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# Development of predictive tenacity models for *Campylobacter* along the raw milk supply chain

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# List of Abbreviations

| | |
|---|---|
| $A_f$ | Accuracy factor |
| ANN | Artificial neural network |
| APC | Aerobic plate count |
| APZ | Acceptable predictions zone |
| $a_w$ | Water activity |
| $B_f$ | Bias factor |
| CC | Clonal complex |
| CFU | Colony-forming units |
| cgMLST | Core-genome multilocus sequence types |
| EHEC | Enterohaemorrhagic *E. coli* |
| EU | European Union |
| FAIR | Findability, Accessibility, Interoperability, and Reuse |
| FAO | Food Agriculture Organization |
| FSKX | Food Safety Knowledge Exchange |
| HACCP | Hazard analysis and critical control points |
| IPIU | Intact and putatively infectious units |
| $k_{max}$ | Maximum specific inactivation rate |
| LAGESO | State Office for Health and Social Affairs |
| MPN | Most probable number |
| MPRM | Modular process risk model |
| $N_{res}$ | Residual population density |
| PMA | Propidium monoazide |
| PRISMA | Preferred Reporting Items for Systematic Reviews and Meta-Analyses |
| QMRA | Quantitative microbial risk assessment |
| $R^2$ | Coefficient of determination |
| RMSE | Root mean square error |
| RoB | Risk of Bias |
| $S_l$ | Duration of shoulder effect |
| ST | Sequence type |
| STEC | Shiga toxin-producing *Escherichia coli* |

| | |
|---|---|
| TACC | Total aerobic colony count |
| $t_{xd}$ | Time required to obtain an x-log reduction |
| VBNC | Viable but non-culturable |
| v-qPCR | Viable quantitative polymerase chain reaction |
| WHO | World Health Organization |
| σ | Time for the first decimal reduction |

# 1 Introduction

The sale of raw cow's milk has become a common practice in many European countries in recent years. However, if the milk is not properly heat-treated before consumption, there is a potential that some pathogenic bacteria from the animal, like *Campylobacter* spp., may reach the consumer. The consumption of raw milk has repeatedly been associated with campylobacteriosis outbreaks (EFSA, 2021). Campylobacteriosis symptoms comprise short-term effects (fever, abdominal cramps and diarrhea) and possible chronic complications including Guillain-Barré syndrome, irritable bowel syndrome and reactive arthritis (Keithlin et al., 2014). The transmission route of *Campylobacter* spp. along the raw milk supply chain from dairy cows is not completely understood. Cross-contamination due to fecal contamination of the raw milk through insufficient housing conditions and milking hygiene might be an important cause of *Campylobacter* spp. transfer to consumers. To estimate the cross-contamination, it is necessary 1) to investigate the prevalence and concentration of *Campylobacter* spp. in dairy cow feces and 2) to investigate feces and raw milk samples in the same setting. Only low concentration and prevalence of *Campylobacter* spp. have been reported in raw milk (BVL, 2020). Therefore, longitudinal studies with frequent sampling are required to get comprehensive insight in contamination events and an estimation of contamination frequency.

*Campylobacter* spp. are known to enter a viable but non-culturable (VBNC) state under harsh environmental conditions (Rollins and Colwell, 1986). Hence, it is assumed that the survival of *Campylobacter* spp. in raw milk is underestimated since culture-dependent methods only detect colony-forming units (CFU) and are not able to detect *Campylobacter* spp. in VBNC state (Wulsten et al., 2020). The pathogenicity of *Campylobacter* spp. in the VBNC state is unknown. However, once favorable conditions recur, *Campylobacter* spp. can recover back from VBNC into CFU within a certain time window (Wulsten et al., 2020) and regain full infectious potential (Baffone et al., 2006). Reliable experimental data on the survival of *Campylobacter* spp. in raw milk, taking into account not only CFU data but also VBNC data and recovery data of VBNC cells, are necessary to estimate the survival during raw milk storage.

Predictive microbiology models that describe the behavior (growth or decay) of foodborne pathogens are helpful tools that give a rapid response to assess food safety (Pérez-Rodríguez and Valero, 2013). To estimate and compare the potential of *Campylobacter* spp. survival in raw milk during storage in the two possible physiological states predictive models, based on CFU and VBNC data are needed.

Microbial risk assessment allows an *a priori* assessment of the effect of intervention measures along the whole food chain, or combinations of intervention measures on public health

(Havelaar et al., 2008). Current microbial risk assessments for *Campylobacter* spp. in raw milk have focused on the vending machines and lacked detailed data from housing conditions and the milking hygiene (Giacometti et al., 2015; Anonymous, 2009). To reduce consumer exposure to *Campylobacter* spp. by identifying potential intervention measures QMRAs are needed over the whole food chain.

The aim of this PhD project was to conduct a detailed investigation on the presence of *Campylobacter* spp. in the raw milk production chain with the goal of identifying measures at the farm level to mitigate the risk of consumer exposure. In detail, the objectives were 1) to describe the presence and concentration of *Campylobacter* spp. in feces of dairy cows based on information contained in the literature, 2) to analyze the occurrence and transmission of *Campylobacter* spp. on a dairy farm during a longitudinal field study, 3) to obtain experimental data and predict the survival of *C. jejuni* in raw milk during storage and 4) to evaluate different risk mitigation strategies at the farm level to support risk managers. The following corresponding research steps were conducted:

I. Systematic review on *Campylobacter* spp. in feces of dairy cows

A systematic review and meta-analysis was performed to summarize previous research on *Campylobacter* spp. in feces of dairy cows worldwide. Prevalence and concentration data were extracted and important data gaps as well as limitations in current studies were highlighted.

II. Longitudinal study to investigate the occurrence of *Campylobacter* spp. along the raw milk supply chain on one small German dairy farm over one year

In a longitudinal study, different samples were collected directly from the cows, during milking and from the dairy farm environment over one year. The samples were fecal samples directly from the rectum of dairy cows, boot sock samples from the barn, teat skin swab samples, raw milk samples from the individual cows, milk filter samples and swab samples from milking equipment. Samples were analyzed for the presence and concentration of *Campylobacter* spp., *E. coli*, total aerobic colony count (TACC) and for *Pseudomonas* spp. A scoring was performed for the consistency of cows´ feces and the level of cleanliness of the teat skin swab samples.

III. Survival studies and predictive models for *C. jejuni* in raw milk at different temperatures

To investigate the survival of *C. jejuni* in raw milk, a culture-dependent and culture-independent method were used to collect data on different *C. jejuni* strains in inoculated raw milk at 5°C, 8°C, and 12°C. A cultural detection method was used to detect CFU and viable quantitative polymerase chain reaction (v-qPCR) was applied to obtain intact and putatively infectious units (IPIU) comprising CFU and VBNC data. The generated data were used to develop predictive models on the survival of *C. jejuni* in raw milk based on CFU and IPIU data between 5°C to 12°C.

IV. Quantitative microbial risk assessment (QMRA) model

A QMRA model for *Campylobacter* spp. along the raw milk supply chain was developed based on data from the longitudinal study and scientific literature. Different uncertainty analysis and risk mitigation scenarios along the supply chain were evaluated to identify data gaps and support risk managers in controlling *Campylobacter* spp.

# 2 Literature review

## 2.1 Sale and consumption of raw milk

In recent years, consumer demand for fresh and unprocessed products has increased, which has affected the food supply chain. In addition, the abolishment of the milk quota in 2015 has influenced milk production and intensified the direct sale of raw milk to consumers (TMR, 2016). Regulation (EC) No 853/2004 characterizes the product “raw milk” as “milk produced by the secretion of the mammary gland of farmed animals that has not been heated to more than 40°C or undergone any treatment that has an equivalent effect”. In Europe, the microbial criteria for raw milk of cow’s are ≤ 100 000 aerobic plate count (APC)/ml via enumeration on agar plates at 30°C (Regulation (EC) No 853/2004).

Many European countries, including Germany, France, the Netherlands, Belgium, Denmark, Italy, Ireland and parts of the United Kingdom allow controlled direct sale of raw cow’s milk to consumers on-farm (EFSA, 2015). Self-service and automatic vending machines are used for delivering raw milk. Certain requirements are necessary for the direct sale of raw milk. The conditions include that the delivery must take place at the farm producing the milk and the consumer must be advised to boil the raw milk before consumption. The competent authority must be informed about the provision of raw milk to consumers (BMJV, 2018). Raw milk for sale in vending machines should be cooled to below 4°C after milking and this temperature must be maintained during storage and transportation (EFSA, 2015). However, a study has shown that nearly one third of the raw milk samples (31.2%) investigated on site at farms in Germany had a temperature higher than 8°C (Böhnlein et al., 2020). In addition, surveys in Italy demonstrated that 13.9% to 43% of consumers ignore the advice of boiling raw milk before consumption (Giacometti et al., 2013).

In Germany nearly 850 raw milk sale outlets were registered at the end of 2019 based on a query of the respective state and county veterinary authorities (Labohm et al., 2021; Böhnlein et al., 2020). Raw milk sales through vending machines (n= 154) were on average 14,505 liters/year, with a range of 2,200 to 70,000 liters per year in Germany in 2019 (Labohm et al., 2021).

For the consumer the enhanced nutritional qualities, taste and health benefits, as well as support of regionally produced products were reasons for increased interest in raw milk consumption (Crotta et al., 2016; Claeys et al., 2014; Oliver, 2009). However, studies have shown that raw milk can be contaminated by a variety of pathogens, of which some are associated with human illness and disease (e.g. *Campylobacter* spp., Shiga toxin-producing *Escherichia coli* (STEC) or *Salmonella* spp.) (Oliver, 2009).

## 2.2 Campylobacteriosis – a zoonotic disease

Campylobacteriosis is a zoonotic disease caused by bacteria of the genus *Campylobacter*. It is the most commonly reported foodborne gastrointestinal infection in humans in the European Union (EU) since 2007 (EFSA, 2022). In 2021, 127,840 cases of human illness were reported in the EU (EFSA, 2022). Most of these cases were associated with undercooked poultry meat and an insufficient kitchen hygiene related to raw meat handling and cross-contamination on ready-to-eat food. In total, four campylobacteriosis outbreaks with overall 174 human cases were associated with milk and milk products in 2020 in EU (EFSA, 2021). Among them, three outbreaks occurred in Germany associated with the consumption of raw milk, which led to 13 human cases and 1 hospitalization.

A relatively low number of *Campylobacter* spp. can induce clinical gastrointestinal symptoms (Black et al., 1988; Robinson, 1981). Human infections usually lead to a self-limiting gastroenteritis with acute symptoms including vomiting, fever, abdominal cramps and watery or bloody diarrhea (Negretti et al., 2019). In some cases, chronic complications involve reactive arthritis, inflammatory bowel disease, and neurological disorders such as the Guillain-Barré syndrome or its variant, the Miller Fisher syndrome (Jackson et al., 2014; Keithlin et al., 2014; Poropatich et al., 2010; McCarthy and Giesecke, 2001). The Guillain-Barré syndrome and Miller Fisher syndrome are rare, but potentially fatal autoimmune diseases of the peripheral nerves usually triggered by infections (Leonhard et al., 2019; Wakerley et al., 2014).

Thermotolerant *Campylobacter*, mainly *C. jejuni* und *C. coli*, are responsible for most human campylobacteriosis cases. *Campylobacter* species information was provided by 22 Member States for 65.1% of confirmed cases reported in Europe in 2021 (EFSA, 2022). In detail, of the cases 88.4% were *C. jejuni*, 10.1% *C. coli*, 0.18% *C. fetus*, 0.12% *C. upsaliensis* and 0.09% *C. lari* (EFSA, 2022). However, other *Campylobacter* species such as *C. hyointestinalis* have also been reported in the literature to cause human disease (Kim et al., 2015; Edmonds et al., 1987).

*Campylobacter* spp. infections show a characteristic seasonality with a peak in the summer months (EFSA, 2022) and have been positively associated with temperature during early to mid-summer (Lake et al., 2019). Another smaller but distinct winter peak, around the Christmas and New Year period has recently also become apparent. The transmission of *Campylobacter* spp. might be promoted through meat fondues or table-top grilling investigated in a case-control study (Rosner et al., 2021; Bless et al., 2017).

### 2.3 Contamination of raw milk along the supply chain

Thermotolerant *Campylobacter* spp. are, often asymptomatically, carried in the intestinal tracts of numerous wild and domesticated bird and mammal species, like pig, bovine, sheep and goat (Mughini Gras et al., 2012; Waldenström et al., 2010; Humphrey et al., 2007). They have also been isolated from natural environment water samples (Mughini Gras et al., 2012; Humphrey et al., 2007).

Several transmission routes for *Campylobacter* spp. to humans have been suggested, mainly occurring via contaminated food, direct contact with colonized animals and through untreated water (Igwaran and Okoh, 2019; Rosner et al., 2017; Mughini-Gras et al., 2016; Kaakoush et al., 2015; Bronowski et al., 2014).

Contamination of raw milk along the supply chain is thought to be predominantly of fecal origin from cows carrying *Campylobacter* spp. (Del Collo et al., 2017; Modi et al., 2015; Bianchini et al., 2014; Schildt et al., 2006). It is unknown which mechanisms underlie this contamination and how frequently raw milk is contaminated during milking (Giacometti et al., 2015; Bianchini et al., 2014; Giacometti et al., 2013; Giacometti et al., 2012; Anonymous, 2009). Prevalences of *Campylobacter* spp. in dairy cow feces in different studies were 53% and 68% among all samples investigated (Idland et al., 2022; Jaakkonen et al., 2019). The reported concentrations of *Campylobacter* spp. ranged from 2.1 ± 0.45 to 4.2 log CFU/g (Ramonaitė et al., 2013; Nielsen, 2002; Waterman et al., 1984). Possible direct excretion of *Campylobacter* spp. via the mammary gland was reported in only one study (Orr et al., 1995). Prevalence and concentration data of *Campylobacter* spp. from the early stage of the raw milk supply chain are important to estimate the risk for consumers by drinking raw milk (Anonymous, 2009).

Transmission of *Campylobacter* spp. in raw milk is mainly assumed to originate from cow feces via the teats during the milking process. It is currently not clear how this contamination takes place (Giacometti et al., 2015; Bianchini et al., 2014; Giacometti et al., 2013; Anonymous, 2009). The amount of dirt transmitted to milk via the exterior of teats was estimated across farms to average approximately 59 mg/l with a range from 3 – to 300 mg/l (Vissers et al., 2007). *Campylobacter* spp. was detected in 13% of teat skin swab samples during six sampling time points from 18 dairy herds from four different geographical areas of Norway (Idland et al., 2022). Samples from the 18 dairy herds were analyzed together and only separate prevalence data for the six different sampling time points were available. The prevalence ranged from 6% to 21%. The highest prevalence in May (21%), January (17%) and February/March (17%) and lowest in August/September (6%), June (8%) and November/December (11%) (Idland et al., 2022).

*Campylobacter* spp. was only rarely detected in raw milk samples taken at retail with a prevalence of 0.47% (n= 212) in the EU 2021 (EFSA, 2022). Using the most probable number (MPN) method, the mean *C. jejuni* level was calculated as 0.1660 ± 0.3 MPN/ml (Humphrey and Beckett, 1987). This value was affected by one single sample cell count with a high concentration of 1 MPN/ml, whereas the other four samples had levels below 0.05 MPN/ml. One positive *Campylobacter* spp. bulk tank sample (0.34%) was reported in a study from New Zealand in 2007-2008 (Hill et al., 2012). The *Campylobacter* spp. level for that sample was 0.047 MPN/ml (95% CI, 0.0069 to 0.33 MPN/ml). In a Finish study, the concentration of thermotolerant *Campylobacter* spp. in bulk tank milk samples ranged from 0.007 to 35 MPN/ml (Jaakkonen et al., 2020). Milk filters, which were installed at the end of the milk pipeline so that the entire raw milk from all cows passes the filter, were a more suitable sampling target for monitoring pathogenic bacteria than raw milk (Jaakkonen et al., 2019; FSAI, 2015). Prevalences of 14%, 4% or 1% were detected in this sample matrix, with concomitant lower prevalence or lack of detection in the raw milk or raw milk bulk tank (Idland et al., 2022; Hansson et al., 2020; Jaakkonen et al., 2019).

To estimate the transmission of *Campylobacter* spp. from feces to raw milk, it is necessary to investigate feces and raw milk samples in the same setting. Longitudinal studies with frequent samplings are required to detect contamination events and to estimate their frequency. One study from Norway provided concurrent prevalence data on *Campylobacter* spp. in cows' feces (68%), on teat skin (13%), in teat milk (3%), milk filter (4%) and bulk tank milk (3%) samples (Idland et al., 2022). No quantification of *Campylobacter* spp. was performed.

### 2.4 Survival strategies of *Campylobacter* spp.

*Campylobacter* spp. generally show low tenacity due to low oxygen tolerance and sensitivity to unfavorable environmental conditions including temperature and pH (Park, 2002). They also have a low tolerance to drying (Oosterom et al., 1983) and osmotic stress (Doyle and Roman, 1982).

It has been shown that thermotolerant *Campylobacter* spp. cannot grow below 30°C and lose cultivability after prolonged incubation at 4°C (Kim et al., 2021; Baffone et al., 2006). Nevertheless, inactivation of *Campylobacter* spp. by oxygen is slower at lower temperatures (Boleratz and Oscar, 2022; Yoon et al., 2004). The pH optimum and water activity ($a_w$) value for *Campylobacter* spp. are between pH 6.5 and pH 7.5 and an $a_w$-value of 0.997 (Silva et al., 2011).

Although *Campylobacter* spp. are fastidious organisms *in vitro*, they succeed in colonizing farm animals and spread effectively in flocks (Idland et al., 2022; Rawson et al., 2020; Hakkinen

and Hänninen, 2009). *C. jejuni* of multi-locus sequence type (MLST) ST-883 has been shown to persist on a dairy farm and contaminate bulk tank milk for seven months or longer (Jaakkonen et al., 2020). This indicates an adaptability of *Campylobacter* spp., allowing them to tolerate various stress conditions, e.g. adverse environmental factors and different matrices (Kim et al., 2021). The stress adaption mechanisms of *Campylobacter* spp. mainly involve the ability to adopt a VBNC status, but also include a more long-term strategy of adaptive potential based on high genetic diversity (Golz and Stingl, 2021; Burnham and Hendrixson, 2018). The VBNC status is induced by osmotic stress (Lv et al., 2019), starvation (Magajna and Schraft, 2015), cold-stress (Chaisowwong et al., 2012), and probably also aerobic stress (Oh et al., 2015). In this state, the bacteria have a coccoid shape, are unable to multiply (Poursina et al., 2018; Ramamurthy et al., 2014) and are no longer detectable by cultural methods (Krüger et al., 2014; Bovill and Mackey, 1997). The infectivity of *Campylobacter* spp. in the VBNC state is not known. It has been shown that when using a specific gas mixture with a low oxygen level the cells can be recovered from VBNC into CFU in raw milk at 5°C within a limited time window (Wulsten et al., 2020). VBNC cells that are retrieved back into CFUs can regain full infectious potential (Baffone et al., 2006). A v-qPCR with propidium monoazide (PMA) detecting IPIU indicated that the survival of *C. jejuni* (DSM 4688 and BfR-CA-13290) and *C. coli* (DSM 4689) as compared to CFU data in raw milk at 5°C was underestimated (Wulsten et al., 2020). Since the v-qPCR quantifies IPIU, comprising both VBNC and CFU, it provides currently a more complete picture of the survival of *Campylobacter* spp. in raw milk.

### 2.5 Predictive microbiology

Predictive microbiology is a broad research field within food microbiology that provides mathematical models for predicting microbial behavior (growth and decay) in foods (Pérez-Rodríguez and Valero, 2013). These models describe the behavior of the bacteria in real systems by using mathematical equations, which are simplifications of the corresponding system, to predict microbial growth and inactivation in response to certain environmental conditions (Ross and McMeekin, 1994). In order for a model to be “complete”, i.e. to be able to accurately predict the behavior of a microorganism, all essential environmental parameters must be included. Environmental parameters taken into account during modelling might be intrinsic factors, such as pH, $a_w$, salt and other microorganisms in the food matrix or extrinsic factors, e.g. atmosphere and temperature (Baird-Parker and Kilsby, 1987). Predictive microbiology provides information for two main areas, namely prevention of microbial food spoilage and the protection of the consumer against hazards in foods (Mossel and Drion, 1979). Effective predictive models allow researchers to predict the behavior of microorganisms in foods under foreseeable conditions in advance and become important decision support tools

(Pérez-Rodríguez and Valero, 2013). Current applications of predictive microbiology in an industrial context can be summarized into three groups: 1) Product innovation, e.g. new products and processes are developed; 2) Operational support, e.g. implementing or running a food manufacturing operation; 3) Incident support, impacts on consumer safety or product quality are estimated based on reports of problems with products on the market (Membré and Lambert, 2008).

### 2.5.1 Model development

Predictive models can be divided into three groups, namely: primary, secondary and tertiary models (Pérez-Rodríguez, 2014). Primary models describe how microbial counts change over time and estimate kinetic parameters (e.g. maximum growth rate, lag phase, inactivation rate). Secondary models predict the changes in the kinetic parameters of primary models as a function of the effect of environmental conditions (e.g. pH or $a_w$) (Pérez-Rodríguez, 2014). Finally, tertiary models identify patterns in the parameters of the secondary models as a function of the organism and the nutrient source (as proposed by Baranyi et al. (2017)). They were first introduced as software programs that provide simplified user interfaces (Buchanan, 1993). Multiple programs are available e.g. ComBase, DMFit or the food safety and spoilage predictor (FSSP).

In the literature, a broad range of primary and secondary models are available and the selection of an appropriate model is essential to achieve adequate fitting of the parameters. The main groups of primary models are growth models, inactivation models or interaction models. Interaction models consider the effect of the food microbiota on the growth of microorganisms in food. These interactions can either be direct through competition for space and nutrients, or indirect, e.g. change in food characteristics (Pérez-Rodríguez and Valero, 2013).

In the following, primary inactivation models will be further addressed. A known quantitative microbiology tool for microbial inactivation is the Geeraerd and Van Impe Inactivation Model Fitting Tool (GInaFiT). GInaFiT is a freeware Add-in for Microsoft Excel, which includes different types of microbial survival models employing linear and nonlinear regression approaches. The tool is useful for bridging the gap between developers of predictive modelling approaches and end-users in the food industry or research groups who are not familiar with advanced non-linear regression analysis tools (Geeraerd et al., 2005). The first version of the application published by Geeraerd et al. (2005), comprised nine model types: (i) classical log-linear curves, (ii) curves displaying a so-called shoulder before a log-linear decrease is apparent, (iii) curves displaying a so-called tail after a log-linear decrease, (iv) survival curves

displaying both shoulder and tailing behavior, (v) concave curves, (vi) convex curves, (vii) convex/concave curves followed by tailing, (viii) biphasic inactivation kinetics, and (ix) biphasic inactivation kinetics preceded by a shoulder. A tenth model, curves with a double concave/convex shape, extended the software. The model takes into account the development of bacterial resistance during inactivation curves (Coroller et al., 2006).

An overview of primary inactivation model equations is provided in Table 1.

**Table 1. Primary inactivation model equations**.

| Equation name | Equation | | Source |
|---|---|---|---|
| **Log-linear (Bigelow)** | $log(N_t) = log(N_0) - \frac{k_{max} * t}{ln\,(10)}$ | Eq. (1) | Bigelow and Esty (1920); Geeraerd et al. (2005) |
| **Log-linear with tail (Bilinear)** | $log(N_t) = log(N_0) - \frac{k_{max}}{ln\,(10)} * t, t \leq S_t$<br>$log(N_t) = \log(N_{res}), t \geq S_t$ | Eq. (2) | Buchanan and Golden (1995) |
| **Log-linear with tail (Geeraerd without $S_l$)** | $log(N_t) = log[(10^{log\,(N_0)} - 10^{log(N_{res})} * e^{-k_{max} * t} + 10^{log(N_{res})}]$ | Eq. (3) | Geeraerd et al. (2005) |
| **Log-linear with shoulder and tail (Trilinear)** | $log(N_t) = log(N_0), t < S_l$<br>$log(N_t) = log(N_0) - \frac{k_{max}}{ln\,(10)} * (t - S_l), S_l \leq t \leq S_t$<br>$log(N_t) = \log(N_{res}), t \geq S_t$ | Eq. (4) | Buchanan and Golden (1995) |
| **Log-linear with shoulder and tail (Geeraerd)** | $log(N_t) = log\,[(10^{log(N_0)} - log(10^{log\,(N_{res})}) * e^{-k_{max} * t} * \left(\frac{e^{k_{max} S_l}}{1 + (e^{k_{max} S_l} - 1) * e^{-k_{max} t}}\right) + 10^{log_{(N_{res})}}]$ | Eq. (5) | Geeraerd et al. (2005) |
| **Weibull** | $log(N_t) = log(N_0) - (\frac{t}{\delta})^p$ | Eq. (6) | Mafart et al. (2002); van Boekel (2002) |
| **Biphasic** | $log(N_t) = log(N_0) + \log(f * e^{-k_{max}1 * t} + (1 - f) * e^{-k_{max}2 * t}$ | Eq. (7) | Cerf (1977) |

The parameter $N_t$ is the concentration of microorganisms (CFU/ml or IPIU/ml) at a specific time point, $N_0$ (CFU/ml or IPIU/ml) is the initial concentration of microorganisms, $k_{max}$ the maximum specific inactivation rate (1/h), $t$ [h] is the time after inoculation, $N_{res}$ the residual population density (CFU/ml or IPIU/ml), $S_l$ the duration of shoulder effect (h) and $S_t$ the time arriving tail (h), δ the time for the first decimal reduction (h) and $p$ as shape parameter of the Weibull model.

An inactivation model with shoulder and tail and a Weibull model are presented in Figure 1.

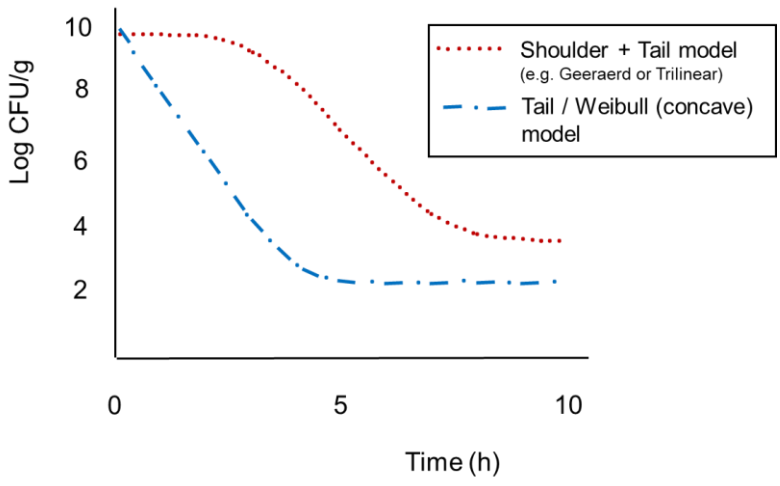


**Figure 1. Illustration of models used for describing the inactivation of pathogens in foods** (modified according to Pérez-Rodríguez and Valero, 2013).

Commonly used secondary models are the square-root-type models (Ratkowsky et al., 1982) or cardinal parameter models (Zwietering et al., 1992), whereby the latter is more complex and requires more input parameters. If applicable, simple linear regressions can also be used to predict the effect of environmental parameters on the parameters of primary models.

Classical predictive models have been developed using a two-step fitting process (Whiting and Buchanan, 1993). For this, a primary regression that estimates kinetic parameters from observed cell counts and a secondary regression that independently fits parameter estimates from the primary regression to experimental variables are necessary. During this separate fitting process errors can accumulate and propagate in each step of the data analysis during the model development process (Huang, 2017). A one-step approach avoids these drawbacks as primary and secondary models are analyzed together during the estimation of kinetic parameters (Huang, 2017).

### 2.5.2 Model evaluation and validation

Statistical “goodness-of-fit indices” are used to assess whether the chosen mathematical function fits well to the data points after a mathematical function is fitted by regression. Not all indices are suitable for all cases and the selection should be made according to the type of function applied during the fitting procedure. A first overview of standardized residuals can be obtained by using simple scatter plots (Pérez-Rodríguez and Valero, 2013).

For model evaluation, the goodness-of-fit indices are mainly the coefficient of determination ($R^2$) and the root mean square error (RMSE) (Pérez-Rodríguez, 2014). The $R^2$ gives information about the percentage of data point variation than can be explained by the model, thus the closer the $R^2$ is to one, the better the model represents observations (Pérez-Rodríguez and Valero, 2013). The RMSE is simple to calculate and easy to interpret. Further, it is suitable for a first approach to the fitted model, as it is a valid index for linear and nonlinear functions (Ratkowsky, 2003). A low RMSE value indicates better adequacy of the model in describing the data, while a large value of RMSE points to a poor fit to the data. A drawback of RMSE is that it is not a standardized (absolute) measure. It depends on the magnitude of the data value, whereby data sets with different units are not directly comparable by RMSE. An F-test can be used to compare the goodness-of-fit for different models, if the models have the same number of regression parameters or are at least mathematically derived from each other (so-called “nested models”) (Zwietering et al., 1990).

Validation is an imperative step in the modelling process, wherby the capacity of a model to predict the behaviour of the real system is assessed. Validation requires model-independent observations from challenge tests or data from the scientific literature (Gibson et al., 1988).

Predictive models should not be applied without previous validation (Dym, 2004). Graphical validation is used to categorized models as “fail-safe models”, if predictions overestimate growth or as “fail-dangerous models”, if predictions underestimate growth. A fail-safe model is maybe preferred for pathogenic bacteria as the predictions are more conservative and therefore safer in terms of public health (Pérez-Rodríguez and Valero, 2013).

The validation indices, bias factor ($B_f$) and accuracy factor ($A_f$), or the acceptable predictions zone (APZ) are used for model validation. The bias and accuracy factor were proposed by Ross (1996) and give a good estimation of the reliability of models. The bias factor indicates whether a model systematically predicts growth to be faster (fail-safe) or slower (fail-dangerous) than observed (Ross, 1996). The accuracy factor is similar to the bias factor and is defined as the absolute value of the ratio between predictions and observations, where $A_f = 1$ indicates perfect agreement between observations and predictions and $A_f = 2$ means that predictions and obervations vary by a factor of 2 (Pérez-Rodríguez and Valero, 2013). Both factors have limitations as they are mean values that might not detect certain forms of prediction bias and are subject to bias by outliers (Ross, 1996; Delignette-Muller et al., 1995). The APZ method overcomes these limitations as it classifies a model as acceptable when 70% of residuals are within the APZ, that can be defined as -1 $\log_{10}$ (fail-safe) to 0.5 $\log_{10}$ (fail-dangerous) (Oscar, 2005). The validation index of the APZ method is defined in the percentage of residuals within the APZ (Oscar, 2005).

### 2.5.3 Predictive models for *Campylobacter* spp. in raw milk

Several predictive models are available for *Campylobacter* spp. inactivation in meat or other food products (Oyarzabal et al., 2010; Lori et al., 2007; Curtis et al., 1995), but few options are available for survival in raw milk. Only two are applicable to survival in liquid (Membré and Lambert, 2008) or raw milk (Boleratz and Oscar, 2022). The predictive model in liquid is based on a meta-analysis including survival data within a temperature range of 0 to 42°C. A log-linear model has been used to describe the survival of *Campylobacter* spp. (Membré and Lambert, 2008). Boleratz and Oscar (2022) developed an artificial neural network model for non-thermal inactivation (without external heat treatment) of *C. jejuni* in raw milk based on CFU data obtained from ComBase. Both models showed a faster non-thermal inactivation at warmer temperatures than at cold temperatures used in the experimental set-up. The models are based on CFU data and did not take VBNC data into account.

## 2.6 Risk analysis

The risk analysis paradigm, according to the Food Agriculture Organization (FAO) and World Health Organization (WHO) (FAO/WHO, 1995) comprises three components: risk management, risk assessment and risk communication (Figure 2).

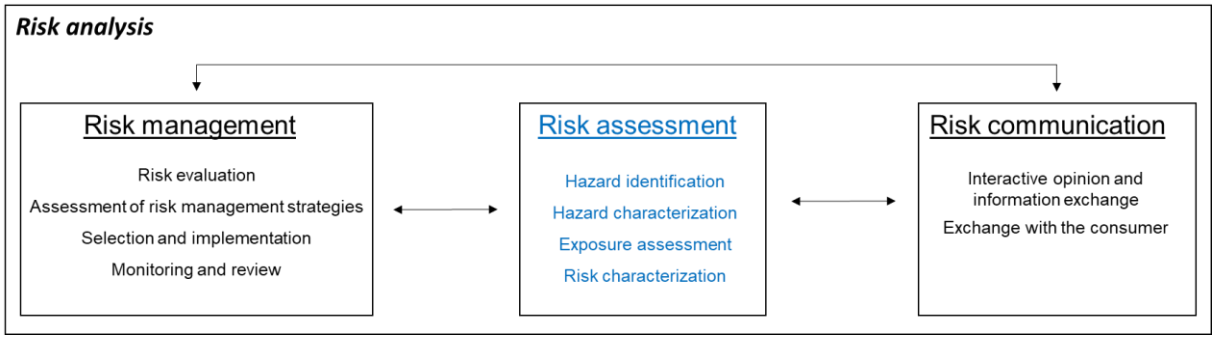


**Figure 2. Interaction between the three elements of risk analysis** (modified according to FAO/WHO, 2006).

Risk assessment is the central scientific part of the risk analysis. It deals with the qualitative and/or quantitative evaluation of the adverse effects linked to a hazard (biological, chemical or physical agent) in the whole farm-to-fork food production chain or the part that is relevant to the problem (FAO/WHO, 2013; Nauta, 2008; Codex Alimentarius Commission, 1999). The term “risk” is defined as the probability of occurrence of an adverse health effect as a consequence of the presence of a hazard in food (FAO/WHO, 2013).

Risk management includes risk evaluation, assessment and selection, implementation of risk management strategies, and monitoring and review (FAO/WHO, 2013). The decision of whether a risk assessment is necessary and the evaluation of whether it is an acceptable risk are the tasks of risk managers, compromising industry, public body representatives and policy makers alike. They also decide which control measures must be implemented in the case of non-acceptable risk (FAO/WHO, 2013).

Risk communication is the interactive exchange of information and options throughout the risk analysis process - including the explanation of risk assessment findings and the basis of risk management decisions - between risk assessors, risk managers and all stakeholders (e.g. industry, academic community and consumer). The exchange with the consumer is additionally attempted in order to enhance trust and confidence in the safety of the food supply (FAO/WHO, 2013).

The three interacting components of risk analysis should be 1) functionally separated and 2) documented systematically to avoid conflicts of interest or bias in the risk assessment process. The risk analysis needs to be performed in a transparent manner. The risk analysis process may be evaluated and reviewed when appropriate, taking into account newly generated scientific data (FAO/WHO, 2013).

## 2.7 Risk assessment

A risk assessment is the qualitative and/or quantitative evaluation of the adverse effects linked to hazards that might be present in foods (FAO/WHO, 2013). In a qualitative risk assessment, the risk is described by descriptive terms (e.g. low or high), while a quantitative risk assessment represents the risk in terms of numerical outcomes.

Quantitative risk assessment can be divided into deterministic and stochastic approaches with regard to how input variables are handled (Campagnollo et al., 2022). In the deterministic approach, point-estimated values are used for variables. However, the outcomes are not representative of real situations. Stochastic models use probability distributions taking variations around the values into account. An iterative calculation process, a so-called Monte Carlo simulation, is used for distribution calculation. During the Monte Carlo Simulation each iteration takes one output value from each of the distributions and calculates the expected number of microorganisms per serving or risk of illness per serving (Schaffner, 2008).

Within the quantitative risk assessment, the quantitative microbial risk assessment (QMRA) has been widely used to assess the human exposure to microorganisms that can cause diseases. As any risk assessment, the QMRA follows a structured approach comprising four fundamental steps: hazard identification, hazard characterization, exposure assessment and risk characterization (Figure 3) (FAO/WHO, 2013).

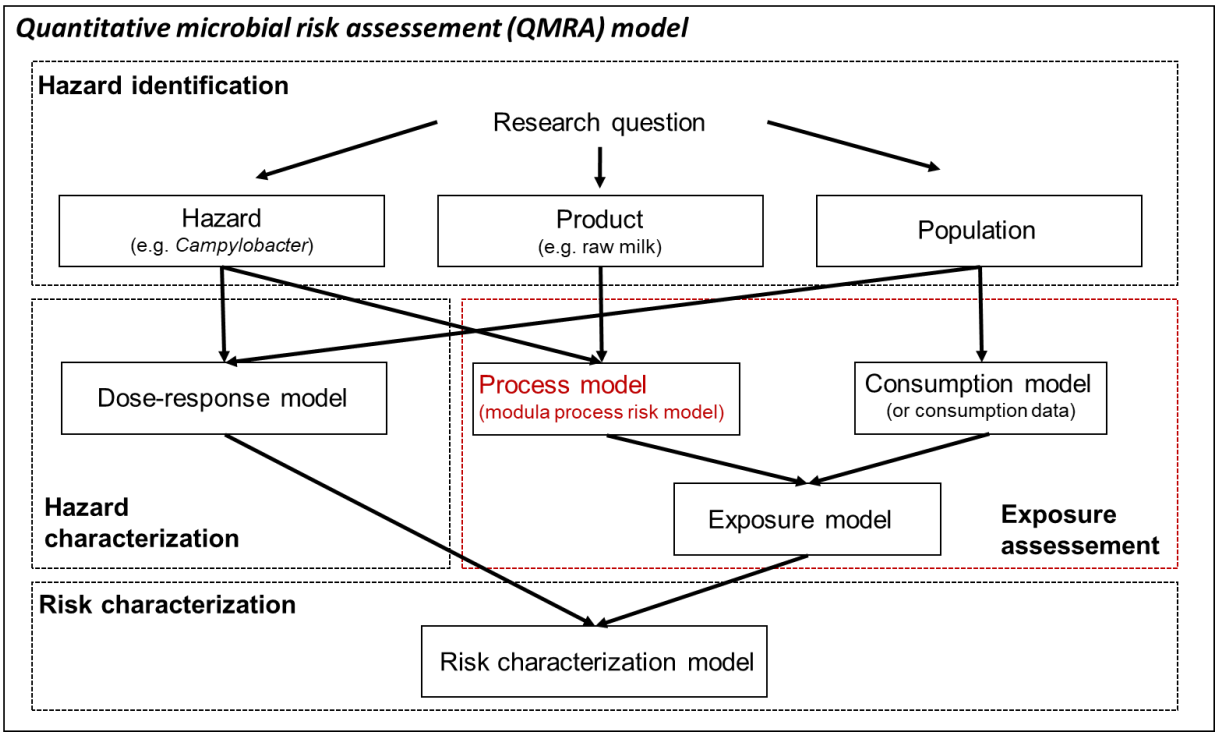


**Figure 3. QMRA model structure.** QMRA model are usually developed along the four elements of a risk assessment (hazard identification, hazard characterization, exposure assessment and risk characterization). The different model classes that belong to the elements can be combined to form a full QMRA model (modified according to Haberbeck et al., 2018).

Before starting the hazard identification, the objective of the QMRA must be defined as a research question regarding the food product, population, food production chain and the microbiological hazard (Nauta, 2008). Generally, risk managers in consultation with risk assessors develop the research question.

The hazard identification is the qualitative description of the microbiological hazard, which may be present in foods (Codex Alimentarius Commission, 1999), e.g. a description of thermotolerant *Campylobacter* spp. in raw milk. After the hazard identification, the characterization of the hazard with focus on the consumer takes place, by description of the adverse health effects of the hazard. The dose-response relation is also part of the hazard characterization. A dose-response assessment determines the relation between the magnitude of exposure (dose) to a hazard and the severity and/or frequency of associated adverse health effects (response) (Codex Alimentarius Commission, 1999). The exposure assessment evaluates the likely intake of a hazard. In the last stage of the risk characterization, the probability of occurrence and severity of an adverse health effect in a population is estimated based on hazard identification, hazard characterization and exposure assessment (Codex Alimentarius Commission, 1999).

The Codex Alimentarius Commission guidelines (1999) for conducting QMRA gives a list of principles and definitions, but does not present a modelling methodology. A modular process risk model (MPRM) approach is recommended (Nauta, 2008). MPRM states that the transmission of the hazard through the food pathway can be regarded as a series of basic processes. In detail, following the MPRM approach, the food pathway describes where the bacteria enter the food pathway and what can happen to the bacteria in terms of either a microbial process (growth and inactivation), often described by predictive models, or product handling processes (cross contamination, mixing, removal or partitioning) (Nauta, 2008). The MPRM structure is determined by this series of basic processes. They may be used in any QMRA study. This can range from industrial food processing to “farm to table” risk assessment models. Besides the evaluation of likelihood of intake of the hazard via food (qualitatively and/or quantitatively estimated), general consumption data of the food product are included (FAO/WHO, 2008). In addition, the amount of product that is consumed has an impact on the likelihood to be exposed to the hazard.

The results of a risk assessment and the estimation of microbial concentration and prevalence in food by the end of the production process are an important management tool. They can help in the detection of critical control points (HACCP) in the food chain and in the assessment of intervention strategies and are consequently of great interest to the industry (FAO/WHO, 2008). The European Food Safety Authority (EFSA) focused on the estimation of microbial

concentration and prevalence in food at the time of consumption. Consumer behavior must therefore be included to assess the final risk for the consumer.

### 2.7.1 Variability and Uncertainty

In the development of stochastic QMRA models, it is essential to address variability and uncertainty, and to consider them independently of each other. In the context of QMRA "variability" represents the true biological heterogeneity in a population. Variability is not influenced and irreducible by additional data or better measurements since it is related to natural randomness (Anderson and Hattis, 1999; Murphy, 1998). To reflect the variability of data, statistical metrics such as standard deviation or quantiles are often used.

On the other hand, the term "uncertainty" represents the lack of perfect knowledge of the true value of a parameter due to a lack of data, analytical limitations or low precision of measurement methods (Anderson and Hattis, 1999). It should be reduced whenever possible by further data generation or, if applicable, adjustment of the experimental set-up. In exposure assessment, the uncertainty is usually quite large based on many unknown steps in the production process as well as at the consumer stage. Surveys addressing consumer behavior are limited. Therefore, the effect of the uncertainty in the consumer phase on the uncertainty of the risk estimate can be very large.

Most data sets include variability and uncertainty at the same time. Two approaches are possible for considering both independently in a stochastic exposure assessment model: the development of a nested set of distributions describing each factor (second order Monte Carlo simulation model) or different scenario analyses (e.g. mean, worst-case, etc.). In a second order Monte Carlo simulation, model uncertainty and variability are considered simultaneously (Nauta, 2000). Scenario analyses are used to consider variability and uncertainty after each other.

### 2.7.2 Dose-response model

The dose-response relationship provides the link between the hazard and population group and is part of the hazard characterization. In detail, this means the relationship between pathogen exposure and the probability of occurrence and severity of an adverse effect (e.g. infection, illness or death) (FAO/WHO, 2003).

Dose-response data are available for many microorganisms based on studies in human volunteers and animal models (Haas, 1983). Some dose-response models are assessed from

outbreak data of the microorganism, if the collected data on exposure are accessible (FAO/WHO, 2003) (Figure 4).

The current understanding is that any single pathogen may be capable of causing infection in the "single-hit concept" (Haas, 1983). It is not assumed that there is a minimum dose, a threshold below which infection cannot occur. Hence, the probability that any pathogen causes infection is independent of number ingested.

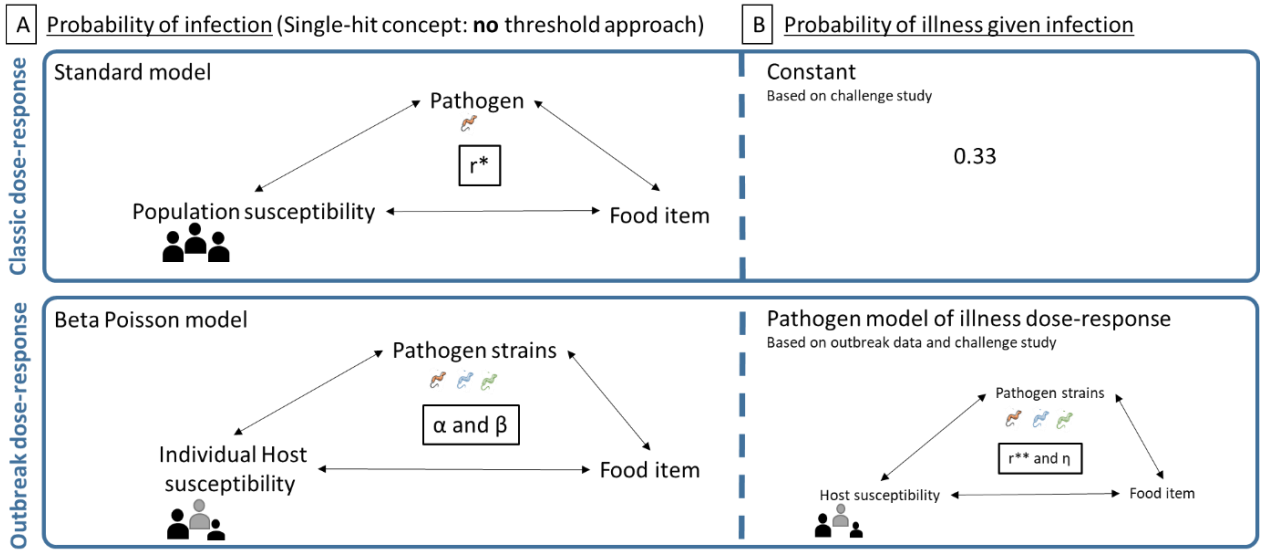


**Figure 4. Overview of classic dose-response model and outbreak dose-response model.** r* is the probability of each single pathogen being individually capable of causing infection in the exposed individual; r** is parameter estimated for pathogen model of illness dose-response.

This "single-hit concept" is a non-threshold approach, calculating the probability of infection ($P_{inf}$) given dose ($D$) of ingested microorganisms as (Eq. (8)).

$$P_{inf} = 1 - (1 - r)^D \qquad \text{Eq. (8)}$$

where $r$ is the probability of each single pathogen being individually capable of causing infection in the exposed individual (by means of independent action).

The probability $r$ depends on pathogen (e.g. pathogenicity, adaption to attack and grow), food item (e.g. fat content) and the host (e.g. microbiome or immune system). This model estimates the risk at the population level as it assumes that the interaction between the pathogen and the host is constant for every individual in the population (Sanaa and Guillier, 2022).

In a Beta Poisson model the variability between strains and the variability between individual consumers are considered (Sanaa and Guillier, 2022) (Eq. (9), Figure 4A, lower part). A Beta distribution of parameters $\alpha$ and $\beta$ is used to describe how different hosts respond to exposure

to a similar dose (Sanaa and Guillier, 2022). The dose parameter represents the mean of Poisson distributed doses (Nauta et al., 2009).

$$P_{inf} = 1 - (1 + \frac{D}{\beta})^{-\alpha} \quad \text{Eq. (9)}$$

Frequently, the Beta Poisson model does not describe the doses as accurately as is necessary for a QMRA food chain model since individuals are exposed to discrete numbers of bacteria. However, it can be accurate for fluid foods, like water, where the Poisson assumption makes sense. A more heterogeneous distribution is assumed in foods (Nauta et al., 2009). Therefore, a better implementation of the dose-response relationship is given by using the Beta Binomial model (Eq. (10)), for which the same parameters can be used as in the Beta Poisson model (Haas, 2002).

$$P_{inf} = 1 - \frac{\Gamma(\alpha+\beta)\Gamma(n+\beta)}{\Gamma(\alpha+\beta+n)\Gamma(\beta)} \quad \text{Eq. (10)}$$

where Γ(.) is Euler's Gamma function (Nauta et al., 2009; Haas, 2002) and n is the discrete dose.

For *Campylobacter* spp. two main dose-response models are available. The "classic" dose-response model with α =0.145 and ß =7.59 (Teunis and Havelaar, 2000) assessed from data from a human challenge study (*C. jejuni* strain A 3249) (Black et al., 1988) and an "outbreak" dose-response model (Nauta et al., 2022; Teunis et al., 2018), which combines data from human and primate challenge studies and data from epidemiological studies on foodborne diseases (raw milk outbreak studies). In recent studies, the median estimates of the model parameters provided by Teunis et al. (2018) for outbreak studies were used in the "outbreak" dose-response model with α =0.38 and ß =0.51 (Nauta et al., 2022).

For the "classic" dose-response model, the probability of illness given infection ($P_{ill/inf}$) is a constant (0.33) (Nauta et al., 2007; Black et al., 1988), independent of the dose. The "outbreak" dose-response model uses a pathogen model of illness dose-response (with parameters *r* and *η* estimated by host species and strain) for $P_{ill/inf}$ (Teunis et al., 2018) (Eq. (11), Figure 4B, lower part).

$$P_{ill/inf} = 1 - (1 + \frac{D}{\eta})^{-r} \quad \text{Eq. (11)}$$

with r= 0.76 and η= 0.0092

In the end, the number of ill consumers ($I_{ll}$) is calculated by multiplying the mean of $P_{inf}$ and $P_{ill/inf}$ (Eq. (12)).

$$I_{ill} = mean P_{inf} * P_{ill/inf} \quad \text{Eq. (12)}$$

The “outbreak” dose-response model indicates that infection with *C. jejuni* occurs at low doses but acute illness requires high doses (Teunis et al., 2018).

### 2.7.3 QMRAs for *Campylobacter* spp. along the raw milk supply chain

There are already existing QMRAs for *Campylobacter* spp. along the raw milk supply chain based on data from Italy and New Zealand (Giacometti et al., 2015; Anonymous, 2009).

The QMRA from New Zealand is based on four pathogens that can occur in raw cow’s milk: *Campylobacter* spp., EHEC, *Salmonella* spp. and *Listeria monocytogenes* (Anonymous, 2009). This model incorporates fecal concentration data for *Campylobacter* spp. based on the MPN method from Stanley et al. (1998). The mean concentration is 1.79 $\log_{10}$ CFU/g ± 1.01 $\log_{10}$ CFU/g feces. For the dose-response relationship, the classical Beta Poisson dose-response model for *Campylobacter* spp. is applied. Three different scenarios are performed: (1) consumption from the bulk milk tank, (2) domestic consumption after farm gate purchase, (3) domestic consumption after packaging, distribution and retail sale, which leads to mean prediction of 19.9, 4.7 and 0.1 cases of illness respectively for adults from *Campylobacter* spp. per 100,000 daily servings of raw milk (Anonymous, 2009).

A recent QMRA from Italy focused on the prevalence of *Campylobacter* spp. in raw milk at vending machines and different raw milk handlings before consumption (boiled and unboiled) (Giacometti et al., 2015). A total of 1.08 x $10^8$ servings per year were estimated whereby the model predicted for the population of the investigated regions 301,785 and 230,776 cases for the best (4°C) and worst time-temperature scenarios (11°C ± 0.5°C), respectively (Giacometti et al., 2015). The best-case scenario was performed at 4°C in order to prevent the growth of pathogenic bacteria. However, *Campylobacter* spp. is inactivated more rapidly at higher temperatures, resulting in lower case numbers in the worst-case scenario (11°C).

The QMRAs for *Campylobacter* spp. in raw milk were mainly focused on the raw milk storage and handling before consumption. Transmission data for *Campylobacter* spp. during the milking process were not integrated. No QMRA for *Campylobacter* spp. in raw milk in Germany is available. In order to reduce consumer exposure to *Campylobacter* spp. by identifying potential intervention measures QMRAs are needed over the whole food chain. Different risk mitigation strategies on farm level and along the supply chain need to be explored to identify data gaps and support risk managers in controlling *Campylobacter* spp.

# 3 Publications

## 3.1 List of publications and own contribution

**Publication 1: Prevalence and concentration of *Campylobacter* in faeces of dairy cows: A systematic review and meta-analysis.**

Knipper A.-D., Ghoreishi N., Crease T.

PLoS ONE 2022; **17**(10): e0276018. https://doi.org/10.1371/journal.pone.0276018



I developed the search strategy for the literature, performed independent title, abstract and full-text screening, created parts of the illustrations and wrote the first draft except for a few small sections. In my function as first and corresponding author, I submitted the article and considered and responded to the reviewer comments on behalf of all co-authors.

**Publication 2: Longitudinal study for the detection and quantification of *Campylobacter* spp. in dairy cows, during milking and in the dairy farm environment**

Knipper A.-D., Göhlich S., Stingl K., Ghoreishi N., Fischer-Tenhagen C., Bandick N., Tenhagen B.-A., Crease T.

*Foods* 2023; **12**(8):1639. https://doi.org/10.3390/foods12081639



I was involved in the experimental design and application of the animal experiment to State Office for Health and Social Affairs (LAGeSo). I performed the sampling in the stable and analyzed samples in the laboratory. Further, I evaluated the extracted data with statistical analyses, visualized the results and wrote the first draft. In my function as first and corresponding author, I submitted the article and considered and responded to the reviewer comments on behalf of all co-authors.

**Publication 3: Modeling the survival of *Campylobacter jejuni* in raw milk considering the viable but non-culturable cells (VBNC)**

Knipper A.-D., Plaza-Rodríguez C., Filter M., Wulsten I. F., Stingl K., Crease T.

Journal of Food Safety 2023, e13077. https://doi.org/10.1111/jfs.13077



I performed the experiments, analyzed and visualized the results, developed the models and wrote the first draft. I also made the developed model available in a Food Safety Knowledge Exchange (FSKX) format (de Alba Aparicio et al., 2018) in order to improve transparency in the model generation process and facilitate the exchange and reusability of the created models. In my function as first and corresponding author, I submitted the article and considered and responded to the reviewer comments on behalf of all co-authors.

**Publication 4: Quantitative microbiological risk assessment model for *Campylobacter* in raw milk of dairy cows in Germany**

Knipper A.-D., Crease T., Guenther T., Filter M., Nauta M.

