium and observed accumulation of iron, calcium and manganese. It is widely known that iron is crucial for cellular processes as it forms complexes with other elements such as oxygen, nitrogen and sulphur and allows catalysation of enzymatic reactions (Palyada et al., 2004). However, if not regulated strictly, reactive iron also generates toxic reactive oxygen species (ROS) (Pomposiello and Demple, 2002; Stahl et al., 2012). In the current study, we did not observe significant upregulation of cytoplasmic and/or membrane-associated protein involved in metal homeostasis during lag phase of reference cells.

Motility

Significant downregulation could be observed for three motility-associated proteins, including an aminopyrimidine aminohydrolase (CJJ81176_0466), that was recently described as a putative transcriptional regulator involved in the regulation of flagellar synthesis (Shabbir et al., 2018). The second highly downregulated protein (log_2-fold

change of -5.7) was the endoribonuclease Rid (CJJ81176_0349). Data from a study using *C. jejuni* strain NCTC 11168 suggested that the related protein RidA plays a role in flagellar biosynthesis, regulation, structure and/or function (Irons et al., 2019; Rahman et al., 2014). The protein-glutamate methyl-esterase CheB (CJJ81176_0931) was downregulated during lag-duration of reference and stressed cells. It was shown to be involved in chemotaxis and 'sensory adaptation' (Chandrashekhar et al., 2017; Kanungpean et al., 2011). Downregulation of motility-associated proteins can be explained by nutrient excess in the fresh medium, additionally providing an energetic advantage, as production of flagellar machinery is an energy costly process (Ni et al., 2020).

Respiration and redox enzymes

Several proteins with functions in electron transfer were significantly differentially expressed. Upregulation of the molybdopterin oxidoreductase family protein SorA (CJJ81176_0031) was observed especially towards the end of lag phase. This protein is part of the sulfite:cytochrome x oxidoreductase system, and SorA catalyses the oxidation of sulfite to sulfate, and electrons are then transferred to the monoheme cytochrome c552 SorB (Myers and Kelly, 2005). In addition, cytochrome c (CJJ81176_0885), an electron carrier in the electron transport chain (Liu and Kelly, 2015), was strongly upregulated (log_2-fold change of 4.69). The transcriptional regulator LysR (CJJ81176_1018) previously reported to activate uptake, production, and respiration of fumarate (Dufour et al., 2013; Van Der Stel and Wösten, 2019), was also upregulated. In line with this, all three subunits (CJJ81176_0463, CJJ81176_0464 and CJJ81176_0465) of the periplasmic methylmenaquinone:fumarate reductase MfrABC were upregulated in reference and stressed cells. Research has shown that the *sdh* operon was previously mislabelled as SdhABC (Guccione et al., 2010), as the enzyme itself does not display any succinate dehydrogenase activity (Weingarten et al., 2009). Under oxygen-limited conditions, fumarate is reduced to succinate through two complexes, namely a cytoplasmic FrdABC complex and a periplasmic MfrABC complex (Guccione et al., 2010, 2017). Our previous study showed that BB contains also fumarate and that *Campylobacter* spp. metabolise this compound during enrichment as fumarate concentrations decreased during the growth phase of *Campylobacter* while succinate concentrations increased (Lanzl et al., 2022). The

proteomics data indicate that fumarate plays an important role as an electron acceptor in anaerobic respiration of *C. jejuni* during lag phase. Three proteins were significantly upregulated during lag phase of both reference and stressed cells which could be associated with redox stress. An upregulation could be observed for the flavohemoprotein Cgb (CJJ81176_1574), possibly associated with nitrosative stress and metal (haem) binding (Pittman et al., 2007). Rubrerythrin Rbr (CJJ81176_0038), which has been recognized to be involved in oxidative stress tolerance and electron transfer (Moura et al., 1994; Sztukowska et al., 2002) was also upregulated and an upregulation could also be observed for a yet uncharacterized protein (CJJ81176_0782). Although its precise function is yet unknown, the protein was also upregulated in *C. jejuni* cultures exposed to nitrosative stress in a study by (Avila-Ramirez et al., 2013). It has been explained that the detoxification catalysed by nitroreductases such as RdxA also leads to the generation of nitrogen sources for metabolism and can play a role in redox balancing and oxidative stress response (Palyada et al., 2009; Roldán et al., 2008; Wang and Maier, 2004).

Unique proteins differentially expressed during lag phase of stressed cells

An additional specific stress-related response was identified in refrigeration-stressed *C. jejuni* cells. **Figure 3** shows in more detail the characterized proteins that were significantly up- or downregulated only in stressed cells. The representative proteins were divided into four relevant themes. A complete list of the log_2-fold change at all time points of all 106 proteins significantly differentially expressed during lag phase of stressed cells can be found in **table S 2 in the supplementary materials**.

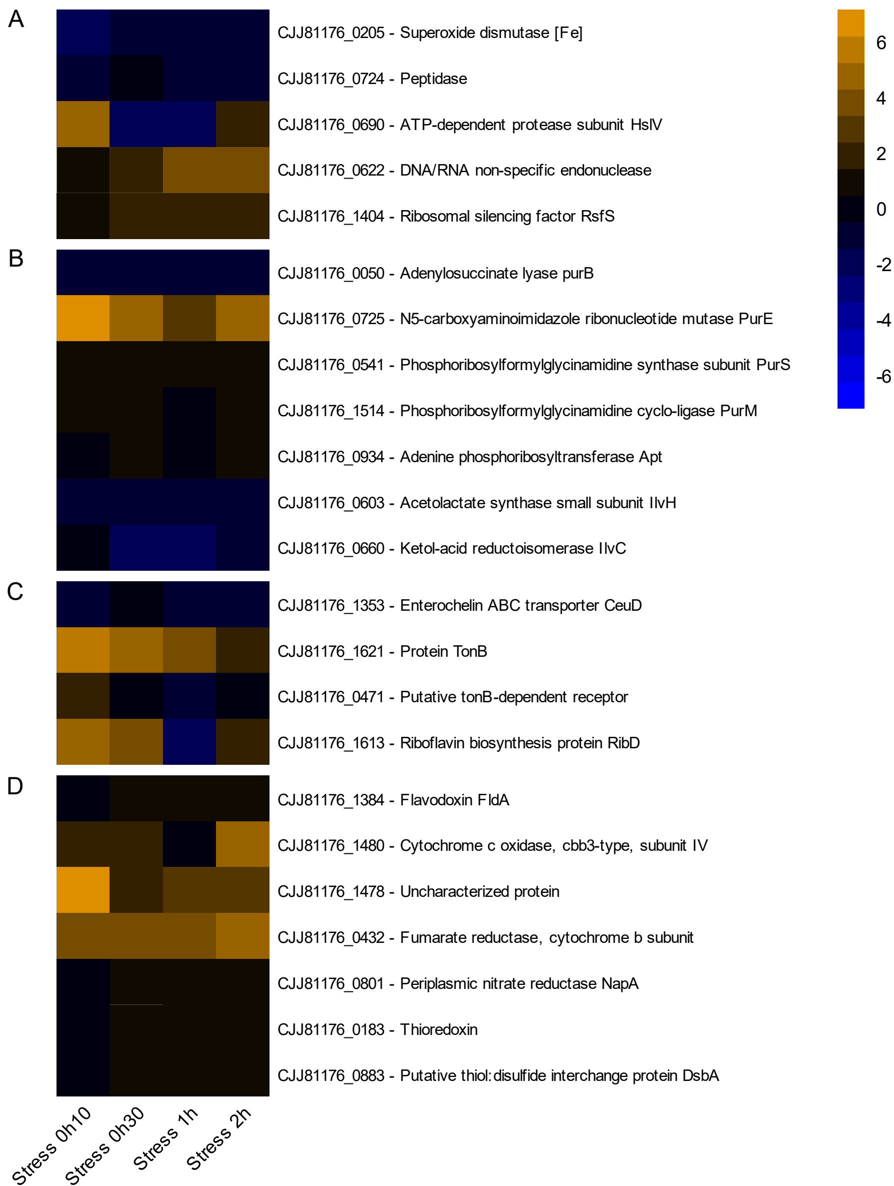

Figure 3: Heatmap of proteins with unique significant differential expression during lag-duration of stressed cells. The heatmap is structured into seven functional groups containing proteins associated with (**A**) stress response and repair, (**B**) metabolism, (**C**) iron acquisition, (**D**) electron transfer and redox stress. The colours indicate the log_2-fold change of the protein at different time points compared to the initial 0h sample.

4

Stress response and damage repair

Five proteins were significantly differentially expressed which can be associated with stress response and damage repair. The superoxide dismutase SodB (CJJ81176_0205) destroys ROS such as superoxide anions, which are naturally produced within cells at higher oxygen concentrations. The observed downregulation of SodB during the lag phase in the enrichment is conceivably linked to induction of the protein during the refrigeration stress treatment under aerobic conditions. A similar trend could be observed for peptidase CJJ81176_0724, with a putative role in protein degradation following exposure to nitrosative stress (reactive nitrogen species [RNS]) (Monk et al., 2008). On the other hand, two proteins were upregulated which are involved in the degradation of damaged proteins and the removal of damaged DNA. The ATP-dependent protease subunit HslV (CJJ81176_0690) is part of a complex which is believed to be a general protein degrading machinery and the DNA/RNA non-specific endonuclease (CJJ81176_0622) can cleave dsDNA/RNA which possibly could remove damaged DNA. Both proteins have been associated with oxidative stress and DNA repair (Gao et al., 2017) and were upregulated during lag phase of stressed cells. This could imply that refrigeration stress under aerobic conditions induced protein and DNA damage which is resolved by the degradation of injured proteins and removal of damaged DNA during lag-duration. In line with this, the ribosomal silencing factor RsfS (CJJ81176_1404) was also downregulated. RsfS prevents the formation of functional ribosomes by hindering the association of the 30S and 50S ribosomal subunits into functional prokaryotic 70S ribosomes, generally observed in stationary phase cells, and as a result translation is repressed. Higher levels of functional ribosomes during lag phase of refrigeration-stressed cells may support synthesis of damage repair proteins and other proteins/enzymes required to get out of lag.

Metabolism

A differential expression could be observed for five proteins associated with purine metabolism. Four of them, PurB, PurE, PurS and PurM (CJJ81176_0050, CJJ81176_0725, CJJ81176_0541 and CJJ81176_1514, respectively) are associated with the *de novo* biosynthesis of inosine 5'-monophosphate (IMP) and the Adenine phosphoribosyltransferase apt (CJJ81176_0934) catalyses a salvage reaction which results in the formation of adenosine monophosphate (AMP). Apart from PurB, all

proteins were upregulated during lag phase of stressed, cells which could indicate that purine metabolism plays an important role during lag phase of stressed cells. A downregulation could be observed for two proteins involved in the biosynthesis and transport of branched-chain amino acids (BCAA). The acetolactate synthase small subunit IlvH and the ketol-acid reductoisomerase IlvC (CJJ81176_0603 and CJJ81176_0660, respectively) are responsible for the first three steps in the biosynthesis of L-isoleucine and L-valine, and downregulation is conceivably linked to availability of these amino acids in the enrichment medium.

Iron acquisition

In stressed cells, several proteins associated with iron transport were upregulated, while the enterochelin ABC-transporter protein CeuD (CJJ81176_1353) was downregulated. The CeuBCDE complex, located in the inner membrane, acts together with the ferric-enterobactin receptor CfrA/CfrB to transport enterochelin, a siderophore which can bind ferric ions with high affinity, and which are used to supply iron for metabolic pathways in iron-limited conditions (Hofreuter, 2014). This may point to roles of other transporters and/or proteins in iron acquisition in the fresh selective BB enrichment medium. Several iron-uptake systems transport iron complexes and require an energy transduction compound comprised of three proteins, namely TonB, ExbB and ExbD. TonB (CJJ81176_1621) was highly upregulated during lag phase of stressed cells, but ExbB (CJJ81176_1619) was not detected and ExbD (CJJ81176_1620) was not significantly differentially expressed. Since TonB is anchored to the inner membrane, while ExbB and ExbD are positioned within the inner membrane (Miethke and Marahiel, 2007; Raymond et al., 2003), detection of the latter two proteins might therefore be less accurate. Since TonB was highly upregulated and only acts in the TonB-ExbB-ExbD complex, it is likely that the whole complex was upregulated during lag phase of stressed cells. A putative TonB-dependent receptor (CJJ81176_0471) was also upregulated but unfortunately, only little is yet known about this protein. The riboflavin biosynthesis protein RibD (CJJ81176_1613) was also highly upregulated during lag phase of stressed cells. Riboflavin is used to reduce Fe^{3+}, which is almost completely insoluble at a pH ≥7, to soluble Fe^{2+}. The latter can then be taken up across the membrane (Crossley et al., 2007). As most of the proteins associated

with iron acquisition were upregulated, this indicates that iron and possibly other metal ions play an important role during lag phase of stressed cells.

Electron transfer and redox stress

In this study, an upregulation of several proteins could be observed which take part in respiration processes. It has been recognized that, within *C. jejuni* electron transport chains, some oxidoreductases prefer flavodoxin over NADH as electron donor (Weerakoon and Olson, 2008). Indeed, flavodoxin FldA (CJJ81176_1384) was upregulated during lag phase of stressed cells in the current study. Reduced FldA is also generated through the oxidation of several carbon sources. In *C. jejuni* strain NCTC 11168, pyruvate and 2-oxoglutarate are oxidized to succinyl-CoA by the pyruvate-flavodoxin oxidoreductase Por (Cj1476c) and the 2-oxoglutarate:acceptor oxidoreductase Oor (Cj0535-38) (Kelly, 2001). However, it has also been recognized that both, Por and Oor are highly sensitive to oxygen but are protected by hemerythrin. Kendall et al. (2014) showed that Por and Oor were protected from oxygen damage by HerA (CJJ81176_0266) and HerB. The former was upregulated during lag phase of stressed cells. An upregulation could also be observed for the cytochrome c oxidase cbb_3-type (subunit IV) CcoQ (CJJ81176_1480) and an uncharacterized protein (CJJ81176_1478); both were recently mentioned together in a publication by Garg et al. (2021) who linked their activity to copper trafficking and homeostasis. Although oxygen has been recognized as the preferred electron acceptor of *C. jejuni,* energy can also be produced through respiration of other terminal electron acceptors such as fumarate and nitrite (Sellars et al., 2002). Indeed, the fumarate reductase cytochrome b subunit FrdC (CJJ81176_0432) required for fumarate respiration and the periplasmic nitrate reductase NapA (CJJ81176_0801) were upregulated. The former reduces fumarate through FrdABC but also acts as a succinate dehydrogenase. Fumarate can also be reduced by the periplasmic methylmenaquinone:fumarate reductase MfrABC which was upregulated in both, reference and stressed cells as discussed before. NapA is part of a complex (NapAB) in which it receives electrons from NapB and subsequently reduces nitrate to nitrite (Pittman et al., 2007). These results indicate that additional electron acceptors are of importance during lag phase of stressed cells. In addition, management of redox stress appears highly relevant, as thioredoxin Trx (CJJ81176_0183), an efficient thiol-disulfide

reductant that can regulate enzyme activity, and the thiol:disulfide interchange protein DsbA (CJJ81176_0883), were also upregulated. Dsb enzymes are responsible for forming disulfide bonds in newly synthesized periplasmic proteins which are subsequently transported into the periplasm (Grabowska et al., 2014; Telhig et al., 2020).

Conclusion

The aim of this study was to gain insight into important cellular processes that take place during lag phase of reference and refrigeration-stressed cells of *C. jejuni* during enrichment in selective BB. Proteomes of reference cells showed upregulation of chaperones involved in maintenance of (membrane) protein quality (ClpB and FtsH-like protease). In addition, upregulation of redox enzymes and anaerobic electron transfer with periplasmic fumarate reductase (MfrABC) acting as terminal electron acceptor was observed during lag phase of reference and stressed cells. The latter cells showed additional upregulation of multiple cell functions including purine metabolism, DNA and protein damage repair, iron acquisition and upregulation of subunits of additional electron acceptors including periplasmic nitrate reductase, cytoplasmic fumarate reductase (FrdABC complex) and cytochrome c oxidase. Activation of multiple (an)aerobic electron transfer chains points to the importance of additional modes of energy generation, conceivably required to support DNA and protein damage repair and outgrowth of stressed *C. jejuni* cells. The outcomes of this study indicated that (an)aerobic respiration was crucial during the lag phase of *C. jejuni* which could indicate that controlled microaerobic oxygenation during *Campylobacter* enrichments might be important.

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enrichment-based detection of foodborne pathogens” with project number 15002, which is partly financed by the Dutch Research Council (NWO).

Supplementary materials

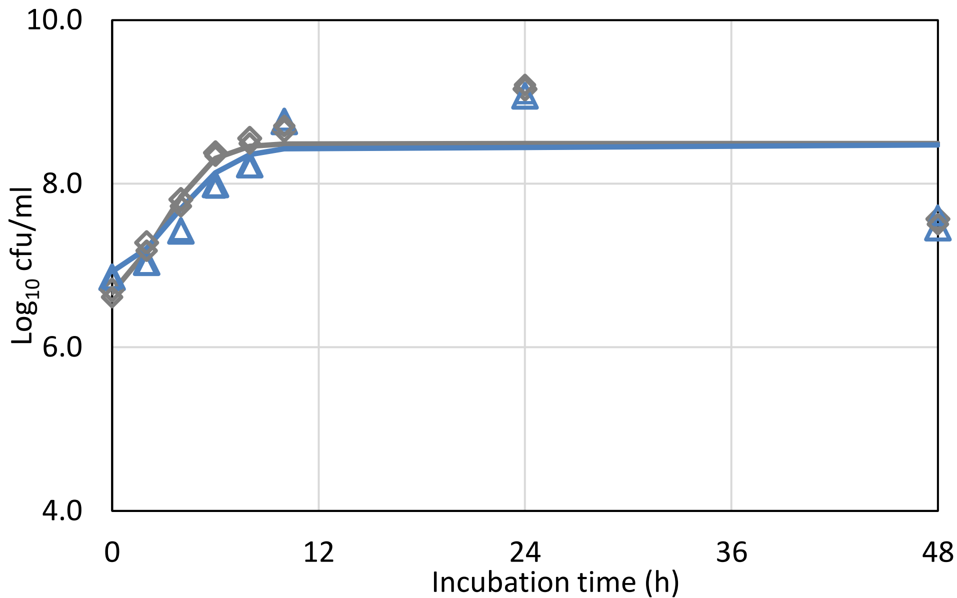

Figure S 1: Growth kinetics of *C. jejuni* strain 81-176 during enrichment in selective BB at high inoculum concentrations of reference cells (grey diamonds) and refrigeration-stressed cells (blue triangles). Error bars depict the standard deviation (n=2). The grey and blue growth curves represent the growth of reference and stressed cells estimated with the Baranyi-model.

Table S 1: Complete list of all 51 proteins with significant differential expression on at least one time point during lag-duration of reference cells. Log_2-fold changes marked in bold indicate significant down- or upregulated compared to t0h. Rows marked in italics indicate proteins which exhibited the same trend in down- or upregulation in stressed cells

Uniprot code	Name	Function	0h10	0h30	1h
A0A0H3P9J3	CJJ81176_0988	Thioesterase family protein	**4.70**	1.28	1.61
A0A0H3PAB3	CJJ81176_1018	Transcriptional regulator, LysR family	3.42	0.26	**3.97**
A0A0H3PAE9	CJJ81176_1477	Uncharacterized protein	**6.11**	4.40	4.19
A0A0H3PAR8	CJJ81176_0554	Endolytic murein transglycosylase (EC 4.2.2.-)	**1.26**	-1.87	-0.23
A0A0H3PAT7	CJJ81176_0784	Serine acetyltransferase (EC 2.3.1.30)	**1.15**	0.90	0.63
A0A0H3PGV0	CJJ81176_0070	Transglycosylase domain protein	**1.09**	0.50	0.81
A0A0H3PGW3	CJJ81176_1177	Uncharacterized protein	**1.18**	-1.00	0.65
A0A0H3PHU7	CJJ81176_1506	Uncharacterized protein	3.57	**3.67**	**4.13**
A0A0H3PI57	CJJ81176_1484	Sensory box sensor histidine kinase, putative	**3.73**	3.27	-1.57
A0A0H3PJ78	CJJ81176_0401	ATPase, AAA family protein	**1.07**	0.93	0.98
A0A0H3PJA6	CJJ81176_0349	Endoribonuclease L-PSP family protein	**-5.67**	-2.04	0.75
A1VY69	CJJ81176_0372	Tryptophan synthase beta chain (EC 4.2.1.20)	**-1.38**	**-1.25**	**-1.41**
A1W094	CJJ81176_1125	ATP-dependent Clp protease adapter protein ClpS	**-4.93**	**-4.68**	-0.14
A1W1D6	CJJ81176_1522	Acetyl-coenzyme A synthetase (AcCoA synthetase)	**1.02**	0.01	0.18
Q2M5Q4	CJJ81176_1320	AccP (Acyl carrier protein, putative)	**-5.61**	**-5.64**	-0.66
A0A0H3P9X6	CJJ81176_1083	Nitroreducatase family protein, authentic frameshift	-0.03	**-1.55**	-0.80
A0A0H3P9Y0	CJJ81176_1228	Peptidase, M23/M37 family	0.22	**-1.01**	-0.14
A0A0H3PAC6	CJJ81176_1013	Delta-aminolevulinic acid dehydratase (EC 4.2.1.24)	-1.19	**-4.59**	-0.10
A0A0H3PAD1	CJJ81176_0466	Aminopyrimidine aminohydrolase (EC 3.5.99.2)	-3.69	**-5.45**	-3.65
A0A0H3PBU0	CJJ81176_0335	Membrane protein, putative	-2.19	-4.46	**-6.18**
A0A0H3PBW9	CJJ81176_0537	ATP-dependent chaperone protein ClpB	0.84	**1.30**	**1.00**
A0A0H3PC37	CJJ81176_0680	Penicillin-binding protein 2	-0.26	**-4.55**	-0.37
A0A0H3PDB4	CJJ81176_0917	Copper chaperone PcuC	0.69	**-1.71**	-0.54
A0A0H3PEV1	CJJ81176_0282	Exodeoxyribonuclease III (EC 3.1.11.2)	-2.23	**-5.01**	-2.52
A0A0H3PEV8	CJJ81176_0536	Penicillin-binding protein 1A	-0.12	**-1.28**	-0.23
A0A0H3PEW2	CJJ81176_0211	Iron ABC transporter, periplasmic iron-binding protein	-0.57	**-2.63**	-1.81

Table S 1: Continued

A0A0H3PIX5	CJJ81176_0480	Uncharacterized protein	0.45	**-3.10**	0.83
A1VY51	CJJ81176_0354	Nucleoside diphosphate kinase (NDK) (NDP kinase) (EC 2.7.4.6) (Nucleoside-2-P kinase)	-0.28	**-1.09**	-0.43
A1VYV0	CJJ81176_0618	Carboxy-S-adenosyl-L-methionine synthase (Cx-SAM synthase) (EC 2.1.3.-)	-0.98	**-3.34**	-2.27
A1W1H0	CJJ81176_1556	NADH-quinone oxidoreductase subunit I (EC 7.1.1.-)	0.45	**-1.26**	0.54
A0A0H3P9M2	CJJ81176_0734	Uncharacterized protein	-2.38	-2.65	**-4.65**
A0A0H3P9X7	CJJ81176_1603	Hemin ABC transporter, ATP-binding protein, putative	-0.38	-2.60	**-1.14**
A0A0H3PAH9	CJJ81176_1530	Flavodoxin-like fold domain protein	-1.10	-0.47	**-1.47**
A0A0H3PAX7	CJJ81176_0816	Mur ligase family protein	-2.53	0.39	**-3.95**
A0A0H3PB49	CJJ81176_1548	Methyl-accepting chemotaxis protein	0.58	0.85	**1.08**
A0A0H3PGV5	CJJ81176_0031	Molybdopterin oxidoreductase family protein	0.18	0.71	**1.05**
A1W1W9	CJJ81176_0014	3-isopropylmalate dehydratase small subunit (EC 4.2.1.33)	-0.49	-2.40	**-3.85**
A0A0H3P997	*CJJ81176_0071*	*Putative integral membrane protein*	*-3.53*	***-5.36***	*-1.93*
A0A0H3P9I1	*CJJ81176_0782*	*Uncharacterized protein*	***1.10***	***2.14***	***3.59***
A0A0H3PA34	*CJJ81176_0931*	*Protein-glutamate methylesterase (EC 3.1.1.61)*	*-0.36*	***-3.40***	*-1.76*
A0A0H3PA58	*CJJ81176_0843*	*Membrane protein, putative*	*2.86*	*2.78*	***4.67***
A0A0H3PA83	*CJJ81176_0885*	*Cytochrome C*	***4.59***	***4.97***	***4.94***
A0A0H3PAG3	*CJJ81176_0465*	*Periplasmic methylmenaquinone:fumarate reductase subunit MfrC*	*0.15*	*0.27*	***1.21***
A0A0H3PBQ2	*CJJ81176_0463*	*Periplasmic methylmenaquinone:fumarate reductase subunit MfrA*	*0.18*	*0.36*	***1.02***
A0A0H3PCH2	*CJJ81176_0038*	*Rubrerythrin*	*-0.51*	*0.32*	***1.33***
A0A0H3PEI3	*CJJ81176_0674*	*Endolytic peptidoglycan transglycosylase RlpA (EC 4.2.2.-)*	*-0.61*	***-2.40***	*-1.79*
A0A0H3PGM1	*CJJ81176_0122*	*Aspartate ammonia-lyase (Aspartase) (EC 4.3.1.1)*	*0.22*	***1.02***	***1.36***
A0A0H3PJB7	*CJJ81176_0464*	*Periplasmic methylmenaquinone:fumarate reductase subunit MfrB*	*0.54*	*0.66*	***1.46***
A1VZL9	*CJJ81176_0892*	*30S ribosomal protein S15*	*-0.42*	*-0.86*	***-1.40***
A1W1S4	*CJJ81176_1668*	*Enolase (EC 4.2.1.11)*	*0.50*	***-1.89***	*-1.16*
Q0Q7H5	*CJJ81176_1574*	*Flavohemoprotein, truncation (Hemoglobin-like flavoprotein-like protein)*	*-0.90*	*-1.81*	***2.70***

Table S 2: Complete list of all 106 proteins with significant differential expression on at least one time point during lag-duration of stressed cells. Log_2-fold changes marked in bold indicate significant down- or upregulated compared to t0h. Rows marked in italics indicate proteins which exhibited the same trend in down- or upregulation in reference cells

Uniprot code	Name	Function	0h10	0h30	1h	2 h
A0A0H3P9E6	CJJ81176_1179	zf-TFIIB domain-containing protein	**1.06**	0.40	**1.32**	**1.42**
A0A0H3P9R0	CJJ81176_1236	DNA-binding response regulator	**4.28**	3.03	3.15	3.07
A0A0H3P9Z2	CJJ81176_0859	Soluble lytic murein transglycosylase, putative	**-1.28**	-1.41	-0.75	**-1.09**
A0A0H3P9Z6	CJJ81176_0695	ABC transporter, ATP-binding protein	**-1.35**	**-1.72**	**-1.53**	**-1.33**
A0A0H3PA65	CJJ81176_1642	Methionine aminopeptidase (MAP) (MetAP) (EC 3.4.11.18) (Peptidase M)	**1.66**	**1.38**	**1.06**	**1.67**
A0A0H3PA86	CJJ81176_0724	Peptidase, U32 family	**-1.27**	-0.24	-0.54	-0.86
A0A0H3PAA1	CJJ81176_1497	UPF0033 domain-containing protein	**1.62**	**1.99**	**1.46**	**2.32**
A0A0H3PAI7	CJJ81176_1559	Uncharacterized protein	**4.16**	1.78	1.88	3.80
A0A0H3PAM6	CJJ81176_1446	tRNA(Ile)-lysidine synthase (EC 6.3.4.19)	**-7.12**	0.12	-1.27	-2.49
A0A0H3PAP1	CJJ81176_0908	4-methyl-5(B-hydroxyethyl)-thiazole monophosphate biosynthesis enzyme	**1.36**	**1.59**	1.04	**1.83**
A0A0H3PAU9	CJJ81176_0809	Uncharacterized protein	**1.15**	0.70	**1.47**	**1.44**
A0A0H3PAW0	CJJ81176_0749	Magnesium transport protein CorA	**3.70**	**3.74**	1.45	3.14
A0A0H3PB55	CJJ81176_0474	Uncharacterized protein	**-1.26**	-0.36	-0.11	**-1.56**
A0A0H3PB69	CJJ81176_1394	MmgE/PrpD family protein	**-1.29**	-0.09	-0.20	-0.60
A0A0H3PBK2	CJJ81176_0676	Uncharacterized protein	**3.39**	0.27	1.60	1.31
A0A0H3PCI2	CJJ81176_0072	Uncharacterized protein	**5.62**	0.00	0.00	0.00
A0A0H3PD11	CJJ81176_1024	MiaB-like tRNA modifying enzyme	**4.62**	**4.46**	2.48	**4.66**
A0A0H3PD29	CJJ81176_1071	NAD-dependent protein deacylase (EC 2.3.1.286) (Regulatory protein SIR2 homolog)	**4.98**	1.59	1.48	1.54
A0A0H3PD90	CJJ81176_0725	N5-carboxyaminoimidazole ribonucleotide mutase (N5-CAIR mutase) (EC 5.4.99.18)	**6.70**	4.73	3.32	4.57
A0A0H3PDI8	CJJ81176_0715	Uncharacterized protein	**4.92**	1.55	1.65	1.48
A0A0H3PDL6	CJJ81176_1459	Flagellar hook-associated protein FlgK, putative	**1.84**	**1.55**	0.02	**1.94**
A0A0H3PDX9	CJJ81176_1613	Riboflavin biosynthesis protein RibD (EC 1.1.1.193) (EC 3.5.4.26)	**4.34**	**4.12**	-1.84	2.37
A0A0H3PE75	CJJ81176_1621	Protein TonB	**5.96**	4.58	4.16	2.35
A0A0H3PEC2	CJJ81176_0189	RNA methyltransferase, TrmH family (EC 2.1.-.-)	**3.03**	0.88	1.76	0.60

Table S 2: Continued

A0A0H3PET5	CJJ81176_0471	TonB-dependent receptor, putative, degenerate	**2.37**	0.08	-0.90	-0.09
A0A0H3PGP1	CJJ81176_0113	L-lactate permease	**3.14**	1.65	1.59	**3.19**
A0A0H3PHL6	CJJ81176_0799	Major antigenic peptide PEB2	**1.55**	0.80	0.71	**1.96**
A0A0H3PHS8	CJJ81176_1412	Capsule polysaccharide export protein KpsS	**4.28**	0.00	0.00	1.49
A0A0H3PIN9	CJJ81176_1478	Uncharacterized protein	**7.19**	1.97	3.01	2.88
A0A0H3PIY7	CJJ81176_0415	Pyruvate kinase (EC 2.7.1.40)	**-1.10**	-0.39	-0.33	0.14
A1VXQ2	CJJ81176_0205	Superoxide dismutase [Fe] (EC 1.15.1.1)	**-1.62**	**-1.35**	-1.04	-0.73
A1VXZ7	CJJ81176_0300	3-hydroxyacyl-[acyl-carrier-protein] dehydratase FabZ (EC 4.2.1.59)	**7.04**	1.80	2.33	2.35
A1VY31	CJJ81176_0334	Peptidyl-tRNA hydrolase (PTH) (EC 3.1.1.29)	**-5.13**	-3.38	-3.13	-1.89
A1VYF9	CJJ81176_0468	Acyl carrier protein (ACP)	**-1.09**	-0.52	0.38	-0.10
A1VYT7	CJJ81176_0605	S-adenosylmethionine:tRNA ribosyltransferase-isomerase (EC 2.4.99.17)	**5.60**	2.11	2.18	**6.29**
A1VZ22	CJJ81176_0690	ATP-dependent protease subunit HslV (EC 3.4.25.2)	**4.88**	-2.06	-2.06	1.89
A1VZ48	CJJ81176_0716	Ribosomal RNA small subunit methyltransferase H (EC 2.1.1.199)	**5.61**	1.80	**4.90**	0.00
A1VZJ8	CJJ81176_0871	Bifunctional protein FolD	**-1.05**	-0.70	-0.48	-0.79
A1W059	CJJ81176_1090	30S ribosomal protein S18	**1.04**	0.05	-0.43	-0.30
A1W0I8	CJJ81176_1219	ATP synthase subunit a (ATP synthase F0 sector subunit a) (F-ATPase subunit 6)	**3.28**	2.13	0.77	**3.38**
A1W1V3	CJJ81176_1697	50S ribosomal protein L16	**1.13**	0.18	-0.25	-0.32
A1W1V5	CJJ81176_1699	50S ribosomal protein L22	**1.21**	0.12	-0.52	-0.26
Q0Q7I1	CJJ81176_1514	Phosphoribosylformylglycinamidine cyclo-ligase (EC 6.3.3.1)	**1.08**	0.97	0.35	**1.00**
A0A0H3PAE1	CJJ81176_0192	Ribosomal RNA small subunit methyltransferase E (EC 2.1.1.193)	2.07	**6.14**	1.81	2.19
A0A0H3PB07	CJJ81176_1268	LPS-assembly protein LptD	0.37	**-3.91**	**-3.72**	**-3.69**
A0A0H3PB56	CJJ81176_1521	UTP--glucose-1-phosphate uridylyltransferase (EC 2.7.7.9) (UDP-glucose pyrophosphorylase)	1.00	**1.00**	0.30	**1.14**
A0A0H3PBK5	CJJ81176_0541	Phosphoribosylformylglycinamidine synthase subunit PurS (FGAM synthase) (EC 6.3.5.3)	0.66	**1.01**	**1.13**	**1.20**
A0A0H3PDD9	CJJ81176_0743	Flagellin subunit protein FlaC	0.39	**1.56**	**1.79**	**1.97**
A0A0H3PEG8	CJJ81176_0642	Phosphate ABC transporter, periplasmic phosphate-binding protein, putative	0.42	**1.01**	**1.33**	**1.10**
A0A0H3P9U1	CJJ81176_1487	Uncharacterized protein	0.21	0.26	**1.03**	0.85
A0A0H3PA10	CJJ81176_1353	Enterochelin ABC transporter, ATP-binding protein	-0.51	-0.45	**-1.31**	**-1.15**
A0A0H3PAC3	CJJ81176_1161	CMP-Neu5Ac synthetase	0.32	0.15	**-4.58**	-2.42
A0A0H3PAJ8	CJJ81176_1033	High affinity branched-chain amino acid ABC transporter, ATP-binding protein	-0.62	-0.59	**-1.14**	-0.90
A0A0H3PB02	CJJ81176_0220	HTH OST-type domain-containing protein	0.00	-2.37	**-4.06**	-0.41

Table S 2: Continued

A0A0H3PB89	CJJ81176_1237	Non-heme iron protein, hemerythrin family	0.58	0.54	**1.24**	-2.08
A0A0H3PBJ8	CJJ81176_0612	DNA polymerase III, delta prime subunit, homolog	0.44	-1.07	**-3.85**	-2.66
A0A0H3PBV0	CJJ81176_0603	Acetolactate synthase, small subunit (EC 2.2.1.6)	-0.78	-0.72	**-1.03**	-0.92
A0A0H3PBZ1	CJJ81176_0414	Uncharacterized protein	3.08	3.26	**6.30**	**7.38**
A0A0H3PCF2	CJJ81176_0965	Permease, putative	1.97	-0.15	**5.01**	**5.03**
A0A0H3PIY4	CJJ81176_0429	Shikimate dehydrogenase (NADP(+)) (SDH) (EC 1.1.1.25)	-0.85	-1.12	**-1.26**	-1.10
A0A0H3PJ11	CJJ81176_0153	PP-loop family protein	0.28	-0.47	**-1.04**	-0.61
A0A0H3PJ70	CJJ81176_0252	Acetylornithine aminotransferase (ACOAT) (EC 2.6.1.11)	-0.88	-0.55	**-1.15**	**-1.29**
A0A0H3PJE6	CJJ81176_0622	DNA/RNA non-specific endonuclease	1.43	1.65	**3.59**	**3.65**
A1VXH8	CJJ81176_0130	50S ribosomal protein L27	0.53	0.26	**-6.40**	-0.52
A1VYZ2	CJJ81176_0660	Ketol-acid reductoisomerase (NADP(+)) (KARI) (EC 1.1.1.86)	0.25	-1.62	**-2.20**	-1.09
A1VZQ4	CJJ81176_0928	Major cell-binding factor (CBF1) (PEB1)	0.28	0.54	**1.08**	**1.14**
A1W0A5	CJJ81176_1136	Chemotaxis protein CheY homolog	0.40	0.81	**1.01**	0.90
A1W1K3	CJJ81176_1588	1-(5-phosphoribosyl)-5-[(5-phosphoribosylamino)methylideneamino] imidazole-4-carboxamide isomerase (EC 5.3.1.16)	0.18	0.79	**1.08**	**1.31**
Q29W30	CJJ81176_0443	YceI family member	-0.11	0.48	**1.17**	0.82
A0A0H3P9H3	CJJ81176_0883	Thiol:disulfide interchange protein DsbA, putative	0.29	0.65	0.88	**1.16**
A0A0H3P9I3	CJJ81176_0793	Lipoprotein, NLPA family	0.38	0.78	0.73	**1.14**
A0A0H3P9M3	CJJ81176_0048	Fumarylacetoacetate hydrolase family protein	2.48	4.86	5.19	**7.92**
A0A0H3P9M5	CJJ81176_0050	Adenylosuccinate lyase (ASL) (EC 4.3.2.2) (Adenylosuccinase)	-0.64	-0.49	-0.68	**-1.19**
A0A0H3PAF3	CJJ81176_0231	Uncharacterized protein	0.00	4.28	4.30	**7.36**
A0A0H3PAI1	CJJ81176_1480	Cytochrome c oxidase, cbb3-type, subunit IV (EC 1.9.3.1)	1.88	1.71	0.17	**4.64**
A0A0H3PAK3	CJJ81176_1587	Imidazole glycerol phosphate synthase subunit HisH (EC 4.3.2.10)	0.91	0.68	0.74	**1.40**
A0A0H3PAL3	CJJ81176_1384	Flavodoxin	0.37	0.58	0.65	**1.31**
A0A0H3PAL9	CJJ81176_1404	Ribosomal silencing factor RsfS	1.17	1.76	2.02	**2.08**
A0A0H3PAP0	CJJ81176_0183	Thioredoxin	0.13	0.56	0.82	**1.11**
A0A0H3PBN2	CJJ81176_0278	Uncharacterized protein	1.41	3.38	1.71	**7.18**
A0A0H3PD19	CJJ81176_1081	Competence/damage-inducible domain protein	-1.07	-0.74	-3.03	**-1.23**
A0A0H3PEG0	CJJ81176_0314	Lipid-A-disaccharide synthase (EC 2.4.1.182)	-0.17	-2.88	-3.13	**-4.64**
A0A0H3PEJ9	CJJ81176_0432	Fumarate reductase, cytochrome b subunit	4.10	3.46	3.99	**5.16**

Table S 2: Continued

A0A0H3PH47	CJJ81176_1185	Uncharacterized protein	1.08	-1.47	-3.76	**-5.84**
A0A0H3PHE9	CJJ81176_0894	Flagellin	-0.54	-3.34	-3.66	**-6.13**
A0A0H3PJF3	CJJ81176_0154	Pyrazinamidase/nicotinamidase, putative	-0.82	-0.47	-0.84	**-1.23**
A1VYG9	CJJ81176_0478	Phosphomethylpyrimidine synthase (EC 4.1.99.17)	0.14	0.40	0.66	**1.30**
A1VYJ0	CJJ81176_0505	50S ribosomal protein L11	-0.09	-0.37	-0.31	**-1.06**
A1VZC8	CJJ81176_0801	Periplasmic nitrate reductase (EC 1.9.6.1)	0.25	0.67	0.89	**1.26**
A1VZR0	CJJ81176_0934	Adenine phosphoribosyltransferase (APRT) (EC 2.4.2.7)	0.36	0.68	0.46	**1.07**
A1W0M1	CJJ81176_1252	Pyridoxine 5'-phosphate synthase (PNP synthase) (EC 2.6.99.2)	0.64	0.07	-0.22	**-5.91**
A1W1U4	CJJ81176_1688	50S ribosomal protein L18	1.07	-0.07	-1.04	**-1.49**
A0A0H3P997	*CJJ81176_0071*	*Putative integral membrane protein*	*-2.75*	*-2.45*	***-4.64***	***-4.69***
A0A0H3P9I1	*CJJ81176_0782*	*Uncharacterized protein*	*0.88*	*1.99*	***3.02***	***3.41***
A0A0H3PA34	*CJJ81176_0931*	*Protein-glutamate methylesterase (EC 3.1.1.61)*	*0.31*	*-0.65*	*-1.02*	***-4.09***
A0A0H3PA58	*CJJ81176_0843*	*Membrane protein, putative*	***4.76***	*1.32*	*0.00*	*1.47*
A0A0H3PA83	*CJJ81176_0885*	*Cytochrome C*	*1.62*	***5.33***	***5.77***	*3.50*
A0A0H3PAG3	*CJJ81176_0465*	*Periplasmic methylmenaquinone:fumarate reductase subunit MfrC*	*-0.15*	*0.54*	*0.84*	***1.45***
A0A0H3PBQ2	*CJJ81176_0463*	*Periplasmic methylmenaquinone:fumarate reductase subunit MfrA*	*-0.48*	*0.25*	*0.66*	***1.14***
A0A0H3PCH2	*CJJ81176_0038*	*Rubrerythrin*	*0.43*	*1.23*	*1.93*	***2.49***
A0A0H3PEI3	*CJJ81176_0674*	*Endolytic peptidoglycan transglycosylase RlpA (EC 4.2.2.-)*	*-0.37*	*-0.54*	***-1.70***	***-1.66***
A0A0H3PGM1	*CJJ81176_0122*	*Aspartate ammonia-lyase (Aspartase) (EC 4.3.1.1)*	*-0.59*	*0.25*	*1.45*	***1.56***
A0A0H3PJB7	*CJJ81176_0464*	*Periplasmic methylmenaquinone:fumarate reductase subunit MfrB*	*-0.24*	*0.20*	*0.74*	***1.14***
A1VZL9	*CJJ81176_0892*	*30S ribosomal protein S15*	*0.59*	*-0.40*	*-0.64*	***-1.26***
A1W1S4	*CJJ81176_1668*	*Enolase (EC 4.2.1.11)*	*0.38*	*-1.06*	***-2.10***	*-0.78*
Q0Q7H5	*CJJ81176_1574*	*Flavohemoprotein, truncation (Hemoglobin-like flavoprotein-like protein)*	*-0.81*	*1.12*	*1.86*	***2.44***

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Combining enrichment with multiplex real-time PCR leads to faster detection and identification of *Campylobacter* spp. in food compared to ISO 10272-1:2017

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Abstract

Conventional protocols for the detection of *Campylobacter* from foods are laborious and time-consuming. This research describes an alternative procedure (EMRT-PCR) for the detection of *Campylobacter* from food by combining ISO 10272-1:2017 enrichment in Bolton broth (BB) with a multiplex real-time (MRT-) PCR assay. Species differentiation was done by targeting *C. jejuni* (*mapA*), *C. coli* (*ceuE*), and both species (*cje*). The detection limit of the MRT-PCR assay was 4.5 and 5.5 $\log_{10}$ cfu/ml in BB and BB containing chicken skin, respectively. A Monte Carlo simulation was conducted to predict the probability that campylobacters reach the MRT-PCR detection threshold throughout enrichment in BB, and results suggested that cold-stressed campylobacters could reach the detection limit after 40 h of enrichment (p=0.99). As a proof of principle, 23 naturally contaminated meat products were enriched according to ISO 10272-1:2017 procedure A, and the EMRT-PCR in parallel. After 24 h, 12 and 11 samples already tested positive for *Campylobacter* with the ISO method and EMRT-PCR, respectively. After 40 h, the 24-h-negative sample was also positive with EMRT-PCR. The EMRT-PCR takes about 2 days to produce reliable results, while results using ISO 10272-1:2017 can take up to 8 days, which demonstrate the potential of the EMRT-PCR method.