Microbial Risk Analysis 2023, 25, 100274. https://doi.org/10.1016/j.mran.2023.100274



I developed the QMRA model, implemented it in @RISK software, performed model analyses, visualized the results and wrote the first draft. I also made the developed model available in the software R 4.2.1 (R Core Team, 2020) and in a Food Safety Knowledge Exchange (FSKX) format (de Alba Aparicio et al., 2018) in order to improve transparency in the model generation process and facilitate the exchange and reusability of the created models. In my function as first and corresponding author, I submitted the article and considered and responded to the reviewer comments on behalf of all co-authors.

## 3.2 Publication 1: Prevalence and concentration of *Campylobacter* in feces of dairy cows: A systematic review and meta-analysis

PLOS ONE

RESEARCH ARTICLE

# Prevalence and concentration of *Campylobacter* in faeces of dairy cows: A systematic review and meta-analysis

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OPEN ACCESS

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**Data Availability Statement:** A pre-specified study protocol was published on the International Prospective Register of Systematic Reviews (PROSPERO) database (CRD42021261914, https://www.crd.york.ac.uk/prospero/display_record.php?RecordID=261914). All relevant data are within the manuscript and its Supporting information files.

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## Abstract

The consumption of raw milk from dairy cows has caused multiple food-borne outbreaks of campylobacteriosis in the European Union (EU) since 2011. Cross-contamination of raw milk through faeces is an important vehicle for transmission of *Campylobacter* to consumers. This systematic review and meta-analysis, aimed to summarize data on the prevalence and concentration of *Campylobacter* in faeces of dairy cows. Suitable scientific articles published up to July 2021 were identified through a systematic literature search and subjected to screening and quality assessment. Fifty-three out of 1338 identified studies were eligible for data extraction and 44 were further eligible for meta-analysis. The pooled prevalence was calculated in two different meta-analytic models: a simple model based on one average prevalence estimate per study and a multilevel meta-analytic model that included all prevalence outcomes reported in each study (including different subgroups of e.g. health status and age of dairy cows). The results of the two models were significantly different with a pooled prevalence estimate of 29%, 95% CI [23–36%] and 51%, 95% CI [44–57%], respectively. The effect of sub-groups on prevalence were analyzed with a multilevel mixed-effect model which showed a significant effect of the faecal collection methods and *Campylobacter* species on the prevalence. A meta-analysis on concentration data could not be performed due to the limited availability of data. This systematic review highlights important data gaps and limitations in current studies and variation of prevalence outcomes between available studies. The included studies used a variety of methods for sampling, data collection and analysis of *Campylobacter* that added uncertainty to the pooled prevalence estimates. Nevertheless, the performed meta-analysis improved our understanding of *Campylobacter* prevalence in faeces of dairy cows and is considered a valuable basis for the further development of quantitative microbiological risk assessment models for *Campylobacter* in (raw) milk and food products thereof.

(Project 1323-104). The funder had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

**Competing interests:** The authors have declared that no competing interests exist.

## Introduction

Since 2005 campylobacteriosis, caused by bacteria of the genus *Campylobacter*, is the most commonly reported foodborne gastrointestinal infection in humans in the EU [1]. The EU Member States reported an overall incidence of 120,946 confirmed cases of human campylobacteriosis, corresponding to an EU notification rate of 40.3 per 100,000 population in 2020. Although a decrease in cases was observed in 2020, the overall campylobacteriosis trend in the last four years was stable [1]. Campylobacteriosis symptoms include fever, vomiting, abdominal cramps and watery or bloody diarrhea. Associated chronic complications involve Guillain-Barré syndrome, irritable bowel syndrome and reactive arthritis [2].

Important animal reservoirs for *Campylobacter* spp. are poultry, in particular chicken, and cattle [3, 4]. However, the bacterium is mainly transmitted through contaminated food, direct contact with animals or untreated water [4–6]. In addition to uncooked poultry meat or poor kitchen hygiene in connection with the handling of raw meat, *Campylobacter* infections are frequently reported in connection with the consumption of raw milk and products thereof [1, 7–10]. From 2011 to 2020 raw milk was one of the food vehicles causing most strong-evidence foodborne campylobacteriosis outbreaks in the EU [1]. This is critical in light of the increasing consumer demand for raw milk [11], the intensification of local sales via raw milk vending machines [12] and the common neglect to boil raw milk before consumption. Surveys in Italy demonstrated that 13.9 to 43% of consumers did not boil raw milk before consumption [13, 14].

It is generally assumed that contamination of raw milk with pathogens is mainly of faecal origin [9, 15–17]. However, it is unclear which mechanisms underlie this contamination and how likely raw milk is to be contaminated during milking [14, 15, 18, 19]. In addition, it is also unclear whether there are seasonal differences in the occurrence and concentrations of *Campylobacter* spp. in faeces of dairy cows, which could potentially help to explain the seasonal trend in campylobacteriosis cases [1]. Different mitigation options along the raw milk supply chain need to be assessed in order to understand the role of faecal contamination and a potential seasonality in the public health risk associated with the consumption of *Campylobacter*-contaminated raw milk. Prevalence and concentration data for *Campylobacter* spp. in faeces form a basis for such a risk assessment.

In microbiology, a risk assessment is the qualitative and/or quantitative evaluation of the adverse effects linked to biological agents that may be present in foods [20]. During a quantitative microbial risk assessment (QMRA) the risk is estimated in terms of numerical outcomes, typically the probability of illness or death [21]. Quantitative data, like the concentration in contamination sources (e.g. faeces) or the food matrix, is needed during exposure assessment for the relation between the dose ingested and the frequency of a given effect. To reduce the risk of human exposure to *Campylobacter* spp. it is essential to assess the prevalence and concentration of *Campylobacter* in faeces of dairy cows'. In this sense, a systematic review is necessary to identify all literature on this particular topic. Further a meta-analysis is a highly valuable statistical tool whose objective is to combine the results of all studies on a particular research question to determine the size and direction of the effect.

This systematic review and meta-analysis aimed to provide and estimate the prevalence and concentration of *Campylobacter* in dairy cow faeces. Moreover, potential data gaps for risk assessments were identified in order to highlight where further research is needed. The knowledge and data generated from this study is ought to contribute to the development of QMRAs and the evaluation of different contamination or exposure scenarios along the raw milk supply chain, thereby helping risk managers to identify mitigation strategies to control *Campylobacter* spp. and to reduce the public health risk associated with the consumption of *Campylobacter*-contaminated raw milk.

## Material and methods

### Literature search and inclusion criteria

A systematic review was performed according to the Preferred Reporting Items for Systematic Review and Meta-Analysis Protocols (PRISMA-P) 2015 statement [22] (S1 Checklist). A pre-specified study protocol was published on the International Prospective Register of Systematic Reviews (PROSPERO) database (CRD42021261914, https://www.crd.york.ac.uk/prospero/display_record.php?RecordID=261914), in order to avoid duplication and to minimize bias. Literature searches were carried out using PubMed, Scopus and Web of Science databases for papers published to July 19th 2021. A detailed overview of search terms per database is provided in Table 1. Synonyms for relevant search terms were identified using the Medical Subject Headings (MeSH) thesaurus by the US National Library of Medicine [23] (https://www.nlm.nih.gov/mesh/meshhome.html).

A title and abstract screening was performed, followed by a full-text screening for eligibility for inclusion and exclusion criteria already defined in the PROSPERO protocol and for the removal of duplicate publications of the same results or study. If the answer to the *a priori* defined exclusion criteria remained unclear during the initial screening the study was forwarded to the full-text screening. All relevant articles were uploaded to the Rayyan Systems Inc. [24] web tool for efficient organization of inclusion and exclusion and to document the reasons for exclusion. Two researchers (ADK, TC) performed both screenings independently in Rayyan. Discrepancies were resolved by a third researcher (NG). Studies were excluded if they met the pre-defined exclusion criteria.

### Data extraction

Full text articles were examined and relevant data was extracted from text and tables into purpose-built tables using Microsoft Excel 2016 (Microsoft Corp., Redmond, WA, USA). Metadata on the general study design and metadata related to each reported outcomes was extracted separately. The following general metadata was extracted from each study: year of publication, country of study, faecal collection method, method for *Campylobacter* detection/ enumeration and species identification, number of dairy cow farms sampled, age class of cows, health status of cows, whether repeated samplings for individual cows or cow farms were performed, whether the repeated outcomes for individual cows or for cow farms were reported, and whether the available repeated outcome were reported by season (i.e. summer, fall, winter, spring).

Each study may comprise more than one prevalence outcome e.g. derived from different sub-groups or sampling conditions (i.e. *Campylobacter* species, age class, health status,

**Table 1. Overview of search strategy and number of articles found specific to the respective datatbase.**

| Date Search performed | Database | Number of articles retrived | Search string/terms and limits |
|---|---|---|---|
| 19. July 2021 | PubMed | 453 | All = (Search #1) AND All = (Search #2) AND All = (Search #3) |
| 19. July 2021 | Scopus | 485 | Abstract, Title, Keyword = (Search #1) AND Abstract, Title, Keyword = (Search #2) AND Abstract, Title, Keyword = (Search #3) |
| 19. July 2021 | Web of Science | 400 | TOPIC = (Search #1) AND TOPIC = (Search #2) AND TOPIC = (Search #3) |
| | | Where: | |
| | | Search #1 | (Campylobacter*) |
| | | Search #2 | (cow) OR (cattle) OR (bovine) OR (ruminant) OR (dairy) OR (heifer) OR (calf) OR (bos indicus) OR (zebu) OR (bos grunniens) OR (yak) OR (bos taurus) |
| | | Search #3 | (feces) OR (faeces) OR (excrement) OR (fecal) OR (faecal) OR (dung) |

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seasons) and outcomes may be reported repeatedly within one study based on different sub-grouping or data aggregation. All relevant prevalence outcomes were extracted and the sub-grouping was documented in the metadata. Each extracted outcome was associated with the following additional metadata: *Campylobacter* species, season, number of faeces samples collected, health status of cows and age class of cows.

The review and data extraction was performed by two researchers (ADK, TC) individually and tables were subsequently merged. Discrepancies were resolved by discussion or consultation of a third researcher (NG). Authors of included articles were not contacted in case of missing data. The created database was double-checked independently by two researchers (ADK, TC).

## Bias assessment

There is currently no validated tool for risk of bias (RoB) assessment in observational animal studies including prevalence studies. The available tools are appropriate for animal experiments (e.g. SYRCLE [25], CAMARADES [26]) or human observational studies (e.g. ROBINS-I [27]). As a result, risk of bias was assessed based on a purpose-built modified RoB tool. Applicable questions from the above mentioned tools were gathered in a table and adapted for prevalence studies (e.g. were rephrased or split into multiple, more study specific criteria). In total ten questions were included in the final tool (S2 Table). During the data extraction, the reviewers also filled the RoB tool for each study, counted the number of "yes", "no" and "unclear" answered questions, and labeled studies with more than four "yes" answers as "low risk of bias". Questions answered with "no" and "unclear" contributed to high risk of bias. No funnel plot was drawn since funnel plots are not appropriate for assessing the publication bias in studies with prevalence outcomes [28].

## Description of data sets for meta-analysis

Study outcomes for pooled faecal samples and outcomes where the number of animals sampled was unclear or not specified were excluded from the meta-analysis. As described in section data extraction, we extracted all relevant prevalence outcomes from each study. This introduced duplications of the same data under different sub-groupings (i.e. *Campylobacter* species, age class, health status, and seasons) in our data set for meta-analysis. To consider the effect of these duplicates on the analysis we chose to work with two different data sets and meta- analytic models. One dataset was reduced to only those prevalence outcomes that were reported as an average across the whole study (e.g. across all potential sub-groups such as age class, health status and seasons). This dataset will hereafter be referred to as aggregated sample (S1 Table). The other dataset included all extracted outcomes, including potential duplications due to different sub-groupings and data aggregation. The method for meta-analytic model was chosen accordingly. This data set will hereafter be referred to as the non-aggregated sample (S1 Table).

Potential influencing factors of interest were, the season during faecal sample collection, the *Campylobacter* species, the age class and the health status of the cows as well as the faecal collection method. The effect of these factors on the prevalence estimates was further investigated via statistical analysis.

## Statistical analysis

We used R Software version 4.1 for statistical analysis [29] and the packages "meta" [30] and "metafor" [31] for the development of the meta-analytic models.

**Meta-analytic models.** Two meta-analytic models were used to estimate the pooled prevalence. In the first model the prevalence outcomes of the aggregated sample were included in a random effect model for proportions with an inverse variance method, which we will refer to as simple model.

In the second model, the non-aggregated sample was included in a multilevel model where prevalence outcomes reported in each study were in one level and studies were compared in the other level. For each level an inconsistency index ($I^2$) was calculated as a measure of heterogeneity which is defined as the percentage of variability in the effect estimates that is not explained by the sampling error. In both models the estimates were double arcsin transformed.

**Subgroup analysis.** For subgroup analysis, we used the aggregated sample prevalence if at least three outcomes from different studies were available. The Q-test was used to test the difference between the subgroups.

**Effect of subgroups on the prevalence.** We performed an analysis on the non-aggregated sample (using all the extracted outcomes) to investigate the effect of subgroups on the pooled prevalence estimate based on a multilevel mixed-effect model with restricted maximum-likelihood estimation (REML). The model features included the *Campylobacter* species, health status and age class of the dairy cows, the season of outcome measurement and the faecal collection method. As with the previous multilevel model, the prevalence outcomes reported for each study were considered as one level and the comparison between the studies was calculated in the other level.

**Meta-regression.** We performed a meta-regression to evaluate the effect of the publication year of studies on the prevalence estimates. For this analysis, we added the publication year as a variable to the simple model regression and created a graph of the prevalence values versus publication year.

**Sensitivity analysis.** The created data table for RoB analysis was used to estimate the pooled prevalence for the high and low risk of bias studies of the aggregated sample and the results were compared using a Q-test. As the second sensitivity analysis, the pooled prevalence estimate from the aggregated sample in the simple model and results from the pooled non-aggregated sample in the multilevel model were compared.

## Results

### Search summary of the systematic review

Fifty-three out of 1338 identified studies were eligible for data extraction after screening and eligibility testing according to PRISMA-P (Fig 1).

Of these, 17 studies were from Europe (32%), 15 from North-America (28.3%), seven from Oceania (13.2%), six from Asia (11.3%), five from South-America (9.5%) and three from Africa (5.7%). Most of the Europe-based studies were from the UK (N = 5; 9.4%). Other European countries i.e. Austria, Denmark, Germany, Latvia, Lithuania and Sweden were represented by one study each, while Finland, Italy and Sweden were represented by two studies.

On average, 432 (± 678) dairy cows and 21 (± 34) farms were sampled in the included studies. The health status of the sampled dairy cows was not specified in a majority of studies (N = 35; 66%), while other studies (N = 18; 34%) gave a clear description of the health status of the dairy cattle (Fig 2a). Different age groups of dairy cows were sampled throughout the included studies (Fig 2b). However, in some studies no description of the age group of cows was given (N = 8; 15%).

The faecal collection methods were the collection of cow pats from the floor (N = 9; 17%) and direct rectal extraction methods (N = 31; 58.5%). In six studies (11.3%) the faecal

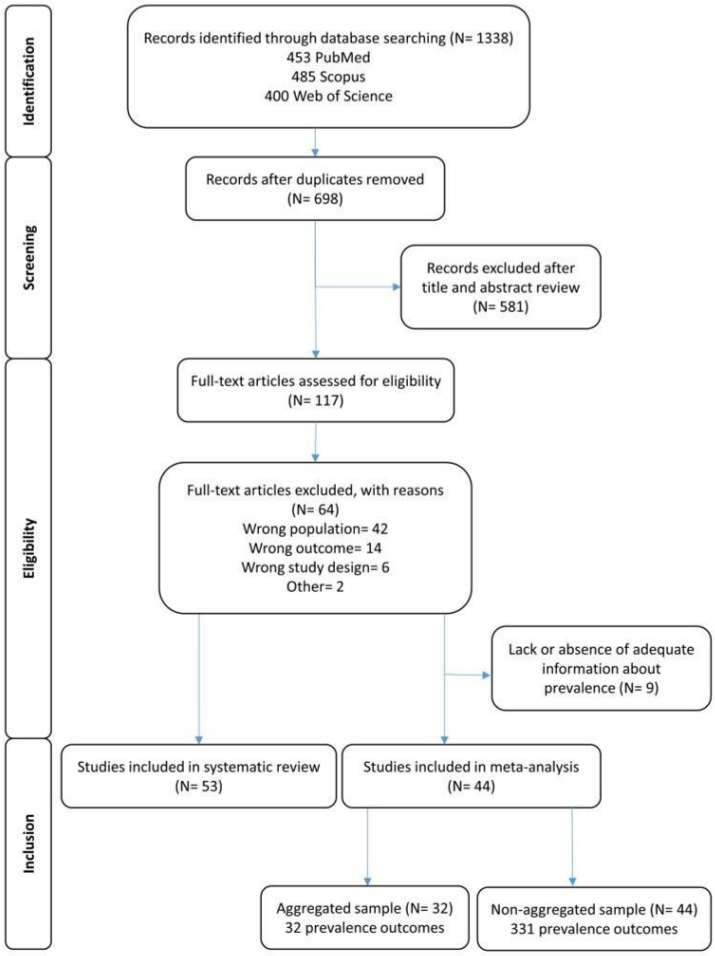


**Fig 1. Flow diagram of selected studies included in the systematic review and meta-analysis.** Aggregated sample means a specific prevalence outcome was reported as an average outcome across the whole study, whereas with non-aggregated sample an outcome was reported for a specific sub-group or condition.

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collection method was not stated and some studies pooled faecal samples before analysis (N = 7; 13.2%) (Fig 2c). *Campylobacter* was mainly detected by culture-based methods (N = 49; 92.5%). Only a few studies used PCR-based methods (N = 3, 5.7%) or a combination of PCR- and culture-based methods (N = 1; 1.9%). The majority of studies (N = 37; 69.8%) tested faecal samples for two or more *Campylobacter* species (including *Campylobacter* spp.). *Campylobacter* spp. (N = 35; 66%) and the species *C. jejuni* (N = 26; 49%) and *C. coli* (N = 11; 20.8%) were most commonly reported in all included studies. Other species such as *C.*

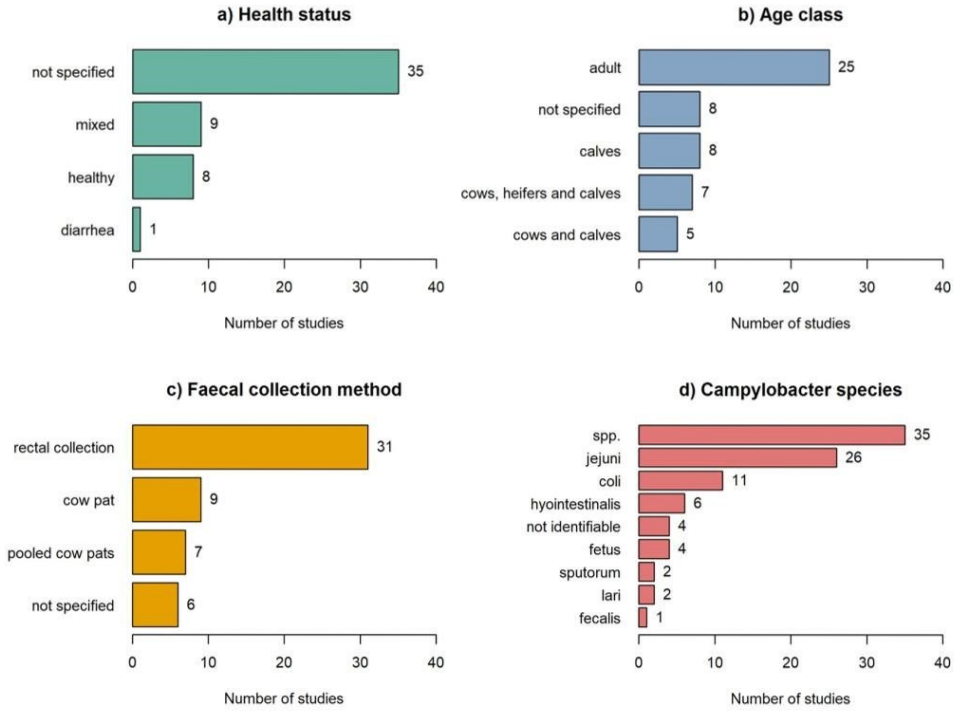


**Fig 2. Number of studies reporting data for potential influencing factors and their subgroups, which are the health status (a) and age class (b) of dairy cows, as well as the faecal collection method (c) and the *Campylobacter* species (d).** In some studies the collected faecal samples were analyzes for more than one species.

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*hyointestinalis*, *C. fetus*, *C. sputorum*, *C. lari*, and *C. fecalis*, were rarely tested for, while some species could not be identified (Fig 2d).

Almost 50% of all studies (N = 26) reported repeated samplings for farms under study. Only some of these (N = 15; 28.3%) were taken according to seasons in temperate regions (i.e. spring, summer, autumn, winter), while others (N = 5; 9.4%) were taken according to rainy and dry season, depending on the geographical location of the country. In general, only few studies (N = 14; 26.4%) made the results of the repeated sampling explicitly available in their publication. This means that although repeated samplings were taken, the results of these samplings were not reported individually, but rather aggregated or not shown at all. Repeated sampling for individual cows were only taken in a small number of studies (N = 5; 9.4%), but none of these studies made the results for individual cattle available in their publication. Data extracted from publications and included in systematic review and meta-analysis are available in S1 Table.

## Risk of bias assessment

The number of "yes", "no" and "unclear" answers for each RoB criteria is shown in S2 Table. No study answered all the RoB criteria with "yes". The highest answer rate was eight out of ten

"yes" answers for one study. Twenty-two studies (42%) had four or more "yes" answers which was considered as low risk of bias. Results of the meta-analysis on prevalence outcomes of RoB sub-groups are further presented in result section sensitivity analysis.

## Findings from the concentration outcomes

Concentration outcomes were only reported in seven (13.2%) of the 53 studies included in the review. The provided concentration outcomes in three of these studies [32–34] was a semi-quantitative estimate, which was determined by the most probable number (MPN) method for *Campylobacter* spp.. Concentration outcomes from another study could not be extracted as they were only presented in a box plot [35]. A meta-analysis for the remaining three studies [36–38] with quantitative concentration outcomes could not be performed, as one of these studies [38] did not provide any standard deviation or confidence intervals for the reported concentration.

The average *Campylobacter* spp. concentration in Danish dairy farms of 120 dairy cows was 2.1 ± 0.45 log colony-forming unit (CFU)/g faeces [36]. In contrast, a Lithuanian study determined for cows higher concentrations of 3.55 ± 0.92, 4.17 ± 0.54, 3.29 ± 0.44 log CFU/g faeces in three different dairy farms [37]. Another study from the United Kingdom found similar average concentrations with seasonal differences of 1 log CFU/g faeces between summer and winter, with an average of 3.2 log CFU/g faeces in summer and 4.2 log CFU/g faeces in winter [38].

## Findings from the meta-analysis on prevalence outcomes

After excluding the studies with prevalence outcomes reported for pooled faecal samples and studies where the number of dairy cows sampled was not clear, 44 studies remained.

Out of these 44 studies, only 32 studies reported a prevalence for the aggregated sample, which equates to 32 prevalence outcomes. For the non-aggregated sample, including these 44 studies, 331 prevalence outcomes for different sub-groups and conditions were reported (S1 Table).

The overall prevalence estimate of the simple model that was based on the 32 prevalence outcomes of the aggregated sample was 29.3%, 95% CI [23–37%] with high heterogeneity $I^2$ = 98.5% [98–99%] and a prediction interval of 1.3% to 73% (Fig 3) [15, 36, 37, 39–67].

The pooled prevalence estimate of the multilevel model that was based on the 44 eligible studies and all their pooled prevalence was 51% with 95% CI [44–57%] and $I^2$ = 97.96% and a prediction interval of 0% to 100%. The sampling error was 2.04%. The heterogeneity within studies was 62.86% and the amount of between study heterogeneity constituted 35.1% of the total variation in our study (S1 Fig).

**Subgroup analysis.** A sub-group analysis of the aggregated sample was performed for the faecal collection method and the age class of cows. All other sub-groups in the aggregated sample could not be analysed because too few prevalence outcomes per group (N<3) were available.

For the faecal collection method, the prevalence outcomes between a rectal faecal extraction (18 studies) and the collection of cow pats (eight studies) from the floor (of the stable or meadow) were compared (Fig 4a). The prevalence estimate for the rectal extraction was 28%, 95% CI [19–38%] and for the cow pat collection 32%, 95% CI [22–44%]. The difference between these prevalence estimates was not significantly different (p = 0.52).

Only the prevalence outcomes of calves (five studies) and adult cows (19 studies) could be compared for the aggregated sample. For heifers, not enough aggregated outcomes were available (N<3) to be included in the analysis. The prevalence estimates for calves and adult cows

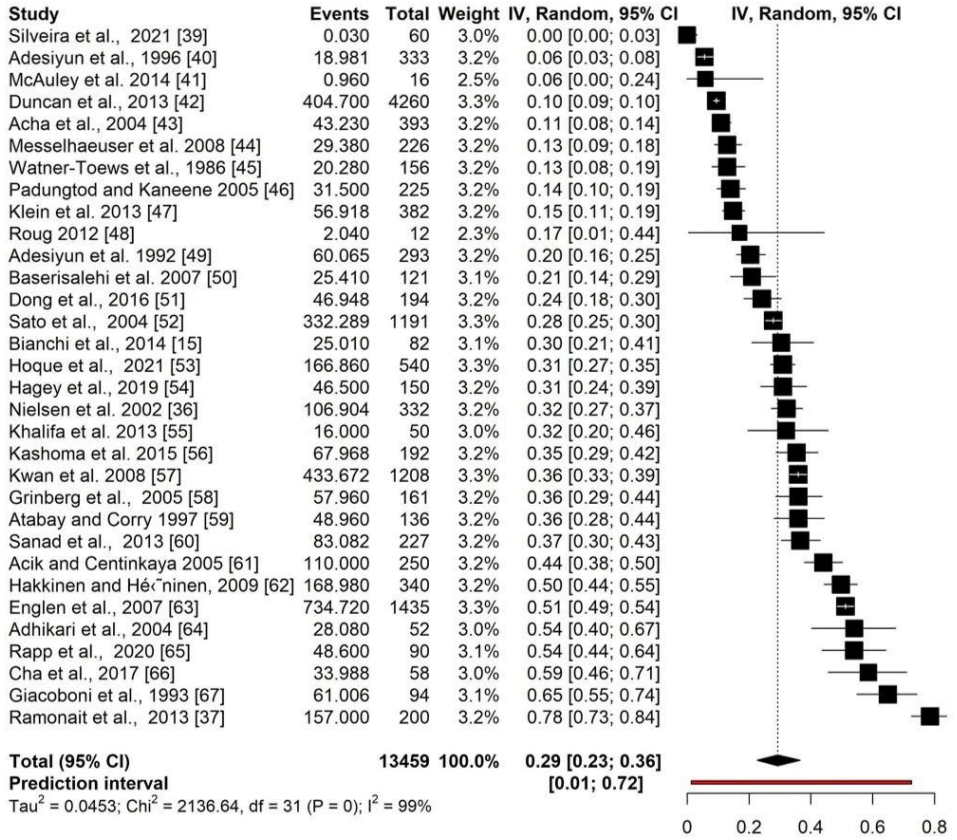


**Fig 3. Forest plot of the aggregated sample estimating the pooled prevalence of *Campylobacter* spp. in cows' faeces from 32 studies.** Event is pooled prevalence times number of individual cattle sampled.

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were 18%, 95% CI [11–27%] and 30%, 95% CI [21–39%] respectively (Fig 4b). No significant difference between these results was found (p = 0.06).

**The effect of subgroups on the prevalence.** The multilevel mixed-effect model showed a variance of 3.7%, 95% CI [1.9–7.3%] between studies and a variance of 3.8%, 95% CI [3.1–4.7%] for within study variance estimates. The variables *Campylobacter* species *C. hyointestinalis* and *C. jejuni* and the rectal faecal collection had a significant impact on the prevalence. The heterogeneity measure within the studies after accounting for the subgroups was 49.46% and the heterogeneity between the studies accounted for 48.29% of the total variability (S1 Fig).

**Meta-regression.** In addition, we assessed the effect of study year of publication on the prevalence estimate in a meta-regression of the aggregated sample. The meta-regression showed that the study year explained less than 1% of the heterogeneity (0.88%) observed in the

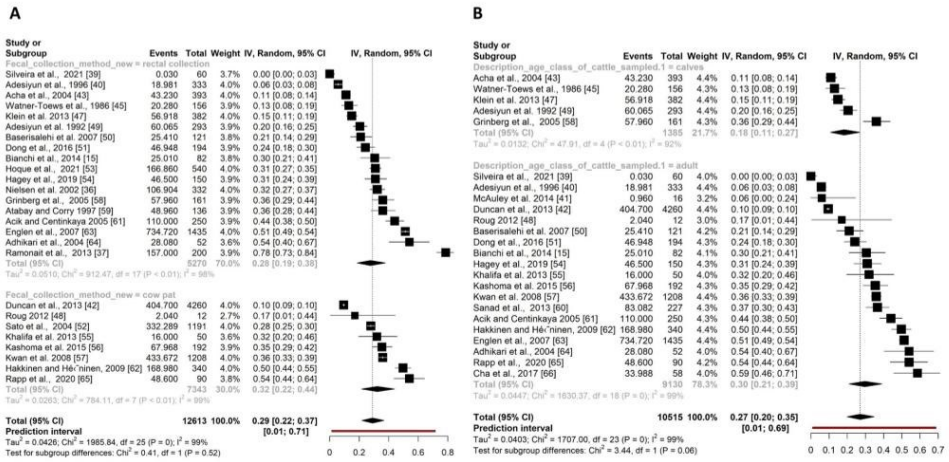


**Fig 4. Forest plot of the sub-group analysis of the aggregated sample comparing the prevalence estimates of *Campylobacter* spp. in faeces of dairy cows between different faecal collection methods (A) and age classes of the dairy cows (B).** Event is pooled prevalence times number of individual cattle sampled.

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prevalence outcomes and was not significantly affecting the prevalence estimate. The bubble plot based on the meta-regression confirms the finding (S2 Fig).

**Sensitivity analysis.** The risk of bias assessment was performed on all studies included in the systematic review (N = 53) and 22 (42%) of these grouped as low risk of bias. In the studies included in the simple meta-analytic model 13 of the 32 studies (41%) were in the low risk of bias group. The pooled prevalence estimate in the simple meta-analytic model was 32.5%, 95% CI [22–44%] and 27%, 95% CI [18–37%] in the low and high risk of bias group, respectively (p = 0.45) (S3 Fig). The second sensitivity analysis was the comparison between the pooled prevalence estimate from the simple and multilevel meta-analytic model. The analysis showed a significant difference between the two models. The estimated prevalence was 29% [23–36%] and 51% [44–57%] and the prediction values were [1–73%] and [0–100%] for the simple and multilevel meta-analytic model, respectively.

## Discussion

Based on the increasing consumer demand for fresh and raw products and the resulting consumption of unboiled raw milk, the raw milk supply chain has become more of a focus in recent decades. Especially because raw milk is one of the top vehicles causing strong-evidence outbreaks in the EU [1]. This might have contributed to the increase in studies focused on prevalence of *Campylobacter* in dairy cows faeces in the last 20 years (S2 Fig). In addition, animal health and farm management are further reasons for increased studies. However, systematic reviews and meta-analysis which allow for an estimation of the prevalence and concentration of *Campylobacter* spp. in cow faeces and identify potential data gaps have not been carried out yet. The assumption that *Campylobacter* contamination of raw milk is mainly caused by faecal contamination highlighted the importance of such systematic review and meta-analysis [9, 15–17]. The prevalence and concentration of *Campylobacter* spp. in faeces of dairy cows form an important basis for the mathematical modelling (via QMRAs) of potential

cross-contamination events and mechanisms along the raw milk supply chain. The development of such QMRAs can furthermore help to identify different mitigation options along the supply chain in order to reduce the public health risk associated with the consumption of *Campylobacter*-contaminated raw milk.

Here, we could only extract quantitative data on the concentration of *Campylobacter* spp. in faeces of dairy cows from three studies, as other studies gave only semi-quantitative estimates of the concentrations or presented results in a figure, which did not enable the extraction of e.g. a mean and standard deviation for the concentration. The average reported concentration of *Campylobacter* in faeces varied between the three studies and a meta-analysis could not be performed due to missing uncertainty measures (e.g. standard deviations). Specifically concentration data (including mean and standard deviation) are an important input for QMRAs, because the risk is the product of the probability that a random serving is contaminated and the probability that a contaminated serving results in disease. To clarify, the probability that a random serving is contaminated is based on the prevalence data and the probability that a contaminated serving results in diseases are calculated with concentration data that are used as input for the dose-response relationship [20, 21, 68]. These results clearly highlight the lack of concentration data (including uncertainty measures), which currently impedes risk assessments and consequently the refinement of mitigation options to reduce the public health risk from contamination of *Campylobacter* in cows' faeces.

The prevalence data for *Campylobacter* in dairy cow faeces were widely available in the scientific literatures, however, the range of prevalence varied highly (0–100%). In addition, some of the studies differed greatly in study design and quality e.g. in the specific and often missing information, e.g., on the health status studied (Fig 2). Subgroup analysis could therefore only be performed for the faecal collection method and the age class of dairy cows. All other subgroups of influencing factors of interest (i.e. the season during faecal sample collection, the *Campylobacter* species, and the health status of the cows) could not be compared because less than three prevalence outcomes per group were available.

Our RoB analysis could have been improved using a validated tool for observational animal studies. We hope future studies develop such a tool to make RoB analysis more standardized among prevalence studies. In addition, the RoB analysis showed that less than half of the studies are having a low risk of bias. It also showed that only five studies explicitly mentioned the application of ISO methods for *Campylobacter* detection and characterization. For most studies (N = 42) it remained unclear (meaning that it was not explicitly mentioned) whether an ISO method (e.g. ISO10272-1:2017 [69] and/or ISO10272-2:2017 [70]) was used. A detailed subgroup analysis of studies with and without the application of ISO methods was also not possible due to too few prevalence outcome in each group. This emphasizes the problem of wide heterogeneity between the studies further, especially since the detection and characterization of a sensitive bacterium such as *Campylobacter* spp. has proven challenging [71, 72].

The meta-analytical models aimed to estimate the pooled prevalence and to subsequently evaluate which influencing factors might affect the prevalence estimates and to some part explain the heterogeneity. The multilevel model offered the opportunity to include all extracted prevalence outcomes (N = 331) from the 44 studies. The pooled prevalence estimate from this model was higher than the estimate from the simple model. The prediction interval was also wider going from zero to one, better reflecting the heterogeneity between the outcomes. When adding the subgroups to the multilevel model the results were in some cases different to subgroup analysis based on aggregated sample (e.g. for faecal collection method). For the mixed-effect multilevel model, the variables of *Campylobacter* species *C. jejuni* and *C. hyointestinalis* (in comparison to *coli*) and rectal faecal collection method (in comparison to cow pat collection) additionally had a significant impact on the pooled prevalence estimate.

The subgroup analysis, in contrast, showed no difference in prevalence between the two faecal collection methods (rectal collection and cow pat) probably due to the remaining heterogeneity between the two subgroups, which have been adjusted for to an extent in the multilevel mixed-effect model.

Heterogeneity between studies was also evident in all meta-analytic models and their high inconsistency index (Fig 3 and S1 Fig). The variation was most likely a result of the different study designs and the subgroup differences. In the multilevel model it was evident that the variation between studies contributed less to the total variation than the within study variance. When subgroups were included in the multilevel model the within study variance decreased from 62.86% to 49.46% and as a result the between study variance accounted for almost half of the total variability (from 35.1% to 48.29%). Thus, making an estimation of the prevalence of *Campylobacter* in faeces of dairy cows difficult based on current studies.

Interestingly, mixed-effect multilevel model showed a significant effect of the faecal collection method on the pooled prevalence estimate. However, the subgroup analysis of aggregated samples in this study showed no significant difference between the prevalence obtained by rectal extraction (28%) or cow pats (32%) (Fig 4a). These findings were contrary to a study by Hoar et al., [73] that showed that the prevalence in cow pats was lower compared to rectal extraction in beef cattle. Nevertheless, the prevalence obtained in this study were quite low with only 5% for rectal faecal samples and 0.5% for cow pats [73]. We assumed that the cow pats in most of the studies included in this review and meta-analysis were examined immediately after shedding, which could explain the high prevalence found in cow pats. Another reason could be that the rectal extraction is not necessary allow for a mixture of a large amount of faeces, but rather supports the extraction of a few grams (e.g. rectal swab), which might not reflect the true prevalence. However, these findings also emphasize that *Campylobacter* already exhibits several survival strategies to adapt harsh conditions, e.g. in cow pats, by genetic exchange [74], by adaption mechanisms [75–77] or undergoing the viable but non-culturable state [78]. Accordingly, the survival of *Campylobacter* in cow pats in the stable environment may have been underestimated in the past.

The subgroup analysis of the aggregated prevalence estimates for calves and adult cows were 18% and 30%. The lower prevalence in calves could possibly be due to the use of straw compared to the stalls of adult cows [79]. Anyway, no significant difference between these results was found based on the subgroup analysis (Fig 4b). The multilevel mixed-effect model also showed no significant effect of the subgroups on the pooled prevalence estimate. In the search for quantitative data, two studies were identified that detected significantly higher concentrations of *Campylobacter* in the faeces of calves compared to dairy cows [36, 37].

In general, thermotolerant *Campylobacter*; mainly *C. jejuni* und *C. coli*, accounted for most human campylobacteriosis cases [80]. Nevertheless, other *Campylobacter* species such as *C. hyointestinalis* have also been reported to cause disease [81, 82]. It is important to mention that different methods of cultivation favour different species of *Campylobacter* [83]. *C. hyointestinalis* mainly colonized cows, but the cultural detection of *C. hyointestinalis* is not always ensured based on the fact that this species is not known to be thermotolerant and higher detection levels would occur after enrichment at 37˚C compared with direct culture [84]. Still, the *Campylobacter* species *C. hyointestinalis* and *C. jejuni* are predominantly found in dairy cows [59, 62]. Accordingly, in the meta-analysis with the multilevel mixed-effect model *C. hyointestinalis* and *C. jejuni* had a significant impact on the pooled prevalence estimate (S4 Fig).

Repeated samplings are needed in order to examine whether the prevalence and concentration of *Campylobacter* in faeces of dairy cows follow a seasonal pattern. In total 14 studies have taken repeated samples according to season in temperate regions and made data available in

their publication. Anyway, this were not enough data for subgroup analysis on the aggregated sample and only the multilevel mixed-effect model could be used to analyse the effect of seasons on the pooled prevalence estimate (S5 Fig). The results from the multilevel mixed-effect model showed no significant effect of seasons on the pooled prevalence estimate which was contrary to results reported by other studies [1, 34, 85]. Seasonal changes in *Campylobacter* concentration in cow faeces were expected based on the observations that the occurrence of *Campylobacter* in the faeces of food-producing animals has been shown to be subject to seasonal changes [3, 86] and that every year a seasonal increase in *Campylobacter* infections is recorded in the warmer months [85, 87, 88]. It has been shown that *Campylobacter* has a characteristic seasonality with a sharp increase of cases in the summer and a smaller but distinct winter peak [1]. Additionally, a distinct peak in the *Campylobacter* concentration in cow faeces in either winter or summer has been reported [89]. However, a bimodal trend with faecal extraction in spring and autumn has also been observed [34].

## Strengths and limitations of the study

This systematic review demonstrates the important data gaps for the meta-analysis of the prevalence and concentration of *Campylobacter* in cow's faeces. The major hurdle in evaluating prevalence data for *Campylobacter* spp. in faeces of dairy cows from the literature was that the data were often made available only in an aggregated state (e.g. average per subgroup). Other identified data gaps were related to the missing metadata regarding the description of the population under study (e.g. age class and health status), the sampling conditions (e.g. season) or the methodology used (e.g. faecal collection method and the use of ISO methods for *Campylobacter* detection). Thus, meta-analysis and evaluation using the specific subgroups was significantly limited. A further limitation was based on the high heterogeneity between studies, which made an estimation of the prevalence difficult. This high heterogeneity was most likely based on the high degree of variability between studies in populations under study, sampling conditions, methodology and so on. In addition, heterogeneity was likely also affected by data aggregation and missing metadata.

Future studies should therefore consider publishing raw data in non-aggregated state in order to provide better re-usability of data and to move towards the Findability, Accessibility, Interoperability, and Reuse (FAIR) data principles for scientific data [90]. Moreover, we are suggesting that authors of future studies carefully consider which metadata to collect and report in their publications to further support re-usability.

In addition, we highlighted the importance of analysing the prevalence and concentration of *Campylobacter* in food-producing animals at farm levels in order to better understand and estimate potential cross-contamination mechanisms along the food chain. Specifically concentration data (including mean and standard deviation) are an important input for QMRAs and this review and meta-analysis emphasizes the need for more studies that collect concentration data for *Campylobacter* in dairy cow faeces.

Nevertheless, the analysis of the extracted prevalence data presented in this study is considered a valuable basis for the further development of QMRAs and different risk mitigation strategies along the raw milk supply chain for *Campylobacter* spp. in (raw) milk and food products thereof.

## Supporting information

**S1 Checklist. PRISMA checklist.**
(DOC)

**S1 Fig. Heterogeneity.** (A) Heterogeneity in the multilevel model, (B) Heterogeneity in the multilevel mixed effects model.
(TIF)

**S2 Fig. Bubbleplot.**
(TIF)

**S3 Fig. Forest plot RoB.**
(TIF)

**S4 Fig. Forest plot species.**
(TIF)

**S5 Fig. Forest plot season.**
(TIF)

**S1 Table. Extraction table.**
(XLSX)

**S2 Table. Risk of bias.**
(XLSX)

## Author Contributions

**Conceptualization:** Anna-Delia Knipper, Tasja Crease.

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**Investigation:** Anna-Delia Knipper, Tasja Crease.

**Methodology:** Anna-Delia Knipper, Narges Ghoreishi, Tasja Crease.

**Project administration:** Tasja Crease.

**Supervision:** Tasja Crease.

**Visualization:** Anna-Delia Knipper, Narges Ghoreishi, Tasja Crease.

**Writing – original draft:** Anna-Delia Knipper, Tasja Crease.

**Writing – review & editing:** Anna-Delia Knipper, Narges Ghoreishi, Tasja Crease.

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## 3.3 Publication 2: Longitudinal study for the detection and quantification of *Campylobacter* spp. in dairy cows, during milking and in the dairy farm environment

foods

MDPI

*Article*

# Longitudinal Study for the Detection and Quantification of *Campylobacter* spp. in Dairy Cows during Milking and in the Dairy Farm Environment

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**Abstract:** Campylobacteriosis outbreaks have repeatedly been associated with the consumption of raw milk. This study aimed to explore the variation in the prevalence and concentration of *Campylobacter* spp. in cows' milk and feces, the farm environment and on the teat skin over an entire year at a small German dairy farm. Bi-weekly samples were collected from the environment (boot socks), teats, raw milk, milk filters, milking clusters and feces collected from the recta of dairy cows. Samples were analyzed for *Campylobacter* spp., *E. coli*, the total aerobic plate count and for *Pseudomonas* spp. The prevalence of *Campylobacter* spp. was found to be the highest in feces (77.1%), completely absent in milking equipment and low in raw milk (0.4%). The mean concentration of *Campylobacter* spp. was 2.43 $\log_{10}$ colony-forming units (CFU)/g in feces and 1.26 $\log_{10}$ CFU/teat swab. Only a single milk filter at the end of the milk pipeline and one individual cow's raw milk sample were positive on the same day, with a concentration of 2.74 $\log_{10}$ CFU/filter and 2.37 $\log_{10}$ CFU/mL for the raw milk. On the same day, nine teat swab samples tested positive for *Campylobacter* spp. This study highlights the persistence of *Campylobacter* spp. for at least one year in the intestine of individual cows and within the general farm environment and demonstrates that fecal cross-contamination of the teats can occur even when the contamination of raw milk is a rare event.

**Keywords:** food safety; food hygiene; raw milk; cattle; risk assessment



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## 1. Introduction

Campylobacteriosis, caused by bacteria of the genus *Campylobacter*, is the most commonly reported bacterial foodborne gastrointestinal infection in humans in the European Union (EU) [1]. Aside from *Salmonella* spp. and the shigatoxin-producing *Escherichia coli* (STEC), *Campylobacter* spp., predominantly *Campylobacter* (C.) *jejuni*, have been regarded as the most notable health hazards, with clear links between drinking raw milk and human illness [2–4]. Between 2011 and 2020, raw milk was one of the main food vehicles causing "strong evidence" for foodborne campylobacteriosis outbreaks reported in the EU [5].

Thermophilic *Campylobacter* spp. colonize the intestinal tract of cattle and are shed intermittently with the feces [6–9]. Therefore, it is assumed that in raw milk, this pathogen mainly originates from fecal cross-contamination during milking. However, it is not clear how this contamination takes place and how often raw milk is contaminated during milking [6,10–12].

The limited number of studies on and low concentrations of *Campylobacter* spp. along the raw milk supply chain have challenged previous risk assessments for raw milk consumption. Some studies investigated the prevalence of *Campylobacter* spp. in the bulk milk tank and milk filter. A meta-analysis of results from North America, Europe and New Zealand provided an estimated mean prevalence of 1.54% for *Campylobacter* spp. in bulk tank milk and 1.75% in milk filters [13]. Two studies attempted to quantify the contamination using the most probable number (MPN) method. They found 16 ± 30 MPN/100 mL in the bulk tank milk [14] and 1 MPN/21 mL in raw milk from farm vats [15]. Despite the low bacterial prevalence and concentration, the consumption of raw milk is considered a high-risk behavior [16]. Consumers are advised to boil raw milk prior to consumption to inactivate pathogens [2]. However, surveys in Italy found that 13.9% to 43% of consumers did not boil raw milk before consumption [17].

The prevalence of *Campylobacter* spp. in the feces of dairy cows vary widely from 0 to 100% [18]. The studies included in the meta-analysis differed in their design and the size of the herds investigated [18]. Seven studies reported quantitative data for *Campylobacter* spp. in cow feces [19–25]. The concentration ranged from 2 $\log_{10}$ colony-forming units (CFU)/g feces to 4 $\log_{10}$ CFU/g feces [20,21]. One study in New Zealand investigated the differences in the fecal concentrations of *C. jejuni* between individual cows based on rectal sampling [23]. Three cows on a pasture and three cows in confinement housing were grouped together as a "high-shedder group", harboring a median concentration of 3–3.6 $\log_{10}$ *C. jejuni* per g of fresh feces [23].

Few studies have focused on the raw milk supply chain and the herd-level epidemiology of *Campylobacter* spp. [6,26–29]. To estimate the transmission of *Campylobacter* spp. from feces to milk, it is necessary to investigate both in the same setting. Limited longitudinal data on cross-contamination with *Campylobacter* spp. from feces to raw milk are available. Frequent sampling is required to detect contamination events and to estimate their frequency because the contamination of milk with *Campylobacter* spp. is expected to occur only sporadically. To the best of our knowledge, only one study from Norway provides concurrent qualitative prevalence data on *Campylobacter* spp. in cows' feces, on teat skin, in raw milk and in environmental samples [30]. However, *Campylobacter* spp. were not quantified in that study.

In our study, the prevalence and concentration of *Campylobacter* spp. in fecal samples, teat swabs, raw milk, milk filters and boot sock samples from the stable alley were determined to close the gaps in the described knowledge and data. We determined the frequency of fecal shedding of *Campylobacter* spp. in individual cows and in different seasons. We compared the occurrence of *Campylobacter* spp. on teat skin, in raw milk and in milk filters with the fecal shedding of this pathogen. *Escherichia coli*, *Pseudomonas* spp. and the total aerobic colony count (TACC) were analyzed as indicators of fecal and environmental contamination throughout the milking process.

## 2. Materials and Methods

### *2.1. Sampling Site*

A Holstein cow herd with 22 lactating animals in Berlin, Germany, was sampled over a period of one year, from the 19th of April 2021 to the 8th of April 2022. Cows were kept in a free-stall barn with a concrete floor and access to an outdoor sand paddock throughout the year. Samples were taken on two consecutive days every two weeks from primiparous ($n$ = 19) and second lactation ($n$ = 3; cow ID 4301, 4317, and 4320) cows. The animals were fed a diet consisting of 27.7% grass silage, 29.5% maize silage, 6.0% straw, 30.1% hay, 6.0% beet pulp, and 0.61% minerals ad libitum throughout the year. Concentrates were provided individually in a transponder-controlled automatic feeder to meet the energy requirements for 25 kg/d of energy-corrected milk. On the first day, samples were collected from raw milk, milk filters, teat skin and milking clusters at 6 a.m. Teat swabs and raw milk samples were taken from twelve lactating cows over the entire period. The same cows were sampled on every occasion with some exceptions: (1) dry cows on specific sampling days

were replaced by lactating cows; (2) two cows had to be treated with antibiotics against mastitis during the trial period. These were excluded once from teat swab and raw milk sampling during treatment. These exceptions resulted in teat swab and raw milk samples from 21 different cows.

On the second day, rectal fecal samples were taken from twelve cows and boot sock samples were obtained from the stable corridor. A mathematical randomization fixed the twelve cows always used for rectal fecal sampling. One cow (6057) left the herd for a reason unrelated to the study and was replaced by a new cow (6005), who was used for rectal fecal sampling. Samples were transported on ice to the laboratory, and microbiological processing was carried out within two hours of sampling at the latest. The entire experiment was approved by the State Office for Health and Social Affairs (LAGESO) (Reg.-No.: G 0215/20).

### *2.2. Sample Preparation*

#### 2.2.1. Teat Swab Samples

Two gauze pads (10 × 10 cm, 8-fold) were placed in a plastic bag and moistened with 8 mL 0.9% NaCl. The bag was closed and stored at 4 °C until sampling. One bag was used per cow. On two occasions, teat swabs could only be collected from 11 cows, resulting in a total of 286 teat swab samples (instead of 288, 24 × 12 cows) during the study.

All four teats of a cow were wiped with the two gauze pads while wearing gloves before being cleaned by the milkers with moist cleaning wipes (udder wipes, clean paper®, Lauchhammer, Germany). The gloves were changed between sampling each individual cow to avoid cross-contamination. In the laboratory, the gauze pads were visually scored to assess the fecal contamination of the teat skin. Four scoring categories were used: K1: gauze pad clean; K2: gauze pad colored yellowish; K3: gauze pad discolored brown, possibly with fecal particles; K4: gauze pad brownish in color, feces clearly visible on the gauze pads.

Twenty-five milliliters of 1% phosphate-buffered peptone water (PW) was added to each teat swab sample (consisting of two gauze pads) and homogenized using a GRINDOMIX GM200 (Retsch GmbH, Haan, Germany) for 120 s at 4000 rpm.

#### 2.2.2. Raw Milk Samples

While wearing gloves and after the teats were cleaned with udder wipes (clean paper®, Lauchhammer, Germany), raw milk samples were obtained from all four teats and pooled in 50 mL falcon tubes. No disinfectant was applied before the raw milk was sampled. On two occasions, raw milk samples could only be collected from 11 cows, resulting in a total of 286 raw milk samples (instead of 288, 24 × 12 cows) during the study.

#### 2.2.3. Milking Clusters Samples

After the completion of the milking process, one pooled sample was taken from each milking cluster (with four teat cups). One swab (nerbe plus, Winsen/Luhe, Germany) was used for each teat cup. Four swabs from the same cluster were pooled into one sample. Four milking clusters were used at the farm. One of the clusters was not used on seven occasions for technical reasons. Therefore, only 89 samples (instead of 96, 24 × 4 clusters) were analyzed.

Each sample was covered with 18 mL PW and homogenized as previously described.

During the study, intermediate disinfection of the teat cups with 3% peracetic acid between cows was introduced on the farm. The disinfection was introduced to achieve better milking hygiene between cows due to the poor udder health of some cows. However, we continued to take samples from the teat cup at the end of the completed milking process of all cows.

#### 2.2.4. Milk Filter Samples

The milk filter was installed at the end of the milk pipeline, at the start of the milking process, i.e., all milk collected on that day passed through the filter. One milk filter was taken on each sampling day ($n$ = 24). The filters measured 6 cm × 52.5 cm, and the pore

size ranged from 100 µm to 250 µm. After the milking process was finished, the milk filter was removed and transferred to a plastic bag. In the laboratory, it was homogenized after being covered with 35 mL PW.

#### 2.2.5. Boot Sock Samples

Socks (romerlabs, surface boot cover swabs, 10001911 (BTSW200BPW)) were placed on shoes before sampling. One pair of boot socks was taken on each sampling day ($n$ = 24). The entire barn corridor was walked with the boot socks (approx. 80 steps), avoiding fresh fecal pats. After sampling, the socks were placed in a stomacher bag and transported to the laboratory in a cool box. At the laboratory, 180 mL PW, enough to cover the socks, was added to the stomacher bag, and the same procedure as described above was applied for homogenization.

#### 2.2.6. Fecal Samples

Fecal samples were obtained from the recta of twelve cows, using gloved hands and a lubricant gel. On one sampling day, only eleven cows were sampled. Therefore, 287 samples were analyzed (instead of 288, 12 cows × 24 samplings). The samples were placed in plastic cups with a screw-on lid. Gloves were changed between individual cows to avoid cross-contamination. In the laboratory, 10 g of feces per sample was transferred into stomacher bags and mixed with 90 mL PW. Three scoring categories were used to assess the consistency of the feces (K1: liquid; K2: mushy (normal) consistency; K3: dry and compact). The samples were homogenized individually for 120 s at 4000 rpm.

### *2.3. Microbiological Analysis*

#### 2.3.1. Detection and Quantification of Microorganisms

The detection limits of all microorganisms in the samples are provided in Table S1.