Introduction

In 2020, the foodborne pathogen *Campylobacter* was the cause of the most reported zoonotic gastroenteritis cases in the European Union (EU) and has been doing so since 2005 (EFSA & ECDC, 2021). The latest EFSA report showed that illness was mostly associated with *Campylobacter jejuni* (88.1% of confirmed cases of which species information was provided) and *C. coli* (10.6 %) followed by *C. fetus, C. upsaliensis* and *C. lari* (associated with less than 0.2 %, each) (EFSA & ECDC, 2021). According to the latest EFSA report, *Campylobacter* was the fourth most frequently reported cause of foodborne outbreak in Europe but fortunately, hospitalization and death rates are rather low (7.1 % and 0.04 % of all reported illness cases, respectively) (EFSA & ECDC, 2021). In the United States of America, 9% of the foodborne illnesses is estimated to be caused by *Campylobacter* spp. annually (Scallan et al., 2011). *Campylobacter* is frequently found in foods such as raw milk, meat and meat products (broiler and turkey meat) (EFSA & ECDC, 2019; Rossler et al., 2019) but, to a lesser extent, was also isolated from fresh fruits and vegetables (Mohammadpour et al., 2018). Conventional methods for the qualitative detection of low levels of *Campylobacter* spp. make use of an enrichment followed by isolation and confirmation steps. Although theoretically, a single cell in 10 g of food product should be detectable, procedures are lengthy. In case of ISO 10272-1:2017 procedure A, a 48-h enrichment is followed by an isolation step of 48 h and confirmation steps of another 72-96 h, which means that negative results can be obtained after 4 days, while confirmed positive results can be obtained only after 7-8 days. Other culturing-based methods (e.g., direct plating on selective agar) can shorten detection times but have a higher detection limit. Furthermore, limited selectivity of the media has shown to complicate reliable detection due to overgrowth of competitors in culturing and plating media and subsequent masking of *Campylobacter* colonies (Chon et al., 2017; Hazeleger et al., 2016). Moreover, insufficient supply of microaerobic conditions during *Campylobacter* enrichment with common competitors resulted in decreased cell concentrations of *C. jejuni* after enrichment and possible detection failures (Lanzl et al., 2022).

Molecular detection approaches such as (quantitative) polymerase-chain reaction (qPCR) or immunological methods like enzyme-linked immunosorbent assays (ELISA) are generally less time-consuming and are often utilized in clinical settings

(Granato et al., 2010; Shams et al., 2017; Valledor et al., 2020). However, it has been suggested that in samples with a complex matrix such as poultry, the utilization of quantitative PCR may be preferable over immunosorbent assays (Reis et al., 2018). Although molecular assays are much faster compared to culturing-based detection of pathogens, their detection limit is also considerably higher; generally, a minimal cell concentration between 10^3-10^5 cfu/ml is required for reliable results using PCR, and slightly lower concentrations are required for real-time PCR (Wang and Salazar, 2016). Furthermore, the sensitivity of qPCR is often affected negatively by the presence of interfering compounds or competing microbiota when food products are used as sample matrix (Jasson et al., 2010). Several approaches to molecularly detect campylobacters have been developed (e.g., Adekunle et al., 2019; De Boer et al., 2015; Melero et al., 2011; Nouri Gharajalar et al., 2020; Overesch et al., 2020; Seliwiorstow et al., 2015), however, in most applications, faecal material was used as sample matrix instead of food in order to identify and monitor highly colonized broiler batches. Often, *Campylobacter* concentrations are quite high in faecal samples (>6 log_{10} cfu/ml) (Seliwiorstow et al., 2015), while *Campylobacter* numbers are often much lower in foods (from less than 1 up to 3 log_{10} cfu/ml) (Sampers et al., 2010).

A considerable amount of research has been conducted to develop protocols utilizing qPCR for the detection of campylobacters from food products and some aimed at excluding the enrichment step completely (Josefsen et al., 2010; Liu et al., 2017; Papić et al., 2017; Schnider et al., 2010; Toplak et al., 2012; Ugarte-Ruiz et al., 2012; Vondrakova et al., 2014; Yang et al., 2003). Although cell concentrations can be already sufficiently high for detection through qPCR, Melero et al. (2011) showed that in 6% of naturally contaminated samples, *C. jejuni* could only be detected by qPCR after a 48-h enrichment, and Vondrakova et al., (2014) showed that food samples spiked with different concentrations of campylobacters could only be detected through qPCR when cell concentrations were above 10^2 cfu/ml. The initial contamination level therefore is a restricting factor for the utilization of direct molecular detection.

Therefore, a preceding enrichment step is often necessary to increase cell concentrations to reach the detection threshold of nucleic-acid-based procedures, and additionally, resuscitate sub-lethally injured campylobacters (He and Chen, 2010; Ivanova et al., 2014; Josefsen et al., 2004; Lanzl et al., 2020; Mayr et al., 2010; Rantsiou

et al., 2010; Sails et al., 2003). Ivanova et al., (2014) demonstrated that *C. jejuni* was detected from slaughtering environment samples through real-time PCR from colonies grown on modified charcoal cefoperazone-deoxycholate agar (mCCDA) after enrichment in BB. Mayr et al. (2010) combined a 40-48 h enrichment in Preston broth (PB) with MRT-PCR to detect different *Campylobacter* species. Other studies applied modified versions of ISO 10272-1:2006, either utilizing blood-free Bolton broth (Rantsiou et al., 2010; Sails et al., 2003) or a modified sample preparation step (He and Chen, 2010) before enrichment in Bolton broth. The ISO 10272-1:2017 protocol states that an enrichment in Bolton broth should be applied when campylobacters are expected to be sub-lethally injured or stressed in examined food products (International Organization for Standardization, 2017), which can be expected for raw meat products which are often transported and stored at refrigeration- or freezing temperatures (Bhaduri and Cottrell, 2004; Lanzl et al., 2020; Maziero et al., 2010) and Bolton broth showed to be sufficiently nutritious for the recovery and growth of freeze-stressed campylobacters (Lanzl et al., 2022). Josefsen et al., (2004) followed the ISO 10272:2006 procedure and were able to detect campylobacters after conducting an enrichment in Bolton broth, however, differentiation between species within the group of thermotolerant campylobacters was not achieved. The aim of this study was to develop a rapid but reliable protocol for the detection and differentiation of *C. jejuni* and *C. coli* from food products combining enrichment in Bolton broth (based on ISO 10272-1:2017 procedure A) with MRT-PCR, to on the one hand allow repair of damaged *Campylobacter* and detect low levels of campylobacters, but on the other hand shorten the detection time compared to the ISO 10272-1:2017 method, with identification included as an additional facet.

Materials and methods

Bacterial strains and preparation of working cultures

17 *C. jejuni* and 12 *C. coli* isolates were used to test the probes- and primer specificity in this study (details of all *Campylobacter* strains used in this study can be found in **table S 1 of the supplementary materials**). For the preparation of working cultures, *C. jejuni* and *C. coli* were plated from the -80 °C vials onto Columbia agar base (CAB, Oxoid, supplemented with 5% (v/v) lysed horse blood (BioTrading Benelux B.V.

Mijdrecht, Netherlands) and an additional 0.5% agar (Bacteriological agar No.1, Oxoid)) and grown for 24 h at 41.5 °C. Subsequently, a single colony was resuspended in Heart Infusion broth (HI; Bacto HI, Becton, Dickinson and Company) and cultured for 24 h at 41.5 °C to obtain stationary phase cultures. Afterward, a 1:500 dilution was made in unselective BB (Oxoid, supplemented with 5% (v/v) sterile lysed horse blood (BioTrading Benelux B.V., Mijdrecht, the Netherlands) without the addition of selective supplements) and cultured for 24 h at 41.5 °C to reach the stationary phase. Cell concentrations of the working cultures were determined by plating appropriate dilutions (made in peptone physiological salt solution (PPS, Tritium Microbiologie)) on CAB plates and incubation for 48 h at 41.5 °C. Throughout this study, *Campylobacter* was cultured under microaerobic conditions (5 % O_2, 10 % CO_2, 85 % N_2) in flushed jars (Anoxomat WS9000, Mart Microbiology, Drachten, Netherlands).

MRT-PCR assay for detecting and differentiating *C. jejuni* and *C. coli*

For the preparation of the primer/probe mix for MRT-PCR (as further explained in section 2.5), primers and probes of four target genes were used, namely an ATP-binding protein (*cje*), a membrane lipoprotein (*mapA*), a periplasmic substrate binding protein (*ceuE*) and an internal amplification control (IAC) (**table 1**). Primer sequences for *cje* were obtained from a publication by Bonjoch et al. (2010), while primer sequences of *mapA* and *ceuE* were obtained from the Dutch Food Safety Authority (NVWA) and modified to ensure similar melting temperatures. All probes as well as the primer sequences of the IAC were developed using SnapGene Viewer (software version 4.2.11). Primers and probes were diluted in sterilized MilliQ to achieve a primer and probe concentration of 500 and 100 nM in the final assay, respectively. iQ Multiplex Powermix (BIORAD) was used to provide buffer, iTaq DNA polymerase and dNTPs. Since primer sequences for *cje*, *mapA* and *ceuE* were taken from recent publications or protocols and therefore, their individual specificity was not further tested.

Table 1: Overview of sequences of primers and probes (cje, mapA, ceuE, IAC) used in this study. Fluorophores used (HEX, FAM, TexasRed and Cy5) are marked in bold while quenchers (Deep dark quencher 1 [DDQ 1], Black hole quenchers 2 and 3 [BHQ2 and BHQ3]) are marked in italics

Target gene	Species	Primer/ probe	Sequence (5'-3')
ATP-binding protein (*cje*)	*C. jejuni & C. coli*	Fw-primer	AGT GCC GAT AAA GGC TCA
		Rv-primer	ACT CGT CGA GCT TGA AGA ATA CG
		Probe	**HEX**-AAG CCA CTC TTT GCA TTT GTC CGC C-*DDQ1*
Membrane lipoprotein (*mapA*)	*C. jejuni*	Fw-primer	CTG GTG GTT TTG AAG CAA AGA TT
		Rv-primer	CAA TAC CAG TGT CTA AAG TGC GTT TAT
		Probe	**FAM**-TTG AAT TCC AAC ATC GCT AAT GTA TAA AAG CCC TTT-*DDQ1*
Periplasmic substrate binding protein (*ceuE*)	*C. coli*	Fw-primer	AAG CTC TTA TTG TTC TAA CCA ATT CTA ACA
		Rv-primer	TCA TCC ACA GCA TTG ATT CCT AA
		Probe	**TexasRed**-ATC ATG AAT GAT TCC AAA GCG AGA TTG AGG TCC A-*BHQ2*
Internal amplification control (*IAC*)	puC18	Fw-primer	CTG GCG TTT TTC CAT AGG CTC C
		Rv-primer	GGG GAA ACG CCT GGT ATC TTT A
		Probe	**Cy5**-CCT GAC GAG CAT CAC AAA AAT CGA CGC TCA A-*BHQ3*

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The specificity of each primer/probe set was validated for four *C. jejuni* (NCTC 11168, 81-176, WDCM 00005 and ATCC 33560) and four *C. coli* isolates (ATCC 33559, WDCM 00004, Ca 2800 and Ca 1607) as well as purified pUC18 DNA in qPCR experiments using four different master mixes, each containing one primer/probe set (*cje, mapA, ceuE* and IAC) (data not shown). After specificity was confirmed in qPCR, the specificity of the multiplex primer/probe set (containing all four primer/probe sets (*cje, mapA, ceuE* and IAC)) was tested by performing MRT-PCR using working cultures of 17 *C. jejuni* and 12 *C. coli* isolates (as prepared in section 2.1). For DNA extraction, 1 ml of working culture of each strain was transferred to a 1.5 ml Eppendorf-tube and cells were lysed in a heat-block at 95 °C for 15 min, cooled down to room-temperature and 10 µl were used as template for MRT-PCR.

Determination of MRT-PCR detection limits

To determine the lower detection limit of MRT-PCR in different matrices and to extract DNA from enrichment samples, a cell lysis and DNA extraction step was performed using the GENE-UP Lysis kit (Biomérieux). The protocol provided by the supplier was followed with one modification, namely a sample volume of 10 µl instead of 20 µl (as stated in the GENE-UP Lysis kit protocol) was used.

The detection sensitivity of the primers and probes was tested with a subset of strains, namely four *C. jejuni* (NCTC 11168, 81-176, WDCM 00005 and WDCM 00156) and four *C. coli* (WDCM 00004, Ca 2800, Ca 1607 and ATCC 33559) isolates. Working cultures were prepared as stated in section 2.1 and 10-fold dilutions were made in three different matrices, namely HI, BB (BB base supplemented with 5% (v/v) sterile lysed horse blood and 0.5% (v/v) selective supplement SR0208) and the pooled liquids of three 48-h-BB-enrichments conducted with chicken skin that was tested *Campylobacter*-free. After DNA extraction, an MRT-PCR assay was conducted. Standard curves were generated for each primer-probe set and species tested by plotting the Ct from each dilution versus cell concentration and quantification cycles (Cq), correlation coefficients (R^2) and linear ranges were determined. The corresponding standard curves can be found in **figure S 1 of the supplementary materials**. This way, thresholds were determined for each primer/probe set and matrix concerning the minimal relative fluorescent units (RFU) and range of Cq-values samples had to comply with, in order to be categorized as positive for *C. jejuni*

or *C. coli*. As a result, it was determined that, for each target gene/fluorophore, an amplification curve had to reach a minimal RFU of 500 during the quantification cycles 15-35 in order to be viewed as positive. Furthermore, for each sample, a positive result was required for the IAC to rule out inhibition by matrix-components. Since this MRT-PCR protocol contained three primer/probe sets (*cje* for the detection of both *C. jejuni* and *C. coli*, and *mapA* and *ceuE* for the specific detection of *C. jejuni* and *C. coli,* respectively), an enrichment sample was only viewed as positive for *C. jejuni*, when both the amplification curves for *cje* and *mapA* complied with the set criteria. Likewise, a sample was only viewed positive for *C. coli*, when the amplification curves for *cje* and *ceuE* were compliant with the settings.

Determination of sampling times for enrichment samples

To determine the sample time points of interest during enrichment, a Monte Carlo simulation was conducted with 100,000 events to predict the probability that campylobacters reached the MRT-PCR detection limit throughout enrichment in BB at various initial cell concentrations varying from -2 to 2 $\log_{10}$ cfu/ml, to simulate contamination levels of 1 to 10^4 cfu/10 g of food that is diluted in 90 ml of BB at the start of the enrichment. For the simulation, the lag-duration (5.4±1.3 h) and growth rate (0.42±0.09 $\log_{10}$ h^{-1}) of freeze-stressed campylobacters during enrichment in BB was used (Lanzl et al., 2020). In the beforementioned study, the growth kinetics of 23 *Campylobacter* strains during enrichment in BB was tested and the variability in lag-duration and growth rate was assessed. For the strain selection, *C. jejuni* and *C. coli* strains isolated from different sources (food, human and environment) were chosen. The data was used for the simulation to predict the probability of severely stressed campylobacters to reach the detection limit of MRT-PCR.

Analysis of naturally contaminated food samples

As food samples, predominantly raw poultry products were used in this study since prevalence of *Campylobacter* is high compared to other foods (Zbrun et al., 2020). In total, 18 poultry and 5 beef products were examined. Samples were purchased at a local butcher and transferred to 4 °C for approximately 2 h before enrichment was conducted. Of each food product, 10 g was collected and used as sample for the enrichment procedure. For poultry products containing skin, as much of the skin was

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collected for the 10 g of sample required for the enrichment procedure. Subsequently, an enrichment was conducted according to ISO 10272-1:2017, procedure A (using BB as enrichment medium). For that, 10 g of food product was transferred into a stomacher bag, diluted with 90 ml of BB and homogenized with a stomacher machine (Seward Stomacher Model 400) for 60 s at 230 rpm. To check whether samples were already highly contaminated at the start of the enrichment, 100 µl of the initial dilution was spread on Rapid *Campylobacter* agar (RCA; BIORAD) plates, which were incubated and subsequently counted to determine the cell concentration. In parallel, the enrichment bag was placed in an Anoxomat jar, which was flushed with a microaerobic gas mixture (5 % O_2, 10 % CO_2, 85 % N_2) using an Anoxomat (WS9000, Mart Microbiology, Drachten, Netherlands) and incubated according to ISO 10272-1:2017 (5 h at 37 °C followed with 43 h at 41.5 °C). After 24, 40 and 48 h samples were taken, and jars were flushed again and re-incubated for the remaining enrichment time. For the ISO detection, 10 µl were taken at each time point and cells were isolated according to procedure A of the ISO 10272-1:2017 protocol with the amendment that RCA was used as a selective isolation medium instead of mCCDA. Typical *Campylobacter* colonies were taken and confirmed through microscopy. Next to that, 1.1 ml of sample was used to determine cell concentrations using RCA-plates. Agar plates were incubated microaerobically for 48 h at 41.5 °C and cell concentrations (range: 1.0-5.5 log_{10} cfu/ml) were determined. For the EMRT-PCR procedure, 10 µl of sample were taken at each time point and cell lysis and DNA extraction were performed using the GENE-UP lysis kit protocol (as explained in section 2.3). Afterwards, 10 µl of each sample was transferred into one well of a 96-well PCR-plate (Hard-Shell 96-well PCR plate HSL9001, BIORAD) filled with 15 µl of iQ Multiplex Powermix (BIORAD), 5 µl of primer/probe mix as prepared according to **table S 3 of the supplementary materials** and 1 µl of IAC sample DNA (250 ng/µl). After filling, the PCR-plate was sealed using the Microseal 'B' PCR Plate Sealing Film (MSB1001, BIORAD) and spun down at 3,800 rpm for 10 s. The plate was then transferred into the PCR-machine (CFX96, BIORAD) and run according to the following settings: Initial denaturation for 2:30 min at 95 °C followed by 40 PCR cycles consisting of 15 s at 95 °C and 60 s at 60 °C.