*Escherichia coli* (beta-glucoronidase-positive), TACC and *Pseudomonas* spp. were quantified according to ISO 16649-2:2001 (using the spread plate method instead of the pour plate method), ISO 4833:2015 and ISO 13720:2010, respectively. The samples were further diluted 1:10 in PW, and 100 µL per dilution step was spread on agar plates. *Escherichia coli* was cultured on tryptone bile X-glucuronide (TBX) agar (Oxoid Deutschland GmbH, Wesel, Germany) for 24 h at 41.5 °C, TACC on plate count agar (carl roth® GmbH + Co., KG, Karlsruhe, Germany) for 72 h at 30 °C and *Pseudomonas* spp. on cephaloridine fucidin cetrimide (CFC) agar (Merck KGaA, Darmstadt, Germany) for 24 h at 25 °C. Thermophilic *Campylobacter* spp. were detected according to ISO 10272-1:2017, using modified charcoal cefoperazone deoxycholate agar (mCCDA, mixture of Merck & Co., Kenilworth, NJ, USA and Oxoid; 48 h, 41.5 °C). For the enrichment of *Campylobacter* spp., Preston broth (Merck KGaA, Darmstadt, Germany; 24 h, 41.5 °C) was used. The enrichment for teat swabs, milk, swabs from milk clusters and milk filters was performed using 5 mL of the sample dilution and 45 mL of Preston broth. For boot sock samples, 1 mL of sample dilution was used in 9 mL of Preston broth. One gram of fecal sample was weighed into a test tube and covered with 9 mL of Preston broth.

Concurrently, ISO 10272-2:2017 was used for the enumeration of thermophilic *Campylobacter* spp. The samples were further diluted 1:10 in PW, and 100 µL per dilution step was spread on agar plates. In addition, 1 mL of the initial sample (raw milk) or sample suspension (all other sample matrices) was spread on agar plates.

#### 2.3.2. Species Identification

One colony from each sample was selected for *Campylobacter* spp. identification. *Campylobacter* spp. colonies were sub-cultured on Columbia blood agar plates with defibrinated sheep blood (Oxoid Deutschland GmbH, Wesel, Germany) in a microaerobic atmosphere for 24 h at 41.5 °C. Afterwards, further analyses were performed according to ISO 10272-1:2017. In short, characteristic morphology and motility were observed via phase contrast microscopy. A catalase activity test was performed by streaking a loop of culture into a drop of hydrogen peroxide

solution on a clean microscope slide. The test was positive if bubbles appeared within 30 s. The detection of cytochrome oxidase activity was performed using a Bactident™ Oxidase test strip (Merck KGaA, Darmstadt, Germany), following the manufacturer's instructions. A color change to violet/blue indicated that hydrolysis had taken place.

In addition, the genus and species identification of the colonies from sheep blood agar plates was performed using a Bruker MALDI-TOF Biotyper System (Bruker Scientific LLC, Billerica, MA, USA). Colonies were transferred to the MALDI-TOF target and covered with 1.0 µL of α-cyano-4-hydroxycinnamic acid, according to the manufacturer's instructions (Bruker Scientific LLC, Billerica, MA, USA). The reference database for species identification was provided by Bruker Scientific LLC (MBT-BDAL-8468).

#### 2.3.3. Somatic Cell Count

Somatic cell counts were determined using a simple cell count meter (DCC, DeLaval; Glinde, Germany). Sixty microliters of raw milk from individual cows was loaded into the cassette, and the measurement was carried out according to the operating manual.

### *2.4. Weather Data*

Weather data were acquired from an official weather station close to the farm (https://openweathermap.org/ (accessed on 22 June 2022)). The meteorological data collected were temperature (°C) (hourly), pressure (hPa), humidity (%), wind (m/s) and rain (mm/h) data.

### *2.5. Statistical Analysis*

Microsoft Excel® 2016 (Microsoft Corp., Redmond, WA, USA) was used to store the data. The software R, version 4.2.1 (Vienna, Austria) [31], was used for data analysis.

#### 2.5.1. Multi-Level Modeling

The effect of the environment data and cross-contamination was evaluated using multi-level modeling, which clusters the observations for each cow (repeated measurements) and offers variation effects on both the sample and cow level.

Due to the large number of zero values in the final results, a multivariate generalized linear regression model was not possible because numerous zero values result in heteroscedasticity and collinearity. They also caused the distribution to be skewed and non-normal. As a result, the *Campylobacter* spp. concentration data were classified as a binary variable (0 and 1), and a multi-level mixed logistic regression was performed. Level one of the model comprised the bi-weekly observations for each cow. Level two consisted of the cows.

Only the teat and feces samples were selected as dependent variables for modeling since the occurrence of *Campylobacter* spp. in almost all other samples was negative. The occurrence of *Campylobacter* spp. in the teats and feces was modeled against weather data (minimum temperature, pressure, wind and humidity), seasons, the concentration values of other microorganisms (*E. coli*, *Pseudomonas* spp. and TACC), teat cleanliness scores, fecal consistency scores and somatic cell count. Each parameter was added to the model in a stepwise manner, and the goodness-of-fit was decided based on the Akaike information criterion (AIC). Due to the relatively small number of observations and the difficulty in merging the model, only parameters with a significant effect were retained in the final model. To test whether the effect of minimum temperature was related to the occurrence of *Campylobacter* spp. in the teat or feces samples, an interaction variable was integrated into the multi-level model.

The intraclass correlation coefficient (ICC) was calculated to evaluate the variation in concentration values between the cows (Level 2) and for each cow throughout the year (Level 1). Multi-level modeling was performed using the lme4 package [32].

#### 2.5.2. Correlation Analysis

The correlation of the concentration of *Campylobacter* spp. on teat skin with its concentration in feces was graphically and statistically assessed using the Spearman rank correlation coefficient for non-parametric data.

## 3. Results

### *3.1. Species Identification*

*Campylobacter* spp. were isolated from 263 of the 997 samples tested. Of the 263 isolates, 256 (97.3%) were identified as *C. jejuni* and 7 as *C. hyointestinalis* (2.7%). The latter was only isolated from feces. Five of the seven isolates were obtained from one cow (6001). The remaining two isolates were obtained from two other cows (4664 and 4652). All *Campylobacter* spp. isolates were positive for catalase activity and cytochrome oxidase activity.

### *3.2. Prevalence and Concentration Data*

An overview of all prevalence data for the specific sample types and analyzed bacteria is shown in Table 1.

**Table 1.** Prevalence data for all sample types and taxa analyzed.

| Sample Type | Total Number of Samples | Proportion of Positive Samples (%) | | |
|---|---|---|---|---|
| | | ***Campylobacter* spp.** | ***E. coli*** | ***Pseudomonas* spp.** |
| Teat swab | 286 | 12.2 | 81.8 | 97.6 |
| Raw milk | 286 | 0.4 | 15.0 | 71.7 |
| Milking cluster | 89 | 0 | 15.7 | 51.7 |
| Milk filter | 24 | 4.2 | 45.8 | 95.8 |
| Feces | 287 | 77.1 | 94.8 | Not tested |
| Boot socks | 24 | 29.2 | 100 | 58.3 |

The prevalence of *Campylobacter* spp. was the highest in fecal samples (77.1%), followed by boot sock samples (29.2%), teat swabs (12.2%), milk filters (4.2%) and raw milk samples (0.4%). No *Campylobacter* spp. were detected in the milking clusters.

*Escherichia coli* was most frequently detected in the fecal samples (94.8%), boot sock samples (100%) and teat swab samples (81.8%). *Pseudomonas* spp. were most frequently detected on the teats (97.5%), in the milk filters (95.8%), the milking clusters (51.7%) and in raw milk (71.7%). Fecal samples were not tested for *Pseudomonas* spp.

The somatic cell count in the 286 milk samples ranged from 3 to 933 $\times$ $10^3$ cells/mL. One sample taken on 7 July 2020 had a somatic cell count of 4.7 $\times$ $10^6$ cells/mL. This sample appeared normal, without signs indicative of inflammation such as flocculation.

An overview of the quantitative data on all bacterial microorganisms in all sample types can be found in Table 2.

*Campylobacter* spp. were only detected in one raw milk sample and one milk filter, with a concentration of 2.37 $\log_{10}$ CFU/mL and 2.74 $\log_{10}$ CFU/filter, respectively. These samples were taken on the same sampling day. Otherwise, the highest mean concentration of *Campylobacter* spp. detected in the boot sock samples was 3.01 $\pm$ 1.05 $\log_{10}$ CFU/2 socks and 2.43 $\pm$ 0.9 $\log_{10}$ CFU/g in the cow feces. The mean concentration of *Campylobacter* spp. at the cow teats was 1.26 $\pm$ 0.75 $\log_{10}$ CFU/4 teats. It is important to note the different units in the concentration data.

**Table 2.** Mean $\log_{10}$ concentration and standard deviation for all sample types and microorganisms analyzed.

| Sample Type [Unit] | Concentration Data (No. of Positive Samples) | | | |
|---|---|---|---|---|
| | *Campylobacter* spp. | *E. coli* | *Pseudomonas* spp. | TACC [1] |
| Teat swab [$\log_{10}$ CFU/4 teats] | 1.26 ± 0.75 (35) | 3.87 ± 0.98 (234) | 8.03 ± 0.62 (279) | 5.36 ± 0.71 (284) |
| Raw milk [$\log_{10}$ CFU/mL] | 2.37 (1) [2] | 2.47 ± 0.53 (43) | 2.7 ± 0.6 (205) | 4.96 ± 0.66 (286) |
| Milking cluster [$\log_{10}$ CFU/4 cups] | 0 (0) | 2.69 ± 0.55 (14) | 2.78 ± 0.46 (46) | 5.07 ± 0.66 (84) |
| Milk filter [$\log_{10}$ CFU/filter] | 2.74 (1) [2] | 3.74 ± 0.76 (11) | 5.16 ± 0.9 (23) | 6.91 ± 0.54 (24) |
| Feces [$\log_{10}$ CFU/g] | 2.43 ± 0.9 (215) | 4.48 ± 1.18 (273) | Not tested | 6.34 ± 0.48 (285) |
| Boot socks [$\log_{10}$ CFU/2 socks] | 3.01 ± 1.05 (4) | 5.39 ± 1.11 (24) | 6.64 ± 0.52 (14) | 9.18 ± 0.63 (23) |

[1] total aerobic colony count; [2] no standard deviation calculation possible.

### 3.3. *Campylobacter* spp. Prevalence and Concentration in Feces

The fecal consistency scores were not related to the *Campylobacter* spp.-positive fecal samples. In total, 26, 191 and 70 teat swab samples were categorized as K1, K2 and K3, respectively. The scoring for the positive *Campylobacter* spp. samples ranged from K1 to K3 (Table S2). A seasonal overview indicated that *Campylobacter* spp. could be detected in the herd's feces throughout the year. The mean concentration of the positive *Campylobacter* spp. samples ranged between 1.9 $\log_{10}$ CFU/g and 2.9 $\log_{10}$ CFU/g (Figure 1A). The proportion of negative samples was lower in the warm months (July and August) compared to colder months (November, January, February and April) except for December and March (Figure 1B).

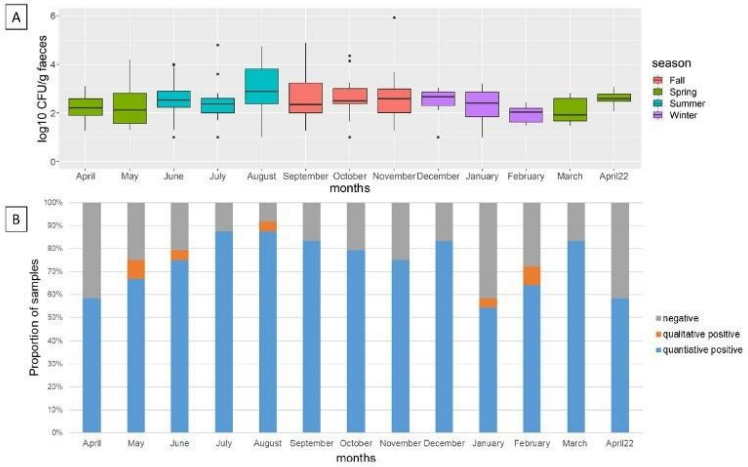


**Figure 1.** (**A**) Concentration of *Campylobacter* spp. in feces per month. (**B**) Proportion of negative, qualitative positive (enrichment) and quantitative positive samples.

The concentration of *Campylobacter* spp. in the feces of individual cows over time is depicted in Figure 2 and Table S2. Occasionally, no *Campylobacter* spp. were detected in individual cows. All cows carried *Campylobacter* spp. in at least two samples. Cow 4317 only tested positive for *Campylobacter* spp. on two consecutive sampling occasions in July. Cows 4320, 4659 and 6005 were always positive.

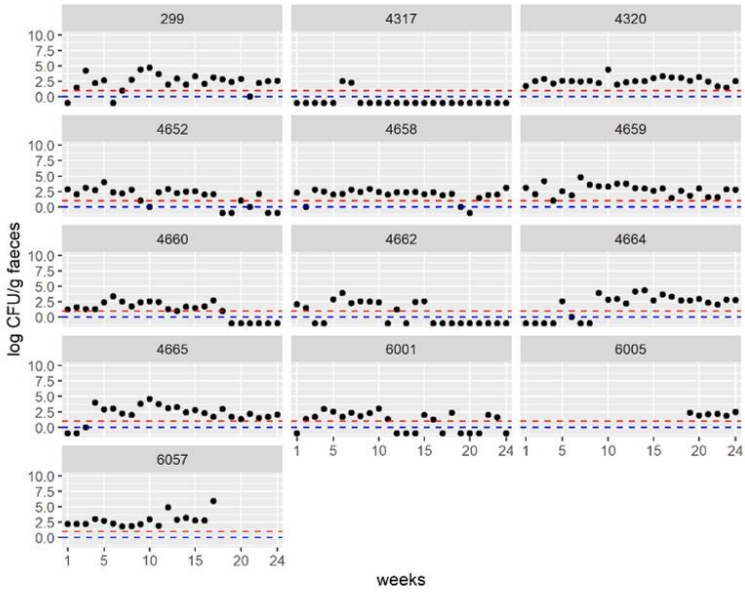


**Figure 2.** Overview of the concentration of *Campylobacter* spp. in feces of individual cows (ID numbers). In total, 24 samplings were performed over one year. The first sampling took place in April (week 1). Cow 6057 was replaced by cow 6005 after the 17th sampling. The limits of quantification and detection for enrichment are depicted as a red and blue line, respectively. Dots below the limit of detection indicate negative samples. Dots at the blue line indicate samples that were qualitatively positive but could not be quantified.

The highest median concentrations in the feces of individual cows shedding *Campylobacter* spp. were 2.9 ± 0.9 $\log_{10}$ CFU/g (4659), 2.8 ± 0.96 $\log_{10}$ CFU/g (4664) and 2.7 ± 1.09 $\log_{10}$ CFU/g (6057) and the lowest were 1.6 ± 0.68 $\log_{10}$ CFU/g (4660) and 2.0 ± 0.57 $\log_{10}$ CFU/g (6001).

### 3.4. *Campylobacter* spp. *in Teat Swab Samples*

Among the 286 teat swab samples, 35 were positive for *Campylobacter* spp. These originated from 15 different cows (Table S3). Teat swab samples from the individual cows were positive for *Campylobacter* spp. on up to three occasions. On one sampling day (14 June 2021), nine teat swab samples were positive for *Campylobacter* spp. On the same day, the positive raw milk sample and the positive milk filter were obtained. The positive raw milk sample was from one cow (6005) tested as a replacement for another cow in the dry period. Therefore, no fecal sample was collected from this cow.

On the other sampling days, a maximum of two teat swab samples were positive for *Campylobacter* spp. The cleanliness scores of the teat samples could not be linked to the *Campylobacter* spp. positive teat swab samples. In total, 23, 117, 93 and 53 teat swab samples were categorized as K1, K2, K3 and K4 respectively. The scoring for the positive *Campylobacter* spp. samples ranged from K1 to K4 (Table S3).

*Campylobacter* spp. positive teat swab samples were detected at minimum outdoor temperatures between −4 °C and 17 °C (Figure 3). The negative samples were observed at all minimum temperatures. The qualitative positive samples occurred within a smaller temperature range. The mean minimum temperature for the detection of negative, qualitative and quantitative positive samples were 5 °C, 4 °C and 7 °C, respectively.

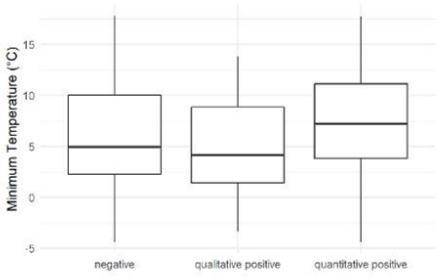


**Figure 3.** Association of minimum temperature with the occurrence of *Campylobacter* spp. on teat skin (*Campylobacter* spp. negative, qualitatively positive (enrichment) and quantitatively positive teat swab samples). The black line indicates the median temperature, and the boxes display the interquartile range.

### *3.5. Multi-Level Model and Correlation Analysis*

The multi-level model (Table 3) with a binary outcome of *Campylobacter* spp. (dependent variable) shows the effective parameters on the occurrence of *Campylobacter* spp. in the teat swab and fecal samples.

**Table 3.** Multi-level model with parameters with an effect on the occurrence of *Campylobacter* spp. in teat swab and feces samples. The confidence intervals (CI) demonstrate the variability of the odds ratio (OR). When confidence interval contains 1, the effect is not significant.

| | **Effect Estimate (OR)** | **Lower CI** | **Upper CI** |
|---|---|---|---|
| Fall | 2.88 | 0.82 | 10.676 |
| Spring | 0.72 | 0.23 | 2.25 |
| Summer | 15.02 | 2.15 | 121.189 |
| Teats | 0.01 | 0.00 | 0.03 |
| $Log_{10}$ *E. coli* | 1.56 | 1.2 | 2.11 |
| Minimum temperature | 0.98 | 0.84 | 1.13 |
| Type Minimum Temperature (interaction term) | 0.79 | 0.67 | 0.92 |

The seasons fall, spring and summer were individually compared to the winter. The effect of the season as a whole also significantly improved the model fit. The effect of the sample type is shown by the comparison of the teat and feces samples.

An integrated interaction term between the minimum temperature and the sample type (feces or teat swab) demonstrated a different temperature effect of the occurrence of *Campylobacter* spp. for the two sample types.

Other parameters tested, including the weather conditions (humidity, wind and rain), other microorganisms (*Pseudomonas* spp. and TACC), the scoring of teat and fecal samples and somatic cell counts, did not show an influence on the occurrence of *Campylobacter* spp.

The ICC of the multi-level model was 0.11, indicating that 11% of the variation in the model comes from the variation between the cows, whereas the rest of the heterogeneity originates from the variation in the measurements for each cow that happened throughout the year.

Correlation analyses were performed for the concentrations of *Campylobacter* spp. in teat swab samples and the concentrations of *Campylobacter* spp. in the feces samples. No correlations were detected in these analyses.

## 4. Discussion

To explore the risk associated with the consumption of raw milk, the occurrence of *Campylobacter* spp. in a small German dairy herd was studied. In our study, the highest

proportion of positive samples was found in the feces (77.1%), and the lowest proportion of positive samples was found in the milking clusters (0%) and raw milk (0.4%, one sample).

The high prevalence of *Campylobacter* spp. in the cows' fecal samples is consistent with some previous studies, which reported a prevalence between 66.7% and 78.5% [21,27,30,33]. A lower prevalence (7–38%) of *Campylobacter* spp. in cow feces was reported in other studies [20,26,34,35]. Two of these studies used comparable study designs and detection methods [26,34]. One study did not mention the interval between sampling and testing [20]. The last study reported that the samples were analyzed one day after sampling [35]. The interval between sampling and testing could have influenced the detection of *Campylobacter* spp. in the samples as *Campylobacter* spp. is a fastidious organism (with a low oxygen tolerance and sensitivity to temperature and pH) [36]. In addition, a low prevalence was observed in a study carried out on smaller farms, while a larger farm displayed a higher prevalence [26].

This is the first study to monitor the concentration of *Campylobacter* spp. in individual dairy cows in Germany over a period of one year using rectal fecal sampling. The concentration of *Campylobacter* spp. in the fecal samples varied between individual cows. It ranged between high concentrations (over 5 $\log_{10}$ CFU/g) and negative samples. Some cows shed *Campylobacter* spp. consistently (4320, 4659), whereas other cows shed *Campylobacter* spp. less often (4662) or only twice (4317) throughout the year. The proportion of negative samples was lower in the warmer months than in colder months, whereas the mean concentration in any individual month was relatively constant, between 1.9 $\log_{10}$ CFU/g and 2.9 $\log_{10}$ CFU/g.

The overall mean concentration of *Campylobacter* spp. in fecal samples determined in this study was $2.43 \pm 0.9$ $\log_{10}$ CFU/g. A similar value of $2.1 \pm 0.45$ $\log_{10}$ CFU/g was reported in a Danish study [20]. A longitudinal study in New Zealand examined the concentration of *Campylobacter* spp. in feces from individual cows on pasture and in confinement housing [23]. The concentration of *C. jejuni* varied between 0 and 6.0 $\log_{10}$ CFU/g in the herd on pasture and between 0 and 5.7 $\log_{10}$ CFU/g in the confinement-housed herd. The median concentration of *Campylobacter* spp. in feces per cow was between 2.9 $\log_{10}$ CFU/g and 1.6 $\log_{10}$ CFU/g for the individual cows. Significant differences in the frequency and range of the *C. jejuni* concentrations occurred among individual cows. At least three cows in the two different herds were identified as high shedders of *Campylobacter* spp., with a median concentration between 3.3 $\log_{10}$ to 3.6 $\log_{10}$ CFU/g [23]. Our study underscores the previous finding that cows excrete *Campylobacter* spp. intermittently [14,23,26], although according to the criteria of the New Zealand study, the cows we sampled would not be classified as high shedders.

Overall, 29.2% of the boot sock samples tested positive for *Campylobacter* spp., with a mean concentration in positive samples of $3.01 \pm 1.05$ $\log_{10}$ CFU/2 socks. The samples were taken in the barn corridor, avoiding direct contact with fresh cowpats. The positive boot sock samples were found once each in January, May, June and July. In November, both boot sock samples were positive. Both the prevalence and the concentration values show that *Campylobacter* spp. can be found in the barn environment, and that this can represent a contamination risk throughout the year.

Another study found a higher prevalence (60%) of *Campylobacter* spp.-positive boot sock samples [27]. However, they did not avoid fresh dung pats, and it was described that all parts of the socks were in contact with the feces [27]. The intensity of the fecal contamination of the boot sock samples could explain the differences in the concentrations found in our study.

The finding of *Campylobacter* spp. in 12.2% of teat swabs with a mean concentration of $1.26 \pm 0.75$ $\log_{10}$ CFU/4 teats underlines that the teat skin can become contaminated with *Campylobacter* spp. The origin of these bacteria is likely the fecal contamination of the environment. The occurrence of *Campylobacter* spp. in teat swab samples was not at a specific minimum temperature (Figure 3), although the multi-level model indicated the minimum temperature as an effective parameter. However, the weak but reliable effect of the temperature on the occurrence of *Campylobacter* spp. was based on feces and teat swab

samples. An interaction term indicated that both sample types were influenced differently. However, the number of positive teat swab samples is too small to effectively analyze the effect. Further, the negative, qualitative positive and quantitative positive samples all lay in the same temperature range. The minimum temperature was used for the analysis since the teat swab samples were taken early in the morning. Previous *in vitro* studies demonstrated a slower inactivation of *C. jejuni* by oxygen at cooler temperatures [37–39]. This was not observed in our study, as all classes of results occurred in the same temperature range.

In this study, the raw milk samples were taken after the teats had been cleaned. No sterile milk sampling with teat disinfection was performed to mimic the routine milking situation. Only one raw milk sample (0.4%) was positive. The *Campylobacter* spp. concentration in the raw milk sample was 2.37 $\log_{10}$ CFU/mL. The contamination of the milk sample indicates that not all *Campylobacter* spp. had been removed from the teat skin by the routine cleaning process. A concentration of *Campylobacter* spp. of 2.74 $\log_{10}$ CFU/filter was detected in the milk filter on the same sampling day. The entry of *Campylobacter* spp. into the milk pipeline, as indicated by the positive milk filter, could have occurred through the transmission of *Campylobacter* spp. from the teat skin to the milk during milking, which is in line with the positive milk sample on the same day. In addition, the nine positive teat swab samples on the same sampling day indicated a cross-contamination event of the raw milk and milk filter samples. The milking clusters did not test positive for *Campylobacter* spp. and were therefore not assumed to be an entry source. A recent meta-analysis estimated the prevalence of *Campylobacter* spp. in raw milk samples at 1.18% [13]. Two studies estimated concentration data for *Campylobacter* spp. in raw milk using the MPN method [14,15]. They found low concentrations of $\leq$5 MPN/100 mL, but one outlier of 100 MPN/100 mL was detected [14]. In the other study, the *Campylobacter* spp. level of one sample was 1 CFU/21 mL of raw milk from the farm vats [15].

A recent study indicated that there is only a limited detection of *Campylobacter* spp. CFU in raw milk, possibly due to the *Campylobacter* spp. entering a viable but non-culturable (VBNC) state [40]. This was underlined by a newly developed viable qPCR using propidium monazide (PMA). This qPCR allows for the detection of intact and putatively infectious units (IPIUs) comprising CFU and VBNC bacteria. It demonstrated an underestimation of the survival of *Campylobacter* spp. with a difference of up to 4.5 $\log_{10}$ between the CFUs and IPIUs. Furthermore, within a certain time period, the CFUs of those IPIUs could be restored using a special "low-oxygen" atmosphere, confirming the viability of the bacteria [40]. However, in field samples, the application of the viable qPCR method is difficult due to the low concentration of *Campylobacter* spp. in raw milk, and the detection limit and application of different atmospheres is beyond the current ISO 10272-1.

Recent studies have shown that milk filter sampling is a potential approach to assessing the risk of *Campylobacter* spp. contamination in milk. The milk filter is installed at the end of the milking line so that all raw milk from all cows passes through it before ending up in the bulk tank. The reported prevalence of positive milk filters ranged from 0–14% [27,28,30,41]. In some studies, none of the concurrently collected bulk milk tank samples were positive, or a low prevalence was detected. In our study, one milk filter was positive, with a concentration of 2.74 $\log_{10}$ CFU/filter. On the same sampling day, nine teat swab samples and one raw milk sample were positive.

In summary, our data indicate a low risk of *Campylobacter* spp. contamination in the raw milk of the herd under study. Still, even in such a small herd, the contamination of milk can occur sporadically. Further research is required to better understand the reasons for sporadic contamination events.

We have used a multi-level logistic model to investigate the effective parameters on the presence of *Campylobacter* spp. in the feces and teat swab samples. The ICC shows that 11% of the variation in the occurrence was between the cows, whereas the rest of the variation happened throughout the year for each cow. The effect of the seasons was confirmed in the multi-level model, indicating the significant difference between the summer and winter.

Based on our model, temperature affects the concentration of *Campylobacter* spp. in teat and feces samples differently. However, other weather data were not shown to have an effect.

We used *E. coli* as a fecal contamination indicator since feces are considered the primary source of milk contamination during or after the milking process [42,43]. *Pseudomonas* spp. were used as an indicator of environmental contamination. We could show that the prevalence of *E. coli* was the highest in the feces, on the teat swab and in the boot sock samples. In contrast, the prevalence of *Pseudomonas* spp. was the highest on the teat swab and in the milk filter, milk equipment and raw milk samples. The *Escherichia coli* concentration data comprised a parameter effecting the occurrence of *Campylobacter* spp. in the feces and teat swab samples. This strengthens the assumption that the cross-contamination of teats with *Campylobacter* spp. had a fecal origin. However, *Pseudomonas* spp. and TACC had no effect on the occurrence. Another study assumed a relationship between the presence of *C. jejuni* in the bulk tank milk and a high load of *Enterobacteriaceae* in the same samples [6]. They found no association and supposed that fecal contamination might not be the only mechanism responsible for the presence of *C. jejuni* in raw milk. It has been suggested that udder infection may play a role in raw milk contamination, whereby the pathogen is directly excreted into the raw milk [6,44,45]. Unfortunately, the somatic cell count was not measured in any of these studies.

The health status of the studied cows is often not reported [18]. *Campylobacter* spp. commonly colonizes the intestine of asymptomatic cows [19,25,35,46–50]. Our data confirm that *Campylobacter* spp. are part of the intestinal microbiota of healthy dairy cattle.

In one sample, 7 July 2022, there was a somatic cell count of $4.7 \times 10^6$ cells/mL. In this sampling event, we found no association between the occurrence of *Campylobacter* spp. and the high somatic cell count. In the following sampling runs, this cow was excluded from the milk samples for as long as the local antibiotic treatment continued. The *Campylobacter* spp.-positive raw milk sample in this study had a low somatic cell count of $11 \times 10^3$ cells/mL. However, there was only one positive sample, and future research might therefore be necessary to determine whether there is an association of *Campylobacter* spp. with high somatic cell counts.

The scoring was used to either monitor whether the cows had diluted feces or a severe fecal contamination of the teats. In the experimental setup of this study, the teat scores were not found to be a parameter that influenced the occurrence *Campylobacter* spp. in the multi-level model. Another study also found no association between scores and the detection of *Campylobacter* spp. in bulk milk tanks, milk filters or feces. They demonstrated an association between cow hygiene and the detection of *Campylobacter* spp. in the teat milk [30]. However, they used a mean score calculated for the herd at each visit and not only a teat skin score directly related to the *Campylobacter* spp. concentration.

This study demonstrated that the contamination of raw milk with *Campylobacter* spp. was a rare event, although the cows were consistently colonized with *Campylobacter* spp. in the intestine, and cross-contamination of the teats with *Campylobacter* spp. did occur. On one sampling day, nine teat swab samples, one raw milk sample and the milk filter tested positive for *Campylobacter* spp. In terms of the annual study, we could not demonstrate a parameter that influenced this sporadic contamination event.

## 5. Conclusions

The obtained data can be integrated into risk assessments for *Campylobacter* spp. along the raw milk supply chain. The occurrence of *Campylobacter* spp. in feces differs between individual cows throughout the year. The season, *E. coli* concentration, minimum temperature and sampling type are effective parameters for the occurrence of *Campylobacter* spp. in feces and teat swab samples. No correlation was observed between the concentrations of *Campylobacter* spp. in feces and teat swab samples. Further research is required to explain sporadic *Campylobacter* spp. contamination in raw milk.

**Supplementary Materials:** The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/foods12081639/s1, Table S1: Detection limits of microorganisms. Table S2: Concentration data for *Campylobacter* spp. in feces of individual cows per sampling. Table S3: Concentration data for *Campylobacter* spp. on teat swab samples of individual cows per sampling.

**Author Contributions:** Conceptualization, A.-D.K., K.S., N.B. and T.C.; formal analysis, A.-D.K. and N.G.; investigation, A.-D.K. and S.G.; resources, C.F.-T.; data curation, A.-D.K., N.G. and T.C.; writing—original draft preparation, A.-D.K.; writing—review and editing, A.-D.K., S.G., N.G., K.S., C.F.-T., N.B., B.-A.T. and T.C.; visualization, A.-D.K., N.G. and T.C.; supervision, T.C.; project administration, T.C. All authors have read and agreed to the published version of the manuscript.

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**Data Availability Statement:** Data is contained within the article or Supplementary Material.

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**Conflicts of Interest:** The authors declare no conflict of interest.

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## 3.4 Publication 3: Modeling the survival of *Campylobacter jejuni* in raw milk considering the viable but non-culturable cells (VBNC)



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ORIGINAL ARTICLE

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# Modeling the survival of *Campylobacter jejuni* in raw milk considering the viable but non-culturable cells (VBNC)

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**Abstract**

*Campylobacter* spp. cannot grow in raw milk, but it is able to transform into a viable but non-culturable (VBNC) state enabling the survival in such harsh conditions. In this study, *Campylobacter jejuni* survival in raw milk was investigated taken into consideration colony-forming units (CFUs) and VBNC cells. CFU from two different strains of *C. jejuni* (DSM 4688 and BfR-CA-18043) were enumerated at three temperatures (5°C, 8°C, and 12°C). In parallel, a viability real-time PCR was conducted to quantify intact and putatively infectious units (IPIUs) (comprising CFU and VBNC bacteria). The data generated were used to model the viability of *C. jejuni* during raw milk storage. Here, a one-step fitting approach was performed using parameter estimates from an intermediate two-step fit as starting values to generate tertiary models. Different primary model equations (Trilinear and Weibull) were required to fit the CFU and the IPIU data. Strain-specific linear secondary models were generated to analyze the effect of storage temperature on the maximum specific inactivation rate of the CFU data. The time of the first decimal reduction parameter of the IPIU models could be modeled by a strain-independent linear secondary model. The developed tertiary models for CFU and IPIU differ significantly in their predictions, for example, for the time required for a one $\log_{10}$ reduction. Taken into consideration that VBNC could revert to a culturable state during the raw milk storage, our results underline the importance of considering IPIU and not only CFU to avoid underestimation of the survival of *C. jejuni* in raw milk.

## 1 | INTRODUCTION

Campylobacteriosis is the most commonly reported foodborne gastrointestinal infection in humans in the European Union (EU) with 127,840 reported cases in 2021 (EFSA, 2022). Typical acute symptoms of campylobacteriosis are diarrhea, abdominal pain, and fever. In addition, in few cases, reactive arthritis, irritable bowel syndrome, and neurological complications such as Guillain Barré syndrome can also occur as long-term complications (Jackson et al., 2014; Keithlin et al., 2014; Leonhard et al., 2019). *Campylobacter* infections in humans primarily originate from contaminated raw meat and raw milk, direct contact with colonized animals or consumption of contaminated untreated water (Kaakoush et al., 2015; Mughini-Gras et al., 2016; Rosner et al., 2017). *C. jejuni* is the most common *Campylobacter* species in human infections and the main route of zoonotic transmission to humans might occur from fecal cross-contamination of raw meat or raw milk (Del Collo

et al., 2017; EFSA, 2011; Knipper et al., 2023). However, the prevalence data of *Campylobacter* spp. in feces of dairy cows herds vary in scientific literatures (0–100%) (Knipper et al., 2022).

In 2020, four campylobacteriosis outbreaks associated with milk and milk products were reported in the EU. The food vehicles had been attributed with "strong-evidence" (EFSA, 2021). These outbreaks might be associated with a high consumer demand for raw milk (Oliver et al., 2009) and the intensification of local sales via raw milk vending machines (TMR, 2016). Raw milk should be cooled down after milking to less than 4°C and maintained at this temperature during transportation and storage in vending machines (EFSA, 2015). Although most vending machines are equipped with a cooling device and an agitator to ensure homogenous cooling of the raw milk (EFSA, 2015), average temperature of raw milk measured directly on site was 7.7 ± 3.8°C and one third of the samples had a temperature exceeding 8°C with the maximum of 18.6°C (Böhnlein et al., 2020). Surveys in Italy illustrated that 13.9%–43% of consumers did not boil raw milk before consumption (Giacometti et al., 2012; Giacometti et al., 2013).

*C. jejuni* only grows under special conditions, that is, at high temperatures and low oxygen levels, and in general, its non-thermal inactivation by oxygen is slower at cold temperatures (Boleratz & Oscar, 2022; Olofsson et al., 2015; Yoon et al., 2004). Additionally, *Campylobacter* spp. is highly adapted to surviving harsh conditions by undergoing the "viable but non-culturable" (VBNC) state. In that state, they are no longer detectable by cultural detection methods (Baffone et al., 2006), but are able to revive its infectious potential under specific conditions (Baffone et al., 2006; Federighi et al., 1998; Rollins & Colwell, 1986; Wulsten et al., 2020).

Only a few studies investigated the survival of different strains of *Campylobacter* spp. in raw, skimmed or unpasteurized milk at distinct temperatures (Boleratz & Oscar, 2022; Christopher et al., 1982; Doyle & Roman, 1982; Jaakkonen et al., 2020; Wulsten et al., 2020), and only one investigated the possibility of survival of *Campylobacter* spp. in VBNC state in raw milk (Wulsten et al., 2020). In this study, Wulsten et al. (2020) used a newly developed viability real-time PCR (v-qPCR) with propidium monoazide (PMA) staining. This method enables to specifically quantify intact and putatively infectious units (IPIU)—that is, all viable bacteria—comprising colony-forming units (CFUs) as well as VBNC bacteria. Consequently, it was shown that *C. jejuni* survival in raw milk would be highly underestimated when using only CFU data, since up to 4.5 $\log_{10}$ more IPIU than classically enumerated CFU were detected. They also demonstrated that these IPIU could be reverted to the culturable state within an experimental time window by extremely lowering the partial pressure of oxygen (Wulsten et al., 2020).

Predictive microbiology models that describe the growth or inactivation of foodborne pathogens and spoilage microorganisms in foods are helpful tools to assess food safety and quality risks and are used to support decisions in regulatory agencies and food industries (Pérez-Rodríguez & Valero, 2013). The high sensitivity of *C. jejuni* to oxygen and other environmental stressors (e.g., pH, temperature) (Al-Qadiri et al., 2015; Christopher et al., 1982; Doyle & Roman, 1982; Kim et al., 2017) together with the limitation of available quantitative detection methods not only makes *C. jejuni* difficult to be cultured and enumerated, but also leads to highly variable results. Therefore, data for model development and validation are still limited in this area and to our knowledge, just one predictive mathematical model describing the survival of *C. jejuni* in milk have been developed so far, but the authors did not take into account the IPIU data, but just the CFU data (Boleratz & Oscar, 2022).

According to the definition proposed by Whiting and Buchanan (1993), the data analysis in predictive microbiology research follows a standard approach of two-step fitting process. In the first step, a primary model describing bacterial growth/inactivation/survival over time under constant environmental conditions (e.g., temperature, humidity and water activity) is generated. In the second step, a suitable secondary model is used to analyze the effect of temperature and other environmental conditions on the kinetic parameters of the primary models (e.g., growth rate, lag time, or *D* value). Once the primary and secondary models are developed, they are combined as a tertiary model to predict the final number of microorganisms over the time under the different environmental conditions (Baranyi et al., 2017). However, a one-step approach has also been widely used in kinetic analysis. In this one-step analysis, the primary and secondary models are analyzed together during the estimation of kinetic parameters (Dolan & Mishra, 2013; Huang, 2017; Jewell, 2012).

The objectives of the present study were:

1. obtain reliable experimental data on the survival of *C. jejuni* in raw milk, taking into consideration not only CFU data but also IPIU data and recovery data of non-culturable cells;
2. model and predict the survival of *C. jejuni* based on the CFU and IPIU data;
3. compare the survival kinetics of CFU and IPIU based models and analyze the differences between the survival estimates.

## 2 | MATERIALS AND METHODS

### 2.1 | Experimental data generation

#### 2.1.1 | Preparation of inoculum

The *C. jejuni* DSM 4688 reference strain was obtained from the DSMZ strain collection (DSMZ, Braunschweig, Germany). The field strain BfR-CA-18043 (Sequence type [ST]-21 according to multilocus sequence typing scheme [Dingle et al., 2001; Jolley et al., 2018]) isolated from feces of dairy cow at the National Reference Laboratory (NRL) for *Campylobacter* at the German Federal Institute for Risk Assessment (BfR). In addition, the strain *C. jejuni* BfR-CA-18040 (ST-61) also obtained from fecal sample from dairy cow, was analyzed for model validation purposes, but just at 5°C.

Strains were stored at −80°C in cryocultures (MAST Group Ltd., Bootle, UK). Bacteria were cultured on Columbia blood agar (ColbA, Oxoid, Thermo Fisher Scientific Inc., Waltham, MA) supplemented with 5% defibrinated sheep blood (Oxoid) at 41.5°C under microaerobic conditions (5% $O_2$, 10% $CO_2$, rest $N_2$) for 24 h. After subculture on ColbA 18 ± 2 h under the same conditions, cells were resuspended in Brucella

broth (Becton Dickinson, Franklin Lakes, NJ) at $OD_{600} = 0.2$, corresponding to ~9 $log_{10}$ cell counts per mL (Krüger et al., 2014). Furthermore, a 10-fold dilution was performed to reach 8 $log_{10}$ cell counts per mL, which was kept on ice before spiking of the raw milk.

### 2.1.2 | Inoculation of raw milk

Raw milk samples were spiked with one of the mentioned *C. jejuni* strains to reach an initial concentration of 6 $log_{10}$ cells/mL raw milk. Inoculated raw milk samples were kept in closed 50-mL tubes and incubated at 5°C ± 0.54°C (accredited refrigerator with Sirius storage monitoring software), 8 ± 0.1°C or 12 ± 0.1°C for up to 97 h under normal atmospheric conditions. A data logger (plug & track, France) monitored the temperature for 8°C and 12°C at 30 min intervals. pH was measured (Mettler Toledo, Columbus, OH) throughout the experiment and was stable at 6.8 ± 0.041 compared to a control sample at each temperature.

### 2.1.3 | Study of the survival of *C. jejuni*

Samples were analyzed at three different temperatures (5°C, 8°C, and 12°C) by quantifying CFU and v-qPCR analysis in parallel. A schematic overview of the experimental set-up is shown in Figure 1. The range of temperatures was selected according to the experiments carried out by Böhnlein et al., 2020. For CFU quantification of thermophilic *Campylobacter* spp. modified charcoal cefoperazone deoxycholate agar (mCCDA) (mixture of Merck & Co., Kenilworth, NJ and Oxoid) was used according to ISO 10272-2:2017 with a theoretical sensitivity of 1 CFU/mL milk. The mCCDA was incubated for 48 h at 41.5°C under microaerobic atmosphere.

In order to verify if the survival of *C. jejuni* in raw cow milk could be reproduced in previously frozen cow milk, some preliminary analyses were necessary. For that purpose, the same batch of raw bulk tank milk obtained from the institute's dairy herd was used. Fresh raw milk was kept at 4°C until processing, but not longer than 4 h after milking. Previously frozen raw milk batches were frozen at −20°C before use up to 100 days. For the preliminary experiments, we used the DSM 4688 strain in fresh and frozen raw milk at 5°C. Comparison of the results showed that there were no difference between them and therefore we carried out our experiments with previously frozen milk. This preliminary data obtained for the DSM 4688 strain at 5°C in fresh raw milk was subsequently used for validation purposes.

### 2.1.4 | Viability real-time PCR (v-qPCR)

Live and dead *C. jejuni* cells were differentiated according to already published methods (Pacholewicz et al., 2019; Stingl et al., 2021; Wulsten et al., 2020), as a previous step to the v-qPCR.

In brief, from each raw milk sample two aliquots of 1 mL were needed; one was processed with the DNA intercalating agent PMA (Biotium Inc., Hayward, CA) (only viable cells) and the other was used without PMA (total amount of cells). First, to enable the PMA staining both aliquots were 10-fold diluted in 9 mL of precooled Brucella broth and centrifuged at 8000 × *g* for 20 min at 4°C using 15 mL centrifugation tubes. Supernatants were discarded, pellets suspended in 1 mL PBS (Wulsten et al., 2020) and transferred to a 1.5 mL tube and stored on ice until PMA staining. Afterwards an internal sample process control (ISPC) of dead *C. sputorum* cells (Pacholewicz et al., 2019) was used at different concentrations (ISPC A $10^8$ copies/mL, ISPC B $10^6$ copies/mL) (Stingl et al., 2021) to confirm reliability of the PMA staining and monitor DNA loss during processing.

The sample and a control (1 mL peptone water) were treated with PMA as follows: 2.5 µL of a 20 mM PMA solution in 20% DMSO (Dimethylsulfoxide, Carl Roth, Karlsruhe, Germany) and 10 µL of ISPC A were added, vortexed and incubated for 15 min at 30°C and 700 rpm in the dark. Crosslinking was performed for 15 min at room temperature using the PhAST Blue photo-activation system (GenIUL, Terrassa, Spain) at 100% light intensity. After crosslinking samples were placed on ice for 2 min, 10 µL ISPC B was added, centrifuged at 4°C at 16,000 × *g* for 5 min and the supernatant was discarded. The cell pellets were stored at −20°C until DNA extraction.

Samples and controls (again 1 mL peptone water) without staining were placed on ice. One of the four controls received 10 µL ISPC A. Ten microliters of ISPC B were added to all controls and the samples, vortexed and centrifuged at 4°C at 16,000 × *g* for 5 min. The supernatants were discarded and cell pellets were stored at −20°C until DNA extraction.

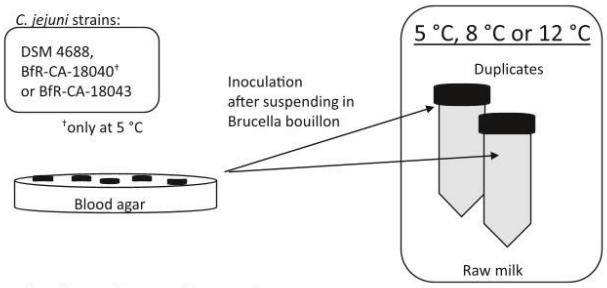
DNA extraction for v-qPCR was performed using the GeneJet Genomic DNA extraction kit (Thermo Fisher Scientific Inc.) according to the manufacturers' protocol using 100 µL elution volume. A negative-DNA-extraction control was included for quality assurance. A volume of 10 µL of the extracted DNA of all samples and controls was analyzed in duplicate by v-qPCR.

Avoiding a competition of the *Campylobacter* signal on the ISPC signal, based on the high load of thermophilic *Campylobacter* in our setting, two duplex v-qPCR were applied (Stingl et al., 2021). One duplex v-qPCR was targeting thermophilic *Campylobacter* and IPC-ntb2 plasmid as an internal amplification control (IAC). The other one was targeting *C. sputorum*, the internal process control (ISPC), and the IAC. Genomic DNA standards from *C. jejuni* NCTC 11168 and *C. sputorum* DSM 5363 as well IPC-ntb2 plasmid as internal amplification control were prepared accordingly. qPCR was performed targeting a fragment of the 16S rRNA gene of *C. jejuni* or of *C. sputorum*. Furthermore, qPCR was applied on a QuantStudio 5 Real-Time PCR system (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA).

Quantitative measurement was ensured by genomic standards, comprising five decile serial dilutions ranging from 50,000 to 5 genomic copies per reaction included in each qPCR run and results were analyzed using the excel sheet for analysis (Stingl et al. (2021), Suppl. Information 3).

### 2.1.5 | Recovery of viable but non-culturable cells

Wulsten et al. (2020) demonstrated that *C. jejuni* VBNCs could return into a cultivable state (recovery) within a certain time period after incubation in raw milk. However, their experiments were carried without covering the whole range of temperatures included in our study,




**Quantification methods and sampling points**

| | | | 0 h | 3h | 6 h | 7 h | 9 h | 10 h | 24 h | 25 h | 29h | 30 h | 31 h | 48 h | 49 h | 72 h | 73 h | 80 h | 97 h |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| ISO 10272-2 | DSM 4688 / BfR-CA-18043 | 5°C | | | | | | | | | | | | | | | | | |
| | | 8°C | | | | | | | | | | | | | | | | | |
| | | 12°C | | | | | | | | | | | | | | | | | |
| | BfR-CA-18040‡ | 5°C | | | | | | | | | | | | | | | | | |
| Recovery of VBNC | DSM 4688 / BfR-CA-18043 | 8°C | | | | | | | | | | | | | | | | | |
| | | 12°C | | | | | | | | | | | | | | | | | |
| v-qPCR | DSM 4688 / BfR-CA-18043 | 5°C | | | | | | | | | | | | | | | | | |
| | | 8°C | | | | | | | | | | | | | | | | | |
| | | 12°C | | | | | | | | | | | | | | | | | |
| | BfR-CA-18040‡ | 5°C | | | | | | | | | | | | | | | | | |

‡ Just for validation purposes

**FIGURE 1** Overview of experimental set-up for spiking of raw milk and culture-dependent plus culture-independent method for quantification of viable *Campylobacter jejuni* in raw milk.

but just at 5°C. In order to verify, if the recovery of VBNC could be possible under our experimental conditions and to know the percentage of VBNC that could be recovered, the raw milk samples were spiked as described before and controlled by platting on mCCDA under standard incubation condition. Recovery of CFU in *C. jejuni* was reached using a gas mixture with extremely low oxygen partial pressure, by incubation for 4–5 days at 37°C under 3.5% $H_2$, 1% $O_2$, 10% $CO_2$, and rest $N_2$ (Wulsten et al., 2020). Samples of 100 µL were taken at different time points up to 80 h after inoculation, leading to a theoretical detection limit of 10 CFU/mL per mL of milk.

Recovery rates were calculated as the percentage of cells that are culturable compared to the inoculum as follow:

$$\text{Recovery rate} = \frac{\text{Concentration of culturable cells}\left(\frac{\log_{10}\text{CFU}}{\text{mL}}\right)}{\text{Inoculum}\left(\frac{\log_{10}\text{CFU}}{\text{mL}}\right)} * 100 \quad (1)$$

## 2.2 | Data analysis and modeling

### 2.2.1 | Model generation process

Experimental CFU and IPIU data from the DSM 4688 and the BfR-CA-18043 strains (at 5°C, 8°C, and 12°C) were included for the model generation process (Figure 1).

To model *C. jejuni* survival in raw milk, a one-step fitting approach was performed analyzing primary and secondary models together during the estimation of kinetic parameters. However, in order to make an initial estimate of the parameters to be used as a starting point for the one-step fitting approach, an intermediate two-step fitting approach was carried out, fitting the primary and secondary models separately. The models created under the one-step fitting approach are referred in this paper as tertiary models (Baranyi et al., 2017).

For the initial two-step fitting approach, different primary model equations were fitted to all individual CFU ($\log_{10}$ CFU/mL vs. time) and IPIU data ($\log_{10}$ IPIU/mL vs. time) obtained from three constant temperatures (5°C, 8°C, and 12°C).

Fitting was performed using R 4.2.1 (R Core Team, 2020) and R package "nlsMicrobio" (Baty & Delignette-Muller, 2014). Generated models were compared pair-wise through an *F*-test using R 4.2.1 (R Core Team, 2020) to identify the least complex primary model equation needed.

The three kinetic parameter models (Geeraerd with $S_l$ [Geeraerd et al., 2005] and Trilinear [Buchanan & Golden, 1995]) had the best goodness-of-fit for the CFU data compared to the two kinetic parameter models without shoulder as indicated by the lower root mean squared error (RMSE) and the higher determination coefficient ($R^2$) values and *F*-test (Table S1). The Geeraerd with $S_l$ and Trilinear models have a similar goodness-of-fit and were able to accurately describe the experimental data. However, the Trilinear model (Equation 2) was simpler and it was therefore selected for the present study as follow:

$$\log_{10}(N_t) = \log_{10}(N_0), t < S_l \quad (2)$$

$$\log_{10}(N_t) = \log_{10}(N_0) - \frac{k_{max}}{\ln(10)} * (t - S_l), S_l \le t \le S_t$$

$$\log_{10}(N_t) = \log_{10}(N_{res}), t \ge S_t$$

where $N_0$ is the initial concentration (CFU/mL), $N_t$ is the bacterial concentration (CFU/mL) at time $t$ (h), $N_{res}$ is the residual population density, $k_{max}$ is the maximum specific inactivation rate (1/h), $S_l$ the duration of shoulder effect (h), and $S_t$ is the time arriving tail (h). This last parameter was calculated using Equation (3):

$$S_t = S_l + (\log_{10}N_0 - \log_{10}N_{res}) * \frac{\ln 10}{k_{max}} \quad (3)$$

For the IPIU data set, the Weibull model (Equation 4) had the best goodness-of-fit compared to the other primary models tested (Table S2) and was used as follow:

$$\log_{10}(N_t) = \log_{10}(N_0) - \left(\frac{t}{\delta}\right)^p \quad (4)$$

where $\delta$ is the time for the first decimal reduction (h) and $p$ is the shape parameter (van Boekel, 2002).

The effect of temperature on the primary inactivation rate parameter of the Trilinear model $k_{max}$, as well as of the Weibull model parameter $\delta$, was modeled by a linear secondary model (Equations 5 and 6):

$$\log_{10}k_{max} = \text{Intercept} + \text{Slope} * T \quad (5)$$

$$\log_{10}\delta = \text{Intercept} + \text{Slope} * T \quad (6)$$

where $T$ is the temperature in °C.

To decide whether temperature influenced the survival of the reference and the field strains differently, independent secondary models were generated and compared using an $F$-test.

Finally, the parameters estimated in the two-step fitting procedure were used as starting values for the one-step fitting approach. In this way, models could be generated that directly link observed concentration data with environmental conditions. In case of the CFU data, we substituted $k_{max}$ in the Trilinear model equation (Equation 2) by the linear secondary model equation (Equation 5).

$$\log_{10}(N_t) = \log_{10}(N_0), t < S_l$$

$$\log_{10}(N_t) = \log_{10}(N_0) - \frac{10^{(\text{Intercept}+\text{Slope}*T)}}{\ln(10)} * (t - S_l), S_l \le t \le S_t$$

$$\log_{10}(N_t) = \log_{10}(N_{res}), t \ge S_t \quad (7)$$

Furthermore, the mean of $S_l$ values obtained in the primary model fitting step on all examined temperatures was used as starting value for fitting the parameter $S_l$ in the tertiary model.

To obtain a tertiary model for all IPIU data the linear model for $\delta$ (Equation 6) was integrated into the Weibull model equation (Equation 4).

$$\log_{10}(N_t) = \log_{10}(N_0) - \left(\frac{t}{10^{(\text{Intercept}+\text{Slope}*T)}}\right)^p \quad (8)$$

The mean of $p$ values obtained in the primary model fitting step on all examined temperatures was used as starting value for fitting the parameter $p$ in the tertiary model.

The value of $N_0$ was determined by the experimental set-up and fixed to 5.7 $\log_{10}$ CFU/mL or 5.54 $\log_{10}$ IPIU/mL, respectively. To simplify the model fitting process we assigned to all data points below the detection limit of 1 CFU/mL and to the residual population density ($\log_{10}$ $N_{res}$) parameter a value of −0.1 $\log_{10}$ CFU/mL. This information and the implications for the interpretation of predicted values below 1 CFU/mL is provided in the annotation of the model files (see below).

The goodness-of-fit of the created tertiary models were documented by calculating RMSE and $R^2$ values. Additionally, visual evaluation of the fitted curves was performed.

To check if different tertiary models were necessary for the different strains (reference and field strain), the obtained tertiary models for the different strains were compared using an $F$-test.

### 2.2.2 | Model validation

The three tertiary models generated for the *C. jejuni* survival in raw milk were validated with the following data:

- Independent experimental data from the DSM 4688 and the BfR-CA-18043 strains not used during the model generation phase (at 8°C and 12°C)
- Data generated in fresh raw milk from the DSM 4688 strain at 5°C
- Data generated from a different *C. jejuni* strain (BfR-CA-18040) at 5°C

In order to decide if these data were eligible for the model validation purpose an unpaired $t$-test was performed. For the DSM 4688 strain no significant difference in $k_{max}$ estimates (for CFU data) or $\delta$ estimates (for IPIU data) could be identified when comparing the experiments carried out with previously frozen raw milk versus fresh raw milk at 5°C. There was also no significant difference between the survival of the two field strains, BfR-CA-18040 and BfR-CA-18043, at 5°C. Therefore, the data for fresh raw milk, that was not previously frozen, and the data from the BfR-CA-18040 strain were considered as

suitable for the validation of the related tertiary models for the DSM 4688 strain and the BfR-CA-18043, respectively.

In addition, a systematic search was carried out in the literature and in ComBase database in order to find suitable data for the external validation of the model.

The model performance was described by a graphical validation and by calculation of RMSE and $R^2$ values for predicted concentration values versus the corresponding validation data.

#### 2.2.3 | Model exchange and reusability

The final CFU data derived models for the *C. jejuni* (DSM 4688 and BfR-CA-18043 strains) as well as the IPIU data derived model were converted into the Food Safety Knowledge Exchange (FSKX) format, with the aim of improving transparency in the model generation process and facilitate the exchange and reusability of the models created. These FSKX files hold all model parameter estimates, the raw experimental data and all relevant metadata, including a description of the model's range of applicability. The model files can be accessed and downloaded via the following model repository: https://knime.bfr.berlin/landingpage/RAKIP-Model-Repository, and executed in the open-source software solution FSK-Lab (de Alba Aparicio et al., 2018).

### 2.3 | Comparison of the survival kinetics

To enable a comparison of the survival kinetics of the CFU and IPIU data derived models, the time required to obtain an x $\log_{10}$ reduction ($t_{xd}$) was calculated (Buchanan et al., 1993). The $t_{xd}$ for the CFU and IPIU models was calculated for x = 1, 2, 3, or 4 $\log_{10}$ based on the fitted parameters of the generated tertiary models.

The $t_{xd}$ for the CFU data was calculated using Equation (9) (Patil et al., 2010).

$$t_{xd} = S_l + (x) * \frac{\ln(10)}{k_{max}} \quad (9)$$

The $t_{xd}$ for the IPIU data was calculated using Equation (10) (Patil et al., 2010).

$$t_{xd} = \delta * (x)^{\frac{1}{\rho}} \quad (10)$$

Normal distribution and homogeneity of variances were tested using Shapiro–Wilk test and Levene-test, respectively. Homogeneity of variances was not found. Therefore, the differences between obtained $t_{xd}$ values from CFU and IPIU data of DSM 4688 or BfR-CA-18043 were compared pairwise performing a Wilcoxon signed-rank test.

For all statistical tests applied in this study the 0.05 significance level was applied.

## 3 | RESULTS

### 3.1 | Experimental data

For both data sets (CFU and IPIU), the measured data points of *C. jejuni* strain BfR-CA-18043 and strain DSM 4688 in raw milk exposed to 5°C, 8°C, and 12°C are shown in Figure 2. CFU survival of DSM 4688 strain was lower compared to the BfR-CA-18043 strain (Figure 2, dots). Only low concentrations up to a maximum of 0.9 $\log_{10}$ CFU/ml of the strain BfR-CA-18043 were detected at 49 h. *C. jejuni* CFU cell concentration for both strains (DSM 4688 and BfR-CA-18043) were below the detection limit from the next sampling time point (72–73 h) onwards.

The IPIU data showed no strain and temperature-specific differences in *C. jejuni* survival (Figure 2, triangles).

Recovery of *C. jejuni* strain DSM 4688 from the VBNC state into a culturable state was shown by extremely lowering the partial pressure of oxygen at 8°C and 12°C. In addition, with the same method strain BfR-CA-18043 displayed time-dependent recovery of CFU from VBNC within 80 h of incubation in raw milk at 8°C and 12°C (Figure S1). At 12°C the BfR-CA-18043 strain was recovered up to 72 h while the DSM 4688 strain was only recovered up to 48 h. Recovery rates varied widely (Table 1) not only between strains and temperatures, but also within strains at the same temperature conditions. Lower recovery rates were observed over time.

### 3.2 | Modeling process

#### 3.2.1 | Intermediate two-step fit: primary models

Figure 2 shows the selected primary models for CFU and IPIU data. Due to the large differences in the survival kinetics of the cell populations, two different primary model equations had to be used to model the survival of *C. jejuni*.

#### 3.2.2 | Intermediate two-step fit: secondary models

For the CFU data, a linear relation could be observed between the $\log_{10}$-transformed survival rates ($\log_{10}k_{max}$) and the storage temperatures. This relationship was modeled in two secondary models with Equation (11) for the DSM 4688 strain (RMSE = 0.05) and Equation (12) for the BfR-CA-18043 strain (RMSE = 0.09).

$$\text{DSM 4688 } \log_{10}k_{max} = -0.35\,(\pm 0.05) + 0.014\,(\pm 0.005) * T \quad (11)$$

$$\text{BfR} - \text{CA} - 18043\ \log_{10}k_{max} = -0.60\,(\pm 0.07) + 0.026\,(\pm 0.008) * T \quad (12)$$

Comparison of the 95% confidence intervals of the estimated slopes and intercepts of these linear secondary models showed overlap in values, requiring an *F*-test (Table S3). The *F*-test indicated that

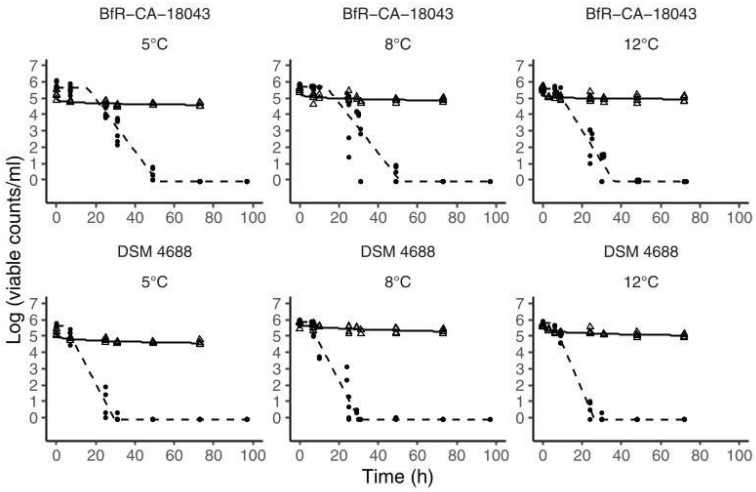



**FIGURE 2** Survival kinetics for *Campylobacter jejuni* strain BfR-CA-18043 (top row) and strain DSM 4688 (bottom row) in raw milk at 5°C, 8°C, and 12°C storage. Data points indicate colony forming units (CFU, •) and intact and putatively infectious units (IPIU, Δ). Lines show the fit with the Trilinear model for CFU data (dashed line) and the Weibull model for IPIU data (solid line). Detection limit of CFU data was 1 CFU/mL.

**TABLE 1** Recovery rates of *Campylobacter jejuni* strains DSM 4688 and BfR-CA-18043.

| | *T* (°C) | 9 h | 24 h | 30 h | 48 h | 72 h | 80 h |
|---|---|---|---|---|---|---|---|
| DSM 4688 | 8 | | 44.3–70% | | 0–40% | 0–23.3% | 0–22% |
| | 12 | 75–100% | | 0–22% | 0–22% | 0% | |
| BfR-CA-18043 | 8 | | 74.5–82% | | 36.67–78.8% | 23.3–60% | 21–38% |
| | 12 | 80–100% | | 0–73.2% | 0–65.83% | 0–28.2% | |

*Note*: The minimum and maximum recovery rates are shown for each time–temperature and strain combination.

two separate models were needed for the CFU data of the DSM 4688 and BfR-CA-18043 strain, which showed the different impact of the temperature on the two *Campylobacter* strains.

As also shown in recent publications, due to the high variability found in the shoulder region, a secondary model for the $S_l$ parameter was not possible (Pérez-Rodríguez & Valero, 2013).

Regarding IPIU data, a linear equation for the secondary model was used to describe the $\log_{10}$-transformed primary model parameter $\delta$. For the DSM 4688 strain, the model in Equation (13) yield an RMSE of 0.8 whereas the model for BfR-CA-18043 data Equation (14) had an RMSE of 0.74.

$$\text{DSM 4688 } \log_{10}\delta = 2.83\,(\pm 0.77) + 0.028\,(\pm 0.09) * T \quad (13)$$

$$\text{BfR} - \text{CA} - 18043\ \log_{10}\delta = 3.07\,(\pm 0.72) + 0.02\,(\pm 0.08) * T \quad (14)$$

The comparison of the 95% confidence intervals of the model parameters from the two strains indicated that there is no significant difference between the two obtained equations (Table S4). Also, an *F*-test indicated that a single model (Equation 15) was sufficient to describe the IPIU data. This model yielded a RMSE and $R^2$ of 0.44 and 0.53 over all data.

$$\log_{10}\delta = 2.95\,(\pm 0.51) + 0.023\,(\pm 0.06) * T \quad (15)$$

### 3.2.3 | One-step fitting: tertiary models

The estimated parameters from the intermediate two step-approach were used as starting point to generate tertiary models. For the CFU data-derived tertiary models the estimated $R^2$ and RMSE values are shown in Table 2. In general, the CFU data-derived tertiary models predicted the inactivation rate of the corresponding strains reasonably well, as indicated the by RMSE and $R^2$ values.

In Figure 3, the relationship between the predictions and the experimental CFU data for DSM 4688 and BfR-CA-18043 are shown. As it can be seen, in the specific case of the strain BfR-CA-18043, the wide scattering of the experimental data led to models that cannot provide an "accurate" prediction for the survival of *C. jejuni* at some time points.

The estimated $R^2$ and RMSE values for the developed IPIU data-derived tertiary model are shown in Table 3. The developed IPIU-derived tertiary model is able to predict the bacterial concentration with a variation of about 0.5 $\log_{10}$ IPIU/mL (Figure 4).

**TABLE 2** Estimated parameters and goodness-of-fit values for the developed CFU data-derived tertiary models of *Campylobacter jejuni* strain DSM 4688 and BfR-CA-18043.

| | | Number of observations for model development | | Model parameters | | | Goodness-of-fit value | |
|---|---|---|---|---|---|---|---|---|
| Tertiary model | Temperature range (°C) | Data points | Survival curves | Intercept | Slope | Shoulder (h) | RMSE | $R^2$ |
| DSM 4688 | 5–12 | 96 | 14 | −0.31 (−0.37 to 0.24) | 0.007 (0.00–0.02) | 5.13 (4.30–5.97) | 0.41 | 0.98 |
| BfR-CA-18043 | 5–12 | 132 | 20 | −0.67 (−0.74 to 0.59) | 0.05 (0.04–0.06) | 15.99 (13.55–18.43) | 0.65 | 0.93 |

*Note*: Numbers in parentheses are 95% confidence intervals.

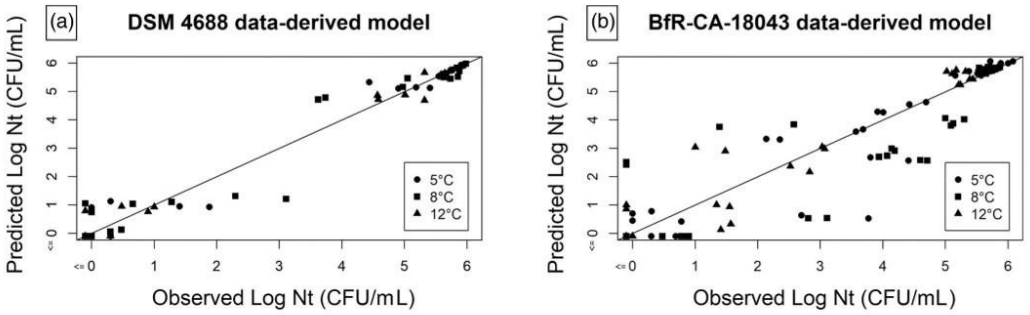



**FIGURE 3** Relationship between predicted-concentrations ($\log_{10} N_t$) from the (a) DSM 4688-derived and (b) BfR-18043-derived tertiary models and the data used for the model development. Non-detects were assigned a value of −0.1 log CFU/mL.

**TABLE 3** Estimated parameters and goodness-of-fit for the developed tertiary model derived from IPIU data of *Campylobacter jejuni* strain DSM 4688 and BfR-CA-18043.

| | | Number of observations for model development | | Model parameters | | | Goodness of-fit-value | |
|---|---|---|---|---|---|---|---|---|
| Tertiary model | Temperature range (°C) | Data points | Survival curves | Intercept | Slope | *p* | RMSE | $R^2$ |
| DSM 4688 and BfR-CA-18043 | 5–12 | 180 | 32 | 0.68 (−0.17 to 1.52) | 0.31 (−0.09 to 0.53) | 0.15 (0.06–0.24) | 0.24 | 0.53 |

*Note*: Numbers in parentheses are 95% confidence intervals.

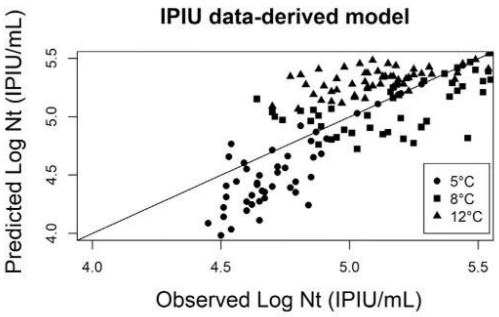



**FIGURE 4** Relationship between predicted-concentrations ($\log_{10} N_t$) from the intact and putatively infectious units (IPIU) derived tertiary model and data used for model development.

### 3.2.4 | Model validation

The results of the model validation are provided in Figures 5 and 6, Tables S5 and S6.

For the DSM 4688-derived models, independent data from *C. jejuni* strain DSM 4688 in raw milk (5°C) and in previously frozen raw milk (8°C, 12°C) were used. The BfR-CA-18043 derived model was validated with data from *C. jejuni* BfR-CA-18043 (8°C, 12°C) in raw milk and BfR-CA-18040 (5°C) in previously frozen raw milk. The variable evaluated for the model validation was $N_t$.

As indicated by the RMSE and $R^2$ values for each experimental run (Tables S5 and S6), the CFU data-derived tertiary models are able to make accurate predictions. Validation of the internal experimental data (Figure 5) showed that most of the CFU data were under the equivalence line for the DSM 4688 data-derived model

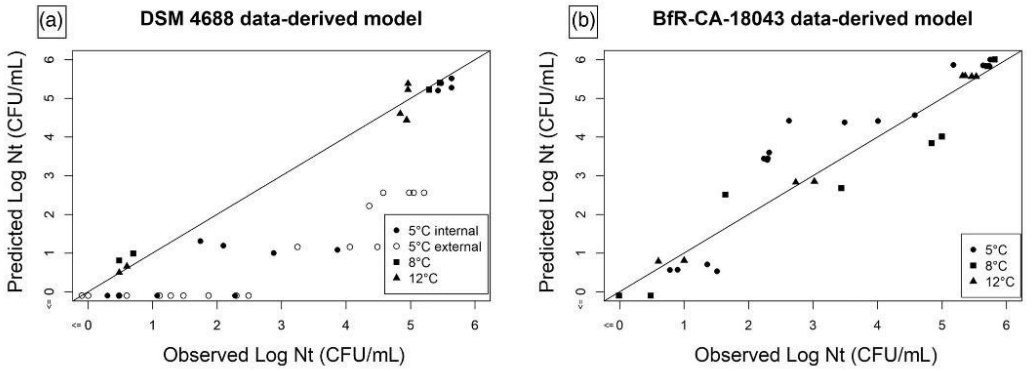



**FIGURE 5** Validation of the predicted-concentrations ($\log_{10} N_t$) from the (a) DSM 4688 and (b) BfR-CA-18043 derived tertiary models and independent (internal and external) experimental validation data. Non-detects were assigned a value of −0.1 log CFU/mL.

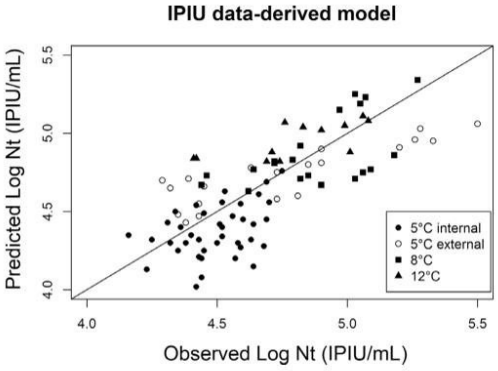



**FIGURE 6** Validation of the predicted-concentrations ($\log_{10} N_t$) from the intact and putatively infectious units (IPIU) derived tertiary model and independent (internal and external) experimental validation data for *Campylobacter jejuni* (DSM 4688, BfR-CA-18040 and BfR-CA-18043) in previously frozen raw milk at different temperatures.

and above the equivalence line for the BfR-CA-18043 data-derived model.

Experimental CFU data available in ComBase could not be used for model validation as these were outside the application range of the generated models.

Only the data from Wulsten et al. (2020), obtained under the same experimental conditions, could be used for external validation of our models. In case of the tertiary model predicting IPIU, RMSE, and $R^2$ values demonstrated a good performance and accuracy of the model predictions (Figure 6 and Table S6). However, results from the model validation using CFU data from Wulsten et al. (2020) were not acceptable (Figure 5a, Table S5), as their experimental data differed greatly from the data obtained in our study.

**TABLE 4** The $t_{xd}$ (time to achieve *x*-log reduction, e.g., $t_{1d}$) values for CFU and IPIU data for *Campylobacter jejuni* strains in raw milk of different temperatures.

| Strain | Data | Temperature (°C) | $t_{1d}$ (h) | $t_{2d}$ (h) | $t_{3d}$ (h) | $t_{4d}$ (h) |
|---|---|---|---|---|---|---|
| DSM 4688 | CFU | 5 | 8.6 | 13.0 | 17.3 | 21.6 |
| DSM 4688 | CFU | 8 | 8.5 | 12.7 | 17.1 | 21.2 |
| DSM 4688 | CFU | 12 | 8.4 | 12.1 | 15.18 | 18.56 |
| BfR-CA-18043 | CFU | 5 | 18.46 | 24.92 | 31.38 | 37.84 |
| BfR-CA-18043 | CFU | 8 | 18.55 | 23.41 | 28.23 | 33.12 |
| BfR-CA-18043 | CFU | 12 | 13.79 | 17.6 | 21.41 | 25.22 |
| DSM 4688 | IPIU | 5 | >100 | >1000 | >1000 | >1000 |
| DSM 4688 | IPIU | 8 | >100 | >1000 | >1000 | >1000 |
| DSM 4688 | IPIU | 12 | >100 | >1000 | >1000 | >1000 |
| BfR-CA-18043 | IPIU | 5 | >1000 | >1000 | >1000 | >1000 |
| BfR-CA-18043 | IPIU | 8 | >1000 | >1000 | >1000 | >1000 |
| BfR-CA-18043 | IPIU | 12 | >1000 | >1000 | >1000 | >1000 |

### 3.3 | Comparison between colony-forming unit and intact and putatively infectious unit predicted survival

An overview of the time required to obtain a 1-, 2-, 3-, and 4- log reductions ($t_{xd}$) is available in Table 4. The $t_{xd}$ from the IPIU data-derived model was significantly higher than for the CFU data-derived model, independent of the strain and temperature. For the CFU data-derived model a significant difference in $t_{xd}$ between strains but not between temperatures for the same strain were found. In contrast, for the IPIU data-derived model no significant difference between strains was evident. For the CFU data, the time needed to reach one log reduction varies between 8.6 and 18.46 hours depending on the strain and the temperature. On the other hand, the predictions of the $t_{xd}$ for IPIU data were outside the application range of the Weibull

model (>100 h), which indicates that *C. jejuni* in VBNC-state can survive until the end of the shelf-life of raw milk, assuming that raw milk is kept in the fridge for a week.

## 4 | DISCUSSION

The consumption of raw milk has repeatedly been associated with *Campylobacter* spp. outbreaks (EFSA, 2015, 2021; Heuvelink et al., 2009; Teunis et al., 2018). It had been shown that fecal cross-contamination of teat skin from dairy cows can occur (Knipper et al., 2023), even though it is a rare detectable event. Traditional cultural methods used for the detection of *Campylobacter* spp. in raw milk are challenging due to rapid decline of CFU (Barrell, 1981; Doyle & Roman, 1982; Humphrey, 1986). Even when *Campylobacter* spp. cannot grow in raw milk filling stations during storage, it has been suggested that it can survive under unfavorable environmental conditions in the VBNC state (Rollins & Colwell, 1986). In that state, they putatively remain pathogenic, once favorable conditions recur (Baffone et al., 2006; Federighi et al., 1998; Rollins & Colwell, 1986; Wulsten et al., 2020). Until now, there was no method capable of detecting cells in the VBNC state in raw milk. However, a recently developed v-qPCR enables the detection of IPIU (comprising CFU and VBNC) of *Campylobacter* spp. in raw milk (Wulsten et al., 2020). This raises the opportunity to study how efficiently *Campylobacter* spp. can survive in raw milk at different temperatures based on CFU and IPIU data.

In our work experimental data were obtained by using a culture-dependent method for detection of CFU (ISO 10272-2:2017) and a culture-independent method for detection of IPIU (v-qPCR, [Wulsten et al., 2020]). The v-qPCR enabled the detection of IPIU while excluding DNA from already dead cells since PMA staining is performed prior to DNA extraction (Wulsten et al., 2020). In contrast to a conventional qPCR without staining, in the v-qPCR *Campylobacter* survival is not overestimated by DNA from dead cells (Krüger et al., 2014; Pacholewicz et al., 2019; Wulsten et al., 2020). According to Wulsten et al. (2020), we could demonstrate an underestimation of the survival of *C. jejuni* by CFU. We were able to confirm the data from Wulsten et al. (2020) for the DSM 4688 at 5°C and furthermore demonstrated that there is also no difference in the IPIU data at 8°C and 12°C. In this study the BfR-CA-18043 field strain showed a prolonged CFU survival compared to the reference strain DSM 4688. Wulsten et al. (2020), observed the same for the "outbreak" strain (BfR-CA-13290) isolated from raw milk compared to the reference strain DSM 4688. This could be explained by the fact that due to the level of stress tolerance, resistance and progression to the VBNC vary between different strains of *C. jejuni* (Lv et al., 2020), with the field strains being more stress tolerant than the reference strains.

The generated experimental data were used for modeling generation purposes. In our work, we applied a one-step fitting approach using parameter estimates from an intermediate two-step fit as starting values for the fitting of tertiary model equations. Traditionally, a two-step fitting approach has been used, in which primary and secondary models are generated in two different steps and then integrated in a software tool as a tertiary model to predict the number of microorganisms under the different environmental conditions. However, this approach is not without drawbacks, as it can accumulate and propagate the errors in each step of the data analysis during the model development process (Huang, 2017). A one-step fitting approach in which the primary and secondary models are analyzed together during the estimation of kinetic parameters, have been also widely used in kinetic analysis as it is believed that the resulting tertiary models give better fits to the data. Directly interpretable regression diagnostics and standard errors can be also obtained with this latter approach (Dolan & Mishra, 2013; Huang, 2017; Jewell, 2012).

During the model design some assumptions were necessary. This is the case, for example, with the handling of non-detects during model fitting. As described before, the detection limit of our method was 1 CFU/mL. After around 70 h, we were not able to detect CFUs in raw milk. To simplify the generation of a predictive model applicable over the whole experimental time span we opted for a trilinear model that contains a tail. As we fixed the values for non-detects at $-0.1$ $\log_{10}$ CFU/mL this model predicts this concentration for all time points after $S_t$. To prevent users from misinterpreting this value we provide a dedicated annotation to the output parameter in the FSKX model file. In addition, we explored if for example, maximum likelihood estimation (MLE)-based method for parameter estimation given censored data would have yielded significantly different results, which was not the case (data not shown). Therefore, we decided to stick to the generated trilinear model for prediction purpose.

The wide scattering of the experimental CFU data led to CFU-derived tertiary models that cannot provide an "accurate" prediction for the survival of *C. jejuni* at some time points. This is the case, for example, of the strain BfR-CA-18043. In addition, the quality of the model prediction on the concentration at a specific time point, especially of the DSM-4688-derived tertiary model, could be improved in the future by integrating further data. This applies specifically to the time range between 9 and 30 h, where there were only few and widely scattered data available.

On the other hand, the developed IPIU-derived tertiary model showed a small variation in the prediction of the IPIU, which could be linked to the more complex method of detecting IPIU by v-qPCR. However, the IPIU-derived model demonstrated to be better able to predict the concentrations than the CFU-derived model, which could be attributed to two reasons: 1. CFU data have a high variability compared to IPIU data, and 2. the inactivation of IPIU occurs in a short period of time, usually within the first few hours. Furthermore, for the IPIU data hardly any reduction on the number of cells will occur after the initial phase.

The validation process carried out with the internally generated validation data indicated that CFU data-derived tertiary models were able to make accurate predictions indicated by the RMSE and $R^2$ values. Most of the CFU data were under the equivalence line for the DSM 4688 data-derived model and above the equivalence line for the BfR-CA-18043 data-derived model. That means that the DSM 4688 data-derived model predicts a lower population of microorganisms than observed, meanwhile the BfR-CA-18043 data-derived model, predicts a higher population of microorganisms than observed.

Experimental data at temperatures other than those used in the experiments should be included in the future for further internal validation.

Experimental CFU data-sets available in ComBase strongly deviated from the measured values obtained in our experiments and could not be used for the external model validation because were outside the application range of the generated models. Comparison of model predictions with data from other studies that do not match the range-of-model-applicability is difficult because the matrix and strain effects on the survival vary strongly. This underlines the importance of proper model annotations to clearly inform on the application range of each model.

Only data from Wulsten et al. (2020) could be used for the external model validation. A good performance and accuracy of the model predictions for the IPIU model were confirmed. This could be explained by the fact that IPIU levels in milk seem to remain unchanged irrespective of the specific matrix or strain used. This is also in line with the results from Wulsten et al. (2020), which showed that the developed v-qPCR is robust against different milk batches. The validation of CFU models with data from Wulsten et al. (2020) was, however, not acceptable. The large deviation from our data and therefore from the predictions of our model could be attributed to the different batch of the raw milk used. Even when the raw milk in both studies came from the same herd, our experiments were carried out 3 years later. Animal feed and therefore the amount and percentage of the components in raw milk, like protein and fat, may have changed. Further experiments with milk from different herds or even milk from different batches within the same herd should be carry out in order to evaluate the impact of the variations of milk composition in the CFU survival data.

The calculated time needed to reach one log reduction, demonstrated big differences between CFU and IPIU data, as for CFUs it varied between 8.6 and 18.46 h (depending on the strain and the temperature) while for IPIU data was predicted to be greater than 100 h (independent of the temperature and the strain used). This large difference again shows the underestimation of the concentration of *C. jejuni*, if only CFU are taken into account.

Regarding the recovery of CFU from VBNC, Wulsten et al. (2020) showed that it is possible to recover *C. jejuni* strain DSM 4688 from the VBNC state into a culturable state in raw milk by extremely lowering the partial pressure of oxygen at 5°C. We reproduced this phenomenon for the DSM 4688 strain in a wider range of temperature (from 8°C to 12°C) and furthermore, could demonstrate that for a recently isolated field strain derived from cow feces, BfR-CA-18043, displayed time-dependent recovery of CFU from VBNC. The recovery of CFU from VBNC showed that *C. jejuni* was viable at least during 80 h (both strains) at 8°C and 48 h (DSM 4688) and 72 h (BfR-CA-18043) at 12°C.

Due to the recovery of CFU from VBNC, the CFU concentration of *C. jejuni* in raw milk varies dependent on specific conditions (beyond standard incubation procedures), and therefore might range between our model predictions of CFU and IPIU, but so far it is unknown. Therefore, further studies including the modeling of the recovery data under different conditions should be carried out in order to be able to get more realistic predictions on the concentration of CFU in raw milk.

CFU recovery required very specific conditions (Li et al., 2014), and depended on many factors such as the strain used, the age of VBNC cells, the conditions that induced the VBNC state and, of course, the conditions provided for recovery (Pinto et al., 2011). CFU recovery from VBNC has been reported to happen in microaerobic conditions (Bovill & Mackey, 1997), in embroynated chicken eggs (Cappelier et al., 1999) and in vivo using mouse infection models (Baffone et al., 2006). So far, it has not been confirmed if this recovery occurs in the human gut, but if so, this would mean that the risk of exposure to infective cells through consumption of raw milk, leading to human disease, may be underestimated.

It was assumed that VBNC cells are avirulent, as they have a coccoid shape and a reduced rate of gene expression and protein translation required for pathogenesis (Ramamurthy et al., 2014). However, VBNC cells that are recovered back into CFUs can regain full infectious potential (Baffone et al., 2006). For example, it was demonstrated that *Campylobacter* cells recovered after embryonated-egg passage—considered as an animal model with reduced animal defense properties—since they regained attachment capacity to HeLa cells (Cappelier et al., 1999).

Maintenance of adhesion potential was used as pathogenicity indicator and suggested that the VBNC state of *Campylobacter* does, in fact, constitute a public health concern (Cappelier et al., 1999). This capacity of recovery with no apparent loss of virulence potential evidently raises concerns regarding the presence of VBNC bacteria in food (Li et al., 2014).

Nevertheless, the infectivity of VBNC cells is not known. One approach to test the infectivity of VBNC cells would be to test VBNCs within and beyond the "recovery window" in animal models. It should be noted that the "recovery window" is based on current knowledge on conditions for in vitro recovery of VBNCs, which might be extended in future. Hence, it is worth investigating if all VBNC cells are infectious or only those that can be recovered by special treatment into CFUs.

We conclude that as long as the infectivity and pathogenicity factors from VBNC and recovered CFU are not entirely understood in raw milk, the IPIU-derived model should be considered as a worst-case scenario, assuming that these VBNC might also be infectious. Nevertheless, even when the developed model could help to quantify the consumer exposure to *C. jejuni* through consumption of raw milk, the results presented in this study should be interpreted with caution, as they are based on a specific scenario, data and assumptions.

## 5 | CONCLUSION

Our study corroborate that the survival of *C. jejuni* is largely underestimated if only based on CFU data. This work confirmed the potential for underestimation of *C. jejuni* concentrations in raw milk, not only at 5°C but also at 8°C and 12°C. Furthermore, we confirmed that VBNCs in raw milk could be successfully recovered within a

relevant time window by drastically reducing the oxygen partial pressure. Three mathematical models were developed on the basis of the newly generated experimental data that can predict the effect of temperature on the survival of *C. jejuni* DSM 4688 and BfR-CA-18043 in raw milk. For the IPIU survival of the *C. jejuni* our model predicts that not even a one log reduction will be observed within 100 h while the same reduction is predicted to occur at least after 18 h for CFUs. The obtained tertiary models clearly demonstrate the potential for underestimation of the survival of *C. jejuni* in raw milk. As the degree of infectivity of cells in VBNC status is still unknown, IPIU data should be taken into account as a worst-case scenario, as these VBNCs still might be infectious. To improve model-based predictions of IPIU and CFU concentrations it is necessary to collect more data, as the variability in *C. jejuni* survival in the complex matrix of raw milk is very high. In addition, it must be investigated weather VBNCs remain infectious even beyond the "recovery window." Despite of these considerations, the newly developed models might become valuable resources for food managers and risk assessors.

## AUTHOR CONTRIBUTIONS

Anna-Delia Knipper: Data Curation, Formal Analysis, Investigation, Methodology, Visualization, Writing – original draft, Writing – review & editing. Carolina Plaza-Rodríguez: Writing – review & editing. Matthias Filter: Writing – review & editing. Imke F. Wulsten: Writing – review & editing. Kerstin Stingl: Writing – review & editing. Tasja Crease: Conceptualization, Data Curation, Formal Analysis, Methodology, Project Administration, Supervision, Visualization, Writing – review & editing.

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## CONFLICT OF INTEREST STATEMENT

The authors declare that they have no known competing financial interests or personal relationship that could have appeared to influence the work reported in this paper.

## DATA AVAILABILITY STATEMENT

The developed models are available in the model repository: https://knime.bfr.berlin/landingpage/RAKIP-Model-Repository. All relevant data are within the manuscript and its supporting information files.

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## SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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## 3.5 Publication 4: Quantitative microbiological risk assessment model for *Campylobacter* in raw milk of dairy cows in Germany

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# Quantitative microbiological risk assessment model for *Campylobacter* in raw milk of dairy cows in Germany

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ABSTRACT

The consumer demand for raw milk from dairy cows has increased and local sales via vending machines have been intensified. Therefore, this study aimed to assess the risk associated with the consumption of unboiled raw milk contaminated with *Campylobacter* by estimating the number of campylobacteriosis cases. For this a stochastic quantitative microbial risk assessment (QMRA) model was developed that covered the whole supply chain. Information and data for model parametrization were obtained from research publications. Different probability distributions were used to represent the data whenever possible and probabilistic risk estimation was performed using Monte Carlo simulations. Simulations for outbreaks from single vending machines were performed using the developed QMRA baseline model. Further, different risk mitigation scenarios along the supply chain were evaluated to support risk managers in controlling *Campylobacter*. The analysis suggest a role for *Campylobacter* infections due to fecal contamination of cows' udder. The model can easily be adapted and extended when additional data become available as it is provides in the harmonized exchange Food Safety Knowledge Exchange (FSKX) format.

## 1. Introduction

*Campylobacter* causes campylobacterisosis a diarrheal disease and represents one of the leading causes of zoonotic enteric infections worldwide. The 27 European Union (EU) Member States reported an overall incidence of 127,840 confirmed cases of human campylobacteriosis, corresponding to an EU notification rate of 41.1 cases per 100,000 population in 2021 (EFSA, 2022).

From 2011 to 2020 raw milk was one of the food vehicles causing most strong-evidence foodborne *Campylobacter* outbreaks in the EU (EFSA, 2021). In recent years, zoonoses monitoring in Germany reported 1 to 2.5% of bulk tank samples positive for *Campylobacter* (BVL, 2010, 2012, 2016a, 2016b, 2020). Further, in 2018 most of the outbreaks were caused by *Campylobacter* and associated with the consumption of unboiled raw milk (9/38 outbreaks) (BVL, 2019).

Direct sale of raw milk for human consumption by self-service and automatic vending machines is conditionally allowed in many European countries, such as Germany, France, the Netherlands, Belgium, Denmark, Italy, Ireland and parts of the UK (EFSA, 2015). A survey in 2019 of state and county veterinary agencies for registered raw milk sale outlets in Germany indicated nearly 850 raw milk sale outlets on farm (Böhnlein et al., 2020). Websites based on farmers' voluntary entries capture currently just about half of the raw milk supply in Germany (Liebers, 2013).

A sign on the vending machines stating that the raw milk must be boiled before consumption is legally required (BMJV, 2018). Nevertheless, data from Italy demonstrated that up to 43% of consumers did not boil raw milk before consumption (Giacometti et al., 2013).

Quantitative microbial risk assessment (QMRA) has emerged in the area of food safety as a comprehensive and systematic approach which allows an *a priori* assessment of the effect of intervention measures along the whole food chain, or combinations of intervention measures on public health (Havelaar et al., 2008). QMRA is based on the principles for microbial risk assessment defined within the Codex Alimentarius and comprises four different stages: hazard identification, exposure assessment, hazard characterization and risk characterization (Codex Alimentarius Commission, 1999). Of these, exposure assessment often requires the development of a food supply chain model that describes changes of prevalence and concentration of the microbial hazard, during food production, processing and handling. Such models are often designed using the modular process risk model (MPRM) approach for exposure assessment. It describes where the bacteria enter the food

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pathway and what can happen to the bacteria in consecutive modules, in terms of either a microbial process (growth and inactivation) or product handling processes (cross-contamination, mixing, removal or partitioning) (Nauta, 2008). The output of one module then serves as the input for the following module. The calculated probability and amount of exposure are then used in combination with a dose-response model to calculate the individual risk per serving. Further, to capture the true heterogeneity in a population (variability) and the lack of knowledge related to e.g. low precision of measurement methods (uncertainty), probability distributions are used in the different modules of a QMRA to estimate the risk for the population (Membré and Boué, 2018).

The aim of this study was to model the transfer of *Campylobacter* along the raw milk supply chain and to assess the impact of potential mitigation options that may reduce the public health risk associated with the consumption of *Campylobacter*-contaminated raw milk in Germany. For that, a food chain modeling approach was applied using the MPRM methodology. First a baseline model was built describing a "normal" raw milk production, distribution and consumption scenario. The changes in prevalence and concentrations of *Campylobacter* at different steps of the supply chain were simulated. Next, alternative scenarios were defined to identify the most important data gaps (uncertainties) and to evaluate the effects of potential interventions. Two dose-response models were used: the "classic" model for the dose-response (Teunis and Havelaar, 2000), based on a human challenge study (Black et al., 1988) and a novel raw milk outbreak dose-response model (Teunis et al., 2018).

## 2. Material and methods

### 2.1. Description of the food pathway and model implementation

The MPRM methodology (Nauta, 2008) was used to design the model structure. Fig. 1 displays the elements of the food pathway covered in the model.

To parameterize the QMRA experimental data from scientific literature were used, e.g. Knipper et al. (2023), Giacometti et al. (2015), Vissers et al. (2007) and Böhnlein et al. (2020). The entire model was implemented and executed in @Risk software (version 8.2 Pro, Palisade Corporation, Newfield, NY, U.S.) using the Monte Carlo simulation technique with 100,000 iterations. In addition, the final model was implemented in software R 4.2.1 (R Core Team, 2020) and converted into the Food safety Knowledge Exchange (FSKX) format, with the aim of improving transparency in the model generation process and facilitate the exchange and reusability of the model created. The FSKX files hold all model parameter estimates, the raw experimental data and all relevant metadata. The model files can be accessed and downloaded via the following model repository: https://knime.bfr.berlin/landingpage/RAKIP-Model-Repository, and executed in the open-source software solution FSK-Lab (de Alba Aparicio et al., 2018).

### 2.2. Baseline model

A detailed description of the distributions and parameters used is shown in Table 1. First, a baseline model was developed to estimate the number of *Campylobacter* cases through the consumption of raw milk from vending machines in Germany. This model includes the variability as explained below.

*Step 1: Initial contamination of cows' feces*

*Campylobacter* concentration in cows' feces (log colony-forming units (CFU)/g) was modelled by a normal distribution describing the variability between infected cows:

$$C_{feces} \sim \text{Normal}\left(m_{feces}; sd_{feces}\right) \quad (1)$$

where "$m_{feces}$" is the mean concentration and "$sd_{feces}$" the standard deviation.

*Step 2: Fecal cross-contamination of udders*

The amount of feces per udder ($G_{feces}$) was assumed to be 0.059 g (Vissers et al., 2007). To describe the variability between fecal cross-contamination of udders by different cows, a triangle distribution was used:

$$G_{feces} \sim Triangle\left(G_{feces;min}; G_{feces;most\ likely}; G_{feces;max}\right) \quad (2)$$

"$G_{feces;\min}$" the minimum amount, "$G_{feces;most\ likely}$" being the most likely amount of feces per udder, and "$G_{feces;\ \max}$" the maximum value.

The prevalence of udders contaminated with feces ($P_{udder}$) had a fixed value.

*Step 3: Milking process and bulk tank*

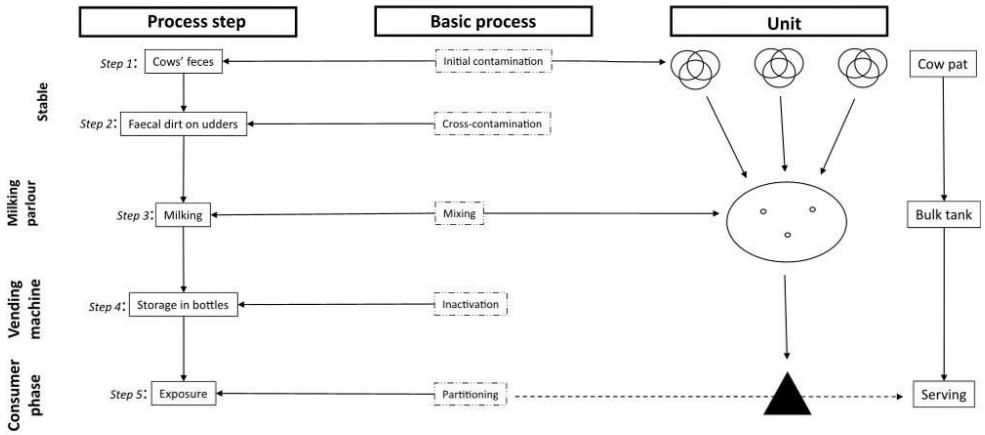


**Fig. 1.** Baseline model and an overview of the different processes and units used to describe the concentration and prevalence of *Campylobacter* in the raw milk pathway.

**Table 1**
Overview of variables and parameters in the baseline model enabling to estimate the number of *Campylobacter* cases from consuming raw milk.

| Process step | Variable | Description | Data/Equation | Unit | References |
|---|---|---|---|---|---|
| 1 | $m_{feces}$ | Mean of *Campylobacter* concentration in cows feces | 2.4 | (Log CFU)/g | Knipper et al. (2023) |
| | $sd_{feces}$ | Standard deviation of *Campylobacter* concentration in cows feces | 0.9 | (Log CFU)/g | Knipper et al. (2023) |
| | $C_{feces}$ | Concentration of *Campylobacter* in cows feces | ~ Normal ($m_{feces}$; $sd_{feces}$) [Variability per cow] | (Log CFU)/g | Calculated |
| 2 | $G_{feces;min}$ | Minimum amount of feces per udder | 0.003 | g | Assumption based on Vissers et al. (2007) (5% quantile of triangle distribution) |
| | $G_{feces;most\ likely}$ | Most likely amount of feces per udder | 0.059 | g | Vissers et al. (2007) |
| | $G_{feces;max}$ | Maximum amount of feces per udder | 0.3 | g | Assumption based on Vissers et al. (2007) (90% quantile of triangle distribution) |
| | $G_{feces}$ | Gram feces per udder | ~ Triangle ($G_{feces;min}$; $G_{feces;most\ likely}$; $G_{fecesmax}$) [Variability per cow] | g | Calculated |
| | $P_{udder}$ | Prevalence of contaminated udders with *Campylobacter* | 12 | % | Knipper et al. (2023) |
| 3 | $m_{cow\_liter}$ | Mean amount of milk per cow | 30 | l | Own data |
| | $sd_{cow\_liter}$ | Standard deviation of amount of milk per cow | 10 | l | Own data |
| | $A_{herd,\alpha}$ | Value α parameter Gamma distribution | 1.2 | Variable | Estimated based on BMEL (2022) |
| | $A_{herd,\beta}$ | Value β parameter Gamma distribution | 59 | Variable | Estimated based on BMEL (2022) |
| | $A_{herd}$ | Number of cows contributed to bulk tank | ~ 1+(round(Gamma($A_{herd,\alpha}$; $A_{herd,\beta}$);0) [Variability per bulk tank] | Cow | Calculated |
| | $V_{bulk\_tank}$ | Raw milk liter volume in bulk tank | ~ $A_{herd}$ x Normal($m_{cow\_liter}$; $sd_{cow\_liter}$/ $\sqrt{A_{herd}}$) [Variability per bulk tank] | l | Calculated |
| | $A_{contaminated}$ | Number of cows with contaminated udder | ~ Binomial (round($A_{herd}$;0);$P_{udder}$) [Variability per bulk tank] | Cow | Calculated |
| | $N_{cow}$ | Number of *Campylobacter* a single cow contributed to bulk tank | = G $_{feces}$ $x\ 10^{\ C\ feces}$ | CFU | Calculated |
| | $N_{bulk\_tank}$ | Number of *Campylobacter* in bulk tank | = sum($N_{cow}$) sampled from $A_{contaminated}$ cows | CFU/bulk tank | Calculated |
| | $C_{liter}$ | Concentration of *Campylobacter* per liter in bulk tank | = Log ($N_{bulk\_tank}$ / $V_{bulk\_tank}$) | (Log CFU/l) in bulk tank | Calculated |
| 4 | $S_{time;min}$ | Minimum storage time | 0.5 | h | Giacometti et al. (2015) |
| | $S_{time;most\ likely}$ | Most likely storage time | 24 | h | Giacometti et al. (2015) |
| | $S_{time;max}$ | Maximum storage time | 120 | h | Giacometti et al. (2015) |
| | $S_{time}$ | Time of raw milk storage | ~ Pert ($S_{time;min}$; $S_{time;most\ likely}$; $S_{time;max}$) [Variability per serving] | h | Giacometti et al. (2015) |
| | $S_{reduction;min}$ | Minimum decimal reduction time based on 12 °C data | 113 | h | Giacometti et al. (2015) |
| | $S_{reduction;most\ likely}$ | Most likely decimal reduction time based on 12 °C data | 132 | h | Giacometti et al. (2015) |
| | $S_{reduction;max}$ | Maximum decimal reduction time based on 12 °C data | 151 | h | Giacometti et al. (2015) |
| | $S_{reduction}$ | Decimal reduction time based on 12 °C data | ~ Pert ($S_{reduction;min}$; $S_{reduction;most\ likely}$; $S_{reduction;max}$) [Variability per serving] | h | Giacometti et al. (2015) |
| | $C_{raw\ milk}$ | Concentration of *Campylobacter* at consumption | = $C_{liter}$ - ($S_{time}$/ $S_{reduction}$) | (Log CFU/l) | Calculated |
| 5 | $m_{consumed}$ | Mean volume of raw milk consumed | 0.21 | l | Assumption (a mug) |
| | $sd_{consumed}$ | Standard deviation of volume of raw milk consumed | 0.05 | l | Assumption |
| | $U_{consumed}$ | Portion of raw milk consumed per serving | ~ Lognormal ($m_{consumed}$; $sd_{consumed}$) [Variability per serving] | l | Estimated (one mug) |
| | $D_{ingested}$ | Dose | ~Poisson(10 $^{Craw\ milk\ *\ Uconsumed}$) [Variability per doses] | CFU/serving | Calculated |
| Dose-response | $P_{inf}$ | Probability of infection (dose-response model) | = 1-Gamma ($\alpha+\beta$) x Gamma ($\beta+D_{ingested}$)/ Gamma ($\alpha+\beta+D_{ingested}$)/ Gamma ($\beta$) | | Teunis and Havelaar (2000) |
| | $\alpha$ | Dose-response model | 0.145 | | Teunis and Havelaar (2000) |
| | $\beta$ | Dose-response model | 7.59 | | Teunis and Havelaar (2000) |
| | $P_{inf,\ mean}$ | Mean probability of infection | = Mean ($P_{inf}$) [mean for all servings (= iterations)] | | Calculated |
| | $P_{ill/inf}$ | Probability of illness given infection | = 1/3 | | Black et al. (1988); Havelaar et al. (2000); Nauta et al. (2007) |
| | $P_{ill,\ mean}$ | Mean probability of illness | = $P_{inf,\ mean}$ x $P_{ill/inf}$ | | Calculated |

(*continued on next page*)

**Table 1** (*continued*)

| Process step | Variable | Description | Data/Equation | Unit | References |
|---|---|---|---|---|---|
| | $N_{served}$ | Raw milk's servings per year | 44,365,750 | | Estimated based on Giacometti et al. (2013); Böhnlein et al. (2020); Labohm et al. (2021) |
| | $N_{served\_unboiled}$ | Unboiled raw milk's servings per year | 13,309,725 | | Estimation based on Giacometti et al. (2013) |
| | *Output* | Cases per year | $= P_{ill,\ mean} * N_{served\_unboiled}$ | | Calculated |

The variability in the number of cows contributing to the milk tank was included using a gamma distribution ($A_{herd}$). In Germany 2020, the average herd size on the farms was 72 cows, where 56% of all dairy cows were kept on farms with more than 100 animals (BMEL, 2022). This was fitted to a Gamma distribution, yielding a mean of about 71.8 cows per farm, a minimum of 1 cow per farm and about 55.7% of the cows in a farm with 100 cows or more:

$$A_{herd} \sim 1 + (round(Gamma(A_{herd\_\alpha}; A_{herd\_\beta}); 0) \quad (3)$$

We assumed that each cow gives 30 liters per day with a standard derivation of 10 liters based on our own data (*not shown*). To take into account that the amount of milk per cow is independent between the cows, the volume of raw milk liter in bulk tank ($V_{bulk\ tank}$) was calculated as:

$$V_{bulktank} \sim A_{herd} x \text{Normal}\left(m_{cow\_liter}; sd_{cow\_liter} / \sqrt{A_{herd}}\right) \quad (4)$$

where "$m_{cow\_liter}$" is the mean amount of milk per cow and "$sd_{cow\_liter}$" the standard deviation.

The number of cows contributing to the bulk tank with contaminated udders is:

$$A_{contaminated} \sim \text{Binomial}\left(\text{Round}(A_{herd}); P_{udder}\right) \quad (5)$$

Each of the $A_{\text{contaminated}}$ cows contributes a different amount of feces and a different concentration, so the CFU added to the milk tank per cow is:

$$N_{cow} = G_{feces} x 10^{Cfeces} \quad (6)$$

and the total amount in the bulk tank is:

$$N_{bulk\_tank} = \Sigma_{A_{contaminated}} N_{cow.}$$

Next, the amount of *Campylobacter* per liter was calculated and expressed on a log scale

$$C_{bulk\_tank} = \log(N_{bulk\_tank} / V_{bulk\_tank}) \quad (7)$$

The storage in the bulk tank is assumed to be short (up to 5 h) and at 4 °C, and considered to have negligible effect in comparison to the storage in vending machines (see below).

*Step 4: Storage (Vending machine)*

The decimal reduction time of *Campylobacter* is higher at 4 °C (>200 h) than 12 °C (>100 h) (Giacometti et al., 2015). Therefore, we assumed storage at 12 °C in the baseline model to study the effect of *Campylobacter* concentration within the storage time of raw milk. Nevertheless, we have also considered other storage conditions in scenarios analysis (see below).

The variability of storage time ($S_{time}$) was included by a pert distribution, with a minimum 0.5 h, most likely 24 h and maximum 120 h, based on data from Giacometti et al. (2015). The decrease of *Campylobacter* concentration during storage in a vending machine ($C_{raw\ milk}$) was calculated based on a time-temperature profile. The decimal reduction time of *Campylobacter* in raw milk was adopted from Giacometti et al. (2015).

To include the variability in the decimal reduction time a pert distribution was used:

$$S_{reduction} \sim \text{Pert}\left(S_{reduction;\min}; S_{reduction;mostlikely}; S_{reduction;\max}\right) \quad (8)$$

Afterwards the concentration of *Campylobacter* (log CFU/l) after storage was calculated:

$$C_{rawmilk} = C_{liter} - (S_{time} / S_{reduction}) \quad (9)$$

*Step 5: Exposure*

A lognormal distribution was used to cover the variability of the portion size ($U_{consumed}$):

$$U_{consumed} \sim \text{Lognormal}(m_{consumed}; sd_{consumed}) \quad (10)$$

The ingested dose ($D_{ingested}$) in a raw milk portion should be an integer value, as bacteria are discrete units. Assuming the bacteria are homogeneously distributed in the raw milk, it is obtained as:

$$D_{ingested} \sim \text{Poisson}\left(10^{Crawmilk * Uconsumed}\right) \quad (11)$$

Additionally, we assumed the number of raw milk portions (210 ml, =one mug) in Germany is about $4.44 \times 10^7$ per year, whereby 30% of the servings are not boiled prior to consumption (Giacometti et al., 2013) resulting in $1.33 \times 10^7$ unboiled raw milk servings per year. This is calculated assuming that 850 vending machines in Germany (Böhnlein et al., 2020), operate 365 days a year, selling at least 30 litres per day (Labohm et al., 2021), which corresponds to 143 servings of 210 ml of milk.

### 2.3. Dose-response (DR)

As part of the hazard characterization, a dose-response model needs to be applied in a risk assessment. In the baseline model, we are using the Beta-Poisson model developed by Teunis and Havelaar (2000), which has been adopted widely before (EFSA, 2011, 2021; Nauta et al., 2009).

It was implemented as:

$$P_{inf}(D;\ \alpha,\ \beta) = \frac{\Gamma(\alpha+\beta)\Gamma(D+\beta)}{\Gamma(\alpha+\beta+D)\Gamma(\beta)} \quad (12)$$

where Γ(.) is Euler's Gamma function (Haas, 2002; Nauta et al., 2009), $D = D_{ingested}$, $\alpha = 0.145$ and $\beta = 7.59$ (EFSA, 2011; Teunis and Havelaar, 2000).

The probability of illness given infection is assumed to be $P_{ill|inf} = 1/3$ (Black et al., 1988; Havelaar et al., 2000).

### 2.4. Probability of a daily outbreak from a single vending machine

We used the developed model to get an estimate of the probability that a campylobacteriosis outbreak will occur from the consumption of unboiled raw milk from one vending machine.