Results and discussion

Validation of primer/probe sets

All 17 *C. jejuni* and 12 *C. coli* strains were identified correctly using the primer/probe sets as depicted in table 1. The primer/probe set for *cje* gave amplification curves for all *Campylobacter* strains, while the primer/probe sets for *mapA* and *ceuE* gave only amplification curves for *C. jejuni* and 12 *C. coli*, respectively (detailed results are depicted in **table S 1 in the supplementary materials**).

For this MRT-PCR assay, the primer/probe set *cje* (specific for *C. jejuni* and *C. coli*) was used in combination with two primer/probe sets specific for each of the two species. This served as double confirmation since food samples were only labelled positive for a species when amplification results were positive for *cje* as well as for one of the species-specific primer/probe sets (*mapA* or *ceuE*). The target species of the current assay were *C. jejuni* and *C. coli*, since these two species are most associated with campylobacteriosis (EFSA & ECDC, 2021). A further assay extension may also include less prominent species such as *C. lari* or even *C. upsaliensis* (Klena et al., 2004; Wang et al., 2002). Research suggests that primer/probe sets of *gyrA* or *peptT* could be used to reliably detect *C. lari* in rt-PCR assays (Chapela et al., 2015; He and Chen, 2010). However, when designing primers and probes the lengths of the newly designed primer pairs should be comparable to the other primer-sets in the assay (<100 bp differences between amplicons) (Ricke et al., 2019), to ensure that the time-temperature settings of the assay remain the same. Also, the choice of fluorophore and quencher is important to avoid fluorescence resonance energy transfer (FRET). After adaptation of the current setup, the assay should be revalidated to reconfirm species specificity and sensitivity of the assay (On et al., 2013).

The detection limits of the MRT-PCR in spiked BB were 4.5 $\log_{10}$ cfu/ml, for both *Campylobacter* species. However, when chicken skin was added as food sample in both media, the detection limited increased to 5.5 $\log_{10}$ cfu/ml. It has been recognized that food matrices can contain substances which can inhibit (q)PCR reactions (Schrader et al., 2012). A study by Schnider et al., (2010) found, that Cq values corresponding to *C. jejuni* and *C. coli* in a real time-PCR assay were more than five times higher in spiked buffered peptone water (BPW) containing broiler skin samples than in spiked BPW

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without the food matrix, indicating the presence of PCR inhibitors in the food matrix. Consequently, sample preparation protocols and DNA isolation methods have been developed to remove such inhibitors. In this study, different DNA extraction and isolation protocols have been tested such as the application of thermal cell lysis (95 °C, 10 min), Chelex-100, the DNA isolation kits WIZARD Genomic DNA Purification Kit (Promega), GenElute^{TM} Bacterial Genomic DNA Kit (Sigma-Aldrich), DNeasy Blood &Tissue Kit (Qiagen) and the GENE-UP lysis protocol. In pure cultures, a thermal cell lysis step of ten min was sufficient for subsequent detection of campylobacters through MRT-PCR, and longer (15, 20, 30 min) lysis steps did not enhance the MRT-PCR signal (no increase in RFU or lower Cq-values). In more complex matrices such as chicken meat products, the thermal lysis step was not sufficient to remove PCR inhibitors. The GENE-UP lysis protocol, however, proved to be fast and user-friendly to isolate bacterial DNA from an enriched food sample for subsequent MRT-QPCR analysis.

Determining enrichment sampling times for MRT-PCR detection

According to ISO 10272-1:2017 procedure A, enrichment in BB is conducted for 48 h before the detection step takes place. To determine possible earlier detection time points, a Monte Carlo simulation was conducted to predict the probability that campylobacters reached the MRT-PCR detection limit throughout enrichment in BB at various initial cell concentrations. Data on the growth kinetics of freeze-stressed campylobacters was used to simulate enrichment of severely stressed cells, which need a longer recovery time resulting in a later reach of the required cell concentrations for the application of MRT-PCR. The probability of reaching the detection limit of 5.5 log_{10} cfu/ml throughout the course of enrichment for different initial cell concentrations is depicted in **figure 1**. The detection limit was reached already after 22 h of enrichment with a probability of 0.99 when the initial cell concentration was high (2 log_{10} cfu/ml), while for the lowest inoculum (-2 log_{10} cfu/ml, simulating 1 cell per 10 gram of food sample) an enrichment duration of 40 h was needed. Apart from the 48-h sampling point, which is the initial duration of the ISO-A enrichment step, two additional sampling points were selected based on the simulation outcomes. In case a food product initially already contained relatively high amounts of *Campylobacter*, detection through MRT-PCR should be possible after 24

h (50% of the original enrichment time), while on the other hand, in a worst-case scenario (1 cell per 10 gram of food), cell concentrations should reach the MRT-PCR detection limit within 40 h.

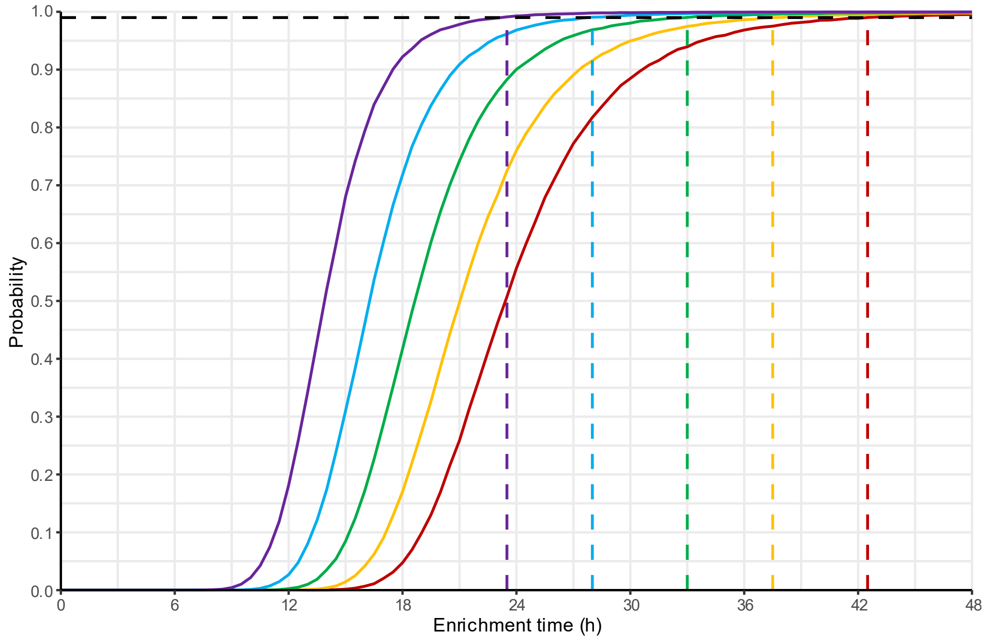

Figure 1: Estimation of probability of campylobacters to reach the detection limit of MRT-PCR (5.5 $\log_{10}$ cfu/ml) throughout enrichment in BB at different cell concentrations at the start of enrichment. A Monte Carlo simulation was conducted utilizing the average lag-duration and growth rate of campylobacters during enrichment in BB after freeze-stress (5.4±1.3 h and 0.42±0.09 $\log_{10}$ h^{-1}, respectively). The Initial cell concentrations used for the simulation were 10^4, 10^3, 10^2, 10 and 1 cell in 10 g of food product, corresponding to 2, 1, 0, -1 and -2 $\log_{10}$ cfu/ml in enrichment, respectively. The respective line colours in the figure are depicted in purple, blue, green, orange and red). The detection probability of 0.99 is depicted in a striped, black line. For each initial cell concentration simulated, the enrichment time (h) was calculated, at which the probability of detection is 0.99 and depicted as vertical stripes lines in the respective colour.

Detection of campylobacters from food samples using ISO 10272-1:2017 procedure A and EMRT-PCR

As a proof of principle, 23 meat products were purchased, and 10 g of each sample were used to confirm the reliability of the EMRT-PCR by comparing it to the detection outcomes obtained by using the standard protocol, namely the ISO 10272-1:2017 procedure A. After preparing the initial dilution in 90 ml of selective BB, the contamination level was assessed through spread-plating (100 µl). For 78% (18/23) of the samples, the initial cell concentration was below the detection limit of 1 log_{10} cfu/ml and therefore, cell concentrations in the food products itself were below 2 log_{10} cfu/g. The initial concentration in the other samples ranged from 1.3 to 1.9 log_{10} cfu/ml, yet still below the detection limit of the MRT-PCR detection limit. This highlights the necessity of enrichment since initial contamination levels are generally low. Samples were taken during the enrichment after 24, 40 and 48 h and as part of the ISO-A procedure a streak plate was done onto RCA. The ISO protocol states that the isolation step should be done on both modified charcoal cefoperazone-deoxycholate agar (mCCDA) and another solid medium with different selective principles (International Organization for Standardization, 2017). However, various studies have shown that Extended-spectrum beta-lactamase-producing *Enterobacteriaceae*, which are regularly present on especially broiler (Bortolaia et al., 2010; Depoorter et al., 2012) can also grow on mCCDA, thereby masking *Campylobacter* (Hazeleger et al., 2016; Jasson et al., 2009) and resulting in false-negative detection outcomes. Since RCA has proven to be sufficiently selective for the isolation of campylobacters (Lanzl et al., 2020, 2022; Seliwiorstow et al., 2016) only RCA was used for isolation. Since the detection limit of ISO-A is quite low (1 cfu per loop (~10 µl); 2 log_{10} cfu/ml), *Campylobacter* concentrations were also determined at each sampling point to assess whether cell numbers were high enough to reach the detection limit of 5.5 log_{10} cfu/ml of the MRT-PCR method. After 24 h of enrichment, 52% (12/23) and 48% (11/23) of the food samples tested positive and negative for *Campylobacter* spp., respectively, when ISO-A was applied. Samples which were positive after 24 h were also positive at the later sampling points (at 40 and 48 h). When EMRT-PCR was applied, only one of the samples (Nr. 14), which tested positive with ISO-A after 24 h, tested negative with MRT-PCR after 24 h of enrichment. However, the cell concentration in this sample determined with ISO-A was 2.7 log_{10} cfu/ml, which was indeed lower than the

detection limit of MRT-PCR. For all other samples, detection outcomes with EMRT-PCR were the same as with ISO-A. Note that, for one sample (No. 13) the EMRT-PCR results were positive at the beginning of enrichment although cell concentration was below the MRT-PCR detection limit of 5.5 log_{10} cfu/ml. The outcomes of this comparison can be found in **table 2**. Further shortening of the detection times may be obtained by lowering the detection limit of the MRT-PCR assay. The detection limit of the MRT-PCR was approximately one log_{10} higher in selective BB containing chicken than in selective BB. Further research to minimize PCR inhibitors and improve DNA isolation and purification may result in a lower detection limit which is inherent with shorter enrichment times. The experimental results obtained in this study showed that *Campylobacter* could be detected reliably from naturally contaminated food samples after 40 h of enrichment. This is in line with the predictions obtained through the Monte Carlo analysis, which was conducted based on experimental growth kinetics data of severely cold-stressed campylobacters during enrichment in BB. So, although the simulation results were experimentally confirmed, EMRT-PCR could be further tested for a higher number of food products also from different origins to strengthen the statistical power of the detection outcomes.

Table 2: Detection outcomes of food samples using the traditional ISO 10272-1:2017 procedure A protocol and EMRT-PCR consisting of an enrichment step in BB followed by MRT-PCR. Cell concentrations were assessed at the beginning of enrichment (0 h) and samples were observed as negative (-) when cell concentrations were below the detection limit of 1 log_{10} cfu/ml BB. For ISO 10272-1:2017 procedure A, detection outcomes were observed as negative (-) when cell concentrations were below 1 log_{10} cfu/ml BB, whereas outcomes were observed as positive (+) when cell concentrations were above 5.5 log_{10} cfu/ml BB. For food products 13 and 14, cell concentrations of enrichment samples after 24 h were positive, but did not reach the set detection limit of MRT-PCR. For the alternative procedure, EMRT-PCR assays were carried out for all samples and outcomes were observed as negative (-), if the MRT-PCR curves did not meet the set assay

No.	Food samples	0 h	24 h		40 h		48 h	
		Cell concentration (log_{10} cfu/ml BB)	ISO 10272-1:2017 Procedure A	EMRT-PCR	ISO 10272-1:2017 Procedure A	EMRT-PCR	ISO 10272-1:2017 Procedure A	EMRT-PCR
1	Chicken filet	-	-	-	-	-	-	-
2	Chicken drumstick	**1.8**	+	+ (Cj + Cc)	+	+ (Cj + Cc)	+	+ (Cj + Cc)
3	Chicken wing (marinated)	-	-	-	-	-	-	-
4	Chicken shawarma	-	-	-	-	-	-	-
5	Chicken liver	-	-	-	-	-	-	-
6	Minced beef	-	-	-	-	-	-	-
7	Chicken wing	-	+	+ (Cj)	+	+ (Cj)	+	+ (Cj)
8	Chicken hearts	-	-	-	-	-	-	-
9	Chicken thigh filet	-	-	-	-	-	-	-
10	Beef steak	-	-	-	-	-	-	-
11	Chicken shawarma	-	-	-	-	-	-	-
12	Minced beef	-	-	-	-	-	-	-
13	Chicken strips (marinated)	-	+ **(4.7 log_{10} cfu/ml BB)**	+ (Cj)	+	+ (Cj)	+	+ (Cj)
14	Chicken saté	-	+ **(2.7 log_{10} cfu/ml BB)**	-	+	+ (Cj)	+	+ (Cj)
15	Merguez sausage	**1.5**	+	+ (Cj)	+	+ (Cj)	+	+ (Cj)
16	Beef liver	-	+	+ (Cj)	+	+ (Cj)	+	+ (Cj)
17	Chicken liver	**1.9**	+	+ (Cj)	+	+ (Cj)	+	+ (Cj)
18	Chicken drumstick (marinated)	-	-	-	-	-	-	-
19	Chicken drumstick	**1.5**	+	+ (Cj + Cc)	+	+ (Cj + Cc)	+	+ (Cj + Cc)
20	Turkey leg	-	+	+ (Cj + Cc)	+	+ (Cj + Cc)	+	+ (Cj + Cc)
21	Chicken leg	-	+	+ (Cj + Cc)	+	+ (Cj + Cc)	+	+ (Cj + Cc)
22	Chicken wing	**1.3**	+	+ (Cj + Cc)	+	+ (Cj + Cc)	+	+ (Cj + Cc)
23	Chicken thigh filet	-	+	+ (Cj + Cc)	+	+ (Cj + Cc)	+	+ (Cj + Cc)

Application of EMRT-PCR allowed species differentiation, demonstrating that half of the *Campylobacter*-positive samples (6/12) were contaminated with *C. jejuni* only, while the other half was contaminated with both *C. jejuni* and *C. coli*. A study by Kramer et al. (2000) showed, that almost 30% of the meat samples examined in that study were contaminated by more than one strain of *Campylobacter*, and research conducted by Zhao et al. (2001) also revealed that meat sample were contaminated by more than one *Campylobacter* species. In a study by Schnider et al. (2010) a total 351 meat samples were examined for the presence of *C. jejuni* and *C. coli* in a qPCR assay utilizing primers for *hipO* and *ceuE*. Results showed that approximately 36% and 18% of the samples were contaminated by either *C. jejuni* only or by both species, respectively, which is lower than results obtained in this research. However, samples for qPCR were taken without prior enrichment, and the choice of both liquid and solid media can have an impact on detection outcomes and strain diversity (Habib et al., 2011; Hayashi et al., 2013; Newell et al., 2001; Paulsen et al., 2005; Ugarte-Ruiz et al., 2013; Williams et al., 2012). Goossens et al. (1986) demonstrated that *C. coli* showed higher susceptibility to polymyxin B, a component of PB and was subsequently less successfully isolated from the medium compared to BB. Another study showed that campylobacters in naturally contaminated food samples grew significantly better in BB compared to PB (Baylis et al., 2000). Next to the enrichment medium, also the choice of isolation medium can affect the recovery of *Campylobacter* strains, as it has been found that the diversity of *Campylobacter* isolates was higher when isolated onto CampyFood agar compared to mCCDA, irrespective of a preceding enrichment step (Ugarte-Ruiz et al., 2013). Currently, BB is recommended by ISO for the recovery of sub-lethally injured campylobacters, while PB is advised when the presence of ESBL-producing *Enterobacteriaceae* is expected. Both media utilize a cocktail of different antibiotics to suppress the growth of competing microbiota, however, these compounds can also cause a selection bias between *Campylobacter* species. Studies by Ugarte-Ruiz et al. (2013) and Newell et al. (2001) showed that the enrichment step (using different selective enrichment media) reduced the diversity of *Campylobacter* isolates and that some strain types were recoverable by direct plating from the samples but could not be recovered after enrichment.

Comparison of steps required for detection of *Campylobacter* spp. using ISO and EMRT-PCR

The detection of *Campylobacter* spp. using procedure A of ISO 10272-1:2017 contains an enrichment step in BB for 4-6 h at 37 °C + 44±4 h at 41.5 °C for cell repair and growth of campylobacters, followed by an isolation step on selective solid media (mCCDA and another selective plate) of 44±4 h. Presumptive colonies are streaked onto non-selective blood agar plates and incubated for 24-48 h, dependent on the size of the colonies. Then, presumptive *Campylobacter* colonies are confirmed by examination of morphology and motility, the presence of oxidase activity and absence of aerobic growth at 25 °C. To test the latter, colonies grown on non-selective blood agar are streaked onto non-selective blood agar again and incubated aerobically for 44±4 h. All these steps combined, a negative detection outcome can be determined after 4 days (2 days of enrichment and 2 days of isolation), while a confirmed positive detection outcome takes 7-8 days (2 days of enrichment and 2 days of isolation and 3-4 days of confirmation [1-2 days for growth on non-selective blood agar and 2 days to test for absence of aerobic growth at 25 °C). EMRT-PCR, consisting of an enrichment in BB for 40-48 h, followed by detection and species differentiation through a 1 h sample preparation and 2 h MRT-PCR run provides results in about 2 days. In comparison to ISO procedure A, the application of EMRT-PCR shortens the detection of *Campylobacter* spp. from food samples by approximately two days for *Campylobacter*-negative and 5-6 days for *Campylobacter*-positive results. The flowchart in **figure 2** gives an overview concerning the timeline of the two procedures.

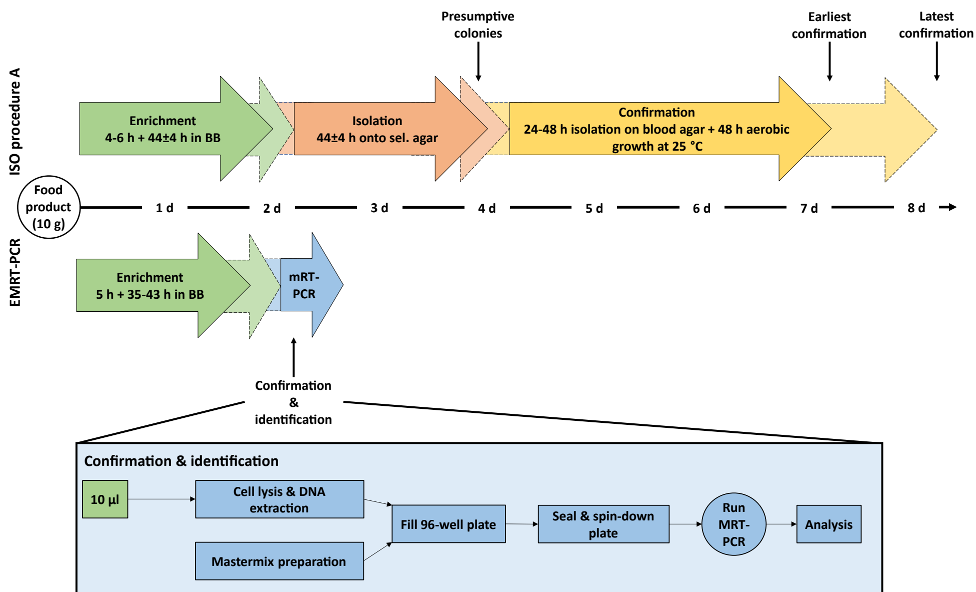

Figure 2: Flowchart illustrating the detection procedure of *Campylobacter* spp. using ISO 10272-1:2017 (ISO procedure A) and the combination of enrichment with MRT-PCR (EMRT-PCR). Traditional detection using the ISO-A procedure takes between 7-8 days (±48 h of enrichment + ±48 h of isolation + 72-96 h of confirmation) while EMRT-PCR provides detection and species differentiation within ~2 days (40-48 h of enrichment + 3 h of confirmation and identification).