If we assume $n_{unb}$ servings of $U_{consumed}$ l of unboiled raw milk are consumed from a vending machine on one day, and the *Campylobacter* concentration in the machine is $C_{raw\ milk}$, so the expected (mean) dose is $D_{exp} = 10^{U\_consumed\ C\_raw\ milk}$, the number of servings containing $D = 0,1,2,\ldots$ CFU, N(D = x), can be obtained by sampling from a Multinomial distribution

$$N(D = x) \sim \text{Multinomial}(n_{unb}, \{p_x\})$$

for x = 0,1,2,…, where $p_x$ has the Poisson density

$$p_x = \frac{D_{exp}{}^{x} e^{-D_{exp}}}{x!}$$

From this, the number of raw milk consumers that actually get infected is obtained from

$$N_{inf}(x) \sim \text{Binomial}\left(N(D=x), P_{inf}(x;\alpha,\beta)\right)$$

with the number of cases

$$N_{ill} = P_{ill \mid inf} \sum_{all\ x} N_{inf}(x)$$

In a Monte Carlo simulation, we estimate the probability that $N_{ill} \geq 2$ or $N_{ill} \geq 5$, i.e. the probabilities that more than 2 or 5 people would get ill from the consumption of unboiled raw milk from the same vending machine on the same day. Based on the data presented above we assume that 30% of 143 servings of raw milk from a vending machine are consumed unboiled, $n_{unb} = 43 \approx 0.3*143$ and that the serving size is $U_{consumed} = 0.21$ l. The distribution of $C_{raw\ milk}$ is obtained from the baseline model.

### 2.5. Scenario analyses

In this assessment, uncertainties were taken into account for a selection of variables considered in the model through different scenario analyses as e.g. in Benamar et al. (2021) or Tirloni et al. (2020). We used variables were we thought are important and cover different aspects over the whole food chain. The results from the scenario analyses were further used to evaluate the implementation of different intervention strategies. An overview of the different variables that were modified to evaluate different scenarios is shown in Table 2.

Scenario analyses were performed for the initial contamination (step 1) of the baseline model using a mean concentration of $m_{feces}$ of 0.92 CFU/g and 3.88 CFU/g, lower or higher than in the baseline model. To simulate the effect of the cross-contamination (step 2) lower or higher values of variables (5% quantile or 95% quantile) for the triangle distribution of $G_{feces}$ and prevalence of contaminated udders ($P_{udder}$) were used. The effect of the mixing (step 3) in the bulk tank was simulated using lower and higher mean amount of milk per cow ($m_{cow\_liter}$). In

**Table 2**
Overview of the parameters to evaluate uncertainty and their effect on the estimated campylobacteriosis risk due to raw milk consumption, relative to the baseline model. The effect is given as the relative risk (RR, Eq. 13) for each scenario (see Fig. 2).

| Process step according to baseline model | Scenario | Description of the scenario | Model parameter changed | Alternative value/ distribution | Source | Estimated cases/year | RR in $Log_{10}$ (scenario/ baseline) |
|---|---|---|---|---|---|---|---|
| | | | Baseline | | | 9906 | 0 |
| 1 | 1A | Lower initial mean concentration in cow feces | Low $m_{feces}$ | 0.92 | Assumption based on Knipper et al. (2023) (5% quantile) | 360 | -1.44 |
| | 2A | Higher initial mean concentration in cow feces | High $m_{feces}$ | 3.88 | Assumption based on Knipper et al. (2023) (95% quantile) | 173,414 | 1.24 |
| 2 | 1B | Lower amount of feces per udder | Low $G_{feces}$ | ~ Triangle (0; 0.0318; 0.059) | Assumption (5% quantile of $G_{feces;min,\ Gfeces;most\ likely}$, $G_{feces;max}$) based on Vissers et al. (2007) | 2660 | -0.57 |
| | 2B | Higher amount of feces per udder | High $G_{feces}$ | ~ Triangle (0.059; 0.24; 0.3) | Assumption (95% quantile of $G_{feces;min,\ Gfeces;most\ likely}$, $G_{feces;max}$) based on Vissers et al. (2007) | 173,414 | 1.24 |
| | 1C | Lower prevalence of contaminated udders with *Campylobacter* | Low $P_{udder}$ | 0.05 | Assumption based on Knipper et al. (2023) (5% quantile) | 4202 | -0.37 |
| | 2C | Higher prevalence of contaminated udders with *Campylobacter* | High $P_{udder}$ | 0.95 | Assumption based on Knipper et al. (2023) (95% quantile) | 73,818 | 0.87 |
| 3 | 1D | Lower mean amount of milk per cow | Low $m_{cow\_liter}$ | 20 | Assumption | 14,525 | 0.17 |
| | 2D | Higher mean amount of milk per cow | High $m_{cow\_liter}$ | 40 | Assumption | 7614 | -0.11 |
| | 1E | Less cows contributed to bulk tank | Low $A_{herd}$ | ~ 1+(round (Gamma (0.3;14);0) | Assumption based on BMEL (2022) (5% quantile of $A_{herd_\alpha}$and $A_{herd\_\beta}$) | 7439 | -0.12 |
| | 2E | More cows contributed to bulk tank | High $A_{herd}$ | ~ 1+(round (Gamma (2;100);0) | Assumption based on BMEL (2022) (95% quantile of $A_{herd_\alpha}$and $A_{herd\_\beta}$) | 10,496 | 0.03 |
| 4 | 1F | Less reduction during storage based on 4 °C data | High $S_{reduction}$ | ~ Pert (225; 625; 1023) | Based on Giacometti et al. (2015) | 14,790 | 0.17 |
| | 2F | No reduction during storage | $S_{time}$/ $S_{reduction}$ | 0 | Based on Wulsten et al. (2020) | 16,928 | 0.23 |
| 5 | 1G | Lower mean volume of raw milk consumed | Low $m_{consumed}$ | 0.1 | Assumption (5% quantile of $m_{consumed}$ (a mug) | 4904 | -0.31 |
| | 2G | Higher mean volume of raw milk consumed | High $m_{consumed}$ | 0.5 | Assumption 95% quantile of $m_{consumed}$ (a mug) | 22,227 | 0.35 |
| Dose-response | 1H | Less raw milk's servings per year | Low $N_{served}$ | 2699,175 | Assumption minimum value (2.200 liter per year/per machine) based on Labohm et al. (2021) | 2023 | -0.69 |
| | 2H | More raw milk's servings per year | High $N_{served}$ | 85,070,550 | Assumption maximum value (70.000 liter per year/per machine) based on Labohm et al. (2021) | 63,595 | 0.81 |
| | 1I | Less unboiled raw milk's servings per year | Low $N_{served\_unboiled}$ | 6211,205 | Based on Giacometti et al. (2013) (14% unboiled) | 4670 | -0.33 |
| | 2I | More unboiled raw milk's servings per year | High $N_{served\_unboiled}$ | 19,077,272 | Based on Giacometti et al. (2013) (43% unboiled) | 14,163 | 0.16 |

addition, lower and higher values as input for the distribution of number of cows contributed to a bulk tank ($A_{herd}$) were investigated. The impact of storage of raw milk (step 4) was determined using the decimal reduction time ($S_{reduction}$) at 4 °C based on Giacometti et al. (2015) and a scenario without reduction of *Campylobacter* (Wulsten et al., 2020). The effect of the exposure (step 5) was simulated with lower and higher mean volume of raw milk consumed ($m_{consumed}$).In a last step the values of the parameter used for the dose-response were taken into account using lower or higher values for raw milk's servings ($N_{served}$) and unboiled raw milk's servings per year ($N_{served\_unboiled}$).

The scenario analyses were compared by calculating the log of the relative risks:

$$\text{Relative risk} = log\left(\frac{Risk_{alternative}}{Risk_{baseline}}\right) \quad (13)$$

$Risk_{baseline}$ is the value for "Output" as presented in Table 1. For the $Risk_{alternative}$ one parameter value in the model was changed into a value that represents a low or high end of the uncertainty interval around the value chosen in the baseline model. This was performed to analyze the sensitivity of the risk estimate for some of the uncertain model parameters. Additionally the impact on the risk of different intervention strategies during milking were evaluated.

## 3. Results and discussion

### 3.1. Model output

After simulation of the baseline model, the mean probability of infection is 0.22% and the mean probability of illness is 0.07% for consuming a random raw milk portion (a mug). Assuming that $1.33 \times 10^7$ *Campylobacter* risk servings without boiling prior to consumption were consumed in Germany per year, this results in an estimate of over 9300 campylobacteriosis cases per year. In general, the model estimates high prevalence and low concentrations of *Campylobacter*. Considering that a bulk tank is contaminated with *Campylobacter* when $A_{contaminated} > 0$, i.e. as soon as one cow with contaminated udders contributes milk to the tank, the model predicts that the true prevalence of contaminated bulk tanks is 93.3%. The distribution of the concentrations in contaminated bulk tanks, $C_{liter}$, is a skewed empirical distribution with a mean of -0.41 log (CFU/l) and sd 0.67. The 93.3% prevalence is very high compared to the 1 to 2.5% of bulk tank samples found positive for *Campylobacter* in the German zoonoses monitoring in recent years (BVL, 2010, 2012, 2016a, 2016b, 2020). However, with the limit of detection of 1 CFU/25 ml raw milk, as applied in this monitoring, by sampling from a Poisson ($10^{C_{liter}}/40$) distribution in the Monte Carlo simulations to account for the discrete nature of bacteria, we obtained an observed prevalence of 2.3% contaminated bulk tanks in the model, which shows the model results do not contradict those of the monitoring.

The estimated mean ingested dose of *Campylobacter* in contaminated raw milk is $D_{ingested} = 0.13$ CFU/portion, which means a mean concentration of *Campylobacter* per ml of raw milk from the bulk tank is equal to 0.00062 ± 0.076 CFU/ml. Assuming a homogeneous distribution and taking into account that bacteria are discrete units, it is found that 91.3% of the portions is uncontaminated (contains 0 CFU), 8.7% contains one or more *Campylobacter* cells and 0.046% contains ten or more.

The simulations for outbreaks from single vending machines, applying the baseline model, showed that the probability that more than two out of 43 consumers of raw milk get ill, $P(N_{ill} \geq 2)$, equals 0.22%, whereas this probability for 5 out of 100, $P(N_{ill} \geq 5)$, equals 0.01%. For 850 vending machines in Germany, this would be 2.2 and 0.05 daily outbreaks respectively, equivalent to about 800 and 20 annual outbreaks. These numbers are much larger than what is observed, which, next to uncertainty associated to the model, may be explained by the presumed acquired immunity of frequent consumers of raw milk and underreporting of campylobacteriosis. Further, we assumed a homogeneous distribution of *Campylobacter* in the raw milk at every time point (e.g. in bulk tank or vending machine). We used this approach as a basic assumption as the real distribution is not known. This could be another reason why our estimation is higher than what is observed.

The developed QMRA model captured the contamination route of raw milk by *Campylobacter* through feces via the udder. This indirect contamination is assumed to be the most important route (Bianchini et al., 2014; Del Collo et al., 2017; Knipper et al., 2023; Modi et al., 2015; Schildt et al., 2006). One study reported a direct excretion of *Campylobacter* via the mammary gland (Orr et al., 1995). However, this knowledge is only based on one study and for one cow investigated in that study. Therefore, this direct contamination has not been considered in this QMRA model to date.

To our knowledge, this is the first QMRA for *Campylobacter* in raw milk in Germany, but the results can be compared with similar studies in Italy and New Zealand (Anonymous, 2009; Giacometti et al., 2015). In the study from Italy the number of expected cases of campylobacteriosis in the Italian population per year due to raw milk consumption were 301,785 for the best time-temperature scenario (4 °C), whereby the mean concentration of *Campylobacter* were estimated as $1.42 \times 10^{-4}$ CFU/ml and standard deviation $1.93 \times 10^{-5}$ (Giacometti et al., 2015). The number of expected cases in adult consumers linked to raw milk consumption in relation to the percentages of consumers that do not boil milk before consumption and different time-temperature storage conditions ranged between 79.4/100,000 population/year and 333.1/100,000 population/ year using the Beta Poisson dose-response model ($\alpha = 0.145$ and $\beta = 7.589$). For young consumers (≤5 years old) the Betabinomial model ($\alpha = 0.024$ and $\beta = 0.011$) was applied and estimated cases for this sensitive population between 1013.7/100,000 population/year and 8110.3/100,000 population/year (Giacometti et al., 2015).

The QMRA from New Zealand predicted a mean number of cases of illness for adults from *Campylobacter* of 19.9 or 4.7 per 100,000 daily servings of raw milk based on consumption from the bulk milk tank or domestic consumption after farm gate purchase respectively (Anonymous, 2009). We estimate 69.5 cases per 100,000 daily unboiled servings and 20.9 cases per 30,000 daily unboiled servings (assuming that 30% of the 100,000 servings are unboiled).

Only few concentration data for *Campylobacter* in raw milk are available. These have been generated by the most probable number (MPN) method due to the often very low concentration of *Campylobacter* in raw milk. One study reported 0.16 ± 0.30 MPN/ml as mean *Campylobacter* level from five samples (Hill et al., 2012). Another one indicated approximately 0.047 MPN/ml (Humphrey and Beckett, 1987). The measured concentrations of *Campylobacter* in raw milk samples are higher than the mean estimated concentrations from QMRAs. This may be due to the fact that *Campylobacter* is difficult to detect by cultural methods and its survival is often underestimated by plate counts (CFU) (Wulsten et al., 2020).

Campylobacteriosis outbreak data based on raw milk consumption in Germany are available from 2017 (EFSA, 2018). In total, 15 outbreaks including 201 human cases were reported. Two of the outbreaks were attributed to direct consumption of raw milk on the farm. The other occurred due to consumption of raw milk from automatic distribution system for raw milk or inadequate heating at home. However, normally it is assumed that the number of cases in an outbreak associated with a farm visit, e.g. a total school class, is higher than in a 4–6 person household drinking raw milk at home (Heuvelink et al., 2009).

More recent data on campylobacteriosis outbreaks related to milk and milk products are available from the entire EU 2020 (EFSA, 2021). Only four outbreaks with in total 174 human cases were reported. Among them, three outbreaks occurred in Germany associated with the consumption of raw milk.

There are several reasons for the differences between the number of reported and predicted cases. Campylobacteriosis symptoms include mainly short-term complications such as fever, vomiting, abdominal

cramps and diarrhea. Only in few cases chronic complications occur (Jackson et al., 2014; Keithlin et al., 2014; Leonhard et al., 2019). This may lead to an underreporting in official numbers of campylobacteriosis, since people with short-term complications might not consult a doctor (Bouwknegt et al., 2014).

Further, there are many uncertainties around the values integrated in the baseline model. These are based on lack of data or detection limits of methods. To get an understanding on the impact of these values for the relative risk different uncertainty analysis were applied. Another hypothesis is that people who drink unboiled raw milk regular could get immune. Higher immunity would lead to overestimation of risk, since such immunity is not captured in the "classic" dose-response model (Havelaar and Swart, 2014).

### 3.2. Scenario analysis

Eighteen hypothetical scenarios were compared to the baseline model which can be used to evaluate the impact of parameter uncertainty on the relative risk and/or to explore the potential effect of different mitigation strategies along the raw milk supply chain (Fig. 2).

For every scenario a higher (e.g. 95% quantile) and lower (e.g. 5% quantile) value for the parameter were used, except for the decimal reduction time during storage. Here two scenarios with a lower decimal reduction time or no reduction time during storage were applied.

When comparing different risk mitigation options, the strongest reduction of campylobacteriosis cases associated with the consumption of unboiled raw milk estimated per year are obtained by a lower concentration of *Campylobacter* in cows feces (scenario 1A), a lower amount of fecal dirt on udders (scenario 1B) and a lower prevalence of contaminated udders (scenario 1C). On consumer side, a smaller portion size (scenario 1G), less raw milk portions consumed (scenario 1H) and a smaller amount of unboiled portions for consumption (scenario 1I) indicated the highest reduction. The amount of milk per individual cow as well as the amount of cows contributing to the bulk tank had no high impact on the relative risk (scenario 1,2D and 1,2E).

There was only a small difference in the increase of the relative risk compared to a lower decimal reduction time (scenario 1F and 2F). This could be explained by the fact that the storage time is smaller than the decimal reduction time (time needed to achieve one log reduction).

### 3.3. Effect of dose-response model choice

In addition to the "classic" DR model (Teunis and Havelaar, 2000), which is based on a human challenge study (Black et al., 1988) and used in the baseline model, an alternative DR model was applied. The alternative model (Nauta et al., 2022; Teunis et al., 2018) was defined by the median estimates of the model parameters for outbreak studies. As in the baseline model, the Beta-Poisson model was applied (Eq. (12)), with $\alpha = 0.38$ and $ß = 0.51$.

Whereas in the "classic" DR model $P_{ill/inf}$ is a constant, independent of the dose, in the outbreak dose-response model it is calculated as:

$$P_{ill/inf} = 1 - \left(1 + \frac{D}{\eta}\right)^{-r} \tag{15}$$

with $r = 0.76$ and $\eta = 0.0092$, $D = D_{ingested}$

Using the outbreak model, 895,891 campylobacteriosis cases are predicted for consuming raw milk in Germany. This value is much higher than the prediction from the baseline model. *Campylobacter* might survive better in raw milk due to the protecting fat content. In addition, in the outbreak dose-response model specific *Campylobacter* strains are considered that are very virulent.

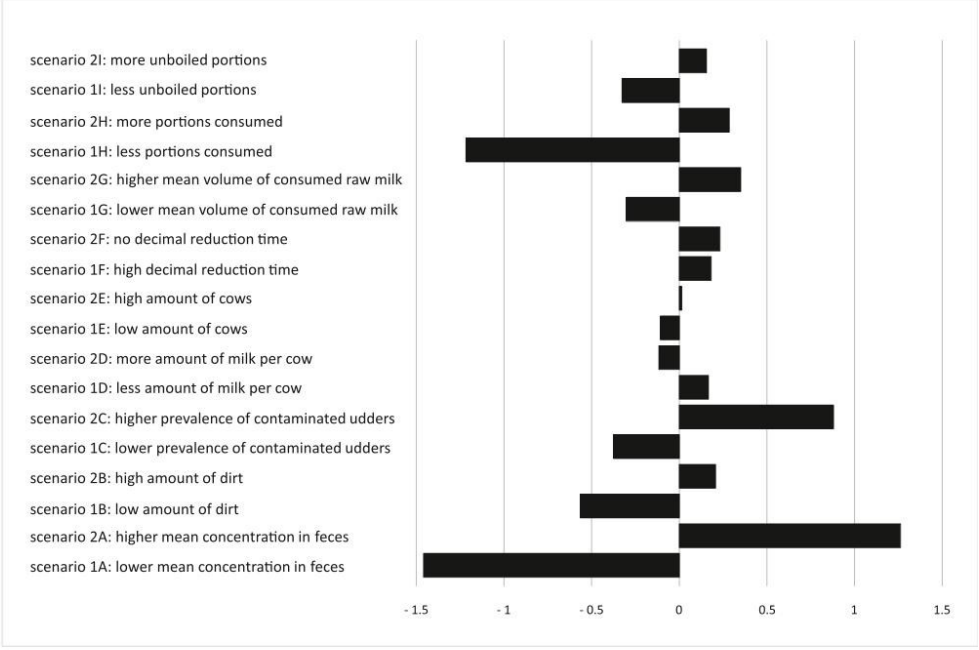


**Fig. 2.** Results of different scenario analysis to investigate the uncertainty around the model parameter values on estimated campylobacteriosis risk due to raw milk consumption, relative to the baseline model.

### 3.4. Implications for risk management

The QMRA performed provides insights in the effects of potential mitigation strategies to reduce the risk of campylobacteriosis from drinking raw milk. Additionally, it identified gaps of knowledge.

The results of the different scenario analyses confirmed that clean udders are of crucial importance as improper hygiene can lead to fecal cross-contamination during milking (Oliver et al., 2005). This confirms that if an inadequate udder hygiene practice is applied, a higher risk for *Campylobacter*-contaminated raw milk occurs (Beumer et al., 1988).

For example, surveillance data on the amount of raw milk sold and consumed, as well as on the prevalence and concentrations of *Campylobacter* in vending machines in Germany would be helpful to improve the assessments.

## 4. Conclusion

This study presents a QMRA to investigate the public health risk of human infection with *Campylobacter* from the consumption of raw milk in Germany. This is the first QMRA model for *Campylobacter* that took into account data from early steps in the supply chain including cow feces concentration data and prevalence data of contaminated udders. Farmers should focus on performing good farm hygiene practices as this will reduce the prevalence and number of *Campylobacter* on cow's udder and by this the risk for consumers. Further, to maintain sales of raw milk via vending machines the consumers are still encouraged to heat-treat raw milk prior to consumption. However, the consumer practices cannot be changed easily and risk management should take place on the farm side. In addition, consumer data on the amount of raw milk consumed in Germany is urgently needed to provide a more accurate risk assessment.

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## CRediT authorship contribution statement

**Anna-Delia Knipper:** Formal analysis, Methodology, Software, Visualization, Writing – original draft, Writing – review & editing. **Tasja Crease:** Conceptualization, Project administration, Writing – review & editing. **Taras Günther:** Software, Writing – review & editing. **Matthias Filter:** Writing – review & editing. **Maarten Nauta:** Conceptualization, Formal analysis, Methodology, Software, Writing – review & editing, Visualization, Supervision.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationship that could have appeared to influence the work reported in this paper.

## Data availability

The developed model is available in the model repository: https://knime.bfr.berlin/landingpage/RAKIP-Model-Repository. All relevant data are within the manuscript and its supporting information files.

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# 4 Discussion

Campylobacteriosis cases have been associated with the consumption of raw milk (Kenyon et al., 2020; Davis et al., 2016). In the years 2011 to 2020, raw milk was one of the food vehicles causing most “strong-evidence” foodborne campylobacteriosis outbreaks (EFSA, 2021). Thermotolerant *Campylobacter* spp. frequently colonize the intestines of dairy cows, without obvious negative health effects to host animals (Açik and Çetinkaya, 2005). Nevertheless, *Campylobacter* from cow feces can cross-contaminate teats and subsequently the raw milk, thereby posing a health risk to consumers (EFSA, 2015). The sale of raw milk is permitted in Germany and other countries, as long as guidelines are followed. These include the need for a label on vending machines to boil raw milk before consumption (BMJV, 2018). However, in a survey from Italy it became evident that 13.9% to 43% of consumers do not boil raw milk before consumption (Giacometti et al., 2013).

For a better understanding of the potential of raw milk to be contaminated by *Campylobacter* spp., prevalence and concentration data on *Campylobacter* spp. in feces of dairy cows were summarized from scientific literature in this PhD project. Furthermore, the occurrence and transmission of *Campylobacter* spp. on a small dairy farm was investigated for the duration of one year. The data obtained from the dairy farm samples and from literature raised the research question of for how long and in which status *C. jejuni* can survive in raw milk during storage. Therefore, additional experimental data were generated and modelled for the survival of *C. jejuni* in raw milk at different temperatures. Finally, the obtained data were used in a developed QMRA model for *Campylobacter* in raw milk to gain insight on the impact and usefulness of different intervention strategies along the supply chain.

## 4.1 Cow feces: The initial contamination source

A systematic literature review with meta-analysis on *Campylobacter* spp. prevalence and concentration in feces of dairy cows was performed (Knipper et al., 2022). *Campylobacter* spp. prevalence and concentration were summarized and data gaps were analyzed.

In the systematic review, 53 studies were included. Meta-data were extracted, and it was evident that there were substantial data gaps in the description and investigation of the individual studies. Most of the studies did not mention the health status of the cows investigated (n= 35) and did not further specify the *Campylobacter* species (n= 35). Samples were obtained primarily by rectal collection (n= 31). Different age groups of dairy cows were sampled with the most frequent being adult cows (n= 25). The age class and fecal collection method were not specified for eight and six studies, respectively (Knipper et al., 2022).

No meta-analysis could be performed for concentration data since only three studies provided this data (Ramonaitė et al., 2013; Nielsen, 2002), and one did not include a measure of variation (Waterman et al., 1984). Other studies gave only semi-quantitative estimates of the concentrations (Rapp et al., 2014; Moriarty et al., 2008; Stanley et al., 1998) or presented results in a boxplot without providing raw data (Rapp et al., 2012). The reported *Campylobacter* mean concentrations ranged between 2.1 ± 0.45 and 4.17 ± 0.54 log CFU/g feces (Ramonaitė et al., 2013; Nielsen, 2002; Waterman et al., 1984).

Only studies that included adequate information about prevalence could be included in the meta-analysis (n= 44). A total of 32 studies were identified that reported an aggregate prevalence value (average across the whole study). Based on that, the overall prevalence estimate was 29.3% with a prediction interval of 1.3% to 73% (Knipper et al., 2022). A multi-level model was used to investigate the pooled prevalence estimate based on the 44 studies, taking into account potential duplications due to different sub-groupings and data aggregation. The pooled prevalence was 51%, with a prediction interval of 44% to 57% (Knipper et al., 2022). The prevalence of *Campylobacter* spp. in cow feces varied widely. Some studies investigated flies or birds as additional contamination sources (Sanad et al., 2013; Adhikari et al., 2004). Adhikari and colleges (2004) found the highest prevalence in feces of cows (54%), followed by feces from sparrows (40%). Only a low prevalence was observed for whole flies (9%) (Adhikari et al., 2004). In contrast, another study found a significantly ($p<0.01$) higher prevalence of *C. jejuni* in birds than in dairy cattle (Sanad et al., 2013). Birds are also assumed to play a role as contamination source in the transmission of *Campylobacter* spp. along the raw milk supply chain. However, it is important to take into account the husbandry of the animals in order to evaluate the influence of birds. Birds often practically live in the barns. Therefore, stables cannot be cleaned that fast to avoid their droppings having an effect. The amount of feces produced per animal is significantly lower for most birds than for cows. Hence, we assumed that the greatest influence on *Campylobacter* spp. prevalence in raw milk is from cow feces.

Sub-group analyses were performed on the aggregated sample prevalence if three or more outcomes were available per subgroup. It was performed for the fecal collection method and the age class of cows (Knipper et al., 2022). No significant difference ($p= 0.52$) was observed between the prevalence estimates for rectal extraction (28%) and cow pat collection (32%). For heifers, not enough aggregated outcomes were available for sub-group analysis ($n<3$). Therefore, the prevalence estimates for calves (18%) and adult cows (30%) were compared. No significant differences were found ($p= 0.06$). The results from the multi-level model including sub-groups differed partly from the sub-group analysis. Here, the rectal fecal collection method in comparison to cow pat collection was significantly associated with higher

prevalence data (Knipper et al., 2022). This is probably due to the heterogeneity between the two sub-groups, which have been adjusted in the multi-level mixed-effect model. It is clear that effective comparisons of raw data from the literature are only possible when sufficiently detailed descriptions of sub-groupings are reported.

Based on this study, cow feces can be an initial source to consider that can lead contamination of raw milk. For this cross-contamination, *Campylobacter* positive feces must be excreted by the cow. Subsequently, the same or other cows must lie down in the cow pat to cause a potential contamination of the udders. Insufficient housing conditions and milking time hygiene can finally lead to contamination of raw milk.

It is apparent that concentration data of *Campylobacter* spp. in cow feces is scarce. Prevalence data are available to provide an overview of the occurrence of *Campylobacter* in cow feces. The data from the literature were often only available in aggregated state. However, it is important for the further use of the data that the individual data are retained and available. Authors of future studies should move towards the Findability, Accessibility, Interoperability, and Reuse (FAIR) data principles for scientific data and also deposit raw data in a suitable format to enable further open-access analysis (Wilkinson et al., 2016).

### 4.2 Transmission of *Campylobacter* spp. along the raw milk supply chain

Longitudinal studies covering the raw milk supply chain are needed for assessing the potential of *Campylobacter* spp. contamination in raw milk. Concentration and prevalence data from the barn, teat skin and milking environment are particularly helpful in assessing the potential of possible transmission.

A study was conducted to assess the potential of *Campylobacter* spp. contamination in raw milk through collection of feces samples from dairy cows and samples from the environment and during milking over one year (Knipper et al., 2023a). The highest *Campylobacter* spp. prevalence (77.1%) was found in cow feces, which supports the assumption that cow feces are an initial source of contamination (Knipper et al., 2023a; Knipper et al., 2022). The mean concentration of *Campylobacter* spp. in cow feces was 2.43 ± 0.9 log CFU/g (n=215) (Knipper et al., 2023a). In total, 29.2% of the boot sock samples taken from the entire barn corridor, avoiding fresh fecal pats, were positive for *Campylobacter* spp. For four boot sock samples quantitative data could be obtained for *Campylobacter* spp. with a mean concentration of 3.01 ± 1.05 log CFU/2 socks. Among the teat skin samples, 12.2% were positive for *Campylobacter* spp. The mean concentration of *Campylobacter* spp. in cow teat skin samples was 1.26 ± 0.75 log CFU/4 teats (n=35). None of the swab samples of milking clusters (with four teat cups) was positive. To achieve better milking hygiene between cows due to the poor udder health of some

cows an intermediate disinfection of the teat cups between cows was introduced on the farm. However, the swab samples of milking equipment were performed at the end of the completed milking process of all cows. Therefore, in this study the milking clusters were not assumed to be an entry source of *Campylobacter* spp. in the raw milk supply chain. Raw milk samples were taken after the teats had been cleaned. No further disinfection of the teats was performed to map the routine milking situation. One raw milk sample (0.4%) from an individual cow and one milk filter were positive on the same day with 2.37 log CFU/ml and 2.74 log CFU/filter respectively. In addition, on the same sampling day, nine teat swab samples were tested positive (Knipper et al., 2023a).

Rectal fecal extraction from 12 cows over the period of one year allowed examination of fresh feces and assignment of fecal samples to individual cows over time (Knipper et al., 2023a). Examination of fresh feces allows a higher chance of detection of oxygen sensitive *Campylobacter* spp. (Hoar et al., 1999). There were differences in *Campylobacter* spp. concentration in feces between cows and variations within the same cows at different sampling points (Knipper et al., 2023a). A study from New Zealand showed that cows intermittently excrete *Campylobacter* spp. with their feces (Rapp et al., 2012). This should be considered in the risk assessment of cross-contamination of raw milk by fecal origin using probability distributions.

This is the first longitudinal study that generated quantitative data for *Campylobacter* spp. on cow teats in Germany (Knipper et al., 2023a). To our knowledge, there is only one study from Norway that found a similar prevalence with 13% (Idland et al., 2022). However, the positive samples were not quantitative analyzed for *Campylobacter* spp.

A multi-level logistic model was used to investigate the association parameters on the presence of *Campylobacter* spp. in the feces and teat skin swab samples (Knipper et al., 2023a). Season as a whole had a significant impact on the occurrence of *Campylobacter* spp. The model showed a significant difference between the summer and winter. The minimum temperature influenced the occurrence of *Campylobacter* spp. significantly where the temperature effects in teat and feces samples differ. *Escherichia coli* was used as fecal contamination indicator. An association was observed between *E.coli* and the occurrence of *Campylobacter* spp. (Knipper et al., 2023a). This supported the assumption that the cross-contamination of teats with *Campylobacter* spp. had a fecal origin. The consistency of feces was not associated with the C*ampylobacter* spp. occurrence. The teat scores for cleanliness of teats were not found to be a parameter that influenced the presence of *Campylobacter* spp. (Knipper et al., 2023a). Another study found also no association between the cow hygiene score (three zones: the udder, lower and upper portions of the hind limbs/flanks summarized

in one total score) and detection of *Campylobacter* spp. in feces and teat swabs (Idland et al., 2022). Still, they observed an association between hygiene score and detection of *Campylobacter* spp. in teat milk samples (Idland et al., 2022). No association was found between *Pseudomonas* spp. (indicator of environmental contamination) or TACC and the occurrence of *Campylobacter* spp. (Knipper et al., 2023a).

A correlation analysis was performed for the concentrations of *Campylobacter* spp. in feces samples and in teat skin swab samples (Knipper et al., 2023a). No correlation was detected.

In conclusion, the results showed that sporadic cross-contamination of raw milk with *Campylobacter* spp. can occur. Although on one sampling day, nine teat swab samples, one raw milk sample and the milk filter was tested positive for *Campylobacter* spp. none investigated parameter was found that influenced this sporadic contamination event (Knipper et al., 2023a). The results suggested that there are still unknown factors not covered in this study influencing the occurrence of *Campylobacter* spp. in raw milk. However, the data can provide a basis for the development of a QMRA for *Campylobacter* spp. in the context of raw milk.

The obtained *C. jejuni* isolates from the longitudinal study should be sequenced to perform cluster analysis to investigate which core-genome multilocus sequence types (cgMLST) are related to the different sampling matrices. The different time points of the samples should be considered in cluster analyses to analyze changes of the cgMLST over time. This could indicate whether adaptations of *C. jejuni* strains occurred over the year in which the investigation occurred or whether strains persisted.

### 4.3 Survival of *C. jejuni* in raw milk

*C. jejuni* and *C. coli* are the most important human pathogenic species of *Campylobacter*. *C. jejuni* occurs more frequently in poultry and cattle, while *C. coli* is more likely to be detected in pigs (BVL, 2022). In contrast to animals that are asymptomatically colonized by *Campylobacter* spp., severe infections can occur in humans (EFSA, 2022). Data from zoonotic monitoring in Germany demonstrated that between 1% and 2.5% of bulk tank milk samples have been *Campylobacter* positive in recent years (BVL, 2020, 2016, 2012, 2010). Only low concentrations of *Campylobacter* spp. are reported in raw milk (Jaakkonen et al., 2020; Hill et al., 2012; Humphrey and Beckett, 1987).

In order to evaluate how high the risk is from the consumption of *C. jejuni* contaminated raw milk for the consumer, it is also necessary to investigate the survival of *C. jejuni* in raw milk. *Campylobacter* is a fastidious organism with the need for a reduced oxygen and increased

$CO_2$ atmosphere and cannot multiply at temperatures below 30°C (Kim et al., 2021). It can survive harsh conditions in a VBNC state in which it is no longer detectable by cultural detection methods (Baffone et al., 2006). Comparing IPIU data obtained by a v-qPCR to CFU data, an underestimation of survival of up to 4.5 $\log_{10}$ CFU/ml by CFU data became evident for *Campylobacter* strains in raw milk stored at 5°C (Wulsten et al., 2020). Wulsten and colleagues (2020) showed this underestimation for the reference strains *C. jejuni* DSM 4688 and *C. coli* DSM 4689, as well as for an outbreak strain isolated from raw milk (BfR-CA-13290). Cells in VBNC status could be converted back to CFU status within a time window of 96 h for *C. coli* DSM 4689, 120 h for *C. jejuni* DSM 4688 and 144 h for BfR-CA-1290, using a gas mixture with extremely low oxygen partial pressure (Wulsten et al., 2020).

Two cultural detection methods, the first according to ISO 10272-2 (enumeration of CFU) and the second with the modified gas mixture at 37°C and with prolonged incubation time (recovery of VBNC) were used. A molecular method, v-qPCR (detection of IPIU), was applied to further explore the survival of *C. jejuni* (Knipper et al., 2023b; Wulsten et al., 2020). The pH of the raw milk was stable during the experiment with 6.8 ± 0.041 at each temperature, measured in uninoculated control samples.

The same *C. jejuni* reference strain DSM 4688 as in Wulsten et al. (2020) was used. *C. jejuni* DSM 4688 was obtained from the German Collection of Microorganisms and Cell Cultures GmbH (DSMZ) and was originally isolated from bovine feces in Belgium. In addition, two field strains were used, BfR-CA-18040 (sequence type (ST)-61 according to multilocus sequence typing scheme (Jolley et al., 2018; Dingle et al., 2001)) and BfR-CA-18043 (ST-21), previously isolated from feces of dairy cows from the BfR farm.

*C. jejuni* strain DSM 4688 and BfR-CA-18043 recovered from the VBNC state into a culturable state at 8°C and 12°C (Knipper et al., 2023b). The recovery time periods for both strains were longer at 8°C compared to 12°C, with up to 80 h and 72 h, respectively. At 12°C, the BfR-CA-18043 strain showed a longer recovery time (up to 72 h) compared to the DSM 4688 strain (up to 48 h). Wulsten and colleagues (2020) demonstrated recovery up to 120 h for *C. jejuni* (DSM 4688 and BfR-CA-1290) at 5°C. It was evident that the time window for recovery of VBNC cells decreases with increased temperature.

The recovery of VBNC at low oxygen levels led to the assumption of the presence of a yet unknown oxidative stress response sensor (Wulsten et al., 2020). Our data suggest that this oxidative stress response sensor might be influenced by temperature. A high temperature leads to a shorter period in which *C. jejuni* can be recovered from VBNC to CFU.

Survival studies were performed at 5°C, 8°C, and 12°C, corresponding to the variation of temperature in raw milk stored in vending machines (Böhnlein et al., 2020). As previously noted

by Wulsten and colleagues (2020) at 5°C in raw milk, we could not detect a difference in survival between the *C. jejuni* strains based on IPIU data and tested temperatures. In contrast, the CFU data showed a reduced survival of DSM 4688 compared to BfR-CA-18043. The CFU data also showed a reduced survival of both strains at higher compared to lower temperatures. This is in line with previous studies indicating a slower non-thermal inactivation of CFUs at cold temperatures (Boleratz and Oscar, 2022; Christopher et al., 1982).

The obtained CFU data and IPIU data confirmed the suspicion of an underestimated survival of *Campylobacter* spp. in raw milk from Wulsten et al. (2020). The recovery of VBNC cells into CFUs showed that *C. jejuni* was viable at least during the time periods of recovery (Baffone et al., 2006). Other studies reported CFU recovery from VBNC in microaerobic conditions (Bovill and Mackey, 1997), in embryonated chicken eggs (Cappelier et al., 1999) and *in vivo* using mouse infection models (Baffone et al., 2006). The capacity of recovery from the VBNC to the CFU state with no apparent loss of virulence potential of the resulting CFU highlights the potential regarding the presence of VBNC bacteria in food (Li et al., 2014). Nevertheless, the degree of direct infectivity of VBNC is not known, and awaits in-depth investigation. Further studies are needed to determine the infectious potential of *Campylobacter* spp. in the VBNC status. One approach would be to test VBNCs within and beyond the "recovery period" in animal models. Furthermore, the infectious doses of VBNC and CFU might be different and would need to be investigated in more detail.

### 4.4 Modelling the survival of *C. jejuni* in raw milk

Predictive models are tools for predicting microbial behavior in food environments (e.g. growth or decay). They enable a rapid response to specific food quality and food safety questions (Pérez-Rodríguez and Valero, 2013). The CFU and IPIU data sets were used to develop three tertiary models to predict the concentration of *C. jejuni* DSM 4688 and BfR-CA-18043 in raw milk at given time points depending on temperature (5°C to 12°C).

The primary trilinear model equation best described the CFU data. The primary Weibull model equation had the best goodness of fit for the IPIU data (Knipper et al., 2023b). The need for different primary models to describe the CFU and IPIU data sets has already become evident from the graphical representation. The CFU data of the two strains required two secondary models to determine the effect of temperature on $k_{max}$, whereas the IPIU data were independent of the strain and could be described in one secondary model for $\delta$.

During the model design of the CFU data an assumption was included in the design. The tail of the trilinear model was set to -0.1 log CFU/ml. This was an assumption based on the detection limit of the experimental setup of 1 CFU/ml. Other approaches to deal with non-

detects values have been already proposed, including fitting a distribution for differentiation of artificial and true zeroes (Duarte et al., 2015), or using the maximum likelihood estimation (MLE) or Bayesian models (Bahk and Lee, 2021). An analysis was performed to include censored data in the model development using a MLE with a normal distribution. The inactivation rate did not change significantly. However, in our model, the predictions of the values from the tail must not be seen as numerical values, but only as very low concentrations below the detection limit (Knipper et al., 2023b).

Models were validated with independent internal data not used for model development at 5°C, 8°C and 12°C. The CFU and IPIU data-derived tertiary models were able to make accurate predictions (Knipper et al., 2023b). External data from other studies often do not match the range-of-mode-applicability and are therefore difficult to use for comparison of model predictions. Experimental CFU data from ComBase (Christopher et al., 1982; Doyle and Roman, 1982) could not be used for model validation as these were outside the application range (5°C to 12°C) of the generated models. Christopher and colleagues (1982) determined the effect of temperature and pH on the survival of *Campylobacter fetus* in skim milk. The storage temperatures of the available data in ComBase of the survival studies were 1°C, 10°C, 20°C and 30°C (Christopher et al., 1982). Even though the 10°C data fit the temperature range of the developed model, a different strain (*C. fetus*) and matrix (skim milk) were used. Another study determined *C. jejuni* survival in unpasteurized milk at 4°C (Doyle and Roman, 1982) where the temperature is outside of the application range of the generated models. The CFU and IPIU data of *C. jejuni* DSM 4688 from Wulsten et al., 2020 could be used for external validation as they were collected under the same experimental conditions. The tertiary model predicting IPIU demonstrated a good accuracy and performance of the model predictions using IPIU data (Knipper et al., 2023b). The outcome of the validation of the CFU DSM 4688-derived model with CFU data was not acceptable. The large deviation from the predictions of the model and the CFU data from Wulsten et al., 2020 are assumed to be attributed to the different batches of the raw milk used. Raw milk is a complex matrix made up of water, protein, fat, lactose, vitamins and minerals, with proportions of each being influenced by animal breed, feed, age and phase of lactation (Hudson et al., 2015). The results indicated that CFU data are more affected by environmental factors (e.g. matrix and temperature) than IPIU data. To make the developed models even more robust, further survival data at temperatures between 5°C and 12°C should also be considered for validation. In addition, different milk batches should be used to clarify the influence of the composition of the complex milk matrix.

An already existing model for survival of *C. jejuni* in liquid based on CFU data was developed based on a meta-analysis (Membré and Lambert, 2008). They used a log-linear model to fit the curves obtained from the literature. The log-linear model did not consider a possible

stationary phase (shoulder before log reduction). In our CFU data-derived models, we clearly demonstrated the need of a shoulder to accurately describe the data. Therefore, the model based on the meta-analysis underestimated the survival ability of *Campylobacter* spp. Raw milk is a complex matrix and CFU survival differed when using the same strain together with milk batches obtained from different years (Knipper et al., 2023b). Therefore, it can be assumed that the survival of *C. jejuni* differs remarkably in different liquid matrices. Data from ComBase were recently used to develop an artificial neural network (ANN) model for non-thermal inactivation of *C. jejuni* in raw milk (Boleratz and Oscar, 2022). The ANN model is a different approach than the traditional regression method used in this study (Najjar et al., 1997). Even though this approach can be used to model data that are not possible to model by traditional regression methods, some disadvantages (parameters lacking biological meaning or poor interpolation) are given. However, they could demonstrate, similar to our case, that CFU survival was better at lower temperatures than at ambient temperatures. The validation of the model failed because of data gaps. No IPIU data were considered in the model.

The developed CFU data-derived models and IPIU data-derived tertiary models differed significantly in the time required to achieve an x log reduction ($t_{xd}$) (Knipper et al., 2023b). For the CFU data it varied between 8.6 h and 18.46 h depending on the strain and the temperature. For the IPIU data it was outside of the application range of the Weibull model (>100 h). This demonstrated that *C. jejuni* can survive in VBNC state until the end of the shelf-life of raw milk, assuming that raw milk is stored for one week (Knipper et al., 2023b).

The survival of *C. jejuni* is largely underestimated if only based on CFU data. In accordance with the recovery data of VBNC, the real potential of the presence of infective *C. jejuni* in raw milk should be at some point between the predictions of CFU and IPIU (Knipper et al., 2023b). The degree of infectivity of cells in VBNC status is unknown, IPIU data should be taken into account as a worst-case scenario, as these VBNCs still might be infectious. In addition, further recovery data should be collected and a predictive model developed on these data.

### 4.5 Risk mitigation strategies

QMRAs are a helpful tool for assessing different risk mitigation strategies of bacteria along the food chain. Two different QMRAs for *Campylobacter* spp. in milk are found in the literature. These are focused on the bulk tank milk and storage in vending machines (Giacometti et al., 2015; Anonymous, 2009).

A QMRA model for *Campylobacter* spp. along the raw milk supply chain in Germany was developed with focus on the farm level (Knipper et al., 2023c). The data used in the model were obtained during a longitudinal study over one year (Knipper et al., 2023a). Only CFU

concentration data of *Campylobacter* were considered in this model. A QMRA from Australia (Anonymous, 2009) used fecal concentration data of *Campylobacter* spp. from Stanley et al. (1998), where they used the MPN method (1.79 ± 1.01 log CFU/g). The other QMRA from Italy did not take into account fecal concentration data (Giacometti et al., 2015).

The QMRA model predicts a mean probability of infection of 0.22% and a mean probability of illness of 0.07% for consuming a random raw milk portion (a mug) (Knipper et al., 2023c). We assumed that per year, 4.44 x $10^7$ raw milk portions (210 ml; one mug) were consumed in Germany per year. The servings are calculated assuming that 850 vending machines in Germany (Böhnlein et al., 2020), operate 365 days a year, selling at least 30 litres per day (Labohm et al., 2021), which corresponds to 143 servings of 210 ml of milk. Of these servings 30% are not boiled prior to consumption (Giacometti et al., 2013). This results in 1.33 x $10^7$ *Campylobacter* risk servings without boiling prior to consumption consumed in Germany.

The model estimates over 9,300 campylobacteriosis cases per year. These are 69.5 cases per 100,000 daily unboiled servings. A QMRA from Italy yielded between 79.4/100,000 population/year and 333.1/100,000 population/ year of expected cases for adult consumers based on percentages of consumers that do not boil milk before consumption and different time-temperature storage conditions (Giacometti et al., 2013). In a study from New Zealand, a mean number of cases of illness for adults of 19.9 or 4.7 per 100,000 daily servings of raw milk were predicted based on consumption from the bulk milk tank or domestic consumption after farm gate purchase respectively (Anonymous, 2009).

Overall, the model estimates a high prevalence but low concentration of *Campylobacter* spp. in bulk tank milk. The model predicts that 93.3% of the bulk tanks are *Campylobacter* contaminated with a mean concentration of -0.41 $\log_{10}$ (CFU/l). In comparison with the German zoonoses monitoring in recent years which monitored 1 to 2.5% of bulk tank samples positive the 93.3% prevalence obtained from the model is very high. For zoonosis monitoring, samples should be analysed in the laboratory as soon as possible, so that CFU is expected not to have considerably declined. However, in the baseline model it is assumed that as soon as one cow with contaminated udders contributes milk to the tank the bulk tank becomes contaminated. Nevertheless, with the applied limit of detection of 1 CFU/25 ml raw milk in the monitoring the observed prevalence in the model is 2.3% contaminated bulk tanks. This underlines the model results do not contradict those of the monitoring (Knipper et al., 2023c).

We took into account that bacteria are discrete units (integer values), whereby it is found that 91.3% of the portions are uncontaminated, 8.7% contain one or more *Campylobacter* spp. cells and 0.046% contain ten or more (Knipper et al., 2023c). In the literature only few concentration data for *Campylobacter* in raw milk are available. These have been generated by the MPN

method due to the often very low concentration of *Campylobacter* in raw milk. Studies reported 0.16 ± 0.30 MPN/ml as mean *Campylobacter* level from five samples or indicated approximately 0.047 MPN/ml (Hill et al., 2012; Humphrey and Beckett, 1987).
The model was applied for simulation for outbreaks from single vending machines. It was assumed that 30% of 143 servings of raw milk per year from a vending machine are consumed unboiled ($n_{unb}$= 43 ≈ 0.3*143). The probability that more than two out of 43 consumers of raw milk get ill, P($N_{ill}$ ≥ 2), equals 0.22%, whereas this probability for 5 out of 100, P($N_{ill}$ ≥ 5), equals 0.01%. This would be 2.2 and 0.05 daily outbreaks respectively, equivalent to about 800 and 20 annual outbreaks in Germany for 850 vending machines (Knipper et al., 2023c). In 2018 most of the reported outbreaks were caused by *Campylobacter* spp. associated with the consumption of unboiled raw milk (9/38 outbreaks) (BVL, 2019).

In general, the estimated outbreak numbers obtained from the model are much larger than what is observed. There are several reasons for it. In general, campylobacteriosis cases are assumed to be underreported, since people with self-limiting complications might not go to a doctor (Bouwknegt et al., 2014).

Furthermore, besides to uncertainty associated to the model, frequent consumers of raw milk may acquire immunity and this is not captured in the “classic” dose-response model (Havelaar and Swart, 2014; Teunis and Havelaar, 2000). We considered an alternative dose-response model which is defined by the median estimates of the model parameters for outbreak studies (Nauta et al., 2022; Teunis et al., 2018). This resulted with 895,891 campylobacteriosis cases in a higher value than the prediction from the baseline model. However, this dose-response model considered specific *Campylobacter* strains that are very virulent.

Different scenarios were compared to the developed baseline model to evaluate the impact of parameter uncertainty on the relative risk and to explore the potential effect of mitigation strategies along the raw milk supply chain. The underestimation of survival of *Campylobacter* spp. in raw milk by CFU (Knipper et al., 2023b; Wulsten et al., 2020) was considered using a scenario were no inactivation of *Campylobacter* spp. during storage occurs. However, whether *Campylobacter* spp. is inactivated or not has only a low impact on the relative risk assuming that infections occur from rare high level contaminations. If there is already only a low concentration in the bulk tank, the inactivation no longer has a major influence. It was demonstrated that clean udders are of crucial importance (Knipper et al., 2023c).

Nevertheless, important surveillance data for the amount of sold and consumed raw milk in Germany are lacking. Furthermore, concentrations of *Campylobacter* in vending machines in Germany are unknown, since only prevalence data for *Campylobacter* spp. are available (BVL, 2017).

The developed QMRA model is the first QMRA model for *Campylobacter* spp. along the raw milk supply chain in Germany. It is the only existing one that includes concentration data for *Campylobacter* spp. in dairy cow's feces and prevalence data from contaminated teats through fecal cross-contamination. A risk assessment model can give insights in levels below the microbiological detection limits. This is useful for the understanding of the microbiology and low risk situations. Risk is often in the tail (i.e. rare high concentrations) – for these more samples may be needed than is practically feasible.

### 4.6 Limitations

There were many prevalence data for *Campylobacter* spp. in cow feces from dairy cows in scientific literature (Knipper et al., 2022). However, there was hardly any quantitative data. No meta-analysis on quantitative data was possible.

A longitudinal study was performed to detect and quantify *Campylobacter* spp. in different samples along the raw milk supply chain (Knipper et al., 2023a). Only one raw milk sample and one milk filter tested positive for *Campylobacter* spp. A high concentration could be detected in these samples. However, the concentration of *Campylobacter* spp. is usually very low at the end of the supply chain. The ISO 10272-1 detection method reaches its detection limit. Therefore, no statement can be made whether we still have very low concentrations of *Campylobacter* spp. or whether there is no contamination. The v-qPCR developed by Wulsten et al. (2020), which was used for the survival study, could not be applied in the longitudinal study. Because of the PMA staining and for DNA extraction in general, dilution of the samples must occur, which further dilutes the already low concentration of *Campylobacter* spp. The dilution steps reaching the detection limit of the method. The longitudinal study was performed on a small dairy farm (Knipper et al., 2023a). Good housing conditions and milking time hygiene were found. Only limited comparison to conventional farms is possible.

The survival of *C. jejuni* was investigated and modelled for different temperatures in raw milk (Knipper et al., 2023b). Two different data sets for CFU and IPIU data were obtained. Since the infection potential of VBNC is unknown, the IPIU data can be used as a worst-case scenario for the survival of *C. jejuni* in raw milk. However, the true values are probably somewhere between the worst-case scenario and the CFU data. The recovery data obtained with a specific gas mixture with a lower oxygen level suggest a first hint as to how many cells are capable to reach cultivable state with potential infectivity. The recovery method should be used as a valid method in addition to the ISO 10272-2 method at low *Campylobacter* spp. concentrations. All existing dose-response models used in QMRAs are based on CFU data. Therefore, the use of IPIU data in QMRAs remains challenging. As soon as knowledge about

the infectivity of *Campylobacter* in VBNC status is obtained or about recovery of CFU within environmental niches (e.g. in conjunction with oxygen-consuming microbiota), the establishment of dose-response models for IPIU data can be initiated and discussed.

The developed models are a first step towards a more accurate prediction of the survival of *Campylobacter* spp. in raw milk. Still, there is a need to extend the developed predictive models with further influencing parameters such as temperatures and differences in raw milk batches. When appropriate, other strains should also be used to improve and expand the models. For distinct outcome of the model, it is important to ensure that these do not differ significantly in survival. Likewise, two *C. jejuni* strains from the clonal complex CC-21 BfR-CA-18040 (ST-61) and BfR-CA-18043 (ST-21) could be combined in one model (Knipper et al., 2023b).

The developed QMRA model is the first one to include farm-level data from Germany and give information about different risk mitigation strategies and control options (Knipper et al., 2023c). QMRAs also rely on surveys of the population, so consumption and storage data of raw milk in households in Germany are essential. There should also be an official platform or survey of raw milk outlets. Both sets of data are not yet publicly available. Only prevalence data for *Campylobacter* spp. in vending machines in Germany are accessible (BVL, 2017).

# 5 Conclusion

*Campylobacter* are fastidious bacteria with high requirements for survival. However, *C. jejuni* have been shown to persist on a dairy farm and contaminate bulk tank milk for seven months or longer (Jaakkonen et al., 2020). The transmission routes of *Campylobacter* spp. from fecal cross-contamination into raw milk is unknown.

Scientific literature showed that cow feces are often contaminated with *Campylobacter* spp. A longitudinal study demonstrated that transmission of *Campylobacter* spp. can occur due to cross-contamination of teats by cow feces into raw milk even though it is a rare event. Survival of *C. jejuni* in raw milk is underestimated by CFU data. Therefore, IPIU data should be used as a worst-case scenario even though the infectivity of VBNC or its return into CFU is not yet clarified. VBNC can be recovered in CFU within a certain time window *in vitro*. During these periods VBNC might still be infectious. However, further experiments are necessary to investigate the infectivity of VBNC within and beyond the recovery period. A QMRA model indicated clean udders as crucial importance to lower the risk of *Campylobacter*-contaminated raw milk. However, consumers should primarily heat their raw milk before consumption to reduce exposure to *Campylobacter* spp. from raw milk consumption and the risk of campylobacteriosis.