Conclusion

This study aimed to develop a rapid but reliable protocol for the detection and differentiation of *C. jejuni* and *C. coli* from food products and combines an enrichment with MRT-PCR (EMRT-PCR). The study demonstrated through predictive modelling and experimental results that 40 h of enrichment according to ISO 10272-1:2017 procedure A followed by MRT-PCR allowed the detection of, and differentiation between *C. jejuni* and *C. coli* in naturally contaminated meat samples. EMRT-PCR takes approximately 2 days, while confirmed positive detection outcomes using traditional ISO protocols can take up to 8 days.

Acknowledgments

This study aimed to develop a rapid but reliable protocol for the detection and differentiation of *C. jejuni* and *C. coli* from food products and combines an enrichment with MRT-PCR (EMRT-PCR). The study demonstrated through predictive modelling and experimental results that 40 h of enrichment according to ISO 10272-1:2017 procedure A followed by MRT-PCR allowed the detection of, and differentiation between *C. jejuni* and *C. coli* in naturally contaminated meat samples. EMRT-PCR takes approximately 2 days, while confirmed positive detection outcomes using traditional ISO protocols can take up to 8 days.

Supplementary materials

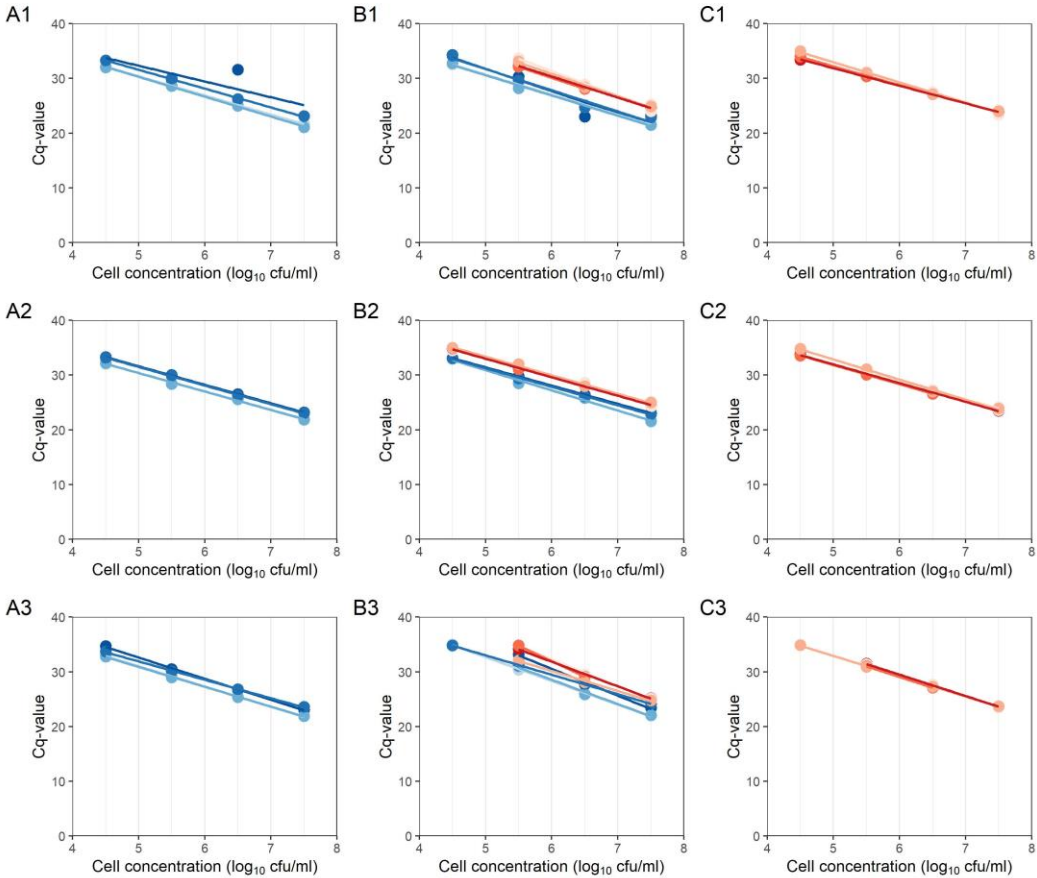

Figure S 1: Standard curves of *Campylobacter* strains with three fluorophores and in three different media for the determination of detection limits of MRT-PCR assay. Graphs A1-A3 depict the standard curves of 4 *C. jejuni* strains (shades of blue) with mapA (fluorophore FAM). Graphs B1-B3 depict the standard curves of 4 *C. jejuni* (shades of blue) and 4 *C. coli* strains (shades of red) with cje (fluorophore HEX) and graphs C1-C3 depict the standard curves of 4 *C. coli* strains (shades of red) with ceuE (fluorophore TexasRed). Graphs A1, B1 and C1 show the standard curves of the strains in Heart Infusion broth (HI), graphs A2, B2 and C2 show the standard curves of the strains in Bolton broth (BB) and graphs A3, B3 and C3 show the standard curves in the food matrix (three pooled enrichment fluids of *Campylobacter*-free chicken skin). In BB, the lower detection limit of the assay was 4.5 $\log_{10}$ cfu/ml, while in the food matrix, the detection limit was 5.5 $\log_{10}$ cfu/ml.

Table S 1: Information of all strains used in this study.

Species	Isolate name	Isolate history (source, country & year [if reported])
C. jejuni	ATCC 33560	Bovine faeces
C. jejuni	Ca 1087	Chicken meat, NL
C. jejuni	Ca 1095	Chicken meat, NL
C. jejuni	Ca 1352	Chicken meat, NL
C. jejuni	Ca 1430	Chicken meat, NL
C. jejuni	Ca 1597	Manure, NL
C. jejuni	Ca 1781	Chicken meat, NL
C. jejuni	Ca 1809	Chicken meat, NL
C. jejuni	Ca 2348	Goat manure, N
C. jejuni	Ca 2426	Sheep manure, NL
C. jejuni	DSM 24306	Chicken faeces, NL
C. jejuni	NCTC 11168	Human stool, UK, 1977
C. jejuni	WDCM 00005	Human faeces
C. jejuni	WDCM 00156	Human stool, USA
C. jejuni	480	Human stool, UK, 1987
C. jejuni	81116	Human stool, UK, 1981
C. jejuni	81-176	Human stool, USA, 1985
C. coli	ATCC 33559	Pig faeces
C. coli	Ca 121	Pig manure, NL
C. coli	Ca 932	Pig manure, NL
C. coli	Ca 1607	Chicken manure, NL
C. coli	Ca 2654	Turkey meat, NL, 2017
C. coli	Ca 2711	Bovine faeces, NL, 2017
C. coli	Ca 2771	Bovine faeces, NL, 2017
C. coli	Ca 2800	Chicken meat, NL, 2017
C. coli	Ca 2804	Lamb meat, NL, 2017
C. coli	Ca 2852	Chicken meat, NL, 2017
C. coli	Ca 3115	Lamb meat, NL, 2017
C. coli	WDCM 00004	Marmoset faeces

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General discussion

Introduction

Campylobacter is a zoonotic bacterial pathogen and the leading cause for foodborne gastroenteritis in the European Union (EU) (EFSA & ECDC, 2021). In 2020, 88.1 % and 10.6% of all confirmed human campylobacteriosis cases were caused by *C. jejuni* and *C. coli,* respectively (EFSA & ECDC, 2021). This makes the reliable detection of these two species from food products crucial to ensure food safety (Kaakoush et al., 2015). To detect low levels of *Campylobacter* from food, the enrichment-based procedures described by the International Organization for Standardization (ISO) 10272-1:2017 are applied (International Organization for Standardization, 2017). *Campylobacter* cells can be damaged by food processing and storage conditions and to detect injured cells, procedure A is advised. It consists of an enrichment in selective Bolton broth (BB) followed by isolation and confirmation steps, which all together takes 7-8 days to yield confirmed *Campylobacter*-positive food samples. Furthermore, it has been shown that the selectivity of BB was impaired by the presence of ESBL-producing *Enterobacteriaceae* causing growth inhibition of *Campylobacter* during enrichment (Hazeleger et al., 2016) which, in turn, can lead to false-negative detection outcomes. Nevertheless, ISO is still viewed as the 'golden standard' (Stingl et al., 2021). However, relatively little is known concerning the behavior of damaged campylobacters during enrichment so the objective of this thesis was to shed light on the black box of enrichments in order to assess whether procedure A is adequate or if possible, changes to the procedure could be proposed to improve the detection of *Campylobacter* spp. from food.

In **chapter 2** we investigated the variability in lag phase of *C. jejuni* and *C. coli* during enrichment in BB with and without prior cold stress treatment and found that lag phase and the variability in lag phase increased as severity of cold stress increased. We further observed that the variability in lag phase was mainly due to strain- and less due to biological variability and that lag phase was also stress type-dependent. We conducted a scenario analysis using data on the *Campylobacter* growth kinetics, which indicated that other factors such as competitive microbiota might be the reason for the false-negative detection outcomes. This was investigated in **chapter 3** where we focused on the utilization of BB compounds by *Campylobacter* spp. and ESBL-producing *Escherichia coli*. The outcomes showed that BB is a rich medium supporting

the growth of campylobacters in the presence of ESBL-*E. coli* and that the growth inhibition during co-culture enrichments was not due to the production of antimicrobial compounds or competition for growth substrates. Instead, we observed that constant microaerobic oxygen availability increased the competitive fitness of *C. jejuni* during co-culture enrichments, indicating the critical role of oxygen availability during enrichment. We then focused more in depth on the cellular processes that take place during lag phase of reference and refrigeration-stressed *C. jejuni* cells in selective BB (**chapter 4**). Proteomic analysis at different lag timepoints revealed that the maintenance of (membrane) protein quality was important in the lag phase of reference cells, while redox enzymes and anaerobic electron transfer were crucial during enrichment of both, reference and stressed cells. On the other hand, proteins associated with purine metabolism, DNA and protein damage repair, iron acquisition and additional electron acceptors were upregulated during lag phase of refrigeration-stressed cells, exclusively. The outcomes of this study demonstrated that protein quality and oxidative stress management were important factors during the recovery of *C. jejuni* cells during enrichment in selective BB and confirmed the supposition that the role of oxygen and the provision of optimal microaerobic conditions during enrichment was important. The observations and results of **chapters 2 to 4** helped to shed light on the black box of enrichments and led to the conclusion that, as long as a continuous microaerobic environment could be provided, selective BB was a suitable enrichment medium for *Campylobacter*. We then used a biomolecular approach to detect campylobacters after enrichment as a possible alternative to culturing-based detection as described in the ISO 10272-1:2017. In **chapter 5** we developed a multiplex real-time PCR (MRT-PCR) protocol, which allowed the detection and differentiation of *C. jejuni* and *C. coli.* The protocol was then combined with an enrichment of food samples in selective BB, which was crucial in order to obtain *Campylobacter* concentrations high enough for the application of MRT-PCR. We estimated that 40 h of enrichment followed by MRT-PCR would allow the detection of even low amounts of *Campylobacter* in foods and tested the protocol (EMRT-PCR) on naturally contaminated food samples. The outcomes showed that the assay was reliable and shortened the detection of *Campylobacter* spp. in food products from 7-8 days to two days.

Although this thesis achieved to shed light on the black box of enrichment and could give recommendations to shorten the enrichment-based detection procedure, the research conducted in chapters 2 to 5 also raised questions and some challenges remained that will be addressed in the upcoming sections.

Challenges of the ISO 10272-1:2017 protocol for the detection of *Campylobacter* spp.

Before 2017, the detection protocol for *Campylobacter* spp. from food products (ISO 10272-1:2006) consisted of an enrichment in BB followed by isolation onto modified charcoal-cefoperazone-deoxycholate agar (mCCDA). The latter contains the antibiotics cefoperazone and amphotericin B while BB also contains vancomycin and trimethoprim (Merck, n.d.; ThermoFisher Scientific, n.d.). However, ISO 10272-1:2006 protocol increasingly showed false-negative detection outcomes due to the emergence and increased prevalence of extended-spectrum beta-lactamase (ESBL-) producing bacteria on especially raw meats (Randall et al., 2017). The ability of ESBL-producing bacteria to hydrolyze the beta-lactam ring of cephalosporins such as cefoperazone (Bradford, 2001) lead to decreased selectivity of BB and mCCDA and consequently to (over-) growth of ESBL-producers in/on BB and mCCDA (Jasson et al., 2009; Moran et al., 2009). Subsequently, the detection protocol was revised and the latest version (ISO 10272-1:2017) therefore offers three procedures and the choice of application depends on several factors, namely the number of campylobacters and background microbiota and the expected health-state of *Campylobacter* cells. This means that lab personnel must make a series of assumptions in order to pick a (supposedly) suiting detection procedure. **Figure 1** gives a schematic overview of the ISO procedures as well as an overview of the steps of the alternative procedure. Unfortunately, although the changes to the 2006 version of ISO were certainly well-intended, offering three detection procedures can also cause confusion and uncertainty, especially due to the assumption that have to be made. This will now be explained by means of an example, namely fresh poultry meat.

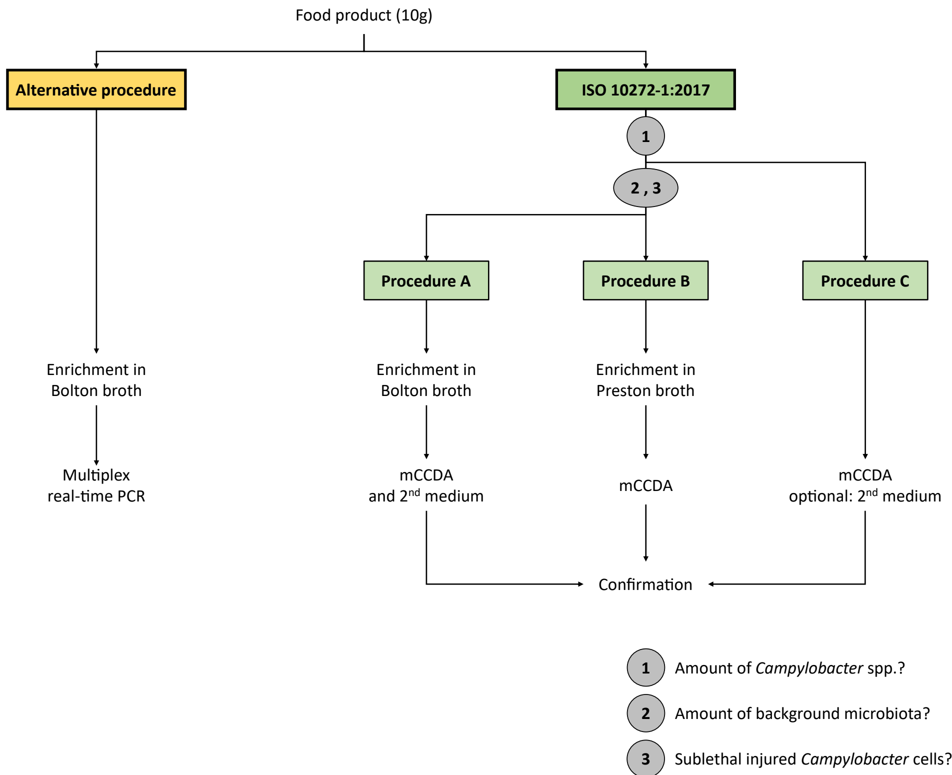

Figure 1: Schematic overview of major steps in the detection of campylobacters using the alternative procedure and the current ISO 10272-1:2017 protocol.

As mentioned above, the choice of procedure is based on several assumptions and the first one is an estimation of the expected number of campylobacters in the food product. According to ISO, raw poultry meat would be expected to contain high amounts of campylobacters. In that case, **procedure C** should be used, as no enrichment is required. However, if the user is in doubt about the expected *Campylobacter* concentrations, procedure C should be applied in combination with either procedure A or B, which would increase the workload. Moreover, the direct plating approach must be done on mCCDA with the optional choice to also take along another selective isolation medium. In the example of raw poultry meat, one could expect also high amounts of background microbiota, thus there is a risk of false-negative detection outcomes if *Campylobacter* cells are overgrown by ESBL-producing

E. coli on mCCDA, which are known to grow faster than campylobacters. To increase the chances of reliable detection outcomes, ISO's advice is followed and next to procedure C, also one of the other procedures should be followed. In order to choose between the two remaining procedures, the user must estimate the expected amount of background microbiota. In fresh poultry meat, high amounts of background microbiota can be expected. In that case, **procedure B** is advised, which consists of an enrichment step in PB followed by isolation onto mCCDA. However, it has been observed that PB is highly selective and therefore not optimal for the recovery of stressed campylobacters (Seliwiorstow et al., 2016), as could also be observed during the heterogeneity experiments in this thesis. This was considered in the current protocol by requiring the user to make a third assumption, namely, to estimate whether campylobacters in the fresh poultry meat sample are expected to be injured. *Campylobacter* is known to be susceptible to a range of environmental conditions such as heat, ambient temperature, chilling, freezing, aerobic or anaerobic atmospheric oxygen and thus, some level of injury can be expected in fresh poultry meat. Consequently, **procedure A** should be used, consisting of enrichment in BB, followed by isolation onto mCCDA and another isolation medium with different selective principles than mCCDA. So even if competitors should grow during enrichment, the isolation of campylobacters would be possible through increased selectivity of the second medium. However, while several media are more selective than mCCDA, some of them still allow the growth of some competitors. Moreover, the exact composition of the medium or selective supplement is often not publicly available. For example, RCA was used throughout this thesis and was highly selective for campylobacters but since the composition of the selective supplement is not published, it is questionable if it would be allowed to be used as second isolation medium. After plating out on selective media, the suspected colonies still need to be confirmed through microscopy, detection of oxidase activity and absence of aerobic growth at 25 °C.

For food safety reasons, the most cautious approach should be chosen when assumption making is required thus if competition and sublethal damage cannot be ruled out without a doubt, procedure A should be followed. The failing selectivity of BB undoubtedly required changes in the detection procedure, and the addition of a medium other than mCCDA was crucial, yet none of the three procedures is optimal. Moreover, the many assumptions that the user must make can also cause confusion

and doubts. Similarly, because there are no clear guidelines for the application of the procedure and use of second selective isolation medium, it is difficult to compare results. If different approaches are chosen between EU countries for the detection of *Campylobacter* spp. in a certain food product, for example, it is not possible to reliably compare the prevalence of *Campylobacter* in said food product within the EU. A clear uniform guideline could be a step towards a harmonized detection approach in EU countries.

Advantages and drawbacks of ISO 10272-1:2017 and the alternative procedure

When comparing ISO 10272-1:2017 procedure A to the alternative procedure, EMRT-PCR, both have their advantages and drawbacks. The big advantage of the ISO protocol is that it is relatively affordable and easy to perform in the lab. However, the protocols are quite laborious since several steps must be followed, especially when it comes to the isolation and confirmation of suspected colonies. Dependent on the protocol which was chosen (by making several assumptions), confirmed *Campylobacter*-positive detection results are reached only after 6-8 days when enrichment-based procedures are chosen. The alternative procedure on the other hand is more expensive since MRT-PCR requires specifically made primer/probe sets, PCR kits and a suitable PCR machine, although the latter is a one-time expense. Also, training of personnel to properly conduct MRT-PCRs is required. However, samples are confirmed *Campylobacter*-positive or -negative after two days, so the procedure is also shorter, thus less workload is required and less personnel costs are made. Currently, the biggest drawback of ISO 10272-1:2017 is the uncertainty when it comes to the choice and outcomes of each procedure. This is no concern with the alternative procedure since one protocol is followed for all samples and campylobacters are reliably detected through MRT-PCR in the alternative procedure. Since species-specific primers are used in combination with different fluorescent probes, the alternative procedure allows for species differentiation as well. This also means that information can be gathered on single or mixed-species contamination. On the other hand, through the isolation step in all procedures of the ISO 10272-1:2017 protocol, confirmed *Campylobacter* strains can be isolated and included in a strain collection for further research. This is not currently the case for the alternative procedure but could be addressed if further improvements were to be made to the EMRT-PCR.

Similarly, the current protocol has shown to work well for the samples tested in chapter 5, but fine-tuning is required to ensure reliable outcomes also in other products.

Intra-species heterogeneity

Firstly, after having quantified the lag phase of different strains on a population-level (chapter 2), we were interested to know, whether the observed outgrowth was similar for the whole population or whether there was heterogeneity in the lag phase between single cells within the same population. When initial contamination levels are low, differences in single-cell outgrowth capacity could lead to a failure to reach the necessary detection threshold which could negatively affect reliable detection. To answer this question, we conducted experiments using fluorescence-activated cell sorting (FACS). Staining of *Campylobacter* cells was needed for single cell sorting to discriminate the small cell size from the background noise. Since double-staining using the LIVE/DEAD BacLight bacterial viability kit (Invitrogen, USA) did not yield reliable results, we stained reference, refrigeration- and freeze-stressed *Campylobacter* cells with a low concentration (500 nM) of SYTO 9. We confirmed that dye concentrations did not affect growth in a separate experiment. Since no distinction could be made between live and dead cells, this meant that also dead cells would be sorted. This was not a big concern for reference and refrigeration-stressed cells since the effect of refrigeration stress, investigated in chapter 2, was negligible (reduction of 0.0 to 0.2 log_{10} cfu/ml). Thus, for these two conditions, single cells were sorted in each well of three 96-well plates containing either Heart-Infusion (HI-) broth, selective BB or selective Preston broth (PB). Hi-broth was chosen as an unselective enrichment medium to assess whether cells would grow better in a less selective enrichment medium compared to selective BB. Likewise, PB was included to compare the outgrowth potential also in a more selective enrichment medium. Freeze stress on the other hand significantly affected viability (mean reduction of 1.5±0.05 log_{10} cfu/ml for 23 tested strains) which means that, if single cells were sorted, it could be expected that approximately 97% of the wells contained dead cells. Therefore, freeze-stressed cells were sorted by one, ten and 100 cells into the 96-well plates. The plates were then incubated microaerobically at 41.5 °C for 48 h and samples were taken throughout enrichment. Preferably, the growth of each cell would be observed

continuously through optical density (OD_{600}) measurements in a spectrophotometer, as was done for *Listeria monocytogenes* in a similar setup (Bannenberg et al., 2021). However, this was not possible for *Campylobacter* for several reasons. Firstly, the strictly microaerobic growth requirement of *Campylobacter* did not allow continuous measurements in the spectrophotometer. Alternatively, measurements could be taken instead by transferring the 96-well plates from microaerobic incubation after every few h. However, due to their small cell size and consequently low biomass compared to other bacteria, OD_{600} measurements of *Campylobacter* have shown to be quite low. Wright et al. (2009) compared the cell concentrations of *C. jejuni* obtained through plate counting with OD_{600} measurements taken throughout growth in Brain-Heart-Infusion (BHI-) broth and found that in early stationary phase, when cell concentrations were the highest (approximately 8.5 log_{10} cfu/ml), the corresponding OD_{600} value just around 0.1. In comparison, the OD_{600} of *Escherichia coli* in BHI at the same stage of bacterial growth was approximately 1.0 (Ko et al., 2009), so it is questionable if such low values are still reliable. Moreover, BB and PB contain lysed horse blood which affects optical density measurements. For those reasons, OD measurements are not commonly used for *Campylobacter*. Alternatively in our experiment, 10 µl samples were spot-plated onto unselective Columbia blood agar (CAB) and colony-forming units were counted after plate incubation. The first sample was taken after 14 h and every two h after until 24 h as well as after 48 h.

Our results, as depicted in **figure 2,** indicated that for reference cells (figure 2A), all viable *C. jejuni* (WDCM 00005) cells grew from a single cell to above 3 log_{10} cfu/ml (which was the upper detection limit using the spot-plating method) within 14 h of enrichment in BB, PB and HI. Also, the percentage of cells which were able to grow was 100% in BB and 99% in PB and HI.

For refrigeration-stressed cells (figure 2B), the growth-percentage of cells was slightly lower, but highest in BB (97.2%) followed by HI (93.75%) and PB (92.71%), which could be expected as a result of refrigeration stress. As explained earlier, refrigeration stress did not cause a biologically significant reduction in cell viability and for strain WDCM 00005, the percentage of viable cells was determined (in chapter 2) to be 87.1-97.7% (reduction of 0.06±0.05 log_{10} cfu/ml). Regarding the heterogeneity in outgrowth, only one well containing refrigeration-stressed *C. jejuni* reached the upper detection limit

only after 48 h when grown in HI, while all other viable cells reached the detection threshold within 14 h in HI, BB and PB. The increase of cell concentrations by more than 2.4 log_{10} cfu/ml (from 1 cfu/250 µl, which is 0.6 log_{10} cfu/ml to above 3 log_{10} cfu/ml) within the first 14 h of enrichment indicates that cells were out of lag and actively growing.

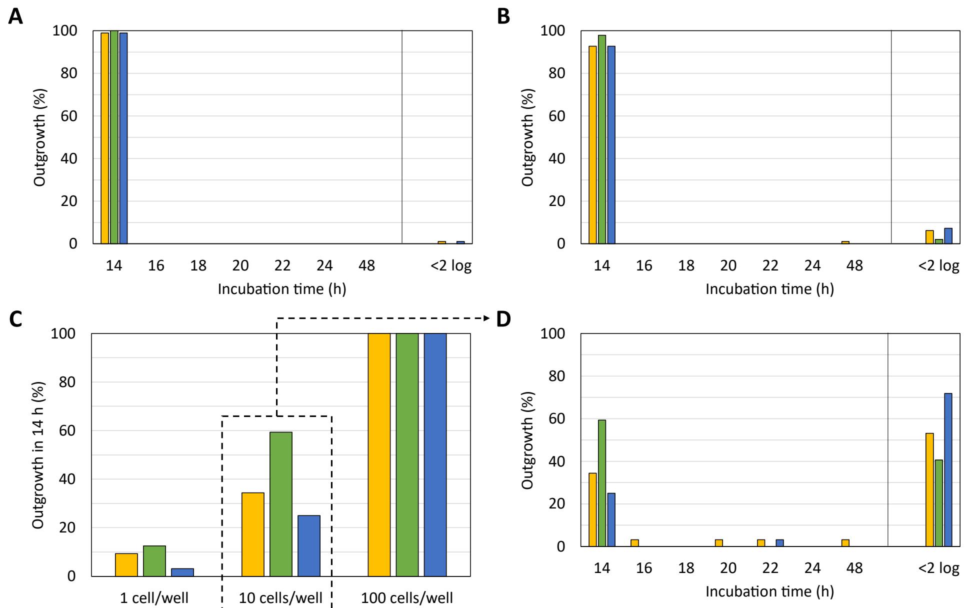

Figure 2: Overview of outgrowth (>3 log_{10} cfu/ml) percentages over time of *C. jejuni* strain WDCM 00005 in HI-broth (yellow), BB (green) and PB (blue). Graphs A and B depict the outgrowth percentage of single reference cells (**A**) and refrigeration-stressed cells (**B**) over time as well as the percentage of cells that did not reach the lower detection limit (2 log_{10} cfu/ml) within 48 h of enrichment. **Graph C** depicts the percentage of freeze-stressed cells that grew above 3 log_{10} cfu/ml within 14 h of enrichment when 1, 10 or 100 cells were sorted in each well. **Graph D** depicts the outgrowth percentage over time when ten freeze-stressed cells were sorted in each well as well as the percentage of cells that did not reach the lower detection limit (2 log_{10} cfu/ml) within 48 h of enrichment.

For freeze-stressed cells (figure 2C), the percentage of cells which were able to grow was much lower, especially when single cells were sorted. In BB, 12.5% of cells were able to grow, followed by HI (9.38%) and PB (3.13%). This can be explained by the significant negative impact of freeze-stress on cell viability. For strain WDCM 00005, the percentage of viable cells was determined to be 11.2-12.9% (reduction of 0.92±0.03

log_{10} cfu/ml), therefore, a growth-percentage of 12.5% in BB could be expected. For HI and PB on the other hand, the growth-percentages in HI and PB were slightly lower, which indicates that both media were not sufficiently sensitive to support the recovery of slightly damaged cells. When ten freeze-stressed cells were sorted per well (figure 2D), a growth-percentage of 59.4% could be observed in BB, followed by HI (46.9%) and PB (28.1%), which is lower than expected. When ten freeze-stressed cells were sorted per well, the probability of each well containing at least one viable cell was determined to be 72% for strain WDCM 00005. This again indicates that BB was more suitable for the recovery of severely injured campylobacters than HI and PB, but still, a small percentage of severely stressed cells failed to grow during enrichment. Heterogeneity in outgrowth could be observed in HI-broth and PB. Although most viable cells grew to the detection limit within 14 h, 12.5% and 6.25% of wells reached the detection limit at a later time point when grown in HI-broth and PB, respectively. For BB on the other hand, all viable freeze-stressed cells grew to the detection limit within 14 h. Since this was the first sampling point and no further outgrowth could be measured at later timepoints up to 48 h, outgrowth heterogeneity could probably be observed in the timespan before 14 h. Therefore, it is possible that there is heterogeneity in outgrowth but not to an extend that could affect reliable detection after 48 h of enrichment when BB is used, since all viable cells already grew above 3 log_{10} cfu/ml, which already is above the detection threshold of ISO 10272-1:2017. Likewise, it is very realistic that cells of WDCM 00005 would reach the detection threshold of the MRT-PCR assay within 40 h since cells were already exponentially growing after 14 h, thus well in stationary phase after 40 h when the scenario analysis of chapter 2 was applied. The experiment also revealed that the outgrowth percentage of freeze-stressed campylobacters was highest in selective BB, followed by HI-broth and PB. This supports ISO's advice to use BB as enrichment medium when stressed campylobacters are expected and could be supported by the findings of chapter 3 and 4.

Effect of different stress treatments on reliable detection

In this thesis, we induced sub-lethal injury to assess the growth kinetics of stressed campylobacters during enrichment. This was done since a scenario in which cells were stressed as a result of the food products' history seemed most realistic. *Campylobacter* is often found on raw meat and other products (Hansson et al., 2015; Walker et al., 2019) which undergo food processing steps and are transported and stored under chilled or frozen conditions (BMT, n.d.). We therefore focused on refrigeration and freeze stress treatments and subsequently gathered more insight into the growth kinetics and behavior of cold-stressed campylobacters during enrichment. We found that lag phase of campylobacters was longer for refrigeration-stressed compared to reference cells, but even longer for freeze-stressed cells and likewise, the variability in lag phase increased as stress increased. Lag phase is described as the bacterial growth phase during which cells adapt to their new environment and repair cellular damages incurred prior to enrichment (Vermeersch et al., 2019). In chapter 2, we found that freeze-stress negatively affected cell viability while no significant change could be observed for refrigeration-stressed cells. Although both stress treatments were related to cold stress, freeze-stress was harsher than refrigeration stress which could explain the increased lag phase. However, chapter 2 also revealed that lag phase was also stress-dependent as strains that recovered comparably fast from freeze-stress recovered significantly slower from oxidative stress at 12 °C. We wondered to what extend lag phase would be influenced by other types of food-related stresses such as heat stress or pH stress and whether this could negatively affect the chance of reaching the minimal detection limit of the MRT-PCR protocol after enrichment. Generally, campylobacters are rapidly inactivated through thorough cooking (Nguyen et al., 2006; Sampers et al., 2010) as heat negatively affects several parts of the cell such as the membrane, RNA and DNA, ribosomes and other proteins (Russell, 2003). Subsequently, it could be argued that heating is one of the harshest stresses encountered by *Campylobacter* spp. in a food product and could very well result in an increased lag phase during enrichment. In chapter 2, a scenario analysis was conducted using the growth kinetics data during enrichment after freeze-stress (figure 2.5). In case of heat-stressed cells, no reliable data is yet available on the lag phase during enrichment. However, several other parameters can be used to estimate the maximum lag phase that would still result in heat-stressed campylobacters reaching

the detection limit of the MRT-PCR assay. Based on the enrichment of a single cell in the whole sample with a *Campylobacter* average specific μ_{max}, the lag phase could be as high as 21.5 h and the MRT-PCR detection limit of 5.5 log_{10} cfu/ml would still be reached within 40 h of enrichment. It is highly unlikely that the lag phase of campylobacters would increase to that extend, however, it cannot be ruled out completely since no experimental evidence has been gathered that quantifies the lag phase of campylobacters during enrichment after heat stress. It would be interesting to investigate the growth kinetics of heat- and otherwise stressed campylobacters during enrichment in BB to be able to shed more light on their behavior during enrichment and substantiate the supposition that cell concentrations of even severely stressed campylobacters would reach the necessary MRT-PCR detection limits.

The balance between sensitivity and specificity of detection methods

Enrichment media

As mentioned above, the ISO 10272-1:2017 protocol advises the use of PB if a high amount of background microbiota is expected in the food sample. PB contains the antibiotics polymyxin B, rifampicin, trimethoprim and amphotericin B and research has shown that the use of PB as enrichment medium indeed inhibited the growth of ESBL-producing *Escherichia coli* (Hazeleger et al., 2016). However, it has also been recognized that the antibiotic composition of PB (containing polymyxin B) might be too harsh and inhibit the growth of (sub-lethally injured) campylobacters (Baylis et al., 2000; Goossens et al., 1986; Paulsen et al., 2005). Seliwiorstow et al. (2016) conducted a comparative study on the detection of campylobacters from frozen poultry meats and found that more samples were *Campylobacter*-positive after enrichment when BB was used compared to PB and Habib et al. (2011) enriched chicken meat using both media and also found that growth in PB was inferior to BB.

According to ISO 10272-1:2017, enrichment in BB entails a pre-enrichment step (4-6 h) at 37 °C aimed at better recovery of injured campylobacters. However, it has been shown that growth kinetics of stressed campylobacters were similar in BB with and without the pre-enrichment step (Hazeleger et al., 2016). In addition, BB also has its drawbacks compared to PB due to decreased selectivity in the presence of ESBL-producers. Efforts have been made to develop alternative enrichment media for

Campylobacter, such as *Campylobacter* Enrichment broth (CEB) (Baylis et al., 2000), Bolton broth supplemented with clavulanic acid (C-BB), triclosan (T-BB), polymyxin B (P-BB) or tazobactam (Tz-BB) (Chon et al., 2018; Hazeleger et al., 2016; Seliwiorstow et al., 2016), CampyFood broth (Habib et al., 2011) and CCPD broth (Chon et al., 2013). Although all of them inhibited the growth of competitors more successful compared to selective BB, it is questionable how these media perform concerning the recovery of sub-lethally injured campylobacters. Baylis et al. (2000) conducted enrichments of naturally contaminated foods in BB and CEB and found that BB yielded more confirmed *Campylobacter* growth than CEB. Similarly, Seliwiorstow et al. (2016) compared the *Campylobacter* recovery rates after enrichment in BB, C-BB, T-BB and P-BB and found that for fresh poultry meats, C-BB and T-BB performed better than BB and P-BB. However, for frozen samples, the recovery rates were much higher in BB and T-BB compared to C-BB and P-BB. This indicates that increased selectivity of alternative enrichment media might hinder the recovery of injured campylobacters as was also observed for PB. Also, the performance of these alternative enrichment media was almost exclusively measured by the percentage of recovered campylobacters on selective media. For example, Chon et al. (2013) compared the *Campylobacter* detection outcomes after enrichment of carcass rinses in BB and the alternative medium CCPD and found that the isolation rate was higher in CCPD, but the isolation was done by streaking onto mCCDA. Similarly, when mCCDA was used as isolation medium, Tz-BB also showed a higher isolation rate of *Campylobacter* compared to BB (Chon et al., 2018). Therefore, not only the choice of enrichment medium is crucial but also the subsequent isolation medium when purely cultural methods are applied.

Isolation media

In the ISO 10272-1:2017 protocol, after enrichment in BB, isolation should be done onto two selective media, namely mCCDA and another *Campylobacter* medium with different selective principles than mCCDA (International Organization for Standardization, 2017). Several of such selective solid media have been developed, among others, CampyFood agar (CFA), Brilliance CampyCount agar (BCA), Campy-Cefex agar and RAPID *Campylobacter* agar (RCA) but efforts have also been made to supplement mCCDA with potassium clavulanate or tazobactam (Chon et al., 2013; Smith et al., 2015). Habib et al. (2011) enriched chicken meat samples in BB and

concluded that subsequent *Campylobacter* recovery on CFA was superior to mCCDA and BCA and similar results were also obtained by Ugarte-Ruiz et al. (2012). Seliwiorstow et al. (2016) conducted enrichments in several liquid media (as mentioned above) and found that in all cases, isolation onto RCA resulted in a higher isolation rate compared to mCCDA. Likewise, in a study by Hazeleger et al. (2016) as well as in this thesis, the growth kinetics of *C. jejuni* during co-culture enrichments in BB with ESBL-producing *E. coli* were investigated through regular enumeration and RCA proved to reliably inhibit the growth of ESBL-producers while still allowing the growth of campylobacters. Hence, RCA would currently be an appropriate alternative to mCCDA if procedure A would be followed even with high numbers of background microbiota.

Selection bias of cultural growth media

As described above, the development of new or modification of existing enrichment and isolation media aimed to restore selectivity generally is achieved through changes to their selective supplements by either exchanging or adding antibiotic agents. Since *C. jejuni* and *C. coli* are the two most pathogenic species, most media also aim to support the growth and detect those two species. However, increased selectivity can also impact the detection outcomes and strain diversity. The most relevant example is the observed growth suppression of *C. coli* in PB due to polymyxin B (Goossens et al., 1986; Ng et al., 1985) which might lead to false-negative detection outcomes. The growth performance of 17 *Campylobacter* species during enrichment in BB was assessed by (Lynch et al., 2010) and results indicated that all species tested, including *C. lari* and *C. upsaliensis* grew during enrichment. However, strains did not undergo any stress history prior to enrichment which could affect their growth performance after all. Additionally, a selection bias could also occur between different subtypes within the same species (Ugarte-Ruiz et al., 2013; Williams et al., 2012). Hetman et al. (2020) investigated how enrichment in BB influenced the selection bias of different mixed cultures containing different *C. jejuni* subtypes and found that BB indeed favoured the growth of certain subtypes above others. In this thesis, we selected *C. jejuni* and *C. coli* strains partly based on their STs as well. We included STs associated with human infection (ST-21, ST-45, ST-828) and animal infection (ST-48 and ST-48) (Colles and Maiden, 2012; De Haan et al., 2010; Dingle et al., 2002; Kärenlampi et al.,

2007; Taboada et al., 2008). However, no correlation could be observed between lag phase and ST. Next to selective enrichment media, also the isolation media can affect the recovery of *Campylobacter* strains. Ugarte-Ruiz et al. (2013) found that strain diversity of campylobacters was higher on CFA than mCCDA, regardless of the prior enrichment step. For detection purposes, it might not be as relevant to recover all subtypes equally well in mixed cultures as long as one strain prevails and grows to detectable concentrations. However, a strong selection bias severely hindering the growth of certain relevant species or subtypes might lead to false-negative detection outcomes. Also, for more in-depth analysis of the prevalence of *Campylobacter* species and sequence types in different food sources for risk assessment purposes or tracing the origin of foodborne campylobacteriosis for food safety reasons for example, a selection bias could lead to the wrong conclusions when purely cultural approaches are applied. Since *Campylobacter* concentrations in food are often too low for direct detection, the enrichment step remains crucial. A selection bias is often associated with the selectivity of the medium, so a less selective enrichment medium might be considered. However, the outcomes of the single-cell growth experiments also showed that *Campylobacter* cells grew better in selective BB compared to unselective HI-broth. Therefore, it would be interesting to investigate the selection bias of BB further, including not only different sequence types of *C. jejuni* and *C. coli* but also those of the other (emerging) pathogenic species such as *C. lari, C. upsaliensis, C. concisus* and *C. ureolyticus* (Igwaran and Okoh, 2019).

Effect of other competitors on the growth of *Campylobacter* spp. during enrichment

The most studied competitor of *Campylobacter* spp. during enrichments is ESBL-*E. coli* (Chon et al., 2017; Hazeleger et al., 2016; Jasson et al., 2009; Moran et al., 2011). However, ESBL enzymes have shown to be present in a variety of *Enterobacteriaceae* and could be transferred into BB-enrichments as well. Their prevalence on animal-based products has been investigated, and while most ESBL-producing bacteria were indeed *E. coli*, ESBL-producing *Serratia fonticola, Klebsiella pneumoniae, Enterobacter cloacae* and *Proteus mirabilis* have also been isolated from red and poultry meat in recent years (Ojer-Usoz et al., 2013; Schill et al., 2017; von Tippelskirch et al., 2018). In this thesis, the substrate utilization of three ESBL-*E. coli* strains during enrichment in

BB was investigated in mono- and co-culture with campylobacters (chapter 3). In parallel, the growth kinetics and inhibiting effect of ESBL-*E. coli* on *Campylobacter* growth were observed. Leading up to that research, we considered including other ESBL-producing species within the *Enterobacteriaceae* family as well, so we screened for the growth of other *E. coli* strains, a *K. pneumoniae, S. fonticola* and *E. cloacae* isolate in selective BB with and without prior freeze-stress treatment. **Figure 3** shows the growth kinetics of the three selected ESBL-*E. coli* strains as well as three of the other ESBL-producers in BHI, unselective and selective BB and PB after freeze-stress. Apart from the three selected ESBL-*E. coli* strains, none of the other tested strains exhibited a considerable growth in selective BB after freeze-stress. Recently, Kim et al. (2019) enriched chicken samples in BB and identified the dominant phyla and genera present after enrichment through microbiota analysis using 16S rRNA sequencing. They found that *Escherichia* was by far the major genus (~70%), followed by *Fusobacterium* (~25%), *Pseudomonas* (~7%) and *Proteus* (~5%). It would be interesting to further investigate the inhibitory potential of isolates of these species on the growth of campylobacters.