# 6 Summary

The consumer demand for raw milk has increased and local sales via raw milk vending machines have intensified in recent years. Alongside, many foodborne outbreaks in Germany were caused by *Campylobacter* spp. in raw milk that had not been properly heat treated prior to consumption. The consumption of not heat-treated raw milk contaminated with *Campylobacter* spp., remains a health risk for consumers. *Campylobacter* spp. often colonize the intestine of cows without causing clinical symptoms. Feces from *Campylobacter*-colonized cows pose a risk for cross-contamination of teats, which can transfer the *Campylobacter* spp. contamination into raw milk during the milking process. To date, it has not been clarified how exactly *Campylobacter* spp. contamination occurs along the raw milk supply chain.

The aim of this PhD project was to identify and fill data gaps regarding *Campylobacter* spp. transfer along the supply chain of raw milk and to investigate its survival in raw milk. Finally, possible intervention strategies to minimize the risk from raw milk consumption were outlined.

Within the framework of this PhD project a systematic review and meta-analysis was performed to summarize previous research on *Campylobacter* spp. in feces of dairy cows worldwide. The prevalence varied widely from 0-100%. Substantial data gaps within the studies, such as the health status of the animals and type of fecal sampling could be identified. Only limited quantitative data on *Campylobacter* spp. in the feces of dairy cows were available. The reported *Campylobacter* mean concentrations ranged between 2.1 and 4.17 log CFU/g feces.

A longitudinal study was performed to investigate the transmission of *Campylobacter* spp. along the raw milk supply chain. Therefore, different samples were taken from a small dairy farm of the BfR over a period of one year. The investigated samples comprised rectal feces, boot socks, teat skin swabs, milking cluster swabs, raw milk and milk filters. Samples were analyzed for the presence and concentration of *Campylobacter* spp., *E. coli*, total aerobic colony count (TACC) and for *Pseudomonas* spp. A scoring was performed for the consistency of cows´ feces and the level of cleanliness of the teat skin swab samples. In total, *Campylobacter* spp. were detected in 77.1% of the fecal samples with an average concentration of 2.43 ± 0.9 log CFU/g. There was variation in the concentration of *Campylobacter* spp. in cow feces within the herd as well as individual cows and over time. The barn environment was tested with boot socks. Of the boot sock samples 29.2% were tested positive with a mean concentration of 3.01 ± 1.05 log CFU/two socks. Contaminated barn environment posed a risk for fecal cross-contamination of the cows' teats. Among the teat swabs, 12.2% tested positive for *Campylobacter* spp. None of the milking clusters were positive. Only one raw milk sample and the milk filter tested positive on the same day. In

addition, on this day nine teat swab samples were tested positive for *Campylobacter* spp. The results showed that contamination of raw milk, albeit rare, can occur.

The data obtained from the dairy farm samples raised the research question about time and physiological state of *C. jejuni* during suvival in raw milk during storage. Therefore, the survival of *C. jejuni* was investigated by two different methods, culturally according to ISO 10272-2 and in addition using a quantitative polymerase chain reaction specifically detecting viable *Campylobacter* spp. in raw milk (v-qPCR). The experiment was performed at three temperatures (5°C, 8°C, and 12°C). While only colony-forming units (CFU) can be detected according to ISO 10272-2, the newly developed v-qPCR (Wulsten et al., 2020) allows detection of intact and potentially infectious units (IPIU), which includes CFU and viable but non-culturable units (VBNC). The survival of three *C. jejuni* strains was tested. According to Wulsten et al. (2020) who performed their experiments at 5°C, the survival was also underestimated in our study by CFU for the strains and temperature tested. To date, the infectious potential of *Campylobacter* spp. in VBNC status is unknown. Wulsten et al. (2020) demonstrated that *C. jejuni* VBNCs developed upon incubation in raw milk at 5°C were recovered into a cultivable state (recovery) within a certain time period (at least 90 h), using a specific gas mixture with a lower oxygen level for 72 h. Here, the recovery was investigated at 8°C and 12°C. The recovery time periods for both strains were longer at 8°C (up to 80 h) compared to 12°C (at least up to 48 h). Hence, the recovery period shortened with increasing temperature.

Predictive models providing a rapid response for predicting microbial behavior in the food environment were developed on the two generated data sets. Three tertiary models consisting of one for all IPIU data and two for the CFU data due to the necessary separation according to the different *C. jejuni* strains, were provided to predict the survival of *C. jejuni* in raw milk between 5°C and 12°C. The time needed to reach one log reduction varied between 8.6 and 18.46 hours for the CFU data depending on the strain and the temperature. For all IPIU data the time was over 100 h.

Finally, a quantitative microbial risk assessment (QMRA) model was developed based on data scientific literature and incorporating the data generated in this PhD study. Through various uncertainty analyses, parameters with the greatest impact on the risk for consumers for drinking *Campylobacter* spp. contaminated raw milk were identified. In addition to the initial concentration of *Campylobacter* spp. in cow feces, the prevalence of contaminated teats had a major impact on the risk of consuming *Campylobacter*-contaminated raw milk.

In summary, transmission of *Campylobacter* spp. can occur due to cross-contamination of teats by cow feces into the raw milk. However, this is a rare event. Clean udders are therefore

of crucial importance. It was shown that survival of *C. jejuni* is underestimated based on CFU data compared to VBNC data at different temperatures in raw milk. The overall data are valuable to support risk managers in controlling *Campylobacter* spp. More CFU and IPIU data are needed to improve the developed predictive models. The significance of the finding on underestimated survival should be determined through studies, e.g., in animal models, on the infectivity of *Campylobacter* spp. in VBNC status, within and beyond the time window in which VBNC can be recovered to CFU.

# 7 Zusammenfassung

**Entwicklung von Modellen zur Vorhersage der Widerstandsfähigkeit von *Campylobacter* entlang der Warenkette Rohmilch**

Die Nachfrage der Verbraucher nach Rohmilch ist in den letzten Jahren gestiegen, und der lokale Verkauf über Rohmilchautomaten wurde intensiviert. Zudem wurden viele lebensmittelbedingte Krankheitsausbrüche in Deutschland durch *Campylobacter* spp. in Rohmilch verursacht, die vor dem Verzehr nicht ordnungsgemäß wärmebehandelt wurde. Der Verzehr von nicht wärmebehandelter Rohmilch, die mit *Campylobacter* spp. kontaminiert ist, stellt weiterhin ein Gesundheitsrisiko für die Verbraucher dar. *Campylobacter* spp. besiedeln häufig den Darm von Kühen, ohne klinische Symptome zu verursachen. Der Kot von *Campylobacter*-besiedelten Kühen stellt ein Risiko für eine Kreuzkontamination der Zitzen dar, wodurch die Kontamination mit *Campylobacter* spp. während des Melkens in die Rohmilch übertragen werden kann. Bis heute ist nicht geklärt, wie genau die Kontamination mit *Campylobacter* spp. entlang der Rohmilchversorgungskette erfolgt.

Ziel dieses Dissertationsprojekts war es, Datenlücken in Bezug auf die Übertragung von *Campylobacter* spp. entlang der Lieferkette von Rohmilch zu ermitteln und zu schließen sowie das Überleben von *Campylobacter* spp. in Rohmilch zu untersuchen. Schließlich wurden mögliche Interventionsstrategien zur Minimierung des Risikos durch Rohmilchkonsum dargelegt.

Im Rahmen dieses Promotionsprojekts wurde eine systematische Übersichtsarbeit und Meta-Analyse durchgeführt, um die bisherige Forschung zu *Campylobacter* spp. im Kot von Milchkühen weltweit zusammenzufassen. Die Prävalenz variierte stark von 0-100%. Es konnten erhebliche Datenlücken in den Studien festgestellt werden, z. B. in Bezug auf den Gesundheitszustand der Tiere und die Art der Kotprobenahme. Es liegen nur begrenzte quantitative Daten über *Campylobacter* spp. in den Fäkalien von Milchkühen vor. Die berichteten mittleren *Campylobacter*-Konzentrationen lagen zwischen 2,1 und 4,17 log CFU/g Kot.

In einer Längsschnittstudie wurde die Übertragung von *Campylobacter* spp. entlang der Rohmilchlieferkette untersucht. Dazu wurden über einen Zeitraum von einem Jahr verschiedene Proben aus einer kleinen Milchviehherde des BfR entnommen. Die untersuchten Proben umfassen rektalen Kot, Stiefelsocken, Zitzenhautabstriche, Melkzeugabstriche, Rohmilch und Milchfilter. Die Proben wurden auf das Vorhandensein und die Konzentration von *Campylobacter* spp. und *E. coli* sowie auf die Gesamtzahl aerober Kolonien (TACC) und *Pseudomonas* spp. untersucht. Die Konsistenz des Kuhkots und die Sauberkeit der

Zitzenhautabstriche wurden bewertet. Insgesamt wurden *Campylobacter* spp. in 77,1 % der Kotproben mit einer durchschnittlichen Konzentration von 2,43 ± 0,9 log KBE/g nachgewiesen. Die Konzentration von *Campylobacter* spp. im Kuhkot variierte sowohl innerhalb der Herde als auch bei einzelnen Kühen und im zeitlichen Verlauf. Die Umgebung des Stalls wurde mit Stiefelsocken getestet. Von den Stiefelsockenproben wurden 29,2 % positiv getestet mit einer mittleren Konzentration von 3,01 ± 1,05 log CFU/zwei Socken. Die kontaminierte Stallumgebung stellte ein Risiko für eine fäkale Kreuzkontamination der Zitzen der Kühe dar. Von den Zitzenabstrichen wurden 12,2 % positiv auf *Campylobacter* spp. getestet. Keines der Melkzeuge war positiv. Nur eine Rohmilchprobe und ein Milchfilter wurden am selben Tag positiv getestet. Außerdem wurden an diesem Tag neun Zitzenabstriche positiv auf *Campylobacter* spp. getestet. Die Ergebnisse zeigten, dass eine Kontamination von Rohmilch, wenn auch selten, vorkommen kann.

Die aus den Proben der Milchviehbetriebe gewonnenen Daten warfen die Forschungsfrage nach dem Zeitraum und dem physiologischen Zustand von *C. jejuni* beim Überleben in Rohmilch während der Lagerung auf. Daher wurde die Überlebensfähigkeit von *C. jejuni* mit zwei verschiedenen Methoden untersucht: kulturell nach ISO 10272-2 und zusätzlich unter Verwendung einer quantitativen Polymerase-Kettenreaktion zum spezifischen Nachweis lebensfähiger *Campylobacter* spp. in Rohmilch (v-qPCR). Der Versuch wurde bei drei Temperaturen (5°C, 8°C und 12°C) durchgeführt. Während gemäß ISO 10272-2 nur koloniebildende Einheiten (CFU) nachgewiesen werden können, ermöglicht die neu entwickelte v-qPCR (Wulsten et al., 2020) den Nachweis von intakten und potenziell infektiösen Einheiten (IPIU), die CFU und lebensfähige, aber nicht kultivierbare Einheiten (VBNC) umfassen. Das Überleben von drei *C. jejuni*-Stämmen wurde getestet. Nach Wulsten et al. (2020), die ihre Experimente bei 5°C durchführten, wurde die Überlebensrate auch in unserer Studie für die getesteten Stämme und Temperaturen um die KBE unterschätzt. Bislang ist das infektiöse Potenzial von *Campylobacter* spp. im VBNC-Status unbekannt. Wulsten et al. (2020) wiesen nach, dass *C. jejuni* VBNCs, die sich bei einer Inkubation in Rohmilch bei 5 °C entwickelt hatten, innerhalb eines bestimmten Zeitraums (mindestens 90 Stunden) wieder in einen kultivierbaren Zustand überführt wurden (Erholung), wobei ein spezifisches Gasgemisch mit einem niedrigeren Sauerstoffgehalt für 72 h verwendet wurde. Hier wurde die Erholung bei 8°C und 12°C untersucht. Die Erholungszeiträume für beide Stämme waren bei 8°C (bis zu 80 h) länger als bei 12°C (mindestens bis zu 48 h). Die Erholungszeit verkürzte sich also mit steigender Temperatur.

Vorhersagemodelle wurden anhand der beiden generierten Datensätze entwickelt, die eine schnelle Reaktion zur Vorhersage des mikrobiellen Verhaltens in der Lebensmittelumgebung ermöglichen. Drei tertiäre Modelle, bestehend aus einem für alle IPIU-Daten und zwei für die

CFU-Daten aufgrund der notwendigen Trennung nach den verschiedenen *C. jejuni*-Stämmen, wurden erstellt, um das Überleben von *C. jejuni* in Rohmilch zwischen 5°C und 12°C vorherzusagen. Die Zeit, die benötigt wird, um eine logarithmische Reduktion zu erreichen, schwankt bei den CFU-Daten je nach Stamm und Temperatur zwischen 8,6 und 18,46 Stunden. Bei allen IPIU-Daten lag die Zeit bei über 100 Stunden.

Schließlich wurde ein Modell zur quantitativen mikrobiellen Risikobewertung (QMRA) entwickelt, das auf Daten aus der wissenschaftlichen Literatur beruht und die in diesen Promotionsprojekt gewonnenen Daten einbezieht. Durch verschiedene Unsicherheitsanalysen wurden die Parameter ermittelt, die den größten Einfluss auf das Risiko für Verbraucher haben, mit *Campylobacter* spp. kontaminierte Rohmilch zu trinken. Neben der Ausgangskonzentration von *Campylobacter* spp. im Kuhkot hatte die Prävalenz von kontaminierten Zitzen einen großen Einfluss auf das Risiko des Konsums von *Campylobacter*-kontaminierter Rohmilch.

Zusammenfassend lässt sich sagen, dass eine Übertragung von *Campylobacter* spp. durch eine Kreuzkontamination von Zitzen mit Kuhkot in Rohmilch erfolgen kann. Dies ist jedoch ein seltenes Ereignis. Saubere Euter sind von entscheidender Bedeutung. Es wurde gezeigt, dass die Überlebensrate von *C. jejuni* auf der Grundlage von KBE-Daten im Vergleich zu VBNC-Daten bei verschiedenen Temperaturen in Rohmilch unterschätzt wird. Die Gesamtdaten sind hilfreich, um Risikomanager bei der Überwachung von *Campylobacter* spp. zu unterstützen. Um die entwickelten Vorhersagemodelle zu verbessern, werden mehr CFU- und IPIU-Daten benötigt. Die Bedeutung des Ergebnisses der unterschätzten Überlebensrate sollte durch Studien, z. B. in Tiermodellen, über die Infektiosität von *Campylobacter* spp. im VBNC-Status innerhalb und außerhalb des Zeitfensters, in dem VBNC zu KBE zurückgewonnen werden kann, ermittelt werden.

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# 9 Appendix

## 9.1 Supplementary material of Publication 1

**S1.** PRISMA checklist.

### PRISMA 2009 Checklist

| Section/topic | # | Checklist item | Reported on page # |
|---|---|---|---|
| **TITLE** | | | |
| Title | 1 | Identify the report as a systematic review, meta-analysis, or both. | 1 |
| **ABSTRACT** | | | |
| Structured summary | 2 | Provide a structured summary including, as applicable: background; objectives; data sources; study eligibility criteria, participants, and interventions; study appraisal and synthesis methods; results; limitations; conclusions and implications of key findings; systematic review registration number. | 2 |
| **INTRODUCTION** | | | |
| Rationale | 3 | Describe the rationale for the review in the context of what is already known. | 3-4 |
| Objectives | 4 | Provide an explicit statement of questions being addressed with reference to participants, interventions, comparisons, outcomes, and study design (PICOS). | |
| **METHODS** | | | |
| Protocol and registration | 5 | Indicate if a review protocol exists, if and where it can be accessed (e.g., Web address), and, if available, provide registration information including registration number. | 5 |
| Eligibility criteria | 6 | Specify study characteristics (e.g., PICOS, length of follow-up) and report characteristics (e.g., years considered, language, publication status) used as criteria for eligibility, giving rationale. | 5-6 |
| Information sources | 7 | Describe all information sources (e.g., databases with dates of coverage, contact with study authors to identify additional studies) in the search and date last searched. | 5-6 |
| Search | 8 | Present full electronic search strategy for at least one database, including any limits used, such that it could be repeated. | Table 1 |
| Study selection | 9 | State the process for selecting studies (i.e., screening, eligibility, included in systematic review, and, if applicable, included in the meta-analysis). | 6-7 |
| Data collection process | 10 | Describe method of data extraction from reports (e.g., piloted forms, independently, in duplicate) and any processes for obtaining and confirming data from investigators. | 5-6 |
| Data items | 11 | List and define all variables for which data were sought (e.g., PICOS, funding sources) and any assumptions and simplifications made. | - |
| Risk of bias in individual studies | 12 | Describe methods used for assessing risk of bias of individual studies (including specification of whether this was done at the study or outcome level), and how this information is to be used in any data synthesis. | 7-8 |
| Summary measures | 13 | State the principal summary measures (e.g., risk ratio, difference in means). | 9-10 |
| Synthesis of results | 14 | Describe the methods of handling data and combining results of studies, if done, including measures of consistency (e.g., $I^2$) for each meta-analysis. | 9-10 |

## PRISMA 2009 Checklist

| Section/topic | # | Checklist item | Reported on page # |
|---|---|---|---|
| Risk of bias across studies | 15 | Specify any assessment of risk of bias that may affect the cumulative evidence (e.g., publication bias, selective reporting within studies). | 7-8 |
| Additional analyses | 16 | Describe methods of additional analyses (e.g., sensitivity or subgroup analyses, meta-regression), if done, indicating which were pre-specified. | 9-10 |
| **RESULTS** | | | |
| Study selection | 17 | Give numbers of studies screened, assessed for eligibility, and included in the review, with reasons for exclusions at each stage, ideally with a flow diagram. | 11, Fig 1 |
| Study characteristics | 18 | For each study, present characteristics for which data were extracted (e.g., study size, PICOS, follow-up period) and provide the citations. | S1 table |
| Risk of bias within studies | 19 | Present data on risk of bias of each study and, if available, any outcome level assessment (see item 12). | 13 |
| Results of individual studies | 20 | For all outcomes considered (benefits or harms), present, for each study: (a) simple summary data for each intervention group (b) effect estimates and confidence intervals, ideally with a forest plot. | 14, Fig 3 |
| Synthesis of results | 21 | Present results of each meta-analysis done, including confidence intervals and measures of consistency. | Fig 4 |
| Risk of bias across studies | 22 | Present results of any assessment of risk of bias across studies (see Item 15). | 13 |
| Additional analysis | 23 | Give results of additional analyses, if done (e.g., sensitivity or subgroup analyses, meta-regression [see Item 16]). | 16-17 |
| **DISCUSSION** | | | |
| Summary of evidence | 24 | Summarize the main findings including the strength of evidence for each main outcome; consider their relevance to key groups (e.g., healthcare providers, users, and policy makers). | 17-22 |
| Limitations | 25 | Discuss limitations at study and outcome level (e.g., risk of bias), and at review-level (e.g., incomplete retrieval of identified research, reporting bias). | 23-24 |
| Conclusions | 26 | Provide a general interpretation of the results in the context of other evidence, and implications for future research. | - |
| **FUNDING** | | | |
| Funding | 27 | Describe sources of funding for the systematic review and other support (e.g., supply of data); role of funders for the systematic review. | - |

*From:* Moher D, Liberati A, Tetzlaff J, Altman DG, The PRISMA Group (2009). Preferred Reporting Items for Systematic Reviews and Meta-Analyses: The PRISMA Statement. PLoS Med 6(7): e1000097. doi:10.1371/journal.pmed1000097

**Fig S1.** Heterogeneity.

(A) Heterogeneity in the multilevel model, (B) Heterogeneity in the multilevel mixed effects model.

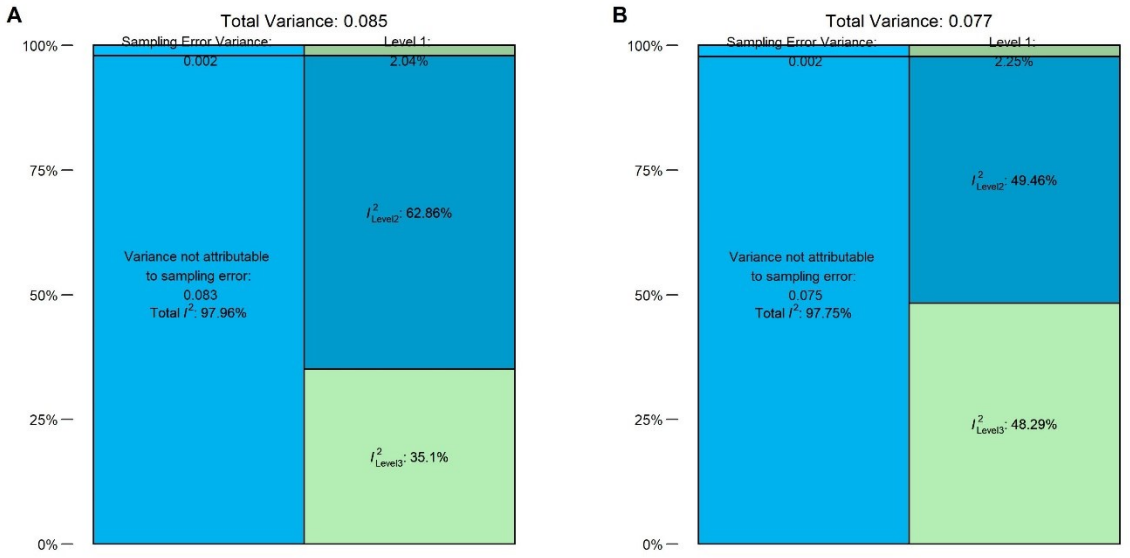


**Fig S2.** Bubbleplot.

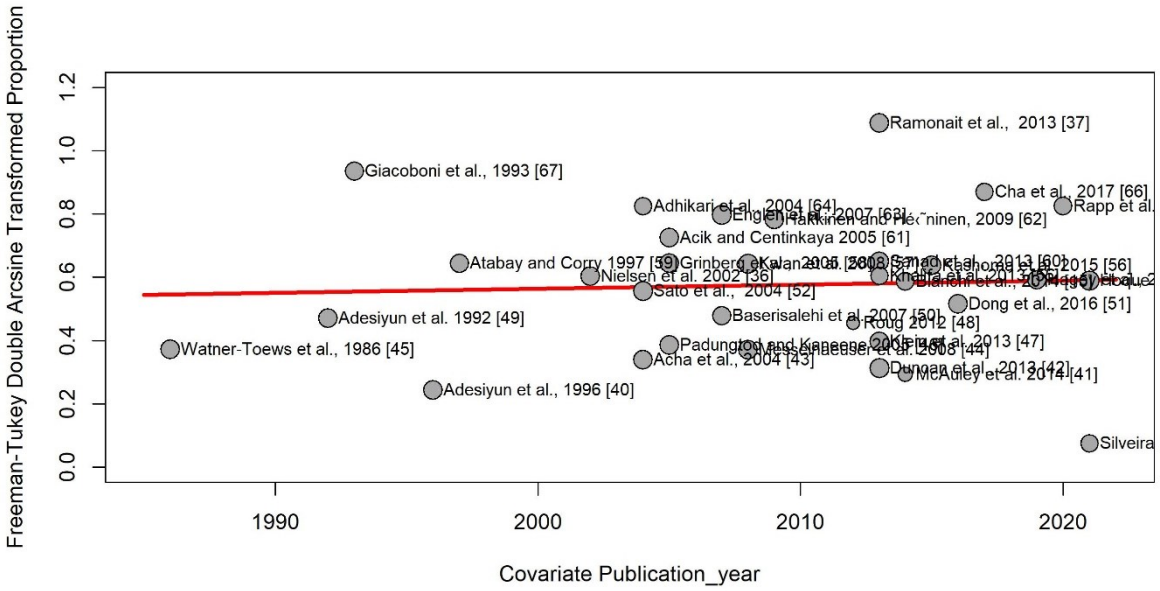

**Fig S3.** Forest plot RoB.

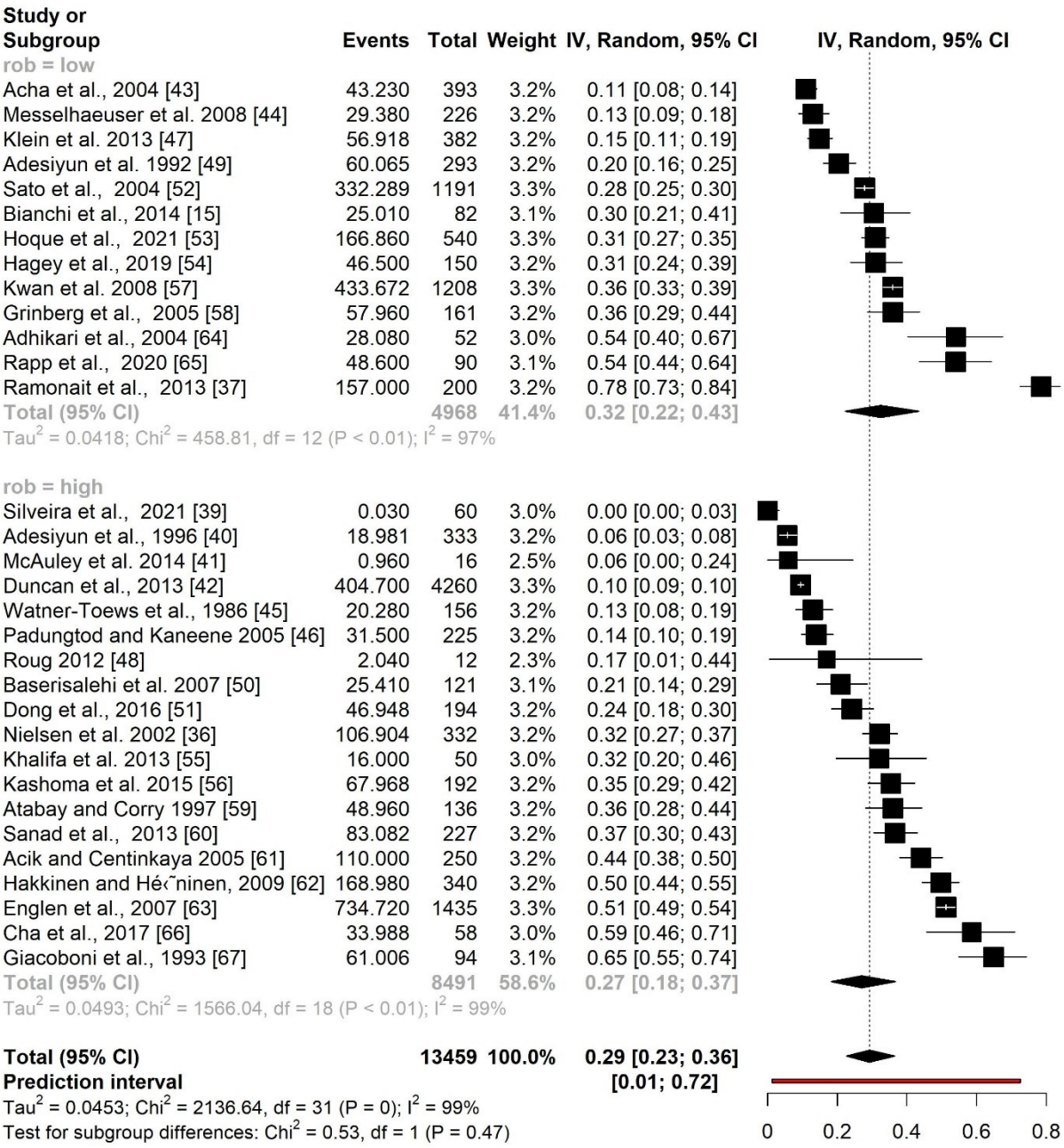

**Fig S4.** Forest plot species.

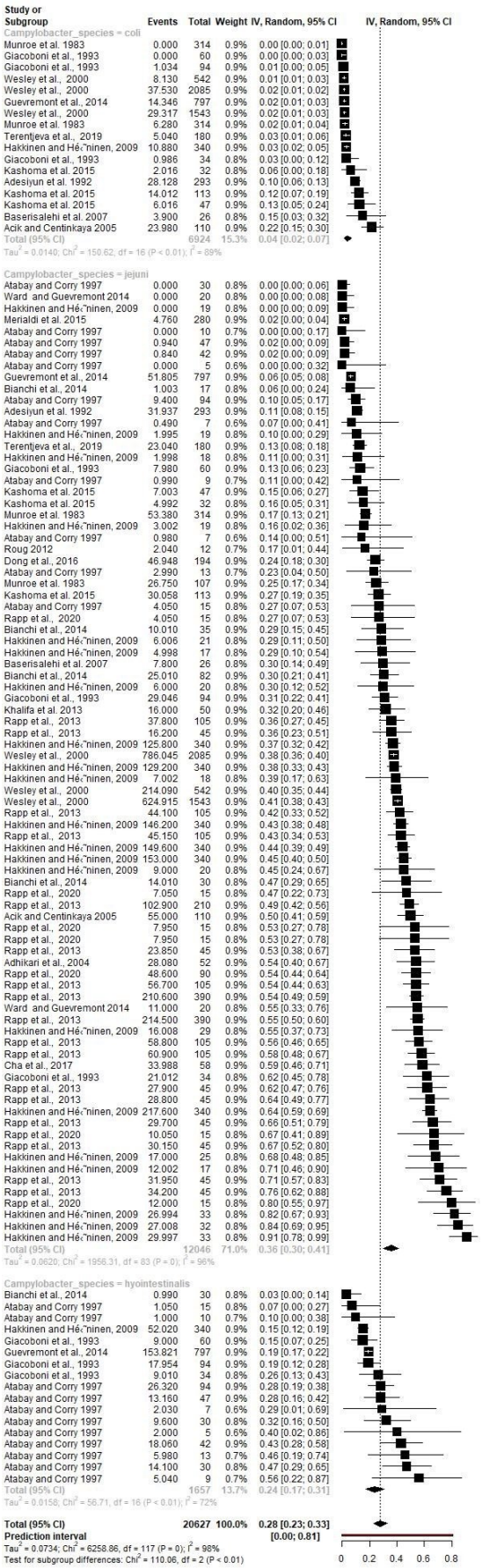

**Fig S5.** Forest plot season.

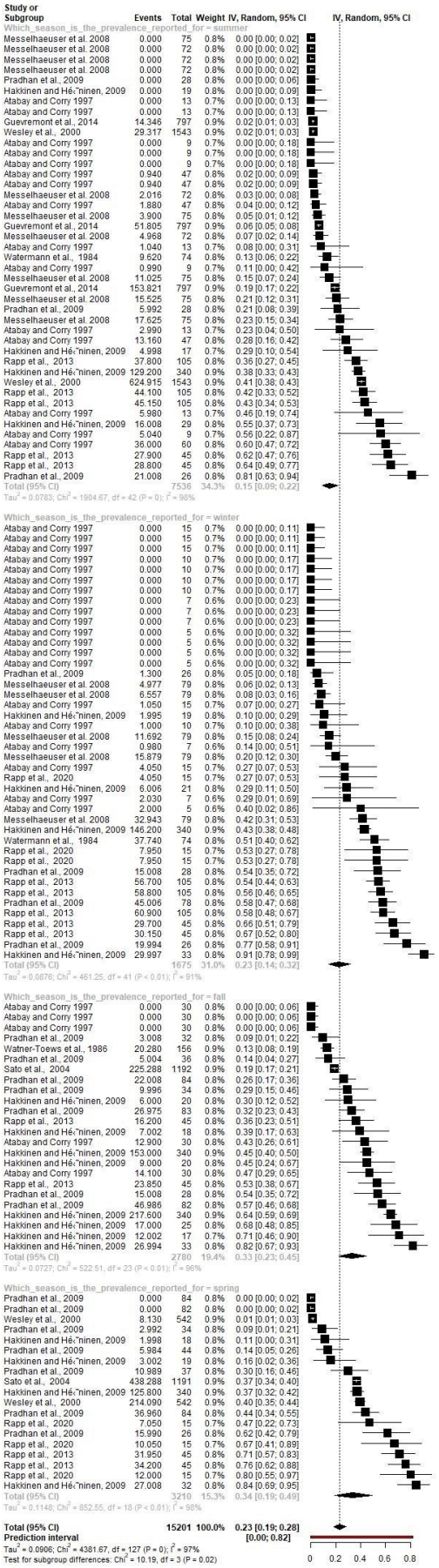

**Table S1.** Extraction table.

(1) Review

| ID | Author | DOI or PMID | Publication_year | Study_year | Fecal_collection_method | Detection_or_quantification_method_for_Campylobacter_spp. | Species_identification_method | Country_of_study | Number_of_total_fecal_samples_taken | Number_of_total_populations_farms_herds_sampled | Description_age_class_of_cattle_sampled | Health_status_of_cattle | Were_repeated_measurements_for_individual_cattle_recorded | Were_repeated_measurements_for_populations_farms_recorded | Are_the_repeated_measurements_split_by_Gregorian_seasons | Are_the_repeated_measurements_for_populations_farms_available | Are_the_repeated_measurements_for_individual_cattle_available | Is_the_concentration_of_Campylobacter_in_faces_given | Is_information_about_the_housing_of_cows_given | Is_information_about_the_feeding_of_cows_given | Is_information_about_organic_or_conventional_farming_given | Was_information_about_the_season_weather_months_given_when_samples_were_taken | Is_information_about_any_other_adjustment_variable_given* | Was_information_given_whether_the_cows_were_selected_randomly_for_sampling | Were_faces_samples_pooled_before_analysis_and_was_the_outcome_reported_for_pooled_samples | Were_samples_or_cows_excluded_from_the_analysis_e.g._due_to_technical_or_methodological_failure | Were_ISO_Methods_applied_for_the_detection_quantification_of_Campylobacter_spp. | Was_the_outcome_reported_for_all_relevant_sub_groups_and_the_whole_population_studied | rob_yes | rob |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | Acha et al., | 10.1186/1751 | 2004 | 1995 | rectal collecti | culture-based | phenotypic | Mozambique | 393 | 8 | calves | mixed | no | yes | yes, but non | no | no | no | yes | yes | unclear | yes | yes | no | yes | unclear | unclear | no | 5 | low |
| 2 | Acik and Cen | 10.1111/j.147 | 2005 | 2003 | rectal collecti | culture-based | PCR-based, | Turkey | 250 | 1 | adult | healthy | no | no | no | no | no | no | no | no | no | no | no | no | yes | unclear | unclear | yes | 2 | high |
| 3 | Adesiyun anc | 10.1016/0167 | 1994 | 1992 | rectal collecti | culture-based | not performe | Trinidad | 304 | 17 | cows, heifers | mixed | no | yes | unclear | no | no | no | yes | yes | unclear | yes | no | no | yes | unclear | unclear | no | 4 | high |
| 4 | Adesiyun et a | 10.1016/0007 | 1992 | 1991 | rectal collecti | culture-based | phenotypic | Trinidad | 293 | 20 | calves | mixed | no | no | no | no | no | no | yes | no | yes | no | yes | no | yes | unclear | unclear | yes | 5 | low |
| 5 | Adesiyun et a | 10.1590/s003 | 2001 | not specified | rectal collecti | culture-based | not performe | Trinidad | 74 | 12 | calves | mixed | no | yes | yes, but non | yes | no | no | no | no | no | yes | no | no | yes | unclear | unclear | no | 2 | high |
| 6 | Adesiyun et a | 9239938 | 1996 | not specified | rectal collecti | culture-based | phenotypic | Trinidad | 333 | 177 | adult | not specified | no | no | no | no | no | no | no | no | no | no | no | yes | yes | unclear | unclear | yes | 3 | high |
| 7 | Adhikari et al | 10.1080/0048 | 2004 | 2002 | rectal collecti | culture-based | other molecul | New Zealand | 52 | 1 | adult | healthy | no | no | no | no | no | no | no | no | yes | yes | yes | yes | yes | unclear | unclear | yes | 6 | low |
| 8 | Atabay and C | 10.1046/j.147 | 1997 | 1995-1996 | rectal collecti | culture-based | phenotypic | UK | 136 | 3 | cows and cal | not specified | yes | yes | yes | yes | no | no | no | no | no | yes | no | no | yes | unclear | unclear | yes | 3 | high |
| 9 | Baserisalehi | 10.3923/pjbs. | 2007 | 2006 | rectal collecti | culture-based | phenotypic | Iran | 121 | | adult | healthy | no | no | no | no | no | no | no | no | no | no | no | no | yes | unclear | no | yes | 2 | high |
| 10 | Bianchini et a | 10.1128/aem. | 2014 | 2010-2012 | rectal collecti | culture-based | PCR-based | Italy | 82 | 3 | adult | not specified | no | yes | no | no | no | no | no | no | no | yes | yes | yes | yes | unclear | unclear | yes | 5 | low |
| 11 | Cha et al., | 10.3389/fmicb | 2017 | 2012 | not specified | culture-based | PCR-based, | USA | 58 | 1 | adult | not specified | no | no | no | no | no | no | no | no | yes | no | no | no | yes | unclear | unclear | yes | 3 | high |
| 12 | Dong et al., | 10.1089/fpd.2 | 2016 | 2012-2014 | rectal collecti | culture-based | PCR-based | South Korea | 194 | 1 | adult | not specified | yes | yes | no | no | no | no | no | no | no | yes | no | yes | yes | unclear | unclear | no | 3 | high |
| 13 | Duncan et al. | 10.1017/s095 | 2013 | not specified | cow pat | culture-based | PCR-based | UK | 4260 | 15 | adult | not specified | no | yes | no | no | no | no | yes | no | no | yes | yes | no | yes | unclear | unclear | no | 4 | high |
| 14 | Englen et al., | 10.1111/j.136 | 2007 | 2002 | rectal collecti | culture-based | PCR-based | USA | 1435 | 96 | adult | not specified | no | no | no | no | no | no | no | no | no | yes | yes | no | yes | unclear | unclear | no | 3 | high |
| 15 | Grinberg et al | 10.1080/0048 | 2005 | 2002 | rectal collecti | culture-based | phenotypic | New Zealand | 161 | 24 | calves | mixed | no | yes | no | no | no | no | yes | yes | no | yes | yes | no | yes | no | unclear | no | 5 | low |
| 16 | Guevremont e | 10.1089/fpd.2 | 2014 | 2011 | rectal collecti | culture-based | PCR-based | Canada | 797 | 40 | adult | not specified | no | no | no | no | no | no | yes | yes | no | yes | yes | yes | yes | unclear | unclear | no | 6 | low |
| 17 | Guevremont e | 10.1089/fpd.2 | 2008 | not specified | pooled cow p | PCR-based | PCR-based | Canada | 185 | | adult | not specified | no | no | no | no | no | no | no | no | no | no | no | no | no | unclear | unclear | unclear | 0 | high |
| 18 | Hagey et al., | 10.3389/fmicb | 2019 | not specified | rectal collecti | PCR-based | other molecul | USA | 150 | 10 | adult | not specified | no | no | no | no | no | no | yes | yes | yes | no | yes | yes | yes | unclear | no | no | 6 | low |
| 19 | Hakkinen anc | 10.1111/j.136 | 2009 | 2006-2007 | cow pat | culture-based | other molecul | Finland | 340 | 3 | adult | not specified | no | yes | yes | yes | no | no | no | no | no | yes | yes | no | yes | unclear | unclear | yes | 4 | high |
| 20 | Hansson et a | 10.1136/vr.10 | 2019 | 2015 | pooled cow p | culture-based | MALDI-TOF | Sweden | 110 | 5 | cows, heifers | not specified | no | yes | yes | no | no | no | no | no | no | yes | yes | no | no | unclear | unclear | no | 2 | high |
| 21 | Harvey et al. | 10.4315/0362 | 2004 | 2001 | rectal collecti | culture-based | other molecul | USA | 720 | 9 | adult | not specified | no | yes | unclear | yes | no | no | no | no | no | yes | yes | no | yes | unclear | no | no | 2 | high |
| 22 | Irshad et al., | 10.1017/S099 | 2016 | 2009 | pooled cow p | culture-based | PCR-based | New Zealand | 72 | 20 | cows, heifers | not specified | no | no | yes | no | no | no | no | no | no | yes | no | no | no | yes | unclear | no | 2 | high |
| 23 | Giacoboni et | 10.1292/jvms | 1993 | not specified | not specified | culture-based | phenotypic | Japan | 94 | 6 | cows and cal | healthy | no | no | no | no | no | no | no | no | no | no | no | no | yes | unclear | unclear | yes | 2 | high |
| 24 | Jaakkonen et | 10.1128/AEM | 2019 | 2014-2015 | pooled cow p | culture-based | PCR-based | Finland | 257 | 3 | cows and cal | not specified | no | yes | yes | yes | no | no | yes | yes | no | yes | yes | no | no | yes | yes | yes | 7 | low |
| 25 | Kashoma et a | 10.3389/fmicb | 2015 | 2013-2014 | cow pat | culture-based | PCR-based | Tanzania | 192 | 3 | not specified | not specified | no | no | no | no | no | no | no | no | no | yes | no | unclear | yes | unclear | unclear | yes | 3 | high |
| 26 | Khalifa et al. | 10.5829/idosi | 2013 | not specified | cow pat | culture-based | PCR-based | Egypt | 20 | | not specified | diarrhea | no | no | no | no | no | | no | no | no | no | no | no | yes | unclear | unclear | yes | 2 | high |
| 27 | Klein et al. | 10.3168/jds.2 | 2013 | 2009-2010 | rectal collecti | culture-based | MALDI-TOF | Austria | 382 | 100 | calves | mixed | no | no | no | no | no | no | yes | yes | yes | no | yes | yes | yes | unclear | yes | no | 7 | low |
| 28 | Kwan et al. | 10.1128/AEM | 2008 | 2003 | cow pat | culture-based | PCR-based | UK | 1208 | 5 | adult | not specified | no | yes | yes | yes | no | no | no | no | yes | yes | yes | no | yes | yes | unclear | no | 5 | low |
| 29 | McAuley et a | 10.3168/jds.2 | 2014 | 2013 and 201 | not specified | culture-based | PCR-based | Australia | 16 | 3 | adult | not specified | yes | no | yes | no | no | no | no | unclear | no | yes | no | no | yes | unclear | yes | no | 3 | high |
| 30 | Merialdi et al. | 10.4315/0362 | 2015 | 2012-2013 | rectal collecti | PCR-based a | phenotypic | Italy | 50 | 1 | cows, heifers | not specified | yes | yes | yes | no | no | no | yes | yes | no | yes | yes | yes | yes | no | unclear | no | 6 | low |
| 31 | Messelhaeus | 10.2376/0003 | 2008 | 2004 | not specified | culture-based | PCR-based | Germany | 226 | 4 | not specified | not specified | no | yes | yes | yes | no | no | yes | yes | no | yes | yes | no | yes | unclear | unclear | yes | 6 | low |
| 32 | Moriarty et al | 10.1111/j.136 | 2008 | not specified | pooled cow p | culture-based | PCR-based | New Zealand | 155 | 4 | adult | healthy | no | yes | yes, but non | no | no | yes | yes | yes | no | yes | yes | no | no | unclear | unclear | no | 4 | high |
| 33 | Munroe et al. | 10.1128/jcm. | 1983 | not specified | rectal collecti | culture-based | phenotypic | USA | 412 | 10 | not specified | mixed | no | no | no | no | no | no | no | no | no | no | no | no | yes | unclear | unclear | no | 1 | high |
| 34 | Murinda et al. | 10.1089/1535 | 2004 | not specified | pooled cow p | culture-based | PCR-based | USA | 98 | 4 | not specified | not specified | no | yes | yes | no | no | no | no | no | no | yes | no | no | no | unclear | unclear | no | 1 | high |
| 35 | Nielsen et al. | 10.1046/j.147 | 2002 | 1999 | rectal collecti | culture-based | other molecul | Denmark | 332 | 24 | cows, heifers | not specified | no | no | no | no | no | yes | no | no | no | yes | no | no | yes | unclear | unclear | no | 2 | high |
| 36 | Oporto et al., | 10.1111/j.136 | 2007 | 2003-2006 | rectal collecti | culture-based | PCR-based | Spain | 493 | 11 | not specified | not specified | no | no | no | no | no | no | yes | yes | yes | yes | yes | | yes | no | unclear | unclear | no | 5 | low |
| 37 | Padungtod an | 10.4315/0362 | 2005 | 2000-2003 | not specified | culture-based | PCR-based | Thailand | 225 | 25 | adult | not specified | no | no | no | no | no | no | no | no | no | no | no | no | yes | yes | unclear | unclear | no | 2 | high |
| 38 | Pradhan et al | 10.3168/jds.2 | 2009 | 2004 | rectal collecti | culture-based | PCR-based | USA | 1010 | 3 | adult | not specified | yes | yes | yes | yes | no | no | yes | yes | yes | yes | yes | yes | no | yes | unclear | unclear | no | 6 | low |
| 39 | Ramonait et a | 10.1186/1751 | 2013 | 2012 | rectal collecti | culture-based | PCR-based | Lithuania | 200 | 3 | cows, heifers | not specified | no | yes | no | no | no | yes | yes | no | | yes | yes | no | yes | unclear | yes | yes | 6 | low |
| 40 | Rapp et al., | 10.1111/jam. | 2013 | 2011-2012 | cow pat | culture-based | PCR-based | New Zealand | 990 | 21 | adult | not specified | no | yes | yes, but non | yes | no | yes | yes | yes | yes | yes | yes | no | yes | unclear | unclear | no | 6 | low |
| 41 | Roug et al., | 10.1016/j.cim | 2012 | 2005 | cow pat | culture-based | PCR-based | USA | 50 | | not specified | not specified | no | no | no | no | no | no | yes | no | no | no | no | no | yes | unclear | unclear | yes | 3 | high |
| 42 | Sanad et al., | 10.1089/fpd.2 | 2013 | 2009 | not specified | culture-based | PCR-based | USA | 227 | 11 | not specified | not specified | no | no | no | no | no | no | no | no | no | yes | no | no | yes | unclear | unclear | yes | 3 | high |
| 43 | Sato et al., | 10.1128/AEM | 2004 | not specified | cow pat | culture-based | not performe | USA | 1191 | 60 | cows and cal | healthy | no | yes | yes | yes | no | no | yes | yes | yes | yes | yes | yes | no | yes | no | no | no | 6 | low |
| 44 | Stanley et al. | 10.1046/j.136 | 1998 | 1993-1995 | pooled cow p | culture-based | phenotypic | UK | 1112 | 4 | cows and cal | not specified | no | yes | yes | no | no | yes | no | no | no | yes | yes | | no | unclear | unclear | no | 2 | high |
| 45 | Terentjeva et | 10.1089/fpd.2 | 2019 | not specified | rectal collecti | culture-based | MALDI-TOF | Latvia | 180 | 18 | calves | not specified | no | no | no | no | no | no | yes | yes | yes | yes | yes | yes | no | yes | unclear | yes | no | 8 | low |
| 46 | Watner-Toew | 3017528 | 1986 | 1982 | rectal collecti | culture-based | not performe | Canada | 156 | 87 | calves | not specified | no | no | no | no | no | no | no | no | no | yes | yes | no | no | unclear | unclear | unclear | no | 2 | high |
| 47 | Ward and Gi | 10.1089/fpd.2 | 2014 | not specified | rectal collecti | PCR-based | other molecul | Canada | 40 | 2 | adult | not specified | no | no | no | no | no | no | yes | yes | no | yes | yes | yes | yes | unclear | unclear | no | 6 | low |
| 48 | Watermann e | 10.1017/s002 | 1984 | not specified | rectal collecti | culture-based | not performe | UK | 74 | | adult | healthy | no | yes | yes | yes | no | yes | no | no | no | yes | no | no | yes | unclear | no | unclear | 2 | high |
| 49 | Wesley et al. | 10.1128/aem. | 2000 | 1996 | rectal collecti | culture-based | PCR-based | USA | 2085 | 31 | adult | healthy | no | yes | no | yes | no | no | no | no | no | yes | no | no | yes | unclear | unclear | no | 2 | high |
| 50 | Hansson et a | 10.1111/jam. | 2020 | 2015-2017 | rectal collecti | culture-based | MALDI-TOF | Sweden | 223 | 7 | calves | mixed | no | yes | no | no | no | no | yes | no | yes | yes | yes | no | no | no | yes | no | 5 | low |
| 51 | Hoque et al., | 10.3390/path | 2021 | 2018-2020 | rectal collecti | culture-based | PCR-based | Bangladesh | 540 | 90 | cows, heifers | mixed | no | no | yes, but non | yes | no | no | yes | yes | yes | yes | yes | yes | yes | unclear | unclear | no | 7 | low |
| 52 | Rapp et al., | 10.3390/micr | 2020 | not specified | cow pat | culture-based | PCR-based | New Zealand | 90 | 1 | adult | not specified | no | yes | yes | yes | no | no | yes | yes | yes | yes | yes | yes | no | yes | unclear | unclear | no | 6 | low |
| 53 | Silveira et al., | 10.5380/avs.v | 2021 | not specified | rectal collecti | culture-based | PCR-based | Brazil | 120 | 12 | adult | not specified | no | no | no | no | no | no | no | no | no | no | no | yes | no | yes | unclear | unclear | yes | 3 | high |

* other adjustment variables were e.g. herd size, management practice, feeding system, milking system, hygiene standard, bedding