Similarly, it might be interesting to include more *C. jejuni* and *C. coli* isolates and expand the dataset of the research conducted in chapter 3. This could help to understand whether the substrate utilization patterns are similar in other *Campylobacter* strains and whether continuous microaerobic oxygen availability would increase the competitive fitness of other *Campylobacter* strains as well during

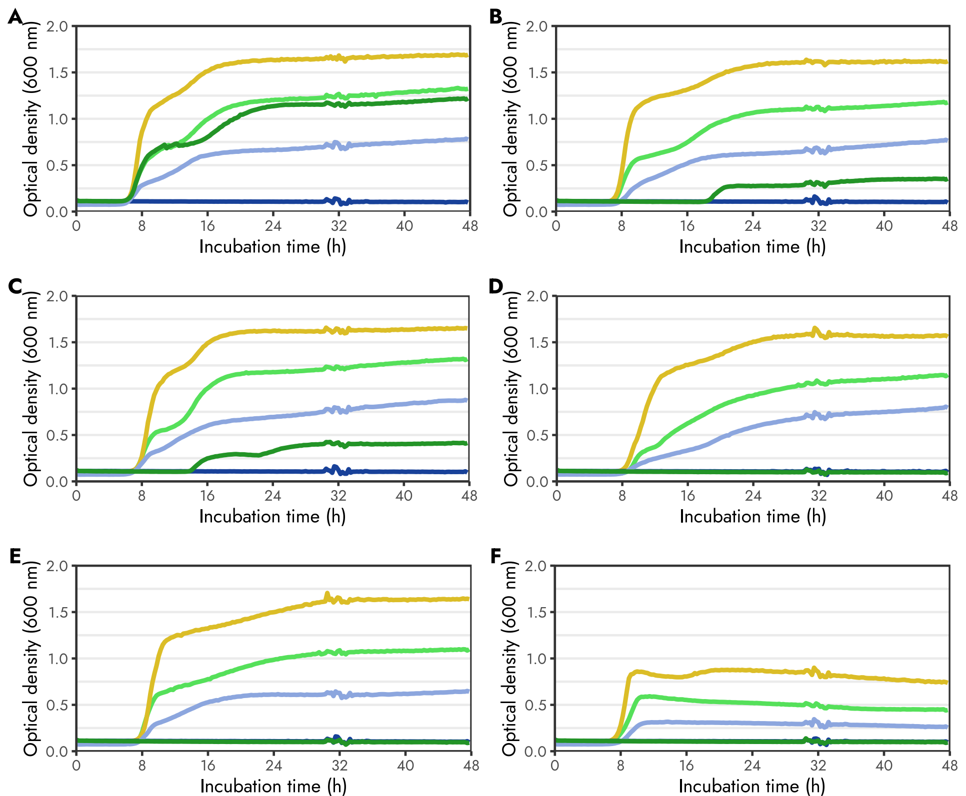

Figure 3: Growth kinetics of ESBL-producers in BHI (yellow), unselective and selective PB (light and dark blue, respectively), unselective and selective BB (light and dark green, respectively). Graphs A-C depict the growth kinetics of the three selected ESBL-*E. coli* strains as those were able to grow in selective BB while graphs D-F show three other strains (*E. coli, S. fonticola* and *K. pneumoniae*) which were unable to grow in selective BB after freeze-stress.

co-culture with ESBL-producers. In chapter 2 we investigated the variability in growth of a bigger strain selection during enrichment but did not observe a lot of variability so it is likely that similar trends could be observed but filling that knowledge gap with experimental data would allow for more reliable conclusions.

Expanding the proteomic research to better understand *Campylobacter* behaviour during enrichment

Likewise, expanding the proteomic research of chapter 4 could contribute to further identifying crucial cellular processes during lag phase and better understanding *Campylobacter* behaviour throughout different stages of cell growth. In our research, we applied refrigerated storage as a stress condition for *C. jejuni* cells and used a proteomics approach to gather insight into the intracellular processes *C. jejuni* requires for getting out of lag with and without a preceding stress treatment. The lag phase of *C. jejuni* (and *Campylobacter* spp. in general) is poorly characterized, especially at a proteomic level as no publication was available describing the lag phase of *Campylobacter* on a proteomic level. Therefore, the research conducted in chapter 4 aimed to identify trends in recovery processes. The outcomes of chapter 4 pointed to oxidative stress management, amongst others, to being an important factor in the recovery of *C. jejuni* during enrichment. In chapter 3, the growth of *C. jejuni* during co-culture with ESBL-producing *E. coli* was investigated and results showed that *C. jejuni* grew best under constant microaerobic oxygen concentrations when competitors were present as cell concentrations in stationary phase were higher compared to other experimental setups with less microaerobic oxygen availability. This indicates that atmospheric oxygen supply might play an important role during enrichment of campylobacters, and it would be interesting to investigate and compare the cellular processes within *C. jejuni* during enrichment under optimal (controlled) microaerobic oxygen conditions and in oxygen-deprived conditions. Linking growth kinetics to proteomic changes might identify crucial intracellular processes related to oxygen availability and might explain and further substantiate the importance of optimal microaerobic conditions during enrichment of food samples where competition for the available oxygen might occur. The effect of an oxygen-enriched environment on the proteome of *C. jejuni* strain NCTC 11168 in chemostat cultures was investigated by Guccione et al. (2017). In our research, we focussed only on *C. jejuni* strain 81-176 since it was used in all of the research questions in this study but moreover because this strain (next to *C. jejuni* strain NCTC 11168) is one of the most characterized strains up to date. Similarly, it would be interesting to investigate whether there are inter- and intraspecies differences in the proteome during lag phase within *C. jejuni* and between *C. jejuni* and *C. coli,* especially with respect to

metabolism-associated proteins. In chapter 3, we focused on the compound utilization of BB during enrichment of *C. jejuni* and *C. coli* and found that utilization patterns were similar with the exception of pyruvate, which was utilized only by reference cells of *C. coli.* It would be worthwhile to combine substrate utilization assays with proteomics to explain interspecies differences through proteomic analysis. In chapter 4, *C. jejuni* cells were exposed to refrigerated storage before transfer to enrichment in BB and changes in the protein expression were compared between stressed and reference cells to identify trends in intracellular recovery processes. The results indicated that, amongst others, DNA and protein damage repair were important processes during recovery from refrigeration stress. Outcomes of chapter 2 showed that decrease in viability was negligible after refrigeration stress, but lag duration increased significantly compared to reference cells. It is likely that the combination of refrigerated storage under aerobic conditions induced DNA and protein damage which was repaired during lag phase. It would be interesting to use the setup of the proteomic research of chapter 4 but expose *C. jejuni* to other food-relevant stresses as well, such as freeze-stress or heat stress. This could provide more insight into crucial recovery processes during lag phase and whether those processes are stress specific. In chapter 2, four *Campylobacter* strains were exposed to freeze-stress and oxidative stress and results showed that lag-duration was not just strain but also stress-dependent. Although for *C. jejuni* 81-176, the difference in lag-duration after both stresses was not significant, for other strains such as WDCM 00005 (*C. jejuni)* Ca 2800 (*C. coli)*, the lag-duration after oxidative stress was significantly longer compared to freeze-stress so it would be interesting to explain this phenotypic observation through proteomic analysis. Lastly, in the proteomic research of chapter 4, the pre-culturing of *C. jejuni* cells was carried out in unselective BB while enrichment was conducted in selective BB. Notably, no significant differential expression of proteins associated with multi-drug or specific resistance mechanisms could be observed after transfer from unselective to selective BB. This suggests that cells either did not have to adapt to the antibiotics present in selective BB or that antibiotic resistance systems were already present and active at the point of transfer. This setup was chosen to be able to compare the phenotypic observations of the other chapters with the proteomic changes during lag phase and also to be able to identify cellular recovery processes after refrigeration stress. However, it would be interesting and relevant, to further investigate the lag

duration and adaptative response of *C. jejuni* precultured for example in (model) foods following transfer to (non-selective) enrichment medium.

Improvements to the EMRT-PCR protocol

The research conducted in chapters 2 to 4 was important to understand the length and variability of the lag phase of *C. jejuni* and *C. coli* to better estimate the growth of different *Campylobacter* strains during enrichment (chapter 2), assess if improvements should be made to BB to stimulate the recovery of injured cells, determine and counteract the impact of competition on *Campylobacter* growth (chapter 3) and understand the cellular processes that take place during lag phase of *C. jejuni* (chapter 4). This gained knowledge allowed us to estimate the minimal enrichment duration needed for foodborne campylobacters to grow to MRT-PCR detectable limits. We then combined these 40 h-long enrichments with a detection step using MRT-PCR, developed the EMRT-PCR protocol and tested it on naturally contaminated food products which yielded promising results with our tested setup (chapter 5).

However, we only tested red and poultry meat samples as they have the highest prevalence of *Campylobacter* spp. (Humphrey et al., 2007; Nesbakken et al., 2003). In order to develop a general detection protocol for campylobacters from food, the performance and reliability of the MRT-PCR assay should be tested also for other food products which have shown to also be contaminated with *Campylobacter* spp. such as raw milk, fruits and vegetables (Mohammadpour et al., 2018; Taghizadeh et al., 2022; Verhoeff-Bakkenes et al., 2011). During the development of the MRT-PCR assay we found that, for some of the primer/probe combinations, the detection limit of *Campylobacter* was approximately 1 log_{10} cfu/ml higher in enrichments containing chicken skin than in those without a food product. This is probably due to the presence of PCR inhibitors originating from the sample. Likewise, it is possible that the sensitivity of the assay is affected by other organic and/or inorganic PCR inhibitors in other food samples. In raw milk samples, for example, calcium has shown to influence the amplification efficiency (Bickley et al., 1996) by competing with magnesium ($MgCl_2$ provided in the PCR mastermix) to the binding site of the Taq polymerase (Kuffel et al., 2021). Fortunately, different methods have been developed to reverse the inhibitory effects of several PCR inhibitors (Schrader et al., 2012). In the

example of calcium in milk, increasing the concentration of magnesium has shown to limit the influence of the PCR inhibitor (Kuffel et al., 2021). Controlling the impact of PCR inhibitors might help to even lower the MRT-PCR detection limit, which in turn, could lead to shorter enrichment times.

Another approach to shorten detection times could be to concentrate the sample DNA to reach the detection limit. Basically, if the sample could be ten-times concentrated, the necessary cell concentrations in the enrichment could be 1 log_{10} cfu/ml lower and thus the enrichment could be shorter. However, this could lead to an increase in PCR inhibitors which should be anticipated. A filtration and/or short centrifugation step might be an option to remove bigger pieces of the food sample, but this remains to be elucidated. For future improvements of the EMRT-PCR protocol, the initial *Campylobacter* contamination level in the food product should also be assessed when a shorter enrichment duration is considered. In chapter 5 we conducted a Monte Carlo analysis (figure 5.1) which predicted the probability of reaching the MRT-PCR detection limit over time at different *Campylobacter* concentrations. At high starting concentrations (3 log_{10} cfu/g of food product), the probability was already very high (p=0.99) after 24 h of enrichment. This is rarely the case as has been shown in chapter 5 where the initial *Campylobacter*-level of naturally contaminated meat samples was tested. Of the twelve *Campylobacter*-positive samples, only one was as high as 2.9 log_{10} cfu/g of food product before enrichment and more than half of the samples were below the detection limit of 2 log_{10} cfu/g. On the other hand, a very low starting concentration might be more realistic, especially with respect to raw fruits and vegetables, which often contain low amounts of *Campylobacter* cells through cross-contamination. In this scenario, the probability of 0.99 was reached after 40 h of enrichment. Experimental data gathered in chapter 5 showed that most meat samples that were positive after 40 h were already positive after 24 h of enrichment (except for one). This indicates that it is realistic that with some fine tuning the enrichment duration could be shorter than 40 h. It would be interesting to test more food samples using the EMRT-PCR with sampling times between 24 and 40 h to close in on the realistic maximum enrichment time needed for reliable outcomes. But above all, the ultimate goal of the EMRT-PCR protocol should be the reliable detection of a single cell in any tested food product, although the average *Campylobacter* concentration in certain food products may be higher in some food products.

Detection and species differentiation was achieved with three primer/probe sets, two of them for the exclusive detection of either *C. jejuni* or *C. coli* and a third one for the detection of both species (*cje* primer). This was done as a double confirmation, but ultimately is not essential for the reliability of the detection outcomes as the other two primers (*hipO* and *ceuE*) also showed to be specific enough for the strains tested. In the future, it might be important to be able to detect also other (emerging) pathogenic *Campylobacter* species. For these purposes, the *cje* primer/probe set could be replaced by an appropriate alternative, such as *gyrA* for *C. lari* (Chapela et al., 2015) or *ups* for *C. upsaliensis* (Fontanot et al., 2014). In that case, the sensitivity and specificity of the method needs to be re-assessed to ensure reliable detection and differentiation.

In the ISO 10272-1 protocol, campylobacters are isolated and confirmed through plating on (selective) isolation media. This also means that *Campylobacter* food isolates can be taken up in strain collections and national reference laboratories for further research. This isolation step would be an interesting addition to the current EMRT-PCR protocol and could be achieved by streak-plating on selective media (such as RCA) either in parallel with the detection step or after positive MRT-PCR results are obtained. In order to obtain a *Campylobacter* colony through streak-plating, cell concentrations after enrichment must be at least 2 log_{10} cfu/ml (equalling 1 cfu/loop). If the MRT-PCR protocol can be followed, cell concentrations are at least 5.5 log_{10} cfu/ml thus sufficiently high for colony isolation.

Concluding remarks and future perspectives

In conclusion, this thesis aimed to shed light on the black box of enrichments to assess whether the current enrichment protocol is adequate or if changes could be proposed to improve the detection procedure of *Campylobacter* spp. in foods. This thesis focused on the variability in lag phase of *C. jejuni* and *C. coli* during enrichment in BB and provided new insights into the substrate utilization of campylobacters and ESBL-*E. coli* and the cellular processes that take place during lag phase of reference and refrigeration-stressed *C. jejuni* cells during enrichment in BB. By better understanding what happens during enrichment, it was concluded that BB is a supportive enrichment medium for campylobacters, but it also became clear that oxygen plays a crucial role during enrichment. These new insights into enrichment could then be used to develop

a rapid and reliable detection method, namely a combination of enrichment and multiplex real-time PCR (EMRT-PCR).

In order to shed more light into the black box of enrichment and gain more knowledge on the processes that take place during the different stages of *Campylobacter* growth during enrichment, both phenotypically and on a proteomic level, it would be interesting to test more food isolates and stress treatments (mimicking food processing). The outcomes of several chapters of this thesis pointed to the importance of microaerobic oxygen during enrichment and more research on the effect of controlled oxygen supply could broaden the understanding of this factor during enrichment, especially if the different techniques of the four research chapters are combined.

The successful testing of naturally contaminated food products for *Campylobacter* with the developed protocol which combines enrichment with multiplex real-time PCR has shown that the protocol works in principle, but there is undoubtedly still much that can and needs to be improved in order for EMRT-PCR to become an interesting alternative to the existing ISO protocol. More *Campylobacter* strains and species should be tested in combination with different food products to assess whether the current protocol is sufficient or needs adaptation. Also, efforts should be made to lower the detection limit through concentration and/or purification steps which could shorten the enrichment duration, perhaps even to 24 h. The current MRT-PCR assay allows for adjustments such as adding primers for the detection of other *Campylobacter* species, which might become even more relevant in the future. On top of that, the addition of a colony isolation step after enrichment, which could be performed in parallel to the MRT-PCR assay would be an invaluable addition to the protocol to obtain colony-material which allows the isolate to be included in a strain collection.

The knowledge gained on the behavior of *Campylobacter* spp. during enrichment in BB can be used to improve the current ISO protocol and the developed EMRT-PCR protocol might serve as a promising base for a new ISO method for the detection of *Campylobacter* spp. from food by combining the ‘old’ (culture-based enrichment) with the ‘new’ (molecular-based detection).

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A

Appendix

Summary

Layman summary for general audiences

Acknowledgements

About the author

Overview of completed training activities

Summary

Campylobacter spp. are the cause of most zoonotic gastroenteritis cases, both in the European Union and worldwide. Food is a major transmission route for the two most pathogenic species, *Campylobacter jejuni* and *Campylobacter coli,* which means that reliable detection procedures need to be in place to help ensure food safety. Since campylobacters are often present on perishable products such as raw poultry meat, the time-to-detect also should be as short as possible to avoid distribution, and possibly also recalls of contaminated products. Currently, the detection procedures described by the International Organization for Standardization (ISO 10272-1:2017) are applied. Low storage temperatures and atmospheric oxygen concentrations during food processing and storage can cause sub-lethal damage or transient non-culturability. Next to that, raw produce often also contains a high amount of background microbiota including extended-spectrum beta-lactamase (ESBL-) producing *Escherichia coli*. Therefore, ISO 10272-1:2017 includes two enrichment-based detection procedures, wherein the enrichment step aims to repair cell damage and suppress the growth of background microbiota to increase *Campylobacter* concentrations, thereby supporting the detection of campylobacters in food. However, the two procedures currently in place have their advantages and disadvantages. Enrichment in selective Bolton broth (BB; procedure A) enables more efficient resuscitation of sub-lethally damaged campylobacters, but often fails to suppress the growth of ESBL-producing bacteria. This can lead to overgrowth on some isolation media which can result in false-negative detection outcomes. Enrichment in selective Preston broth on the other hand, enables suppression of ESBL-producers, but often fails to support the repair and growth of sub-lethally injured campylobacters, which can also result in false-negative detection outcomes. On top of that, both enrichment-based detection methods are time-consuming and positive results are often confirmed only after approximately one week due to especially the isolation and confirmation steps. Since *Campylobacter* spp. are often present on food products in low numbers, an enrichment-step is inevitable to reach reliable detectable cell concentrations from an initial level of one cell in 100 ml. Additionally, food products undergo processing, transport and storage stages which can pose unfavourable environments for *Campylobacter* and can lead to sub-lethal cell injury. In that case, procedure A utilizing selective BB is often used. However, the precise

processes that take place during enrichment of campylobacters are not studied and characterized in detail yet. More insight into the black box of enrichment could identify possibilities for the improvement of media composition or incubation practices which, in turn, could aid *Campylobacter* growth or shorten enrichment durations. Currently, ISO 10272-1:2017 utilizes a culture-based detection approach and the development of a new molecular-based detection method could, in combination with an enrichment step, lead to a shorter and more specific detection procedure.

Therefore, the objective of the thesis was to obtain quantitative and mechanistic insight in the microbial physiology and ecology during enrichment in order to assess whether procedure A of ISO 10272-1:2017 is adequate for the detection of *Campylobacter* spp. from foods or if possible changes to the procedure could be proposed to improve the enrichment-based detection procedure, either in speed or in sensitivity. The second objective of the thesis was to utilize the knowledge gained through the previous research questions and develop a molecular-based method for the detection of food-borne campylobacters after enrichment in BB.

Firstly, the variability in lag-duration of *C. jejuni* and *C. coli* during enrichment after different food-relevant stress treatments was assessed, and its impact on growth kinetics and reliability of detection outcomes was evaluated (**chapter 2**). Therefore, 13 *C. jejuni* and 10 *C. coli* strains were subjected to cold stress during refrigerated and frozen storage. Refrigerated storage did not significantly reduce culturability, but frozen storage reduced cell concentrations by 1.6±0.1 log_{10} cfu/ml for both species. Subsequently, cells were enriched according to ISO 10272-1:2017 procedure A and cell concentrations were determined over time and lag-duration and growth rate were determined by fitting the Baranyi-model. Without prior stress treatment, mean lag-duration for *C. jejuni* and *C. coli* was 2.5±0.2 h and 2.2±0.3 h, respectively. Refrigerated storage increased lag-duration for *C. jejuni* to 4.6±0.4 h and for *C. coli* to 5.0±0.4 h and frozen storage increased lag-duration to 5.0±0.3 h and 6.1±0.4 h for *C. jejuni* and *C. coli*, respectively. Comparison of strain- and biological variability showed that differences in recovery after cold stress can be attributed mainly to strain variability since strain variability after refrigeration and freeze stress increased respectively 3-fold and 4-fold while biological variability remained rather constant. A subset of strains was subsequently subjected to oxidative stress that reduced cell concentrations

by 0.7±0.2 log_{10} cfu/ml and comparison of recovery patterns after oxidative and freeze stress indicated that recovery behaviour was also dependent on the stress applied. A scenario analysis was conducted to evaluate the impact of heterogeneity in outgrowth kinetics of single cells on the reliability of detection outcomes following ISO protocol 10272-1:2017. This revealed that a 'worst-case'-scenario for successful detection by a combination of the longest lag-duration of 7.6 h and lowest growth rate of 0.47 h^{-1} still resulted in positive detection outcomes since the detection limit was reached within 32.5 h. The outcomes of this research question suggested that other factors such as competitive microbiota could act as a causative factor in false-negative outcomes of tested food samples.