## (2) non-aggregated

| ID | Author | Publication_year | Study_year | Fecal_collection_method | Detection_or_quantification_method_for_Campylobacter_spp. | Species_identification_method | Campylobacter_species | Prevalence_in_population | Which_season_is_the_prevalence_reported_for | Number_of_individual_cattle_sampled | Health_status_of_cattle | Description_age_class_of_cattle_sampled | Specific_outcome_reported_for_whole_data_set_or_sub_group | event* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | Acha et al., | 2004 | 1995 | rectal collection | culture-based | phenotypic | spp. | 0 | not specified | 23 | diarrhea | calves | sub-group | 0 |
| 1 | Acha et al., | 2004 | 1995 | rectal collection | culture-based | phenotypic | spp. | 0 | not specified | 55 | healthy | calves | sub-group | 0 |
| 1 | Acha et al., | 2004 | 1995 | rectal collection | culture-based | phenotypic | spp. | 0 | not specified | 78 | mixed | calves | sub-group | 0 |
| 1 | Acha et al., | 2004 | 1995 | rectal collection | culture-based | phenotypic | spp. | 10 | not specified | 230 | healthy | calves | sub-group | 23 |
| 1 | Acha et al., | 2004 | 1995 | rectal collection | culture-based | phenotypic | spp. | 10 | not specified | 239 | mixed | calves | sub-group | 23.9 |
| 1 | Acha et al., | 2004 | 1995 | rectal collection | culture-based | phenotypic | spp. | 11 | not specified | 330 | healthy | calves | sub-group | 36.3 |
| 1 | Acha et al., | 2004 | 1995 | rectal collection | culture-based | phenotypic | spp. | 11 | not specified | 393 | mixed | calves | whole | 43.23 |
| 1 | Acha et al., | 2004 | 1995 | rectal collection | culture-based | phenotypic | spp. | 11 | not specified | 63 | diarrhea | calves | sub-group | 6.93 |
| 1 | Acha et al., | 2004 | 1995 | rectal collection | culture-based | phenotypic | spp. | 16 | not specified | 31 | diarrhea | calves | sub-group | 4.96 |
| 1 | Acha et al., | 2004 | 1995 | rectal collection | culture-based | phenotypic | spp. | 22 | not specified | 9 | diarrhea | calves | sub-group | 1.98 |
| 1 | Acha et al., | 2004 | 1995 | rectal collection | culture-based | phenotypic | spp. | 25 | not specified | 76 | mixed | calves | sub-group | 19 |
| 1 | Acha et al., | 2004 | 1995 | rectal collection | culture-based | phenotypic | spp. | 31 | not specified | 45 | healthy | calves | sub-group | 13.95 |
| 2 | Acik and Centinkaya | 2005 | 2003 | rectal collection | culture-based | PCR-based, flaA-typing, RFLP | coli | 21.8 | not specified | 110 | healthy | adult | sub-group | 23.98 |
| 2 | Acik and Centinkaya | 2005 | 2003 | rectal collection | culture-based | PCR-based, flaA-typing, RFLP | jejuni | 50 | not specified | 110 | healthy | adult | sub-group | 55 |
| 2 | Acik and Centinkaya | 2005 | 2003 | rectal collection | culture-based | PCR-based, flaA-typing, RFLP | not identifiable | 28.2 | not specified | 110 | healthy | adult | sub-group | 31.02 |
| 2 | Acik and Centinkaya | 2005 | 2003 | rectal collection | culture-based | PCR-based, flaA-typing, RFLP | spp. | 44 | not specified | 250 | healthy | adult | whole | 110 |
| 3 | Adesiyun and Kaminjolo | 1994 | 1992 | rectal collection | culture-based | not performed | spp. | 0 | not specified | 16 | mixed | heifers | sub-group | 0 |
| 3 | Adesiyun and Kaminjolo | 1994 | 1992 | rectal collection | culture-based | not performed | spp. | 11 | not specified | 100 | mixed | calves | sub-group | 11 |
| 3 | Adesiyun and Kaminjolo | 1994 | 1992 | rectal collection | culture-based | not performed | spp. | 16.5 | not specified | 121 | healthy | heifers and calves | sub-group | 19.965 |
| 3 | Adesiyun and Kaminjolo | 1994 | 1992 | rectal collection | culture-based | not performed | spp. | 17.1 | not specified | 105 | mixed | calves | sub-group | 17.955 |
| 3 | Adesiyun and Kaminjolo | 1994 | 1992 | rectal collection | culture-based | not performed | spp. | 21.9 | not specified | 183 | diarrhea | heifers and calves | sub-group | 40.077 |
| 3 | Adesiyun and Kaminjolo | 1994 | 1992 | rectal collection | culture-based | not performed | spp. | 33.3 | not specified | 42 | mixed | calves | sub-group | 13.986 |
| 3 | Adesiyun and Kaminjolo | 1994 | 1992 | rectal collection | culture-based | not performed | spp. | 39.1 | not specified | 23 | mixed | calves | sub-group | 8.993 |
| 3 | Adesiyun and Kaminjolo | 1994 | 1992 | rectal collection | culture-based | not performed | spp. | 44.4 | not specified | 18 | mixed | calves | sub-group | 7.992 |
| 4 | Adesiyun et al. | 1992 | 1991 | rectal collection | culture-based | phenotypic | coli | 9.6 | not specified | 293 | mixed | calves | sub-group | 28.128 |
| 4 | Adesiyun et al. | 1992 | 1991 | rectal collection | culture-based | phenotypic | jejuni | 10.9 | not specified | 293 | mixed | calves | sub-group | 31.937 |
| 4 | Adesiyun et al. | 1992 | 1991 | rectal collection | culture-based | phenotypic | spp. | 17.8 | not specified | 118 | healthy | calves | sub-group | 21.004 |
| 4 | Adesiyun et al. | 1992 | 1991 | rectal collection | culture-based | phenotypic | spp. | 20.5 | not specified | 293 | mixed | calves | whole | 60.065 |
| 4 | Adesiyun et al. | 1992 | 1991 | rectal collection | culture-based | phenotypic | spp. | 22.3 | not specified | 175 | diarrhea | calves | sub-group | 39.025 |
| 5 | Adesiyun et al., | 2001 | not specified | rectal collection | culture-based | not performed | spp. | 0 | not specified | 5 | healthy | calves | sub-group | 0 |
| 5 | Adesiyun et al., | 2001 | not specified | rectal collection | culture-based | not performed | spp. | 4.3 | not specified | 23 | healthy | calves | sub-group | 0.989 |
| 5 | Adesiyun et al., | 2001 | not specified | rectal collection | culture-based | not performed | spp. | 12.1 | not specified | 33 | healthy | calves | sub-group | 3.993 |
| 5 | Adesiyun et al., | 2001 | not specified | rectal collection | culture-based | not performed | spp. | 16.6 | not specified | 6 | diarrhea | calves | sub-group | 0.996 |
| 5 | Adesiyun et al., | 2001 | not specified | rectal collection | culture-based | not performed | spp. | 26.8 | not specified | 41 | diarrhea | calves | sub-group | 10.988 |
| 5 | Adesiyun et al., | 2001 | not specified | rectal collection | culture-based | not performed | spp. | 28.1 | not specified | 32 | diarrhea | calves | sub-group | 8.992 |
| 5 | Adesiyun et al., | 2001 | not specified | rectal collection | culture-based | not performed | spp. | 33.3 | not specified | 3 | diarrhea | calves | sub-group | 0.999 |
| 5 | Adesiyun et al., | 2001 | not specified | rectal collection | culture-based | not performed | spp. | 60 | not specified | 5 | healthy | calves | sub-group | 3 |
| 6 | Adesiyun et al., | 1996 | not specified | rectal collection | culture-based | phenotypic | spp. | 5.7 | not specified | 333 | not specified | adult | whole | 18.981 |
| 7 | Adhikari et al., | 2004 | 2002 | rectal collection | culture-based | other molecular method | jejuni | 54 | not specified | 52 | healthy | adult | whole | 28.08 |
| 8 | Atabay and Corry | 1997 | 1995-1996 | rectal collection | culture-based | phenotypic | fetus | 0 | summer | 13 | not specified | adult | sub-group | 0 |
| 8 | Atabay and Corry | 1997 | 1995-1996 | rectal collection | culture-based | phenotypic | fetus | 0 | winter | 15 | not specified | adult | sub-group | 0 |
| 8 | Atabay and Corry | 1997 | 1995-1996 | rectal collection | culture-based | phenotypic | fetus | 0 | winter | 7 | not specified | calves | sub-group | 0 |

| | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 8 | Atabay and Corry | 1997 | 1995-1996 | rectal collection | culture-based | phenotypic | fetus | 0 | summer | 9 | not specified | adult | sub-group | 0 |
| 8 | Atabay and Corry | 1997 | 1995-1996 | rectal collection | culture-based | phenotypic | fetus | 0 | winter | 10 | not specified | adult | sub-group | 0 |
| 8 | Atabay and Corry | 1997 | 1995-1996 | rectal collection | culture-based | phenotypic | fetus | 0 | winter | 5 | not specified | calves | sub-group | 0 |
| 8 | Atabay and Corry | 1997 | 1995-1996 | rectal collection | culture-based | phenotypic | fetus | 2 | all | 94 | not specified | adult | sub-group | 1.88 |
| 8 | Atabay and Corry | 1997 | 1995-1996 | rectal collection | culture-based | phenotypic | fetus | 4 | summer | 47 | not specified | adult | sub-group | 1.88 |
| 8 | Atabay and Corry | 1997 | 1995-1996 | rectal collection | culture-based | phenotypic | fetus | 11 | all | 15 | not specified | cows and calves | sub-group | 1.65 |
| 8 | Atabay and Corry | 1997 | 1995-1996 | rectal collection | culture-based | phenotypic | fetus | 31 | all | 42 | not specified | calves | sub-group | 13.02 |
| 8 | Atabay and Corry | 1997 | 1995-1996 | rectal collection | culture-based | phenotypic | fetus | 43 | fall | 30 | not specified | calves | sub-group | 12.9 |
| 8 | Atabay and Corry | 1997 | 1995-1996 | rectal collection | culture-based | phenotypic | hyointestinalis | 7 | winter | 15 | not specified | adult | sub-group | 1.05 |
| 8 | Atabay and Corry | 1997 | 1995-1996 | rectal collection | culture-based | phenotypic | hyointestinalis | 10 | winter | 10 | not specified | adult | sub-group | 1 |
| 8 | Atabay and Corry | 1997 | 1995-1996 | rectal collection | culture-based | phenotypic | hyointestinalis | 28 | summer | 47 | not specified | adult | sub-group | 13.16 |
| 8 | Atabay and Corry | 1997 | 1995-1996 | rectal collection | culture-based | phenotypic | hyointestinalis | 28 | all | 94 | not specified | adult | sub-group | 26.32 |
| 8 | Atabay and Corry | 1997 | 1995-1996 | rectal collection | culture-based | phenotypic | hyointestinalis | 29 | winter | 7 | not specified | calves | sub-group | 2.03 |
| 8 | Atabay and Corry | 1997 | 1995-1996 | rectal collection | culture-based | phenotypic | hyointestinalis | 32 | all | 30 | not specified | cows and calves | sub-group | 9.6 |
| 8 | Atabay and Corry | 1997 | 1995-1996 | rectal collection | culture-based | phenotypic | hyointestinalis | 40 | winter | 5 | not specified | calves | sub-group | 2 |
| 8 | Atabay and Corry | 1997 | 1995-1996 | rectal collection | culture-based | phenotypic | hyointestinalis | 43 | all | 42 | not specified | calves | sub-group | 18.06 |
| 8 | Atabay and Corry | 1997 | 1995-1996 | rectal collection | culture-based | phenotypic | hyointestinalis | 46 | summer | 13 | not specified | adult | sub-group | 5.98 |
| 8 | Atabay and Corry | 1997 | 1995-1996 | rectal collection | culture-based | phenotypic | hyointestinalis | 47 | fall | 30 | not specified | calves | sub-group | 14.1 |
| 8 | Atabay and Corry | 1997 | 1995-1996 | rectal collection | culture-based | phenotypic | hyointestinalis | 56 | summer | 9 | not specified | adult | sub-group | 5.04 |
| 8 | Atabay and Corry | 1997 | 1995-1996 | rectal collection | culture-based | phenotypic | jejuni | 0 | fall | 30 | not specified | calves | sub-group | 0 |
| 8 | Atabay and Corry | 1997 | 1995-1996 | rectal collection | culture-based | phenotypic | jejuni | 0 | winter | 10 | not specified | adult | sub-group | 0 |
| 8 | Atabay and Corry | 1997 | 1995-1996 | rectal collection | culture-based | phenotypic | jejuni | 0 | winter | 5 | not specified | calves | sub-group | 0 |
| 8 | Atabay and Corry | 1997 | 1995-1996 | rectal collection | culture-based | phenotypic | jejuni | 2 | summer | 47 | not specified | adult | sub-group | 0.94 |
| 8 | Atabay and Corry | 1997 | 1995-1996 | rectal collection | culture-based | phenotypic | jejuni | 2 | all | 42 | not specified | calves | sub-group | 0.84 |
| 8 | Atabay and Corry | 1997 | 1995-1996 | rectal collection | culture-based | phenotypic | jejuni | 7 | all | 7 | not specified | cows and calves | sub-group | 0.49 |
| 8 | Atabay and Corry | 1997 | 1995-1996 | rectal collection | culture-based | phenotypic | jejuni | 10 | all | 94 | not specified | adult | sub-group | 9.4 |
| 8 | Atabay and Corry | 1997 | 1995-1996 | rectal collection | culture-based | phenotypic | jejuni | 11 | summer | 9 | not specified | adult | sub-group | 0.99 |
| 8 | Atabay and Corry | 1997 | 1995-1996 | rectal collection | culture-based | phenotypic | jejuni | 14 | winter | 7 | not specified | calves | sub-group | 0.98 |
| 8 | Atabay and Corry | 1997 | 1995-1996 | rectal collection | culture-based | phenotypic | jejuni | 23 | summer | 13 | not specified | adult | sub-group | 2.99 |
| 8 | Atabay and Corry | 1997 | 1995-1996 | rectal collection | culture-based | phenotypic | jejuni | 27 | winter | 15 | not specified | adult | sub-group | 4.05 |
| 8 | Atabay and Corry | 1997 | 1995-1996 | rectal collection | culture-based | phenotypic | not identifiable | 0 | fall | 30 | not specified | calves | sub-group | 0 |
| 8 | Atabay and Corry | 1997 | 1995-1996 | rectal collection | culture-based | phenotypic | not identifiable | 0 | all | 42 | not specified | calves | sub-group | 0 |
| 8 | Atabay and Corry | 1997 | 1995-1996 | rectal collection | culture-based | phenotypic | not identifiable | 0 | winter | 15 | not specified | adult | sub-group | 0 |
| 8 | Atabay and Corry | 1997 | 1995-1996 | rectal collection | culture-based | phenotypic | not identifiable | 0 | winter | 7 | not specified | calves | sub-group | 0 |
| 8 | Atabay and Corry | 1997 | 1995-1996 | rectal collection | culture-based | phenotypic | not identifiable | 0 | summer | 9 | not specified | adult | sub-group | 0 |
| 8 | Atabay and Corry | 1997 | 1995-1996 | rectal collection | culture-based | phenotypic | not identifiable | 0 | winter | 10 | not specified | adult | sub-group | 0 |
| 8 | Atabay and Corry | 1997 | 1995-1996 | rectal collection | culture-based | phenotypic | not identifiable | 0 | winter | 5 | not specified | calves | sub-group | 0 |
| 8 | Atabay and Corry | 1997 | 1995-1996 | rectal collection | culture-based | phenotypic | not identifiable | 1 | all | 13 | not specified | cows and calves | sub-group | 0.13 |
| 8 | Atabay and Corry | 1997 | 1995-1996 | rectal collection | culture-based | phenotypic | not identifiable | 2 | summer | 47 | not specified | adult | sub-group | 0.94 |
| 8 | Atabay and Corry | 1997 | 1995-1996 | rectal collection | culture-based | phenotypic | not identifiable | 2 | all | 94 | not specified | adult | sub-group | 1.88 |
| 8 | Atabay and Corry | 1997 | 1995-1996 | rectal collection | culture-based | phenotypic | not identifiable | 8 | summer | 13 | not specified | adult | sub-group | 1.04 |
| 8 | Atabay and Corry | 1997 | 1995-1996 | rectal collection | culture-based | phenotypic | spp. | 36 | all | 136 | not specified | cows and calves | whole | 48.96 |
| 8 | Atabay and Corry | 1997 | 1995-1996 | rectal collection | culture-based | phenotypic | spp. | 37 | all | 19 | not specified | adult | sub-group | 7.03 |
| 8 | Atabay and Corry | 1997 | 1995-1996 | rectal collection | culture-based | phenotypic | spp. | 39 | all | 28 | not specified | adult | sub-group | 10.92 |
| 8 | Atabay and Corry | 1997 | 1995-1996 | rectal collection | culture-based | phenotypic | spp. | 40 | all | 5 | not specified | calves | sub-group | 2 |
| 8 | Atabay and Corry | 1997 | 1995-1996 | rectal collection | culture-based | phenotypic | spp. | 43 | all | 7 | not specified | calves | sub-group | 3.01 |
| 8 | Atabay and Corry | 1997 | 1995-1996 | rectal collection | culture-based | phenotypic | spp. | 77 | all | 30 | not specified | calves | sub-group | 23.1 |
| 8 | Atabay and Corry | 1997 | 1995-1996 | rectal collection | culture-based | phenotypic | spp. | 81 | all | 47 | not specified | adult | sub-group | 38.07 |
| 8 | Atabay and Corry | 1997 | 1995-1996 | rectal collection | culture-based | phenotypic | sputorum | 0 | fall | 30 | not specified | calves | sub-group | 0 |
| 8 | Atabay and Corry | 1997 | 1995-1996 | rectal collection | culture-based | phenotypic | sputorum | 0 | all | 42 | not specified | calves | sub-group | 0 |
| 8 | Atabay and Corry | 1997 | 1995-1996 | rectal collection | culture-based | phenotypic | sputorum | 0 | summer | 13 | not specified | adult | sub-group | 0 |
| 8 | Atabay and Corry | 1997 | 1995-1996 | rectal collection | culture-based | phenotypic | sputorum | 0 | winter | 15 | not specified | adult | sub-group | 0 |
| 8 | Atabay and Corry | 1997 | 1995-1996 | rectal collection | culture-based | phenotypic | sputorum | 0 | winter | 7 | not specified | calves | sub-group | 0 |
| 8 | Atabay and Corry | 1997 | 1995-1996 | rectal collection | culture-based | phenotypic | sputorum | 0 | summer | 9 | not specified | adult | sub-group | 0 |
| 8 | Atabay and Corry | 1997 | 1995-1996 | rectal collection | culture-based | phenotypic | sputorum | 0 | winter | 10 | not specified | adult | sub-group | 0 |
| 8 | Atabay and Corry | 1997 | 1995-1996 | rectal collection | culture-based | phenotypic | sputorum | 0 | winter | 5 | not specified | calves | sub-group | 0 |
| 8 | Atabay and Corry | 1997 | 1995-1996 | rectal collection | culture-based | phenotypic | sputorum | 21 | all | 47 | not specified | cows and calves | sub-group | 9.87 |

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|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 8 | Atabay and Corry | 1997 | 1995-1996 | rectal collection | culture-based | phenotypic | sputorum | 30 | all | 94 | not specified | adult | sub-group | 28.2 |
| 8 | Atabay and Corry | 1997 | 1995-1996 | rectal collection | culture-based | phenotypic | sputorum | 60 | summer | 60 | not specified | adult | sub-group | 36 |
| 9 | Baserisalehi et al. | 2007 | 2006 | rectal collection | culture-based | phenotypic | coli | 15 | not specified | 26 | healthy | adult | sub-group | 3.9 |
| 9 | Baserisalehi et al. | 2007 | 2006 | rectal collection | culture-based | phenotypic | jejuni | 30 | not specified | 26 | healthy | adult | sub-group | 7.8 |
| 9 | Baserisalehi et al. | 2007 | 2006 | rectal collection | culture-based | phenotypic | lari | 11 | not specified | 26 | healthy | adult | sub-group | 2.86 |
| 9 | Baserisalehi et al. | 2007 | 2006 | rectal collection | culture-based | phenotypic | not identifiable | 23 | not specified | 26 | healthy | adult | sub-group | 5.98 |
| 9 | Baserisalehi et al. | 2007 | 2006 | rectal collection | culture-based | phenotypic | spp. | 21 | not specified | 121 | healthy | adult | whole | 25.41 |
| 9 | Baserisalehi et al. | 2007 | 2006 | rectal collection | culture-based | phenotypic | sputorum | 7 | not specified | 26 | healthy | adult | sub-group | 1.82 |
| 9 | Baserisalehi et al. | 2007 | 2006 | rectal collection | culture-based | phenotypic | sputorum | 11 | not specified | 26 | healthy | adult | sub-group | 2.86 |
| 10 | Bianchini et al., | 2014 | 2010-2012 | rectal collection | culture-based | PCR-based | hyointestinalis | 3.3 | not specified | 30 | not specified | adult | sub-group | 0.99 |
| 10 | Bianchini et al., | 2014 | 2010-2012 | rectal collection | culture-based | PCR-based | jejuni | 5.9 | not specified | 17 | not specified | adult | sub-group | 1.003 |
| 10 | Bianchini et al., | 2014 | 2010-2012 | rectal collection | culture-based | PCR-based | jejuni | 28.6 | not specified | 35 | not specified | adult | sub-group | 10.01 |
| 10 | Bianchini et al., | 2014 | 2010-2012 | rectal collection | culture-based | PCR-based | jejuni | 30.5 | not specified | 82 | not specified | adult | whole | 25.01 |
| 10 | Bianchini et al., | 2014 | 2010-2012 | rectal collection | culture-based | PCR-based | jejuni | 46.7 | not specified | 30 | not specified | adult | sub-group | 14.01 |
| 11 | Cha et al., | 2017 | 2012 | not specified | culture-based | PCR-based, WGS | jejuni | 58.6 | not specified | 58 | not specified | adult | whole | 33.988 |
| 12 | Dong et al., | 2016 | 2012-2014 | rectal collection | culture-based | PCR-based | jejuni | 24.2 | not specified | 194 | not specified | adult | whole | 46.948 |
| 13 | Duncan et al., | 2013 | | cow pat | culture-based | PCR-based | fetus | 9.5 | not specified | 4260 | not specified | adult | whole | 404.7 |
| 14 | Englen et al., | 2007 | 2002 | rectal collection | culture-based | PCR-based | spp. | 51.2 | not applicable | 1435 | not specified | adult | whole | 734.72 |
| 15 | Grinberg et al., | 2005 | 2002 | rectal collection | culture-based | phenotypic | spp. | 36 | not specified | 161 | mixed | calves | whole | 57.96 |
| 16 | Guevremont et al., | 2014 | 2011 | rectal collection | culture-based | PCR-based | coli | 1.8 | summer | 797 | not specified | adult | sub-group | 14.346 |
| 16 | Guevremont et al., | 2014 | 2011 | rectal collection | culture-based | PCR-based | hyointestinalis | 19.3 | summer | 797 | not specified | adult | sub-group | 153.821 |
| 16 | Guevremont et al., | 2014 | 2011 | rectal collection | culture-based | PCR-based | jejuni | 6.5 | summer | 797 | not specified | adult | sub-group | 51.805 |
| 18 | Hagey et al., | 2019 | not specified | rectal collection | PCR-based | other molecular method | spp. | 31 | not specified | 150 | not specified | adult | whole | 46.5 |
| 19 | Hakkinen and Hänninen, | 2009 | 2006-2007 | cow pat | culture-based | other molecular method | coli | 3.2 | all | 340 | not specified | adult | sub-group | 10.88 |
| 19 | Hakkinen and Hänninen, | 2009 | 2006-2007 | cow pat | culture-based | other molecular method | hyointestinalis | 15.3 | all | 340 | not specified | adult | sub-group | 52.02 |
| 19 | Hakkinen and Hänninen, | 2009 | 2006-2007 | cow pat | culture-based | other molecular method | jejuni | 0 | summer | 19 | not specified | adult | sub-group | 0 |
| 19 | Hakkinen and Hänninen, | 2009 | 2006-2007 | cow pat | culture-based | other molecular method | jejuni | 10.5 | winter | 19 | not specified | adult | sub-group | 1.995 |
| 19 | Hakkinen and Hänninen, | 2009 | 2006-2007 | cow pat | culture-based | other molecular method | jejuni | 11.1 | spring | 18 | not specified | adult | sub-group | 1.998 |
| 19 | Hakkinen and Hänninen, | 2009 | 2006-2007 | cow pat | culture-based | other molecular method | jejuni | 15.8 | spring | 19 | not specified | adult | sub-group | 3.002 |
| 19 | Hakkinen and Hänninen, | 2009 | 2006-2007 | cow pat | culture-based | other molecular method | jejuni | 28.6 | winter | 21 | not specified | adult | sub-group | 6.006 |
| 19 | Hakkinen and Hänninen, | 2009 | 2006-2007 | cow pat | culture-based | other molecular method | jejuni | 29.4 | summer | 17 | not specified | adult | sub-group | 4.998 |
| 19 | Hakkinen and Hänninen, | 2009 | 2006-2007 | cow pat | culture-based | other molecular method | jejuni | 30 | fall | 20 | not specified | adult | sub-group | 6 |
| 19 | Hakkinen and Hänninen, | 2009 | 2006-2007 | cow pat | culture-based | other molecular method | jejuni | 37 | spring | 340 | not specified | adult | sub-group | 125.8 |
| 19 | Hakkinen and Hänninen, | 2009 | 2006-2007 | cow pat | culture-based | other molecular method | jejuni | 38 | summer | 340 | not specified | adult | sub-group | 129.2 |
| 19 | Hakkinen and Hänninen, | 2009 | 2006-2007 | cow pat | culture-based | other molecular method | jejuni | 38.9 | fall | 18 | not specified | adult | sub-group | 7.002 |
| 19 | Hakkinen and Hänninen, | 2009 | 2006-2007 | cow pat | culture-based | other molecular method | jejuni | 43 | winter | 340 | not specified | adult | sub-group | 146.2 |
| 19 | Hakkinen and Hänninen, | 2009 | 2006-2007 | cow pat | culture-based | other molecular method | jejuni | 44 | all | 340 | not specified | adult | sub-group | 149.6 |
| 19 | Hakkinen and Hänninen, | 2009 | 2006-2007 | cow pat | culture-based | other molecular method | jejuni | 45 | fall | 20 | not specified | adult | sub-group | 9 |
| 19 | Hakkinen and Hänninen, | 2009 | 2006-2007 | cow pat | culture-based | other molecular method | jejuni | 45 | fall | 340 | not specified | adult | sub-group | 153 |
| 19 | Hakkinen and Hänninen, | 2009 | 2006-2007 | cow pat | culture-based | other molecular method | jejuni | 55.2 | summer | 29 | not specified | adult | sub-group | 16.008 |
| 19 | Hakkinen and Hänninen, | 2009 | 2006-2007 | cow pat | culture-based | other molecular method | jejuni | 64 | fall | 340 | not specified | adult | sub-group | 217.6 |
| 19 | Hakkinen and Hänninen, | 2009 | 2006-2007 | cow pat | culture-based | other molecular method | jejuni | 68 | fall | 25 | not specified | adult | sub-group | 17 |
| 19 | Hakkinen and Hänninen, | 2009 | 2006-2007 | cow pat | culture-based | other molecular method | jejuni | 70.6 | fall | 17 | not specified | adult | sub-group | 12.002 |
| 19 | Hakkinen and Hänninen, | 2009 | 2006-2007 | cow pat | culture-based | other molecular method | jejuni | 81.8 | fall | 33 | not specified | adult | sub-group | 26.994 |
| 19 | Hakkinen and Hänninen, | 2009 | 2006-2007 | cow pat | culture-based | other molecular method | jejuni | 84.4 | spring | 32 | not specified | adult | sub-group | 27.008 |
| 19 | Hakkinen and Hänninen, | 2009 | 2006-2007 | cow pat | culture-based | other molecular method | jejuni | 90.9 | winter | 33 | not specified | adult | sub-group | 29.997 |
| 19 | Hakkinen and Hänninen, | 2009 | 2006-2007 | cow pat | culture-based | other molecular method | spp. | 49.7 | all | 340 | not specified | adult | whole | 168.98 |

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|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 21 | Harvey et al. | 2004 | 2001 | rectal collection | culture-based | other molecular method | spp. | 0 | not specified | 60 | not specified | adult | sub-group | 0 |
| 21 | Harvey et al. | 2004 | 2001 | rectal collection | culture-based | other molecular method | spp. | 0 | not specified | 60 | not specified | adult | sub-group | 0 |
| 21 | Harvey et al. | 2004 | 2001 | rectal collection | culture-based | other molecular method | spp. | 1.7 | not specified | 120 | not specified | adult | sub-group | 2.04 |
| 21 | Harvey et al. | 2004 | 2001 | rectal collection | culture-based | other molecular method | spp. | 1.7 | not specified | 60 | not specified | adult | sub-group | 1.02 |
| 21 | Harvey et al. | 2004 | 2001 | rectal collection | culture-based | other molecular method | spp. | 3.3 | not specified | 60 | not specified | adult | sub-group | 1.98 |
| 21 | Harvey et al. | 2004 | 2001 | rectal collection | culture-based | other molecular method | spp. | 3.3 | not specified | 60 | not specified | adult | sub-group | 1.98 |
| 21 | Harvey et al. | 2004 | 2001 | rectal collection | culture-based | other molecular method | spp. | 5.8 | not specified | 120 | not specified | adult | sub-group | 6.96 |
| 21 | Harvey et al. | 2004 | 2001 | rectal collection | culture-based | other molecular method | spp. | 6.7 | not specified | 60 | not specified | adult | sub-group | 4.02 |
| 21 | Harvey et al. | 2004 | 2001 | rectal collection | culture-based | other molecular method | spp. | 10 | not specified | 60 | not specified | adult | sub-group | 6 |
| 21 | Harvey et al. | 2004 | 2001 | rectal collection | culture-based | other molecular method | spp. | 10 | not specified | 60 | not specified | adult | sub-group | 6 |
| 23 | Giacoboni et al., | 1993 | not specified | not specified | culture-based | phenotypic | coli | 0 | not specified | 60 | not specified | adult | sub-group | 0 |
| 23 | Giacoboni et al., | 1993 | not specified | not specified | culture-based | phenotypic | coli | 1.1 | not specified | 94 | not specified | cows and calves | sub-group | 1.034 |
| 23 | Giacoboni et al., | 1993 | not specified | not specified | culture-based | phenotypic | coli | 2.9 | not specified | 34 | not specified | calves | sub-group | 0.986 |
| 23 | Giacoboni et al., | 1993 | not specified | not specified | culture-based | phenotypic | fecalis | 0 | not specified | 60 | not specified | adult | sub-group | 0 |
| 23 | Giacoboni et al., | 1993 | not specified | not specified | culture-based | phenotypic | fecalis | 1.1 | not specified | 94 | not specified | cows and calves | sub-group | 1.034 |
| 23 | Giacoboni et al., | 1993 | not specified | not specified | culture-based | phenotypic | fecalis | 2.9 | not specified | 34 | not specified | calves | sub-group | 0.986 |
| 23 | Giacoboni et al., | 1993 | not specified | not specified | culture-based | phenotypic | fetus | 11.7 | not specified | 60 | not specified | adult | sub-group | 7.02 |
| 23 | Giacoboni et al., | 1993 | not specified | not specified | culture-based | phenotypic | fetus | 17 | not specified | 94 | not specified | cows and calves | sub-group | 15.98 |
| 23 | Giacoboni et al., | 1993 | not specified | not specified | culture-based | phenotypic | fetus | 26.5 | not specified | 34 | not specified | calves | sub-group | 9.01 |
| 23 | Giacoboni et al., | 1993 | not specified | not specified | culture-based | phenotypic | hyointestinalis | 15 | not specified | 60 | not specified | adult | sub-group | 9 |
| 23 | Giacoboni et al., | 1993 | not specified | not specified | culture-based | phenotypic | hyointestinalis | 19.1 | not specified | 94 | not specified | cows and calves | sub-group | 17.954 |
| 23 | Giacoboni et al., | 1993 | not specified | not specified | culture-based | phenotypic | hyointestinalis | 26.5 | not specified | 34 | not specified | calves | sub-group | 9.01 |
| 23 | Giacoboni et al., | 1993 | not specified | not specified | culture-based | phenotypic | jejuni | 13.3 | not specified | 60 | not specified | adult | sub-group | 7.98 |
| 23 | Giacoboni et al., | 1993 | not specified | not specified | culture-based | phenotypic | jejuni | 30.9 | not specified | 94 | not specified | cows and calves | sub-group | 29.046 |
| 23 | Giacoboni et al., | 1993 | not specified | not specified | culture-based | phenotypic | jejuni | 61.8 | not specified | 34 | not specified | calves | sub-group | 21.012 |
| 23 | Giacoboni et al., | 1993 | not specified | not specified | culture-based | phenotypic | lari | 0 | not specified | 34 | not specified | calves | sub-group | 0 |
| 23 | Giacoboni et al., | 1993 | not specified | not specified | culture-based | phenotypic | lari | 2.1 | not specified | 94 | not specified | cows and calves | sub-group | 1.974 |
| 23 | Giacoboni et al., | 1993 | not specified | not specified | culture-based | phenotypic | lari | 3.3 | not specified | 60 | not specified | adult | sub-group | 1.98 |
| 23 | Giacoboni et al., | 1993 | not specified | not specified | culture-based | phenotypic | spp. | 1.7 | not specified | 60 | not specified | adult | sub-group | 1.02 |
| 23 | Giacoboni et al., | 1993 | not specified | not specified | culture-based | phenotypic | spp. | 9.6 | not specified | 94 | not specified | cows and calves | sub-group | 9.024 |
| 23 | Giacoboni et al., | 1993 | not specified | not specified | culture-based | phenotypic | spp. | 10 | not specified | 1 | not specified | adult | sub-group | 0.1 |
| 23 | Giacoboni et al., | 1993 | not specified | not specified | culture-based | phenotypic | spp. | 14.7 | not specified | 34 | not specified | calves | sub-group | 4.998 |
| 23 | Giacoboni et al., | 1993 | not specified | not specified | culture-based | phenotypic | spp. | 20 | not specified | 2 | not specified | adult | sub-group | 0.4 |
| 23 | Giacoboni et al., | 1993 | not specified | not specified | culture-based | phenotypic | spp. | 27.2 | not specified | 3 | not specified | adult | sub-group | 0.816 |
| 23 | Giacoboni et al., | 1993 | not specified | not specified | culture-based | phenotypic | spp. | 46.7 | not specified | 60 | not specified | adult | sub-group | 28.02 |
| 23 | Giacoboni et al., | 1993 | not specified | not specified | culture-based | phenotypic | spp. | 50 | not specified | 10 | not specified | adult | sub-group | 5 |
| 23 | Giacoboni et al., | 1993 | not specified | not specified | culture-based | phenotypic | spp. | 64.9 | not specified | 94 | not specified | cows and calves | whole | 61.006 |
| 23 | Giacoboni et al., | 1993 | not specified | not specified | culture-based | phenotypic | spp. | 80 | not specified | 4 | not specified | calves | sub-group | 3.2 |
| 23 | Giacoboni et al., | 1993 | not specified | not specified | culture-based | phenotypic | spp. | 80 | not specified | 4 | not specified | adult | sub-group | 3.2 |
| 23 | Giacoboni et al., | 1993 | not specified | not specified | culture-based | phenotypic | spp. | 80 | not specified | 4 | not specified | adult | sub-group | 3.2 |
| 23 | Giacoboni et al., | 1993 | not specified | not specified | culture-based | phenotypic | spp. | 97.1 | not specified | 34 | not specified | calves | sub-group | 33.014 |
| 23 | Giacoboni et al., | 1993 | not specified | not specified | culture-based | phenotypic | spp. | 100 | not specified | 9 | not specified | calves | sub-group | 9 |
| 23 | Giacoboni et al., | 1993 | not specified | not specified | culture-based | phenotypic | spp. | 100 | not specified | 10 | not specified | calves | sub-group | 10 |
| 23 | Giacoboni et al., | 1993 | not specified | not specified | culture-based | phenotypic | spp. | 100 | not specified | 4 | not specified | adult | sub-group | 4 |
| 23 | Giacoboni et al., | 1993 | not specified | not specified | culture-based | phenotypic | spp. | 100 | not specified | 5 | not specified | calves | sub-group | 5 |
| 23 | Giacoboni et al., | 1993 | not specified | not specified | culture-based | phenotypic | spp. | 100 | not specified | 5 | not specified | adult | sub-group | 5 |
| 23 | Giacoboni et al., | 1993 | not specified | not specified | culture-based | phenotypic | spp. | 100 | not specified | 5 | not specified | calves | sub-group | 5 |
| 25 | Kashoma et al. | 2015 | 2013-2014 | cow pat | culture-based | PCR-based | coli | 6.3 | not specified | 32 | not specified | adult | sub-group | 2.016 |
| 25 | Kashoma et al. | 2015 | 2013-2014 | cow pat | culture-based | PCR-based | coli | 12.4 | not specified | 113 | not specified | adult | sub-group | 14.012 |
| 25 | Kashoma et al. | 2015 | 2013-2014 | cow pat | culture-based | PCR-based | coli | 12.8 | not specified | 47 | not specified | adult | sub-group | 6.016 |
| 25 | Kashoma et al. | 2015 | 2013-2014 | cow pat | culture-based | PCR-based | jejuni | 14.9 | not specified | 47 | not specified | adult | sub-group | 7.003 |

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|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 25 | Kashoma et al. | 2015 | 2013-2014 | cow pat | culture-based | PCR-based | jejuni | 15.6 | not specified | 32 | not specified | adult | sub-group | 4.992 |
| 25 | Kashoma et al. | 2015 | 2013-2014 | cow pat | culture-based | PCR-based | jejuni | 26.6 | not specified | 113 | not specified | adult | sub-group | 30.058 |
| 25 | Kashoma et al. | 2015 | 2013-2014 | cow pat | culture-based | PCR-based | spp. | 25 | not specified | 32 | not specified | adult | sub-group | 8 |
| 25 | Kashoma et al. | 2015 | 2013-2014 | cow pat | culture-based | PCR-based | spp. | 29.8 | not specified | 47 | not specified | adult | sub-group | 14.006 |
| 25 | Kashoma et al. | 2015 | 2013-2014 | cow pat | culture-based | PCR-based | spp. | 35.4 | not specified | 192 | not specified | adult | whole | 67.968 |
| 25 | Kashoma et al. | 2015 | 2013-2014 | cow pat | culture-based | PCR-based | spp. | 40.7 | not specified | 113 | not specified | adult | sub-group | 45.991 |
| 26 | Khalifa et al. | 2013 | not specified | cow pat | culture-based | PCR-based | jejuni | 32 | not applicable | 50 | not specified | adult | whole | 16 |
| 27 | Klein et al. | 2013 | 2009-2010 | rectal collection | culture-based | MALDI-TOF | spp. | 13.2 | not applicable | 303 | healthy | calves | sub-group | 39.996 |
| 27 | Klein et al. | 2013 | 2009-2010 | rectal collection | culture-based | MALDI-TOF | spp. | 14.9 | not applicable | 382 | mixed | calves | whole | 56.918 |
| 27 | Klein et al. | 2013 | 2009-2010 | rectal collection | culture-based | MALDI-TOF | spp. | 21.5 | not applicable | 79 | diarrhea | calves | sub-group | 16.985 |
| 28 | Kwan et al. | 2008 | 2003 | cow pat | culture-based | PCR-based | spp. | 35.9 | not specified | 1208 | not specified | adult | whole | 433.672 |
| 29 | McAuley et al. | 2014 | 2013 and 2014 | not specified | culture-based | PCR-based | spp. | 6 | not applicable | 16 | not specified | adult | whole | 0.96 |
| 30 | Merialdi et al. | 2015 | 2012-2013 | rectal collection | PCR-based and culture-based | phenotypic | jejuni | 1.7 | all | 280 | not specified | cows, heifers and calves | sub-group | 4.76 |
| 30 | Merialdi et al. | 2015 | 2012-2013 | rectal collection | PCR-based and culture-based | phenotypic | spp. | 9.7 | all | 280 | not specified | cows, heifers and calves | sub-group | 27.16 |
| 31 | Messelhaeuser et al. | 2008 | 2004 | not specified | culture-based | PCR-based | spp. | 0 | summer | 72 | not specified | not specified | sub-group | 0 |
| 31 | Messelhaeuser et al. | 2008 | 2004 | not specified | culture-based | PCR-based | spp. | 0 | summer | 72 | not specified | not specified | sub-group | 0 |
| 31 | Messelhaeuser et al. | 2008 | 2004 | not specified | culture-based | PCR-based | spp. | 0 | summer | 72 | not specified | not specified | sub-group | 0 |
| 31 | Messelhaeuser et al. | 2008 | 2004 | not specified | culture-based | PCR-based | spp. | 0 | summer | 75 | not specified | not specified | sub-group | 0 |
| 31 | Messelhaeuser et al. | 2008 | 2004 | not specified | culture-based | PCR-based | spp. | 2.8 | summer | 72 | not specified | not specified | sub-group | 2.016 |
| 31 | Messelhaeuser et al. | 2008 | 2004 | not specified | culture-based | PCR-based | spp. | 5.2 | summer | 75 | not specified | not specified | sub-group | 3.9 |
| 31 | Messelhaeuser et al. | 2008 | 2004 | not specified | culture-based | PCR-based | spp. | 6.3 | winter | 79 | not specified | not specified | sub-group | 4.977 |
| 31 | Messelhaeuser et al. | 2008 | 2004 | not specified | culture-based | PCR-based | spp. | 6.9 | summer | 72 | not specified | not specified | sub-group | 4.968 |
| 31 | Messelhaeuser et al. | 2008 | 2004 | not specified | culture-based | PCR-based | spp. | 8.3 | winter | 79 | not specified | not specified | sub-group | 6.557 |
| 31 | Messelhaeuser et al. | 2008 | 2004 | not specified | culture-based | PCR-based | spp. | 13 | all | 226 | not specified | not specified | whole | 29.38 |
| 31 | Messelhaeuser et al. | 2008 | 2004 | not specified | culture-based | PCR-based | spp. | 14.7 | summer | 75 | not specified | not specified | sub-group | 11.025 |
| 31 | Messelhaeuser et al. | 2008 | 2004 | not specified | culture-based | PCR-based | spp. | 14.8 | winter | 79 | not specified | not specified | sub-group | 11.692 |
| 31 | Messelhaeuser et al. | 2008 | 2004 | not specified | culture-based | PCR-based | spp. | 20.1 | winter | 79 | not specified | not specified | sub-group | 15.879 |
| 31 | Messelhaeuser et al. | 2008 | 2004 | not specified | culture-based | PCR-based | spp. | 20.7 | summer | 75 | not specified | not specified | sub-group | 15.525 |
| 31 | Messelhaeuser et al. | 2008 | 2004 | not specified | culture-based | PCR-based | spp. | 23.5 | summer | 75 | not specified | not specified | sub-group | 17.625 |
| 31 | Messelhaeuser et al. | 2008 | 2004 | not specified | culture-based | PCR-based | spp. | 41.7 | winter | 79 | not specified | not specified | sub-group | 32.943 |
| 33 | Munroe et al. | 1983 | not specified | rectal collection | culture-based | phenotypic | coli | 0 | not specified | 314 | diarrhea | not specified | sub-group | 0 |
| 33 | Munroe et al. | 1983 | not specified | rectal collection | culture-based | phenotypic | coli | 2 | not specified | 314 | diarrhea | not specified | sub-group | 6.28 |
| 33 | Munroe et al. | 1983 | not specified | rectal collection | culture-based | phenotypic | jejuni | 17 | not specified | 314 | diarrhea | not specified | sub-group | 53.38 |
| 33 | Munroe et al. | 1983 | not specified | rectal collection | culture-based | phenotypic | jejuni | 25 | not specified | 107 | healthy | not specified | sub-group | 26.75 |
| 35 | Nielsen et al. | 2002 | 1999 | rectal collection | culture-based | other molecular method | spp. | 9.2 | not specified | 120 | not specified | adult | sub-group | 11.04 |
| 35 | Nielsen et al. | 2002 | 1999 | rectal collection | culture-based | other molecular method | spp. | 20 | not specified | 105 | not specified | heifers | sub-group | 21 |
| 35 | Nielsen et al. | 2002 | 1999 | rectal collection | culture-based | other molecular method | spp. | 22.6 | not specified | 332 | not specified | cows, heifers and calves | sub-group | 75.032 |
| 35 | Nielsen et al. | 2002 | 1999 | rectal collection | culture-based | other molecular method | spp. | 32.2 | not specified | 332 | not specified | cows, heifers and calves | whole | 106.904 |
| 35 | Nielsen et al. | 2002 | 1999 | rectal collection | culture-based | other molecular method | spp. | 42.1 | not specified | 107 | not specified | calves | sub-group | 45.047 |
| 37 | Padungtod and Kaneene | 2005 | 2000-2003 | not specified | culture-based | PCR-based | spp. | 14 | not specified | 225 | not specified | not specified | whole | 31.5 |
| 38 | Pradhan et al., | 2009 | 2004 | rectal collection | culture-based | PCR-based | spp. | 0 | spring | 82 | not specified | adult | sub-group | 0 |
| 38 | Pradhan et al., | 2009 | 2004 | rectal collection | culture-based | PCR-based | spp. | 0 | spring | 84 | not specified | adult | sub-group | 0 |
| 38 | Pradhan et al., | 2009 | 2004 | rectal collection | culture-based | PCR-based | spp. | 0 | summer | 28 | not specified | adult | sub-group | 0 |
| 38 | Pradhan et al., | 2009 | 2004 | rectal collection | culture-based | PCR-based | spp. | 5 | winter | 26 | not specified | adult | sub-group | 1.3 |
| 38 | Pradhan et al., | 2009 | 2004 | rectal collection | culture-based | PCR-based | spp. | 8.8 | spring | 34 | not specified | adult | sub-group | 2.992 |
| 38 | Pradhan et al., | 2009 | 2004 | rectal collection | culture-based | PCR-based | spp. | 9.4 | fall | 32 | not specified | adult | sub-group | 3.008 |
| 38 | Pradhan et al., | 2009 | 2004 | rectal collection | culture-based | PCR-based | spp. | 13.6 | spring | 44 | not specified | adult | sub-group | 5.984 |
| 38 | Pradhan et al., | 2009 | 2004 | rectal collection | culture-based | PCR-based | spp. | 13.9 | fall | 36 | not specified | adult | sub-group | 5.004 |
| 38 | Pradhan et al., | 2009 | 2004 | rectal collection | culture-based | PCR-based | spp. | 21.4 | summer | 28 | not specified | adult | sub-group | 5.992 |
| 38 | Pradhan et al., | 2009 | 2004 | rectal collection | culture-based | PCR-based | spp. | 26.2 | fall | 84 | not specified | adult | sub-group | 22.008 |
| 38 | Pradhan et al., | 2009 | 2004 | rectal collection | culture-based | PCR-based | spp. | 29.4 | fall | 34 | not specified | adult | sub-group | 9.996 |
| 38 | Pradhan et al., | 2009 | 2004 | rectal collection | culture-based | PCR-based | spp. | 29.7 | spring | 37 | not specified | adult | sub-group | 10.989 |

| | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 38 | Pradhan et al., | 2009 | 2004 | rectal collection | culture-based | PCR-based | spp. | 32.5 | fall | 83 | not specified | adult | sub-group | 26.975 |
| 38 | Pradhan et al., | 2009 | 2004 | rectal collection | culture-based | PCR-based | spp. | 44 | spring | 84 | not specified | adult | sub-group | 36.96 |
| 38 | Pradhan et al., | 2009 | 2004 | rectal collection | culture-based | PCR-based | spp. | 53.6 | fall | 28 | not specified | adult | sub-group | 15.008 |
| 38 | Pradhan et al., | 2009 | 2004 | rectal collection | culture-based | PCR-based | spp. | 53.6 | winter | 28 | not specified | adult | sub-group | 15.008 |
| 38 | Pradhan et al., | 2009 | 2004 | rectal collection | culture-based | PCR-based | spp. | 57.3 | fall | 82 | not specified | adult | sub-group | 46.986 |
| 38 | Pradhan et al., | 2009 | 2004 | rectal collection | culture-based | PCR-based | spp. | 57.7 | winter | 78 | not specified | adult | sub-group | 45.006 |
| 38 | Pradhan et al., | 2009 | 2004 | rectal collection | culture-based | PCR-based | spp. | 61.5 | spring | 26 | not specified | adult | sub-group | 15.99 |
| 38 | Pradhan et al., | 2009 | 2004 | rectal collection | culture-based | PCR-based | spp. | 76.9 | winter | 26 | not specified | adult | sub-group | 19.994 |
| 38 | Pradhan et al., | 2009 | 2004 | rectal collection | culture-based | PCR-based | spp. | 80.8 | summer | 26 | not specified | adult | sub-group | 21.008 |
| 39 | Ramonait et al., | 2013 | 2012 | rectal collection | culture-based | PCR-based | spp. | 53.2 | not specified | 21 | not specified | adult | sub-group | 11.172 |
| 39 | Ramonait et al., | 2013 | 2012 | rectal collection | culture-based | PCR-based | spp. | 60 | not specified | 20 | not specified | adult | sub-group | 12 |
| 39 | Ramonait et al., | 2013 | 2012 | rectal collection | culture-based | PCR-based | spp. | 60.6 | not specified | 61 | not specified | adult | sub-group | 36.966 |
| 39 | Ramonait et al., | 2013 | 2012 | rectal collection | culture-based | PCR-based | spp. | 70 | not specified | 20 | not specified | calves | sub-group | 14 |
| 39 | Ramonait et al., | 2013 | 2012 | rectal collection | culture-based | PCR-based | spp. | 70 | not specified | 20 | not specified | adult | sub-group | 14 |
| 39 | Ramonait et al., | 2013 | 2012 | rectal collection | culture-based | PCR-based | spp. | 78.5 | not specified | 200 | not specified | cows, heifers and calves | whole | 157 |
| 39 | Ramonait et al., | 2013 | 2012 | rectal collection | culture-based | PCR-based | spp. | 85 | not specified | 20 | not specified | heifers | sub-group | 17 |
| 39 | Ramonait et al., | 2013 | 2012 | rectal collection | culture-based | PCR-based | spp. | 85 | not specified | 40 | not specified | heifers | sub-group | 34 |
| 39 | Ramonait et al., | 2013 | 2012 | rectal collection | culture-based | PCR-based | spp. | 86.2 | not specified | 80 | not specified | heifers | sub-group | 68.96 |
| 39 | Ramonait et al., | 2013 | 2012 | rectal collection | culture-based | PCR-based | spp. | 86.5 | not specified | 59 | not specified | calves | sub-group | 51.035 |
| 39 | Ramonait et al., | 2013 | 2012 | rectal collection | culture-based | PCR-based | spp. | 89.4 | not specified | 19 | not specified | calves | sub-group | 16.986 |
| 39 | Ramonait et al., | 2013 | 2012 | rectal collection | culture-based | PCR-based | spp. | 90 | not specified | 20 | not specified | heifers | sub-group | 18 |
| 39 | Ramonait et al., | 2013 | 2012 | rectal collection | culture-based | PCR-based | spp. | 100 | not specified | 20 | not specified | calves | sub-group | 20 |
| 40 | Rapp et al., | 2013 | 2011-2012 | cow pat | culture-based | PCR-based | jejuni | 36 | summer | 105 | not specified | adult | sub-group | 37.8 |
| 40 | Rapp et al., | 2013 | 2011-2012 | cow pat | culture-based | PCR-based | jejuni | 36 | fall | 45 | not specified | adult | sub-group | 16.2 |
| 40 | Rapp et al., | 2013 | 2011-2012 | cow pat | culture-based | PCR-based | jejuni | 42 | summer | 105 | not specified | adult | sub-group | 44.1 |
| 40 | Rapp et al., | 2013 | 2011-2012 | cow pat | culture-based | PCR-based | jejuni | 43 | summer | 105 | not specified | adult | sub-group | 45.15 |
| 40 | Rapp et al., | 2013 | 2011-2012 | cow pat | culture-based | PCR-based | jejuni | 49 | all | 210 | not specified | adult | sub-group | 102.9 |
| 40 | Rapp et al., | 2013 | 2011-2012 | cow pat | culture-based | PCR-based | jejuni | 53 | fall | 45 | not specified | adult | sub-group | 23.85 |
| 40 | Rapp et al., | 2013 | 2011-2012 | cow pat | culture-based | PCR-based | jejuni | 54 | winter | 105 | not specified | adult | sub-group | 56.7 |
| 40 | Rapp et al., | 2013 | 2011-2012 | cow pat | culture-based | PCR-based | jejuni | 54 | all | 390 | not specified | adult | sub-group | 210.6 |
| 40 | Rapp et al., | 2013 | 2011-2012 | cow pat | culture-based | PCR-based | jejuni | 55 | all | 390 | not specified | adult | sub-group | 214.5 |
| 40 | Rapp et al., | 2013 | 2011-2012 | cow pat | culture-based | PCR-based | jejuni | 56 | winter | 105 | not specified | adult | sub-group | 58.8 |
| 40 | Rapp et al., | 2013 | 2011-2012 | cow pat | culture-based | PCR-based | jejuni | 58 | winter | 105 | not specified | adult | sub-group | 60.9 |
| 40 | Rapp et al., | 2013 | 2011-2012 | cow pat | culture-based | PCR-based | jejuni | 62 | summer | 45 | not specified | adult | sub-group | 27.9 |
| 40 | Rapp et al., | 2013 | 2011-2012 | cow pat | culture-based | PCR-based | jejuni | 64 | summer | 45 | not specified | adult | sub-group | 28.8 |
| 40 | Rapp et al., | 2013 | 2011-2012 | cow pat | culture-based | PCR-based | jejuni | 66 | winter | 45 | not specified | adult | sub-group | 29.7 |
| 40 | Rapp et al., | 2013 | 2011-2012 | cow pat | culture-based | PCR-based | jejuni | 67 | winter | 45 | not specified | adult | sub-group | 30.15 |
| 40 | Rapp et al., | 2013 | 2011-2012 | cow pat | culture-based | PCR-based | jejuni | 71 | spring | 45 | not specified | adult | sub-group | 31.95 |
| 40 | Rapp et al., | 2013 | 2011-2012 | cow pat | culture-based | PCR-based | jejuni | 76 | spring | 45 | not specified | adult | sub-group | 34.2 |
| 41 | Roug | 2012 | 2005 | cow pat | culture-based | PCR-based | jejuni | 17 | not specified | 12 | not specified | adult | whole | 2.04 |
| 42 | Sanad et al., | 2013 | 2009 | not specified | culture-based | PCR-based | spp. | 5.3 | not specified | 19 | not specified | adult | sub-group | 1.007 |
| 42 | Sanad et al., | 2013 | 2009 | not specified | culture-based | PCR-based | spp. | 17.6 | not specified | 17 | not specified | adult | sub-group | 2.992 |
| 42 | Sanad et al., | 2013 | 2009 | not specified | culture-based | PCR-based | spp. | 24 | not specified | 25 | not specified | adult | sub-group | 6 |
| 42 | Sanad et al., | 2013 | 2009 | not specified | culture-based | PCR-based | spp. | 28 | not specified | 25 | not specified | adult | sub-group | 7 |
| 42 | Sanad et al., | 2013 | 2009 | not specified | culture-based | PCR-based | spp. | 35 | not specified | 20 | not specified | adult | sub-group | 7 |
| 42 | Sanad et al., | 2013 | 2009 | not specified | culture-based | PCR-based | spp. | 36.6 | not specified | 227 | not specified | adult | whole | 83.082 |
| 42 | Sanad et al., | 2013 | 2009 | not specified | culture-based | PCR-based | spp. | 40 | not specified | 20 | not specified | adult | sub-group | 8 |
| 42 | Sanad et al., | 2013 | 2009 | not specified | culture-based | PCR-based | spp. | 40 | not specified | 20 | not specified | adult | sub-group | 8 |
| 42 | Sanad et al., | 2013 | 2009 | not specified | culture-based | PCR-based | spp. | 47.6 | not specified | 21 | not specified | adult | sub-group | 9.996 |
| 42 | Sanad et al., | 2013 | 2009 | not specified | culture-based | PCR-based | spp. | 50 | not specified | 10 | not specified | adult | sub-group | 5 |
| 42 | Sanad et al., | 2013 | 2009 | not specified | culture-based | PCR-based | spp. | 52 | not specified | 25 | not specified | adult | sub-group | 13 |
| 42 | Sanad et al., | 2013 | 2009 | not specified | culture-based | PCR-based | spp. | 60 | not specified | 20 | not specified | adult | sub-group | 12 |
| 43 | Sato et al., | 2004 | not specified | cow pat | culture-based | not performed | spp. | 18.9 | fall | 1192 | healthy | cows and calves | sub-group | 225.288 |
| 43 | Sato et al., | 2004 | not specified | cow pat | culture-based | not performed | spp. | 23.2 | all | 1191 | healthy | adult | sub-group | 276.312 |

| | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 43 | Sato et al., | 2004 | not specified | cow pat | culture-based | not performed | spp. | 27.9 | all | 1191 | healthy | cows and calves | whole | 332.289 |
| 43 | Sato et al., | 2004 | not specified | cow pat | culture-based | not performed | spp. | 32.7 | all | 1191 | healthy | calves | sub-group | 389.457 |
| 43 | Sato et al., | 2004 | not specified | cow pat | culture-based | not performed | spp. | 36.8 | spring | 1191 | healthy | cows and calves | sub-group | 438.288 |
| 45 | Terentjeva et al., | 2019 | not specified | rectal collection | culture-based | MALDI-TOF | coli | 2.8 | not specified | 180 | not specified | calves | sub-group | 5.04 |
| 45 | Terentjeva et al., | 2019 | not specified | rectal collection | culture-based | MALDI-TOF | jejuni | 12.8 | not specified | 180 | not specified | calves | sub-group | 23.04 |
| 46 | Watner-Toews et al., | 1986 | 1982 | rectal collection | culture-based | not performed | spp. | 13 | fall | 156 | not specified | calves | whole | 20.28 |
| 47 | Ward and Guevremont | 2014 | not specified | rectal collection | PCR-based | other molecular method | jejuni | 0 | not specified | 20 | not specified | adult | sub-group | 0 |
| 47 | Ward and Guevremont | 2014 | not specified | rectal collection | PCR-based | other molecular method | jejuni | 55 | not specified | 20 | not specified | adult | sub-group | 11 |
| 48 | Watermann et al., | 1984 | not specified | rectal collection | culture-based | not performed | spp. | 13 | summer | 74 | healthy | adult | sub-group | 9.62 |
| 48 | Watermann et al., | 1984 | not specified | rectal collection | culture-based | not performed | spp. | 51 | winter | 74 | healthy | adult | sub-group | 37.74 |
| 49 | Wesley et al., | 2000 | 1996 | rectal collection | culture-based | PCR-based | jejuni | 39.5 | spring | 542 | healthy | adult | sub-group | 214.09 |
| 49 | Wesley et al., | 2000 | 1996 | rectal collection | culture-based | PCR-based | coli | 1.5 | spring | 542 | healthy | adult | sub-group | 8.13 |
| 49 | Wesley et al., | 2000 | 1996 | rectal collection | culture-based | PCR-based | coli | 1.8 | all | 2085 | healthy | adult | sub-group | 37.53 |
| 49 | Wesley et al., | 2000 | 1996 | rectal collection | culture-based | PCR-based | coli | 1.9 | summer | 1543 | healthy | adult | sub-group | 29.317 |
| 49 | Wesley et al., | 2000 | 1996 | rectal collection | culture-based | PCR-based | jejuni | 37.7 | all | 2085 | healthy | adult | sub-group | 786.045 |
| 49 | Wesley et al., | 2000 | 1996 | rectal collection | culture-based | PCR-based | jejuni | 40.5 | summer | 1543 | healthy | adult | sub-group | 624.915 |
| 51 | Hoque et al., | 2021 | 2018-2020 | rectal collection | culture-based | PCR-based | spp. | 30.9 | not specified | 540 | not specified | cows, heifers and calves | whole | 166.86 |
| 51 | Hoque et al., | 2021 | 2018-2020 | rectal collection | culture-based | PCR-based | spp. | 41.1 | not specified | 180 | not specified | adult | sub-group | 73.98 |
| 51 | Hoque et al., | 2021 | 2018-2020 | rectal collection | culture-based | PCR-based | spp. | 28.3 | not specified | 180 | not specified | heifers | sub-group | 50.94 |
| 51 | Hoque et al., | 2021 | 2018-2020 | rectal collection | culture-based | PCR-based | spp. | 23.3 | not specified | 180 | not specified | calves | sub-group | 41.94 |
| 52 | Rapp et al., | 2020 | not specified | cow pat | culture-based | PCR-based | jejuni | 54 | all | 90 | not specified | adult | whole | 48.6 |
| 52 | Rapp et al., | 2020 | not specified | cow pat | culture-based | PCR-based | jejuni | 27 | winter | 15 | not specified | adult | sub-group | 4.05 |
| 52 | Rapp et al., | 2020 | not specified | cow pat | culture-based | PCR-based | jejuni | 53 | winter | 15 | not specified | adult | sub-group | 7.95 |
| 52 | Rapp et al., | 2020 | not specified | cow pat | culture-based | PCR-based | jejuni | 53 | winter | 15 | not specified | adult | sub-group | 7.95 |
| 52 | Rapp et al., | 2020 | not specified | cow pat | culture-based | PCR-based | jejuni | 80 | spring | 15 | not specified | adult | sub-group | 12 |
| 52 | Rapp et al., | 2020 | not specified | cow pat | culture-based | PCR-based | jejuni | 67 | spring | 15 | not specified | adult | sub-group | 10.05 |
| 52 | Rapp et al., | 2020 | not specified | cow pat | culture-based | PCR-based | jejuni | 47 | spring | 15 | not specified | adult | sub-group | 7.05 |
| 53 | Silveira et al., | 2021 | not specified | rectal collection | culture-based | PCR-based | spp. | 0.05 | not specified | 60 | not specified | adult | whole | 0.03 |
| | | | | | | | | | | | | | | |
| | *event= Prevalence_in_population x Number_of_individual_cattle_sampled | | | | | | | | | | | | | |