It is well-established that ESBL-producing *E. coli* challenge reliable detection of campylobacters during enrichment in selective BB following ISO 10272-1:2017 procedure A, but the cause for the growth suppression was yet unknown. A plausible reason was the competition-induced lack of certain growth substrates. Therefore, it was investigated whether campylobacters and ESBL-*E. coli* compete for the same medium components and whether this was the cause for the observed growth repression (**chapter 3**). The availability of possible growth substrates in BB was determined and changes in their extracellular concentration were measured over time during mono-culture enrichment of *C. jejuni, C. coli* or ESBL-*E. coli* as well as in co-culture enrichments of campylobacters and ESBL-*E. coli*. Comparative analysis showed lactate and fumarate utilization by *C. jejuni* and *C. coli* exclusively, whereas ESBL-*E. coli* rapidly consumed asparagine, glutamine/arginine, lysine, threonine, tryptophan, pyruvate, glycerol, cellobiose, and glucose. Both campylobacters and ESBL-*E. coli* utilized aspartate, serine, formate, a-ketoglutarate and malate. Trends in compound utilization were similar for *C. jejuni* and *C. coli* and trends in compound utilization were rather comparable during enrichment of reference and freeze-stressed campylobacters. Since final cell densities of *C. jejuni* and *C. coli* in co-cultures were not enhanced by the addition of surplus L-serine and final cell densities were similar in fresh and spent medium, growth suppression seems not to be caused by a lack of substrates or production of inhibitory compounds. We hypothesized that oxygen availability was limiting the growth of *Campylobacter* in co-cultures. Indeed, higher oxygen availability increased the competitive fitness of *C. jejuni* 81-176 in co-culture with ESBL-*E. coli* in duplicate experiments, as cell concentrations in stationary

phase were similar to those without competition. The outcomes of this research question indicated the critical role of oxygen availability during the growth of *Campylobacter* during enrichments in the presence of ESBL-producing *E. coli*.

The enrichment step is crucial for the reliable detection of *C. jejuni* in food products to support recovery of potentially stressed cells and to increase the concentration of cells to detectable levels. However, only little was known about the behaviour of *C. jejuni* during the initial lag phase in enrichments. Therefore, a proteomics approach was followed to gain insight into the recovery mechanisms during the lag phase of *C. jejuni* strain 81-176 when enriched in selective BB (**chapter 4**). Cells were pre-cultured to stationary phase in BB-base and either directly transferred into selective BB or first exposed to refrigeration stress under aerobic conditions. Upon transfer of reference cells into selective BB, proteins associated with the maintenance of (membrane) protein quality were upregulated. In addition, redox enzymes and the anaerobic electron transfer protein complex MfrABC were upregulated during the lag phase of reference and refrigeration-stressed cells. The latter cells showed additional upregulation of proteins associated with several cell functions including purine metabolism, DNA and protein damage repair, iron acquisition, and additional electron acceptors, pointing to complementary factors involved in recovery of stressed cells. Outcomes of this study gave insights into the lag phase of *C. jejuni* during enrichment in selective BB, and demonstrated that protein quality and oxidative stress management are important factors in the recovery of reference and refrigeration-stressed *C. jejuni* cells.

The knowledge gained thus far suggested that selective BB was an adequate medium for the enrichment of food-borne campylobacters as it sufficiently supported the resuscitation of stressed cells and increased concentrations to detectable levels well within the enrichment duration of 48 h suggested by ISO 10272-1:2017 provided that a constant microaerobic environment could be ensured. Subsequently, this knowledge was used to combine culture-based ISO 10271-1:2017 enrichment in selective BB with a molecular detection method, and develop a faster detection procedure for food-borne campylobacters, called EMRT-PCR (**chapter 5**). For the detection step of the procedure, a multiplex real-time (MRT-)PCR assay was developed. Species differentiation was achieved by targeting *C. jejuni* (*mapA*), *C. coli* (*ceuE*), and both

species (*cje*). The detection limit of the MRT-PCR assay was 4.5 and 5.5 log_{10} cfu/ml in selective BB and selective BB containing chicken skin, respectively. A Monte Carlo simulation was conducted to predict the probability that campylobacters reach the MRT-PCR detection threshold throughout enrichment in selective BB, and results suggested that cold-stressed campylobacters could reach the detection limit after 40 h of enrichment (p=0.99). As a proof of principle, 23 naturally contaminated meat products were enriched according to ISO 10272-1:2017 procedure A, and the EMRT-PCR in parallel. After 24 h, 12 and 11 samples already tested positive for *Campylobacter* with the ISO method and EMRT-PCR, respectively. After 40 h, the sample that tested negative after 24-h was also positive with EMRT-PCR. The EMRT-PCR takes about 2 days to produce reliable results, while results using ISO 10272-1:2017 can take up to 8 days, which demonstrated the potential of the EMRT-PCR method.

The results from the research chapters of this thesis, the current detection challenges and the impact of the research outcomes on the current detection procedures, as well as future perspectives, are further discussed in **chapter 6**. In conclusion, the knowledge gained on the behavior of *Campylobacter* spp. during enrichment in BB can be used to improve the current ISO protocol and the developed EMRT-PCR protocol might serve as a promising base for a new ISO method for the detection of *Campylobacter* spp. from food by combining the ‘old’ (culture-based enrichment) with the ‘new’ (molecular-based detection).

Layman summary for general audiences

This thesis focusses on the bacteria, which we could describe as tiny, single-celled living organisms. The group of bacteria called *Campylobacter* are the cause of most stomach flu cases that are transmitted from animals to humans, worldwide. Two sub-groups of *Campylobacter* in particular are often associated with illness, namely *Campylobacter jejuni* and *Campylobacter coli*. These pathogenic species can often be found in the organs of animals, especially birds such as chickens. When these animals are slaughtered, the bacteria can transfer onto the food products and can survive on these food products. Humans can ingest these pathogenic bacteria through the consumption of these food products if they are not prepared properly. Oftentimes, the ingestion of only a few hundred cells is enough to cause illness and therefore, it is important to be able to detect them in food products to ensure food safety. Because *Campylobacter* cells can be present on products that spoil easily (such as raw chicken), the detection procedure should also be as fast as possible. Currently, the procedures described by the International Organization for Standardization (ISO) are often used. Although the cells can survive on food products, storage in the fridge or freezer can cause damage to the cells. Similarly, the cells cannot tolerate the oxygen concentrations in the air that surrounds us very well, but also need some oxygen since complete absence of oxygen also is harmful (this need for low amounts of oxygen is called microaerophile). On top of that, there are other bacteria, so-called 'extended spectrum beta-lactamase (ESBL-) producing *E. coli* in the food products which can also interfere with the detection of *Campylobacter*. For those reasons, ISO developed two procedures (A and B), which make use of an enrichment step. In this step, cells are transferred to a liquid medium and placed in conditions which are optimal for cells to recover and grow, which is needed to detect the cells later on. Because *Campylobacter* is often present on food products only in low amounts, using an enrichment is unavoidable to obtain high enough cell numbers to be able to detect the cells. Both procedures have their pro's and con's. Procedure A (enrichment in a medium called 'Bolton broth') works generally better for the recovery of injured cells, but less good for the inhibition of ESBL-producing cells. On the other hand, procedure B (enrichment in a medium called 'Preston broth') inhibits ESBL-producing cells more effectively, but often does not work well for the recovery of injured *Campylobacter* cells. On top of that, both methods are time-consuming and it takes about one week

to get a positive result. In practice, most food products that can contain *Campylobacter* are stored at damage-inducing conditions (for example raw chicken filets in the fridge) and in in those cases, enrichment in Bolton broth (BB) is preferred. We already know from previous research that, after enrichment, cell concentrations are much higher than before, but the exact processes that take place during enrichment of *Campylobacter* are not studied and characterized yet. Studying the enrichment in more detail could identify possibilities for improvement of the enrichment medium or incubation practices, which, in turn could help the growth of *Campylobacter* cells or shorten enrichment times. If enrichment could be combined with a less time-consuming, molecular-based detection method, a shorter and more reliable detection procedure could be developed.

Therefore, the aim of this thesis was to shed light into the blackbox of enrichment to assess if procedure A of the current ISO standard 10272-1:2017 is adequate for detecting *Campylobacter* from food or if possible changes could be proposed to improve the procedure. The second aim was to use this knowledge and develop a molecular-based method to detect *Campylobacter* after enrichment in BB.

In this thesis, four different research questions were worked on, which are described in chapters 2 to 5. In **chapter 2**, we assessed the variability in lag-duration of *C. jejuni* and *C. coli* strains during enrichment. Lag describes a phase during bacterial growth, where actually no growth happens, but cells adapt to the new environment and repair cell damages. We tested 23 different strains, 13 of the species *C. jejuni* and 10 of *C. coli*. We exposed those strains to two stressful environments, namely storage in the fridge (+4°C) and freezer (-20°C). Refrigeration did not have a significant negative effect on the viability (meaning the capacity to grow), but frozen storage reduced cell concentrations by around 97.5%. The strains were then enriched in BB and the lag-duration was estimated for each strain and after each stress treatment. As a control, the lag-duration was also estimated without any stress treatment. The results of this chapter showed, that the lag-duration was shortest in 'unstressed' cells, longer in refrigeration-stressed cells and even longer in freeze-stressed cells. We were interested to find out if this trend was really only due to the stress treatments or if variability in strains could also be the reason. For each strain and treatment, experiments were repeated on different days with newly grown cells and fresh media

and we also were interested if this could account for the variability in lag-duration, as well (biological variability). Our results showed, that the trends was mainly due to the stress treatment. We were then interested to find out if the trend would only be observed for cold stress or if it was the same for different stressful environments. To test that, four strains which had the shortest and longest lag-duration after cold stress were exposed to atmospheric oxygen stress as well and those results showed that lag-duration was also dependent on the stressful environment the cells were exposed to. We used the growth data of all these experiments to conduct a scenario analysis and predicted that even in a 'worst-case'-scenario (with low starting cell concentrations, long lag-duration and slow growth), the cell concentrations would still be high enough to be detected using ISO procedures after an enrichment of 32.5 h. The results of this chapter suggested, that other factors like ESBL-producing E. coli might be a reason for unreliable detection outcomes.

As mentioned earlier, ESBL-producing *E. coli* can interfere with the detection of *Campylobacter*. Previous research has shown that the growth of *Campylobacter* during enrichment is sometimes hindered when ESBL-producers are present, but the exact cause for the growth suppression is still unknown. Enrichments are rich in nutrients and *E. coli* can use a wide range of them to grow, while *Campylobacter* is rather picky in their nutrient use (for example, they don't use sugars for growth which is the favorite nutrient of a lot of other bacteria). In **chapter 3**, we investigated, if competition for nutrients could be the reason for the growth inhibition of *Campylobacter* in the presence of ESBL-*E. coli*. To test this theory, *Campylobacter* and ESBL-*E. coli* were first grown apart from each other and later also together (in so-called 'co-culture') in the same enrichment. The growth over time and the concentration of different nutrients at the same time points was measured and results showed, that *Campylobacter* and ESBL-*E. coli* used some of the same nutrients, especially the amino acid serine. Follow-up experiments showed, that competition for nutrients was most likely not the reason for the growth inhibition, and neither was production of inhibitory compounds by ESBL-*E. coli*. We hypothesized that oxygen availability might be limiting the growth of *Campylobacter* in co-culture. Indeed, constant supply of microaerobic oxygen concentrations did lead to higher *Campylobacter* numbers at the end of enrichment when ESBL-*E. coli* were present.

The outcomes of this chapter indicated that microaerobic oxygen availability is critical during the growth of *Campylobacter* during co-culture enrichments.

We know, that bacterial cells follow the same growth pattern during enrichment – a lag-phase, followed by rapid growth (exponential growth phase) and finally stationary phase, where there is a balance between cell growth and cell death. Of all phases, the lag-phase is the least well described, especially for Campylobacter. Therefore, **chapter 4** focused on the behaviour of *Campylobacter jejuni* during lag-phase in BB-enrichments. We were interested to find out, which processes take place within the cell to find out more about repair and adaptation processes. To do so, a proteomics approach was chosen, which means that we looked at the expression of proteins the *C. jejuni* cells build and how their expression changed during the lag-phase. Cells were grown to stationary phase and either directly transferred to BB or first exposed to refrigeration stress and atmospheric oxygen conditions. By testing both 'unstressed' and stressed cells, we wanted to see if stressed cells express specific proteins that could be linked to cell repair. Results showed, that protein quality and management of oxidative stress (dealing with oxygen in the environment) are important in the recovery of 'unstressed' and stressed *C. jejuni* cells.

The results of the previous chapters suggested that BB was an acceptable medium for the enrichment of *Campylobacter* from foods because it sufficiently supported the recovery of stressed cells and increased cell numbers to detectable levels well within the enrichment time of 48 h suggested by ISO. Our results did show that a constant microaerobic environment should be ensured during enrichment. The next challenge was to combine the enrichment step of ISO with a molecular detection method, and develop a faster detection procedure for *Campylobacter* from food products (described in **chapter 5**). For the detection step, a method was used which detects specific pieces of *Campylobacter* DNA (so-called targets) and copies them over and over until they can be detected by a machine. The method is called PCR (you might recognize the term from the Covid-PCR tests). To test the combination of enrichment and PCR (now called EMRT-PCR), 23 food samples were tested for their *Campylobacter* presence. The outcomes showed that *Campylobacter* could be reliably detected with EMRT-PCR within two days, while results using ISO can take up to 8 days, which demonstrated the potential of the EMRT-PCR method.

In **chapter 6**, the results from the research chapters of this thesis, the current detection challenges and the impact of the research outcomes on the current detection procedures, as well as future perspectives are discussed. In conclusion, the knowledge gained on the behaviour of *Campylobacter* during enrichment in BB can be used to improve the current ISO protocol. The developed EMRT-PCR protocol might serve as a promising base for a new ISO method for the detection of *Campylobacter* from food products by combining the 'old' (culture-based enrichment) with the 'new' (molecular-based detection).

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Finally, I get to write this; the last and (let's be honest) probably most read part of this PhD thesis. I would like to take this moment to sincerely thank everyone who has been a part of my PhD journey, supported me and has given me the opportunities that got me to this point in my career and life.

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again, I'll come and watch a match. **Andy** (the entrepreneur), I hope you're doing well back home in China, we miss you here. Thank you for taking such good care of us in China, we would have been lost and stranded without you on several occasions. **Claire**, you are so cute and funny. Your laugh is contagious and always brightens my mood. Thank you for sharing your hora finita knowledge with me. **Bernard**, thanks for the unforgettable trip to China and singing Backstreet Boys, I'll never forget it. **Diego**, we should have graduated around the same time and I miss you. You should have had more time. **Rebecca**, my passionate Italian lab mate. Due to you, the lab never got boring and I am always excited to hear what's going on in your life. **Linda**, thank you so much for all the tasty treats you brought to the office. If someday you're tired of science, you can always open a patisserie and become a millionaire. **Alberto B.**, I had a great time with you in Athens, thanks for being you. **Xuchuan**, you are such a kind person, great listener and amazing chef. I loved sharing an office with you, you are amazing! **Tamara**, my fellow Baden-Württemberger and Maultaschen-Liebhaberin, **Sylviani**, **Thelma** and **Jasper Z.**, I wish you all the best with your work and PhD journey!

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Dear **friends and family**, thank you very much for the support you gave me in the last few years. You've seen first hand what a PhD project can do to someone – the bad, but also the good parts. You've been there with care packages, tissues, drinks (celebratory and consolatory), open ears and arms and plenty of jokes to cheer me up. I would like to thank my family in particular, for making it possible for me to stand at this point in my life. You provided me with everything I needed, educationally, financially, and mentally and I'm very grateful to you!

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make even more. **Pjotr**, my *Campylobacter*-buddy. You're such a funny guy (not always intentionally, which is even better) and made me almost pee my pants from laughter at the Efteling. You know exactly what you want and always manage to achieve just that, which is something I really admire. Thank you for standing with me during my defence! **Natalia**, my amazing little nerd! You're so smart, patient and light-weight, which has come handy in scary escape rooms on multiple occasions. I'm so happy that we met during the PhD and kept such close contact throughout the years. I can't wait to make some more memories, so let's get planning soon! Thank you for being a great friend! And now, **Jasper**: words can't describe how much I enjoyed sharing a PhD project with you. But numbers can: a solid 6! :D. No, seriously, I am so grateful that we got to do this project together. Without you, I probably would have thought about quitting much more often. You're kind, caring, ambitious and funny and you will achieve great things in life! You've experienced me happy and sad, frustrated and (annoyingly) excited and didn't run away (which you easily could have done because you're a runner and I'm not, although even that you've tried to teach me with unbelievable patience). I really don't know how to thank you for everything you did for and with me in the last years. Thank you for standing with me during my defence!

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About the author

It all started on the 23rd of August 1989, when Maren was born in Herrenberg (Germany). After finishing high school she already knew what her passion was: Food, Food safety and more Food! Therefore, she did the bachelor Food, Nutrition and Hygiene at Albstadt-Sigmaringen University (Sigmaringen, Germany) from 2009 to 2013. During this time, she found out that she enjoys the little things in life and started focussing on microbiology.

Following that, she made what will turn out to be the best decision of her life, which was moving to the Netherlands (or to the Niederlande as they call it). Once in the promised land, she started a master in Applied Food Safety at Wageningen University. During her master she felt like she wasn't challenged enough yet, that's why she started a MSc thesis entitled: Detection of *Campylobacter* spp. and ESBL-producing Enterobacteriaceae. Although working with *Campylobacter* is for many a punishment, inexplicably, Maren's love for this accursed bacteria only grew stronger. Her research focused on the impact of amendments to the ISO 10272-1:2006 detection.

In June 2017, Maren decided to start a PhD project in the same group where she did her master thesis, namely, Food Microbiology. Her project was entitled: "Rapid and reliable detection of *Campylobacter* spp. from food". After many passed seasons, countless tears and sleepless nights, and a pandemic in between, she handed in her PhD thesis on the 17th of January 2023. During her thesis, she became a true *Campylobacter* expert, which is without any doubt the most challenging bacteria to work with. Luckily she had help of some great students, which thought her an important life lesson: "not everyone is made for *Campylobacter*".

In search of a new challenge, Maren turned to the RIVM institute, which she had collaborated with in the past. Currently, Maren is working at the RIVM as Scientist, where she continues her passion for studying pathogens.

Apart from research, Maren has a great love for badminton and escape rooms. Even though she has been playing badminton all her life, she still regularly makes a habit of challenging her less skilled paranymphs, to deliver a much needed lesson in humility (for some of them). Interestingly enough, Maren has only failed escape rooms when one of her paranymphs was missing, suggesting that, when combined, her paranymphs are amazing.

By Pjotr Middendorf (paranymph) and Natalia Crespo (honorary third paranymph).

Overview of completed training activities

A: Discipline specific activities

KNVM Symposium 2018	KNVM	Bilthoven (NL)
IAFP Europe 2019	IAFP	Nantes (FR)
CHRO 2019	Institute of Food Science & Technology	Belfast (UK)
Intestinal Microbiome of Humans and Animals	VLAG	Wageningen (NL)
Food Science Symposium	AFSG Group	Wageningen (NL)
Proteomics workshop	WUR, Biochemistry	Wageningen (NL)
Hot Topics Event	Campden BRI	Online
KNVM Symposium 2021	KNVM	Online
LAS-ICMSF Webinar	ICMSF – Latin America	Online
World Microbe Forum	ASM & FEMS	Online
FoodMicro 2022	ICFMH	Athens (GR)

B: General courses

KNVM Symposium 2018	KNVM	Bilthoven (NL)
PhD Week	VLAG	Baarlo (NL)
Introduction to R	VLAG	Wageningen (NL)
Applied statistics	VLAG	Wageningen (NL)
Flow Cytometry workshop	ThermoFisher Scientific	Wageningen (NL)
HPLC workshop	WUR, Food Microbiology	Wageningen (NL)
Scientific artwork, Data visualization and infographics with Adobe Illustrator	VLAG	Wageningen (NL)
Efficient writing strategies	WGS	Wageningen (NL)
Career perspectives	WGS	Wageningen (NL)
PhD Week	VLAG	Baarlo (NL)
Introduction to R	VLAG	Wageningen (NL)

C: Optional activities

Preparation of research proposal	WUR, Food Microbiology	Wageningen (NL)
PhD trip to China	WUR, Food Microbiology	Wageningen (NL)
Project partner meetings	WUR, Food Microbiology	Wageningen (NL)
Department seminars	WUR, Food Microbiology	Wageningen (NL)
Organization of PhD trip	WUR, Food Microbiology	Wageningen (NL)

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