## (3) aggregated

| ID | Author | Publication_year | Study_year | Fecal_collection_method | Detection_or_quantification_method_for_Campylobacter_spp. | Species_identification_method | Campylobacter_species | Prevalence_in_population | Which_season_is_the_prevalence_reported_for | Number_of_individual_cattle_sampled | Health_status_of_cattle | Description_age_class_of_cattle_sampled | Specific_outcome_reported_for_whole_data_set_or_sub_group | event* | rob_yes | rob |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | Acha et al., | 2004 | 1995 | rectal collection | culture-based | phenotypic | spp. | 11 | not specified | 393 | mixed | calves | whole | 43.23 | 5 | low |
| 2 | Acik and Centinkaya | 2005 | 2003 | rectal collection | culture-based | PCR-based, flaA-typing, RFLP | spp. | 44 | not specified | 250 | healthy | adult | whole | 110 | 2 | high |
| 4 | Adesiyun et al. | 1992 | 1991 | rectal collection | culture-based | phenotypic | spp. | 20.5 | not specified | 293 | mixed | calves | whole | 60.065 | 5 | low |
| 6 | Adesiyun et al., | 1996 | not specified | rectal collection | culture-based | phenotypic | spp. | 5.7 | not specified | 333 | not specified | adult | whole | 18.981 | 3 | high |
| 7 | Adhikari et al., | 2004 | 2002 | rectal collection | culture-based | other molecular method | jejuni | 54 | not specified | 52 | healthy | adult | whole | 28.08 | 6 | low |
| 8 | Atabay and Corry | 1997 | 1995-1996 | rectal collection | culture-based | phenotypic | spp. | 36 | all | 136 | not specified | cows and calves | whole | 48.96 | 3 | high |
| 9 | Baserisalehi et al. | 2007 | 2006 | rectal collection | culture-based | phenotypic | spp. | 21 | not specified | 121 | healthy | adult | whole | 25.41 | 2 | high |
| 10 | Bianchi et al., | 2014 | 2010-2012 | rectal collection | culture-based | PCR-based | jejuni | 30.5 | not specified | 82 | not specified | adult | whole | 25.01 | 5 | low |
| 11 | Cha et al., | 2017 | 2012 | not specified | culture-based | PCR-based, WGS | jejuni | 58.6 | not specified | 58 | not specified | adult | whole | 33.988 | 3 | high |
| 12 | Dong et al., | 2016 | 2012-2014 | rectal collection | culture-based | PCR-based | jejuni | 24.2 | not specified | 194 | not specified | adult | whole | 46.948 | 3 | high |
| 13 | Duncan et al., | 2013 | not specified | cow pat | culture-based | PCR-based | fetus | 9.5 | not specified | 4260 | not specified | adult | whole | 404.7 | 4 | high |
| 14 | Englen et al., | 2007 | 2002 | rectal collection | culture-based | PCR-based | spp. | 51.2 | not applicable | 1435 | not specified | adult | whole | 734.72 | 3 | high |
| 15 | Grinberg et al., | 2005 | 2002 | rectal collection | culture-based | phenotypic | spp. | 36 | not specified | 161 | mixed | calves | whole | 57.96 | 5 | low |
| 18 | Hagey et al., | 2019 | not specified | rectal collection | PCR-based | other molecular method | spp. | 31 | not specified | 150 | not specified | adult | whole | 46.5 | 6 | low |
| 19 | Hakkinen and Hänninen, | 2009 | 2006-2007 | cow pat | culture-based | other molecular method | spp. | 49.7 | all | 340 | not specified | adult | whole | 168.98 | 4 | high |
| 23 | Giacoboni et al., | 1993 | not specified | not specified | culture-based | phenotypic | spp. | 64.9 | not specified | 94 | not specified | cows and calves | whole | 61.006 | 2 | high |
| 25 | Kashoma et al. | 2015 | 2013-2014 | cow pat | culture-based | PCR-based | spp. | 35.4 | not specified | 192 | not specified | adult | whole | 67.968 | 3 | high |
| 26 | Khalifa et al. | 2013 | not specified | cow pat | culture-based | PCR-based | jejuni | 32 | not applicable | 50 | not specified | adult | whole | 16 | 2 | high |
| 27 | Klein et al. | 2013 | 2009-2010 | rectal collection | culture-based | MALDI-TOF | spp. | 14.9 | not applicable | 382 | mixed | calves | whole | 56.918 | 7 | low |
| 28 | Kwan et al. | 2008 | 2003 | cow pat | culture-based | PCR-based | spp. | 35.9 | not specified | 1208 | not specified | adult | whole | 433.672 | 5 | low |
| 29 | McAuley et al. | 2014 | 2013 and 2014 | not specified | culture-based | PCR-based | spp. | 6 | not applicable | 16 | not specified | adult | whole | 0.96 | 3 | high |
| 31 | Messelhaeusser et al. | 2008 | 2004 | not specified | culture-based | PCR-based | spp. | 13 | all | 226 | not specified | not specified | whole | 29.38 | 6 | low |
| 35 | Nielsen et al. | 2002 | 1999 | rectal collection | culture-based | other molecular method | spp. | 32.2 | not specified | 332 | not specified | cows, heifers and calves | whole | 106.904 | 2 | high |
| 37 | Padungtod and Kaneene | 2005 | 2000-2003 | not specified | culture-based | PCR-based | spp. | 14 | not specified | 225 | not specified | not specified | whole | 31.5 | 2 | high |
| 39 | Ramonait et al., | 2013 | 2012 | rectal collection | culture-based | PCR-based | spp. | 78.5 | not specified | 200 | not specified | cows, heifers and calves | whole | 157 | 6 | low |
| 41 | Roug | 2012 | 2005 | cow pat | culture-based | PCR-based | jejuni | 17 | not specified | 12 | not specified | adult | whole | 2.04 | 3 | high |
| 42 | Sanad et al., | 2013 | 2009 | not specified | culture-based | PCR-based | spp. | 36.6 | not specified | 227 | not specified | adult | whole | 83.082 | 3 | high |
| 43 | Sato et al., | 2004 | not specified | cow pat | culture-based | not performed | spp. | 27.9 | all | 1191 | healthy | cows and calves | whole | 332.289 | 6 | low |
| 46 | Watner-Toews et al., | 1986 | 1982 | rectal collection | culture-based | not performed | spp. | 13 | fall | 156 | not specified | calves | whole | 20.28 | 2 | high |
| 51 | Hoque et al., | 2021 | 2018-2020 | rectal collection | culture-based | PCR-based | spp. | 30.9 | not specified | 540 | not specified | cows, heifers and calves | whole | 166.86 | 7 | low |
| 52 | Rapp et al., | 2020 | not specified | cow pat | culture-based | PCR-based | jejuni | 54 | all | 90 | not specified | adult | whole | 48.6 | 6 | low |
| 53 | Silveira et al., | 2021 | not specified | rectal collection | culture-based | PCR-based | spp. | 0.05 | not specified | 60 | not specified | adult | whole | 0.03 | 3 | high |

*event= Prevalence_in_population x Number_of_individual_cattle_sampled

**Table S2.** Risk of bias.

| | Question | yes | No | Unclear |
|---|---|---|---|---|
| 1 | Is information about the housing of cows given? | 23 | 30 | 0 |
| 2 | Is information about the feeding of cows given? | 18 | 34 | 1 |
| 3 | Is information about organic or conventional farming given? | 14 | 36 | 3 |
| 4 | Was information about the season weather months given when samples were taken? | 39 | 14 | 0 |
| 5 | Is information about any other adjustment variable given? | 29 | 23 | 1 |
| 6 | Was information given whether the cows were selected randomly for sampling? | 13 | 38 | 2 |
| 7 | Was the outcome reported for individual samples separately rather than pooled samples? | 43 | 9 | 1 |
| 8 | Was the whole sample included in the analysis? | 3 | 4 | 46 |
| 9 | Were ISO Methods applied for the detection quantification of Campylobacter spp.? | 5 | 6 | 42 |
| 10 | Was the outcome reported for all relevant sub groups and the whole population studied? | 18 | 33 | 2 |

## 9.2 Supplementary material of Publication 2

**Table S1.** Detection limits of microorganisms.

| Detection limit | microorganism | faeces [CFU/ g] | teats [CFU/ 4 teats] | raw milk [CFU/ ml] | milk filter [CFU/ milk filter] | boot socks [CFU/2 boot socks] | milking cluster [CFU/ 4 cups] |
|---|---|---|---|---|---|---|---|
| quantitative | *Campylobacter* spp. | 10 | 25 | 1 | 35 | 180 | 18 |
| quantitative | *E. coli* | 100 | 200 | 100 | 350 | 1800 | 180 |
| quantitative | *Pseudomonas* spp. | 100 | 200 | 100 | 350 | 1800 | 180 |
| quantitative | total aerobic cell count | 1000 | 20000 | 1000 | 3500 | 18000 | 1800 |
| enrichment | *Campylobacter* spp. | 1 | 5 | 0.20 | 7 | 36 | 3.6 |

**Table S2**. Concentration data for *Campylobacter* spp. in feces of individual cows per sampling. Zero indicates a positive sample in enrichment; negative samples are not included.

| Sampling date | Cow_ID | log_campy | scoring consistency of faeces |
|---|---|---|---|
| 20.04.2021 | 4662 | 2.10 | 1 |
| 20.04.2021 | 4660 | 1.26 | 1 |
| 20.04.2021 | 4652 | 2.85 | 2 |
| 20.04.2021 | 4658 | 2.32 | 2 |
| 20.04.2021 | 4659 | 3.1 | 2 |
| 20.04.2021 | 4320 | 1.7 | 2 |
| 20.04.2021 | 6057 | 2.21 | 2 |
| 04.05.2021 | 4320 | 2.55 | 2 |
| 04.05.2021 | 6001 | 1.30 | 2 |
| 04.05.2021 | 4658 | 0 | 2 |
| 04.05.2021 | 4660 | 1.6 | 1 |
| 04.05.2021 | 6057 | 2.21 | 2 |
| 04.05.2021 | 4662 | 1.48 | 1 |
| 04.05.2021 | 299 | 1.48 | 1 |
| 04.05.2021 | 4659 | 2.04 | 2 |
| 04.05.2021 | 4652 | 2.04 | 2 |

| | | | |
|---|---|---|---|
| 18.05.2021 | 4665 | 0 | 3 |
| 18.05.2021 | 4320 | 2.87 | 2 |
| 18.05.2021 | 6001 | 1.7 | 2 |
| 18.05.2021 | 4658 | 2.79 | 2 |
| 18.05.2021 | 4660 | 1.30 | 2 |
| 18.05.2021 | 6057 | 2.21 | 3 |
| 18.05.2021 | 299 | 4.19 | 3 |
| 18.05.2021 | 4659 | 4.17 | 2 |
| 18.05.2021 | 4652 | 3.09 | 2 |
| 01.06.2021 | 4665 | 3.97 | 3 |
| 01.06.2021 | 4320 | 2.16 | 2 |
| 01.06.2021 | 6001 | 2.97 | 2 |
| 01.06.2021 | 4658 | 2.5 | 2 |
| 01.06.2021 | 4660 | 1.30 | 2 |
| 01.06.2021 | 6057 | 3 | 2 |
| 01.06.2021 | 299 | 2.24 | 2 |
| 01.06.2021 | 4659 | 1 | 2 |
| 01.06.2021 | 4652 | 2.74 | 2 |
| 15.06.2021 | 4665 | 2.91 | 3 |
| 15.06.2021 | 4664 | 2.56 | 3 |
| 15.06.2021 | 4320 | 2.6 | 2 |
| 15.06.2021 | 6001 | 2.51 | 2 |
| 15.06.2021 | 4658 | 2 | 2 |
| 15.06.2021 | 4660 | 2.37 | 1 |
| 15.06.2021 | 6057 | 2.7 | 2 |
| 15.06.2021 | 4662 | 2.85 | 2 |
| 15.06.2021 | 299 | 2.65 | 2 |
| 15.06.2021 | 4659 | 2.54 | 2 |
| 15.06.2021 | 4652 | 4 | 2 |
| 29.06.2021 | 4665 | 3.02 | 1 |
| 29.06.2021 | 4664 | 0 | 3 |
| 29.06.2021 | 4320 | 2.49 | 2 |
| 29.06.2021 | 6001 | 1.66 | 2 |
| 29.06.2021 | 4658 | 2.11 | 2 |
| 29.06.2021 | 4317 | 2.5 | 3 |
| 29.06.2021 | 4660 | 3.39 | 2 |
| 29.06.2021 | 6057 | 2.23 | 2 |
| 29.06.2021 | 4662 | 3.88 | 2 |
| 29.06.2021 | 4659 | 1.86 | 2 |
| 29.06.2021 | 4652 | 2.36 | 2 |
| 13.07.2021 | 4665 | 2.26 | 2 |
| 13.07.2021 | 4320 | 2.48 | 2 |
| 13.07.2021 | 6001 | 2.37 | 2 |
| 13.07.2021 | 4658 | 2.80 | 2 |
| 13.07.2021 | 4317 | 2.28 | 2 |

| | | | |
|---|---|---|---|
| 13.07.2021 | 4660 | 2.49 | 2 |
| 13.07.2021 | 6057 | 1.78 | 2 |
| 13.07.2021 | 4662 | 2.3 | 2 |
| 13.07.2021 | 299 | 1 | 2 |
| 13.07.2021 | 4659 | 4.79 | 2 |
| 13.07.2021 | 4652 | 2.16 | 2 |
| 27.07.2021 | 4665 | 2 | 3 |
| 27.07.2021 | 4320 | 2.60 | 2 |
| 27.07.2021 | 6001 | 1.8 | 2 |
| 27.07.2021 | 4658 | 2.43 | 3 |
| 27.07.2021 | 4660 | 1.7 | 3 |
| 27.07.2021 | 6057 | 1.85 | 2 |
| 27.07.2021 | 4662 | 2.6 | 2 |
| 27.07.2021 | 299 | 2.77 | 2 |
| 27.07.2021 | 4659 | 3.6 | 2 |
| 27.07.2021 | 4652 | 2.81 | 2 |
| 10.08.2021 | 4665 | 3.81 | 3 |
| 10.08.2021 | 4664 | 3.91 | 3 |
| 10.08.2021 | 4320 | 2.21 | 3 |
| 10.08.2021 | 6001 | 2.32 | 2 |
| 10.08.2021 | 4658 | 2.88 | 2 |
| 10.08.2021 | 4660 | 2.37 | 2 |
| 10.08.2021 | 6057 | 2.11 | 2 |
| 10.08.2021 | 4662 | 2.53 | 3 |
| 10.08.2021 | 299 | 4.42 | 1 |
| 10.08.2021 | 4659 | 3.32 | 3 |
| 10.08.2021 | 4652 | 1 | 2 |
| 24.08.2021 | 4665 | 4.6 | 2 |
| 24.08.2021 | 4664 | 2.82 | 2 |
| 24.08.2021 | 4320 | 4.41 | 2 |
| 24.08.2021 | 6001 | 3 | 2 |
| 24.08.2021 | 4658 | 2.41 | 2 |
| 24.08.2021 | 4660 | 2.55 | 1 |
| 24.08.2021 | 6057 | 2.95 | 3 |
| 24.08.2021 | 4662 | 2.37 | 3 |
| 24.08.2021 | 299 | 4.74 | 1 |
| 24.08.2021 | 4659 | 3.28 | 2 |
| 24.08.2021 | 4652 | 0 | 2 |
| 07.09.2021 | 4665 | 3.74 | 2 |
| 07.09.2021 | 4664 | 2.92 | 2 |
| 07.09.2021 | 4320 | 2 | 2 |
| 07.09.2021 | 6001 | 1.3 | 2 |
| 07.09.2021 | 4658 | 2 | 3 |
| 07.09.2021 | 4660 | 2.42 | 2 |
| 07.09.2021 | 6057 | 1.9 | 2 |

| | | | |
|---|---|---|---|
| 07.09.2021 | 299 | 3.65 | 3 |
| 07.09.2021 | 4659 | 3.76 | 3 |
| 07.09.2021 | 4652 | 2.36 | 2 |
| 21.09.2021 | 4665 | 3.1 | 2 |
| 21.09.2021 | 4664 | 2.19 | 2 |
| 21.09.2021 | 4320 | 2.34 | 2 |
| 21.09.2021 | 4658 | 2.34 | 3 |
| 21.09.2021 | 4660 | 1.3 | 2 |
| 21.09.2021 | 6057 | 4.88 | 2 |
| 21.09.2021 | 4662 | 1.26 | 3 |
| 21.09.2021 | 299 | 2 | 1 |
| 21.09.2021 | 4659 | 3.74 | 2 |
| 21.09.2021 | 4652 | 2.91 | 2 |
| 05.10.2021 | 4665 | 3.24 | 2 |
| 05.10.2021 | 4664 | 4.14 | 2 |
| 05.10.2021 | 4320 | 2.5 | 2 |
| 05.10.2021 | 4658 | 2.34 | 2 |
| 05.10.2021 | 4660 | 1 | 2 |
| 05.10.2021 | 6057 | 2.9 | 2 |
| 05.10.2021 | 299 | 2.93 | 2 |
| 05.10.2021 | 4659 | 3.06 | 2 |
| 05.10.2021 | 4652 | 2.23 | 2 |
| 19.10.2021 | 4665 | 2.42 | 2 |
| 19.10.2021 | 4664 | 4.35 | 2 |
| 19.10.2021 | 4320 | 2.49 | 2 |
| 19.10.2021 | 4658 | 2.45 | 2 |
| 19.10.2021 | 4660 | 1.66 | 2 |
| 19.10.2021 | 6057 | 3.19 | 2 |
| 19.10.2021 | 4662 | 2.46 | 2 |
| 19.10.2021 | 299 | 2 | 2 |
| 19.10.2021 | 4659 | 2.95 | 3 |
| 19.10.2021 | 4652 | 2.5 | 2 |
| 02.11.2021 | 4665 | 2.75 | 2 |
| 02.11.2021 | 4664 | 2.67 | 3 |
| 02.11.2021 | 4320 | 3 | 2 |
| 02.11.2021 | 6001 | 2 | 2 |
| 02.11.2021 | 4658 | 2.04 | 3 |
| 02.11.2021 | 4660 | 1.48 | 3 |
| 02.11.2021 | 6057 | 2.74 | 3 |
| 02.11.2021 | 4662 | 2.56 | 1 |
| 02.11.2021 | 299 | 3.28 | 2 |
| 02.11.2021 | 4659 | 2.62 | 3 |
| 02.11.2021 | 4652 | 2.55 | 2 |
| 16.11.2021 | 4665 | 2.3 | 2 |
| 16.11.2021 | 4664 | 3.68 | 2 |

| | | | |
|---|---|---|---|
| 16.11.2021 | 4320 | 3.34 | 2 |
| 16.11.2021 | 6001 | 1.26 | 2 |
| 16.11.2021 | 4658 | 2.39 | 2 |
| 16.11.2021 | 4660 | 1.7 | 2 |
| 16.11.2021 | 6057 | 2.76 | 2 |
| 16.11.2021 | 299 | 2.07 | 1 |
| 16.11.2021 | 4659 | 3 | 2 |
| 16.11.2021 | 4652 | 2 | 2 |
| 30.11.2021 | 4665 | 1.7 | 2 |
| 30.11.2021 | 4664 | 3.32 | 3 |
| 30.11.2021 | 4320 | 3.15 | 3 |
| 30.11.2021 | 4658 | 1.86 | 3 |
| 30.11.2021 | 4660 | 2.68 | 3 |
| 30.11.2021 | 6057 | 5.92 | 3 |
| 30.11.2021 | 299 | 3.13 | 2 |
| 30.11.2021 | 4659 | 1.44 | 3 |
| 30.11.2021 | 4652 | 2.07 | 3 |
| 14.12.2021 | 4665 | 3 | 2 |
| 14.12.2021 | 4664 | 2.71 | 2 |
| 14.12.2021 | 4320 | 3.04 | 2 |
| 14.12.2021 | 6001 | 2.37 | 3 |
| 14.12.2021 | 4658 | 2.11 | 3 |
| 14.12.2021 | 4660 | 1 | 2 |
| 14.12.2021 | 299 | 2.81 | 3 |
| 14.12.2021 | 4659 | 2.62 | 2 |
| 11.01.2022 | 4665 | 1.7 | 1 |
| 11.01.2022 | 4664 | 2.68 | 1 |
| 11.01.2022 | 4320 | 2.61 | 2 |
| 11.01.2022 | 4658 | 0 | 2 |
| 11.01.2022 | 299 | 2.41 | 1 |
| 11.01.2022 | 4659 | 1.85 | 2 |
| 11.01.2022 | 6005 | 2.34 | 2 |
| 25.01.2022 | 4665 | 1.3 | 2 |
| 25.01.2022 | 4664 | 2.91 | 2 |
| 25.01.2022 | 4320 | 3.21 | 2 |
| 25.01.2022 | 299 | 2.86 | 1 |
| 25.01.2022 | 4659 | 2.98 | 3 |
| 25.01.2022 | 4652 | 1 | 2 |
| 25.01.2022 | 6005 | 1.9 | 2 |
| 08.02.2022 | 4665 | 2.19 | 2 |
| 08.02.2022 | 4664 | 2.32 | 2 |
| 08.02.2022 | 4320 | 2.44 | 2 |
| 08.02.2022 | 4658 | 1.48 | 3 |
| 08.02.2022 | 299 | 0 | 2 |
| 08.02.2022 | 4659 | 1.6 | 2 |

| | | | |
|---|---|---|---|
| 08.02.2022 | 4652 | 0 | 2 |
| 08.02.2022 | 6005 | 2.11 | 2 |
| 22.02.2022 | 4665 | 1.48 | 2 |
| 22.02.2022 | 4664 | 2.03 | 3 |
| 22.02.2022 | 4320 | 1.65 | 3 |
| 22.02.2022 | 6001 | 2 | 2 |
| 22.02.2022 | 4658 | 1.95 | 2 |
| 22.02.2022 | 299 | 2.2 | 2 |
| 22.02.2022 | 4659 | 1.6 | 2 |
| 22.02.2022 | 4652 | 2.15 | 1 |
| 22.02.2022 | 6005 | 2.19 | 2 |
| 22.03.2022 | 4665 | 1.7 | 1 |
| 22.03.2022 | 4664 | 2.79 | 2 |
| 22.03.2022 | 4320 | 1.48 | 2 |
| 22.03.2022 | 6001 | 1.6 | 2 |
| 22.03.2022 | 4658 | 2 | 2 |
| 22.03.2022 | 299 | 2.54 | 2 |
| 22.03.2022 | 4659 | 2.82 | 3 |
| 22.03.2022 | 6005 | 1.85 | 2 |
| 05.04.2022 | 4665 | 2.07 | 1 |
| 05.04.2022 | 4664 | 2.73 | 3 |
| 05.04.2022 | 4320 | 2.51 | 2 |
| 05.04.2022 | 4658 | 3.08 | 3 |
| 05.04.2022 | 299 | 2.59 | 1 |
| 05.04.2022 | 4659 | 2.81 | 2 |
| 05.04.2022 | 6005 | 2.46 | 2 |

**Table S3.** Concentration data for *Campylobacter* spp. on teat swab samples of individual cows per sampling. Zero indicates a positive sample in enrichment; negative samples are not included.

| **Sampling date** | **Cow_ID** | **log_campy** | **Scoring** |
|---|---|---|---|
| 03.05.2021 | 4662 | 0 | 3 |
| 17.05.2021 | 299 | 2 | 4 |
| 17.05.2021 | 4659 | 0 | 2 |
| 31.05.2021 | 6001 | 0 | 3 |
| 14.06.2021 | 4652 | 0 | 3 |
| 14.06.2021 | 4656 | 2.26 | 3 |
| 14.06.2021 | 6077 | 2.15 | 2 |
| 14.06.2021 | 4658 | 1.78 | 4 |
| 14.06.2021 | 6074 | 0 | 3 |
| 14.06.2021 | 6057 | 1.3 | 2 |
| 14.06.2021 | 4659 | 2.26 | 3 |

| | | | |
|---|---|---|---|
| 14.06.2021 | 4660 | 2.72 | 4 |
| 14.06.2021 | 6005 | 2.3 | 3 |
| 23.08.2021 | 299 | 1.86 | 3 |
| 23.08.2021 | 4660 | 0 | 4 |
| 23.08.2021 | 6005 | 0 | 4 |
| 20.09.2021 | 6057 | 2.68 | 3 |
| 20.09.2021 | 4665 | 1.6 | 4 |
| 04.10.2021 | 299 | 0 | 2 |
| 04.10.2021 | 4652 | 1.3 | 4 |
| 04.10.2021 | 4662 | 0 | 3 |
| 04.10.2021 | 4665 | 2.16 | 4 |
| 18.10.2021 | 6001 | 1.6 | 2 |
| 18.10.2021 | 4665 | 2.26 | 3 |
| 18.10.2021 | 4320 | 1.6 | 2 |
| 01.11.2021 | 4658 | 0 | 3 |
| 29.11.2021 | 4659 | 0 | 3 |
| 13.12.2021 | 4660 | 0 | 2 |
| 10.01.2022 | 4652 | 0 | 3 |
| 24.01.2022 | 4320 | 0 | 1 |
| 07.02.2022 | 299 | 0 | 4 |
| 21.02.2022 | 4320 | 0 | 3 |
| 04.04.2022 | 6005 | 1.3 | 4 |
| 04.04.2022 | 299 | 0 | 2 |
| 04.04.2022 | 4317 | 0 | 2 |

## 9.3 Supplementary material of Publication 3

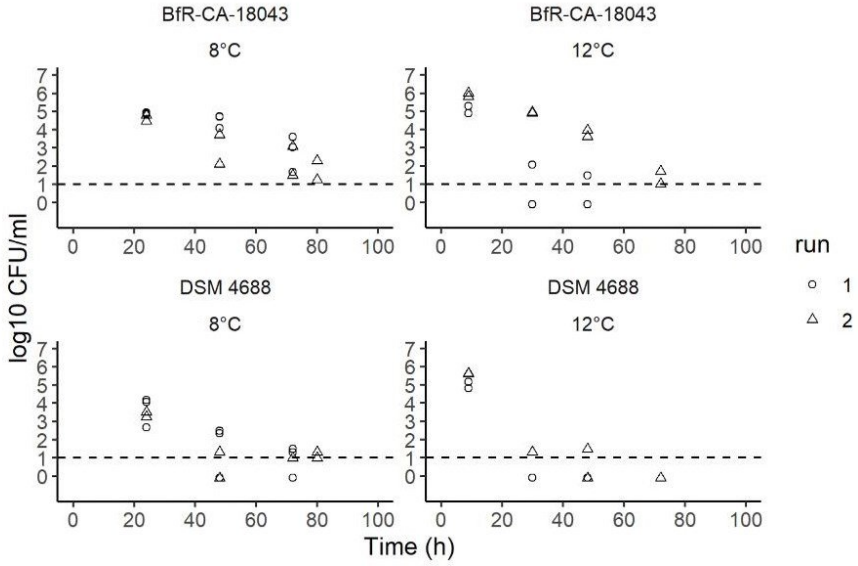


**Fig. S1.** Recovery of non-culturable *C. jejuni* from raw milk. In total, two independent runs (dot or triangle symbol) in duplicates were performed. The dashed line represents the detection limit of 10 CFU/ml.

**Table S1.** Parameter estimates and goodness-of-fit† resulting from fitting different primary model equations to CFU data of survival of *C. jejuni* in inoculated raw milk. The parameter for the tail ($LogN_{res}$) was set to a constant of -0.1.

| Strain | Temp. (°C) | Primary model equation | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Geerard without shoulder (2 parameter) | | | Bilinear (2 parameter) | | | Geeraerd with shoulder and tail (3 parameter) | | | | Trilinear (3 parameter) | | | |
| | | $k_{max}$ | RMSE | $R^2$ | $k_{max}$ | RMSE | $R^2$ | $S_l$ | $k_{max}$ | RMSE | $R^2$ | $S_l$ | $k_{max}$ | RMSE | $R^2$ |
| DSM 4688 | 5 | 0.46 | 0.37 | 0.98 | 0.44 | 0.37 | 0.98 | 4.75 | 0.54 | 0.31 | 0.98 | 4.18 | 0.52 | 0.31 | 0.98 |
| | 8 | 0.48 | 0.59 | 0.95 | 0.47 | 0.58 | 0.95 | 4.38 | 0.55 | 0.53 | 0.96 | 4.21 | 0.52 | 0.52 | 0.96 |
| | 12 | 0.54 | 0.45 | 0.97 | 0.54 | 0.44 | 0.97 | 6.04 | 0.69 | 0.22 | 0.99 | 5.34 | 0.64 | 0.23 | 0.99 |
| BfR-CA-18043 | 5 | 0.25 | 0.49 | 0.96 | 0.25 | 0.49 | 0.96 | 15.93 | 0.39 | 0.32 | 0.98 | 15.21 | 0.36 | 0.33 | 0.98 |
| | 8 | 0.26 | 0.97 | 0.85 | 0.26 | 0.96 | 0.85 | 20.89 | 0.77 | 0.86 | 0.88 | 13.36 | 0.35 | 0.89 | 0.87 |
| | 12 | 0.37 | 0.57 | 0.95 | 0.37 | 0.57 | 0.95 | 8.43 | 0.49 | 0.47 | 0.96 | 6.66 | 0.44 | 0.47 | 0.96 |

† Values obtained from the fit of all individual data points carried out at each temperature.

RMSE: root mean square error; $R^2$: determination coefficient.

**Table S2.** Parameter estimates and goodness-of-fit† resulting from fitting different primary model equations to IPIU data of survival of *C. jejuni* in inoculated raw milk.

| Strain | Temp. (°C) | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Bigelow (1 parameter) | | | Geeraerd without shoulder (2 parameter) | | | | Bilinear (2 parameter) | | | | Weibull (2 parameter) | | | | Biphasic (3 parameter) | | | | |
| | | $k_{max}$ | RMSE | $R^2$ | $k_{max}$ | $Log_{10}N_{res}$ | RMSE | $R^2$ | $k_{max}$ | $Log_{10}N_{res}$ | RMSE | $R^2$ | $\rho$ | $\delta$ | RMSE | $R^2$ | $f$ | $k_{max}1$ | $k_{max}2$ | RMSE | $R^2$ |
| DSM 4688 | 5 | 0.02 | 0.17 | 0.51 | 0.22 | 4.63 | 0.11 | 0.79 | 0.04 | 4.6 | 0.11 | 0.79 | 0.21 | 568.82 | 0.07 | 0.92 | 0.67 | 0.28 | 0.005 | 0.11 | 0.81 |
| | 8 | 0.01 | 0.18 | 0.36 | 0.07 | 5.31 | 0.17 | 0.45 | 0.03 | 5.32 | 0.17 | 0.42 | 0.41 | 470.05 | 0.04 | 0.96 | 0.53 | 0.09 | 0.003 | 0.17 | 0.45 |
| | 12 | 0.02 | 0.16 | 0.51 | 0.35 | 5.14 | 0.11 | 0.77 | 0.03 | 5.05 | 0.12 | 0.74 | 0.21 | 800.5 | 0.08 | 0.88 | 0.59 | 0.55 | 0.008 | 0.09 | 0.82 |
| BfR-CA-18043 | 5 | 0.01 | 0.21 | 0.21 | 0.27 | 4.63 | 0.15 | 0.64 | 0.04 | 4.6 | 0.16 | 0.55 | 0.14 | 2247.65 | 0.15 | 0.63 | 0.71 | 0.29 | 0.002 | 0.15 | 0.64 |
| | 8 | 0.01 | 0.25 | 0.12 | 0.27 | 4.92 | 0.19 | 0.51 | 0.04 | 4.87 | 0.21 | 0.38 | 0.16 | 1098.85 | 0.19 | 0.50 | 0.67 | 0.45 | 0.006 | 0.2 | 0.44 |
| | 12 | 0.02 | 0.25 | 0.29 | 0.96 | 5.0 | 0.14 | 0.78 | 0.04 | 4.94 | 0.22 | 0.44 | 0.07 | 14513.2 | 0.13 | 0.80 | 0.75 | 1.46 | 0.004 | 0.13 | 0.79 |

† Values obtained from the fit of all individual data points carried out at each temperature.

RMSE: root mean square error; $R^2$: determination coefficient.

**Table S3.** Estimated parameters for the linear secondary $k_{max}$ model of *C. jejuni* strain BfR-CA-18043 and strain DSM 4688. Numbers in parentheses are 95% confidence intervals.

| Parameters | Fitted values (for $\log_{10}$ mean $k_{max}$ per trial) | | Fitted values (for all obtained $log_{10}$ $k_{max}$ estimates) | |
|---|---|---|---|---|
| | BfR-CA-18043 | DSM 4688 | BfR-CA-18043 | DSM 4688 |
| Intercept | -0.6 (-0.79 - -0.41) | -0.35 (-0.47 - -0.24) | -0.6 (-0.74- - 0.44) | -0.35 (-0.45 - -0.25) |
| Slope | 0.03 (0.01 - 0.05) | 0.01 (0.00 - 0.03) | 0.03 (0.00 – 0.04) | 0.01 (0.01 – 0.02) |
| RMSE | 0.07 | 0.03 | 0.09 | 0.05 |

**Table S4.** Estimated parameters for the linear secondary $\delta$ model of *C. jejuni* strain BfR-CA-18043 and strain DSM 4688. Numbers in parentheses are 95% confidence intervals.

| Parameters | Fitted values (*for all obtained $log_{10}$ $\delta$ estimates) | | |
|---|---|---|---|
| | BfR-CA-18043 | DSM 4688 | Combination of BfR-CA-18043 and DSM 4688 |
| Intercept | 3.07 (1.46 – 4.68) | 2.83 (1.1 - 4.56) | 2.95 (1.9 - 4.0) |
| Slope | 0.02 (-0.17 - 0.2) | 0.028 (-0.17 - 0.22) | 0.023 (-0.1 – 0.14) |
| RMSE | 0.74 | 0.8 | 0.44 |

**Table S5.** Comparison of observed and predicted concentration at a specific time point ($log_{10}N_t$) for *C. jejuni* CFU data in raw milk at different temperatures obtained by using the tertiary models for CFU data.

| | | | | | DSM 4688 derived model | | | | BfR-CA-18043 derived model | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | $Log_{10}\ N_t$ (CFU/ml) | | Validation values | | $Log_{10}\ N_t$ (CFU/ml) | | Validation values | |
| Ref[a] | Species | Strain | Temp. [°C] | Time [h] | Observed | Predicted | RMSE | $R^2$ | Observed | Predicted | RMSE | $R^2$ |
| 1 | *C. jejuni* | DSM 4688 | 5 | 7 | 5.64 | 5.27 | | | - | - | - | - |
| | | | | | 5.43 | 5.20 | | | | | | |
| | | | | | 5.64 | 5.51 | | | | | | |
| | | | | | 5.48 | 5.39 | | | | | | |
| | | | | 25 | 3.87 | 1.08 | | | | | | |
| | | | | | 2.88 | 1.00 | | | | | | |
| | | | | | 1.74 | 1.31 | | | | | | |
| | | | | | 2.10 | 1.19 | | | | | | |
| | | | | 31 | 2.28 | -0.1 | | | | | | |
| | | | | | 1.07 | -0.1 | | | | | | |
| | | | | | 0.30 | -0.1 | | | | | | |
| | | | | | 0.48 | -0.1 | 1.30 | 0.57 | | | | |
| 2 | *C. jejuni* | DSM 4688 | 5 | 18 | 5.21 | 2.56 | | | - | - | - | - |
| | | | | | 4.98 | 2.56 | | | | | | |
| | | | | | 4.58 | 2.56 | | | | | | |
| | | | | | 5.05 | 2.56 | | | | | | |
| | | | | | 4.36 | 2.22 | | | | | | |
| | | | | 24 | 4.49 | 1.16 | | | | | | |
| | | | | | 4.06 | 1.16 | | | | | | |
| | | | | | 3.25 | 1.16 | | | | | | |
| | | | | 42 | 2.49 | -0.1 | | | | | | |
| | | | | | 2.30 | -0.1 | | | | | | |
| | | | | | 1.48 | -0.1 | | | | | | |
| | | | | | 1.87 | -0.1 | | | | | | |
| | | | | | 1.28 | -0.1 | | | | | | |
| | | | | 48 | 0.60 | -0.1 | | | | | | |
| | | | | | 1.11 | -0.1 | | | | | | |
| | | | | | 0.48 | -0.1 | | | | | | |

| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | 66 | -0.1 | -0.1 | | | | | | |
| | | | | | -0.1 | -0.1 | | | | | | |
| | | | | | -0.1 | -0.1 | | | | | | |
| | | | | | 0.00 | -0.1 | | | | | | |
| | | | | | -0.1 | -0.1 | 1.88 | 0.99 | | | | |
| 1 | *C. jejuni* | DSM 4688 | 8 | 7 | 5.46 | 5.40 | | | - | - | - | - |
| | | | | | 5.29 | 5.22 | | | | | | |
| | | | | 25 | 0.70 | 0.99 | | | | | | |
| | | | | | 0.48 | 0.81 | 0.22 | 0.99 | | | | |
| 1 | *C. jejuni* | DSM 4688 | 12 | 6 | 4.96 | 5.22 | | | - | - | - | - |
| | | | | | 4.96 | 5.38 | | | | | | |
| | | | | 9 | 4.94 | 4.44 | | | | | | |
| | | | | | 4.84 | 4.60 | | | | | | |
| | | | | 24 | 0.48 | 0.50 | | | | | | |
| | | | | | 0.60 | 0.66 | 0.30 | 0.98 | | | | |
| 1 | *C. jejuni* | BfR-CA-18040 | 5 | 7 | - | - | - | - | 5.75 | 6.00 | | |
| | | | | | | | | | 5.64 | 5.85 | | |
| | | | | | | | | | 5.74 | 5.82 | | |
| | | | | | | | | | 5.18 | 5.86 | | |
| | | | | 25 | | | | | 4.58 | 4.56 | | |
| | | | | | | | | | 4.01 | 4.41 | | |
| | | | | | | | | | 3.49 | 4.38 | | |
| | | | | | | | | | 2.63 | 4.42 | | |
| | | | | 31 | | | | | 2.32 | 3.60 | | |
| | | | | | | | | | 2.24 | 3.44 | | |
| | | | | | | | | | 2.29 | 3.41 | | |
| | | | | | | | | | 2.30 | 3.45 | | |
| | | | | 49 | | | | | 1.36 | 0.71 | | |
| | | | | | | | | | 0.78 | 0.56 | | |
| | | | | | | | | | 1.51 | 0.53 | | |
| | | | | | | | | | 0.90 | 0.57 | 0.86 | 0.74 |
| 1 | *C. jejuni* | BfR-CA-18043 | 8 | 7 | - | - | - | - | 5.82 | 6.00 | | |
| | | | | | | | | | 5.71 | 5.83 | | |
| | | | | 25 | | | | | 5.00 | 4.01 | | |
| | | | | | | | | | 4.84 | 3.84 | | |

| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | 31 | | | | | 3.44 | 2.68 | | |
| | | | | | | | | | 1.64 | 2.51 | | |
| | | | | 49 | | | | | 0.48 | -0.1 | | |
| | | | | | | | | | -0.1 | -0.1 | 0.68 | 0.99 |
| 1 | *C. jejuni* | BfR-CA-18043 | 12 | 6 | - | - | - | - | 5.36 | 5.58 | | |
| | | | | | | | | | 5.53 | 5.56 | | |
| | | | | 9 | | | | | 5.32 | 5.58 | | |
| | | | | | | | | | 5.46 | 5.56 | | |
| | | | | 24 | | | | | 3.02 | 2.85 | | |
| | | | | | | | | | 2.73 | 2.83 | | |
| | | | | 30 | | | | | 1.00 | 0.81 | | |
| | | | | | | | | | 0.60 | 0.79 | 0.17 | 0.99 |

[a] References: (1) this publication (2) Wulsten et al., 2020.
- Comparison between the two models was not performed.

**Table S6.** Comparison of observed and predicted concentration at a specific time point ($log_{10}N_t$) for *C. jejuni* IPIU data in raw milk at different temperatures obtained by using the tertiary model for IPIU data.

| | | | | | $Log_{10}\ N_t$ (IPIU/ml) | | Validation values | |
|---|---|---|---|---|---|---|---|---|
| Ref[a] | Species | Strain | Temp. [°C] | Time [h] | Observed | Predicted | RMSE | $R^2$ |
| 1 | *C. jejuni* | DSM 4688 | 5 | 7 | 4.60 | 4.45 | | |
| | | | | | 4.56 | 4.47 | | |
| | | | | | 4.75 | 4.76 | | |
| | | | | | 4.68 | 4.28 | | |
| | | | | 25 | 4.43 | 4.32 | | |
| | | | | | 4.52 | 4.34 | | |
| | | | | | 4.53 | 4.63 | | |
| | | | | | 4.64 | 4.15 | | |
| | | | | 31 | 4.38 | 4.30 | | |
| | | | | | 4.25 | 4.32 | | |
| | | | | | 4.66 | 4.61 | | |
| | | | | | 4.23 | 4.13 | | |
| | | | | 49 | 4.35 | 4.25 | | |
| | | | | | 4.59 | 4.27 | | |
| | | | | | 4.52 | 4.56 | | |
| | | | | | 4.44 | 4.08 | | |
| | | | | 73 | 4.44 | 4.20 | | |
| | | | | | 4.43 | 4.21 | | |
| | | | | | 4.34 | 4.50 | | |
| | | | | | 4.42 | 4.02 | | |
| 1 | *C. jejuni* | BfR-CA-18043 | 5 | 7 | 4.59 | 4.55 | | |
| | | | | | 4.59 | 4.55 | | |
| | | | | | 4.69 | 4.69 | | |
| | | | | | 4.69 | 4.45 | | |
| | | | | 25 | 4.64 | 4.42 | | |
| | | | | | 4.51 | 4.42 | | |
| | | | | | 4.70 | 4.56 | | |
| | | | | | 4.63 | 4.32 | | |
| | | | | 31 | 4.52 | 4.40 | | |
| | | | | | 4.36 | 4.40 | | |
| | | | | | 4.42 | 4.54 | | |
| | | | | | 4.58 | 4.30 | | |
| | | | | 49 | 4.16 | 4.35 | | |
| | | | | | 4.40 | 4.35 | | |
| | | | | | 4.45 | 4.49 | | |
| | | | | | 4.45 | 4.25 | | |
| | | | | 73 | 4.50 | 4.30 | | |
| | | | | | 4.32 | 4.30 | | |
| | | | | | 4.31 | 4.43 | | |
| | | | | | 4.57 | 4.20 | | |
| 2 | *C. jejuni* | DSM 4688 | 5 | 18 | 5.50 | 5.06 | | |
| | | | | | 4.90 | 4.81 | | |
| | | | | | 4.73 | 4.58 | | |
| | | | | | 4.73 | 4.75 | | |
| | | | | | 4.90 | 4.90 | | |
| | | | | 24 | 5.28 | 5.03 | | |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | 4.63 | 4.78 | | |
| | | | | | 4.43 | 4.55 | | |
| | | | | 42 | 5.26 | 4.96 | | |
| | | | | | 4.39 | 4.71 | | |
| | | | | | 4.35 | 4.48 | | |
| | | | | | 4.32 | 4.65 | | |
| | | | | | 4.85 | 4.80 | | |
| | | | | 48 | 5.33 | 4.95 | | |
| | | | | | 4.29 | 4.70 | | |
| | | | | | 4.43 | 4.47 | | |
| | | | | 66 | 5.20 | 4.91 | | |
| | | | | | 4.45 | 4.66 | | |
| | | | | | 4.38 | 4.43 | | |
| | | | | | 4.81 | 4.60 | | |
| | | | | | 4.73 | 4.75 | 0.31 | 0.45 |
| 1 | *C. jejuni* | DSM 4688 | 8 | 7 | 5.18 | 4.86 | | |
| | | | | | 5.27 | 5.34 | | |
| | | | | 25 | 5.09 | 4.77 | | |
| | | | | | 5.03 | 5.25 | | |
| | | | | 31 | 5.06 | 4.75 | | |
| | | | | | 5.07 | 5.23 | | |
| | | | | 49 | 5.03 | 4.71 | | |
| | | | | | 5.05 | 5.19 | | |
| | | | | 73 | 4.90 | 4.67 | | |
| | | | | | 4.97 | 5.15 | | |
| 1 | *C. jejuni* | BfR-CA-18043 | 8 | 7 | 4.82 | 4.92 | | |
| | | | | | 4.72 | 4.82 | | |
| | | | | 25 | 4.79 | 4.83 | | |
| | | | | | 4.85 | 4.73 | | |
| | | | | 31 | 4.72 | 4.81 | | |
| | | | | | 4.82 | 4.71 | | |
| | | | | 49 | 4.64 | 4.77 | | |
| | | | | | 4.44 | 4.67 | | |
| | | | | 73 | 4.46 | 4.73 | | |
| | | | | | 4.62 | 4.63 | 0.20 | 0.21 |
| 1 | *C. jejuni* | DSM 4688 | 12 | 24 | 5.06 | 5.11 | | |
| | | | | | 5.01 | 4.88 | | |
| | | | | 48 | 4.76 | 5.07 | | |
| | | | | | 4.42 | 4.84 | | |
| | | | | 72 | 4.99 | 5.05 | | |
| | | | | | 4.69 | 4.82 | | |
| 1 | *C. jejuni* | BfR-CA-18043 | 12 | 24 | 5.08 | 5.08 | | |
| | | | | | 4.71 | 4.88 | | |
| | | | | 48 | 4.83 | 5.04 | | |
| | | | | | 4.41 | 4.84 | | |
| | | | | 72 | 4.90 | 5.02 | | |
| | | | | | 4.74 | 4.82 | 0.22 | -0.057 |

[a] References: (1) this publication (2) Wulsten et al., 2020.

# 10 List of Publications

**Journal articles:**

Knipper A.-D., Ghoreishi N., Crease T. (2022) Prevalence and concentration of *Campylobacter* in faeces of dairy cows: A systematic review and meta-analysis. PLoS ONE 17(10): e0276018. https://doi.org/10.1371/journal.pone.0276018

Knipper A.-D., Göhlich S., Stingl K., Ghoreishi N., Fischer-Tenhagen C., Bandick N., Tenhagen B.-A., Crease T. (2023) Longitudinal study for the detection and quantification of *Campylobacter* spp. in dairy cows, during milking and in the dairy farm environment. *Foods*. 2023; 12(8):1639. https://doi.org/10.3390/foods12081639

Knipper, A.-D., Plaza-Rodríguez, C., Filter, M., Wulsten, I. F., Stingl, K., & Crease, T. (2023). Modeling the survival of *Campylobacter jejuni* in raw milk considering the viable but non-culturable cells (VBNC). Journal of Food Safety, e13077. https://doi.org/10.1111/jfs.13077

Knipper, A.-D., Crease, T., Günther T., Filter M., Nauta M. (2023). Quantitative microbiological risk assessment model for *Campylobacter* in raw milk of dairy cows in Germany. Microbial Risk Analysis, 25, 100274. https://doi.org/10.1016/j.mran.2023.100274

**Oral presentations:**

Herbstmann A.-D., Buschhardt T., Filter M., Nauta M., Evaluation of different risk mitigation strategies for *Campylobacter* along the raw milk supply chain. 21st International workshop on *Campylobacter, Helicobacter* and Related Organisms (CHRO), 14.-19.11.2022, online

Herbstmann A.-D., *Campylobacter* in Kot von Milchkühen. „Was wurde aus Alma?“-Abschluss-Symposium zum Organ-Sharing-Projekt 2020, 19.10.2022, Berlin, DE

Herbstmann A.-D., Buschhardt T., Filter M., Nauta M., Quantitative microbiological risk assessment model for *Campylobacter* in raw milk. European Symposium - International Association for Food Safety (IAFP), 04.05.-06.05.2022, Munich, DE

Herbstmann A.-D., Buschhardt T., *Campylobacter* along the raw milk supply chain. Junior Scientist Zoonoses Meeting, 03.-04.06.2021, online

Herbstmann A.-D., Development of predictive tenacity models for bacteria along the raw milk supply chain. Pre-Doc Symposium (Tagung der Promovierenden am BfR), 03.12.2020, Berlin, DE

Herbstmann A.-D., Buschhardt T., Predictive tenacity model for *Campylobacter* along the raw milk supply chain. RoKoCon 2020 - Outbreak Response, 30.10.2020, online

**Poster presentations:**

Herbstmann A.-D., Buschhardt T., *Campylobacter* along the raw milk supply chain. Junior Scientist Zoonoses Meeting, 03.-04.06.2021, online

Herbstmann A.-D., Ghoreishi N., Buschhardt T., Prävalenz und Konzentration von *Campylobacter* spp. in Fäzes von Milchkühen: Eine systematische Literaturrecherche und Metaanalyse. Arbeitstagung des Arbeitsgebietes Lebensmittelsicherheit und Verbraucherschutz (DVG), 28.09.-30.09.2021, online

Herbstmann A.-D., Wulsten I. F., Göhlich S., Stingl K., Buschhardt T., Viability qPCR and CFU data acquisition for modelling survival of *Campylobacter jejuni* in raw milk. International Symposium on Zoonoses Research, 13.-15.10.2021, online, 1st poster price

Herbstmann A.-D., Filter M., Wulsten I. F., Stingl K., Buschhardt T., Development of predictive survival models for *Campylobacter jejuni* in raw milk based on viability qPCR and CFU data. International Symposium on Zoonoses Research, 05.-07.10.2022, Berlin, DE, 3rd poster price

Herbstmann A.-D., Buschhardt T., Filter M., Nauta M., Evaluation of risk mitigation strategies for *Campylobacter* in raw milk. 4. LGL-Kongress Lebensmittelsicherheit und Tiergesundheit, 25.10.-27.10.2022, Erlangen, DE

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# 13 Interessenskonflikte

Im Rahmen dieser Arbeit bestehen keine Interessenskonflikte durch Zuwendungen Dritter.

# 14 Selbstständigkeitserklärung

Hiermit bestätige ich, dass ich die vorliegende Arbeit selbstständig angefertigt habe. Ich versichere, dass ich ausschließlich die angegebenen Quellen und Hilfen in Anspruch genommen habe.

Berlin, den 16.02.2024 Anna-Delia Knipper