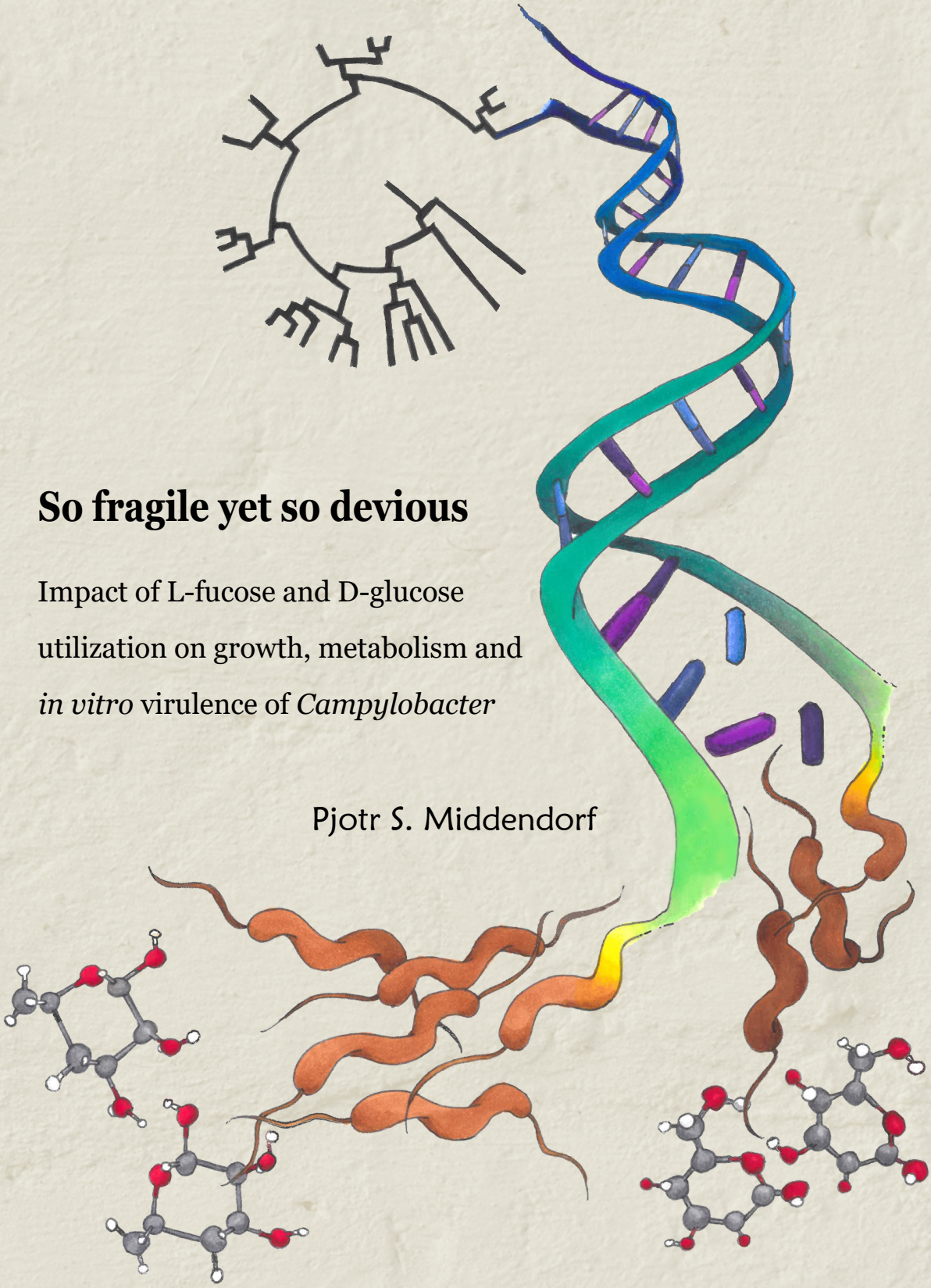

So fragile yet so devious

Impact of L-fucose and D-glucose utilization on growth, metabolism and *in vitro* virulence of *Campylobacter*

Pjotr S. Middendorf

Propositions

1. L-fucose utilization reshapes the cell surface of *Campylobacter jejuni* by modulating the glycosylation system.
   (this thesis)

2. Acquisition of the D-glucose utilization cluster does not provide competitive advantage for *Campylobacter jejuni*.
   (this thesis)

3. There are no dogmas in science.

4. The high specificity of bacteriophage therapy offers opportunities to target antibiotic resistant bacteria with low risk of unwanted side effects.

5. Use of ChatGPT enhances scientific productivity.

6. Cancel culture stifles free speech.

Propositions belonging to the thesis, entitled

So fragile yet so devious – Impact of L-fucose and D-glucose utilization on growth, metabolism and *in vitro* virulence of *Campylobacter*

Pjotr S. Middendorf
Wageningen, 12 September 2023

So fragile yet so devious

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Thesis committee

Promotors

Prof. Dr T. Abee
Personal chair at the Laboratory of Food Microbiology
Wageningen University & Research

Prof. Dr H.M.W. Den Besten
Personal chair at the Laboratory of Food Microbiology
Wageningen University & Research

Co-promotor

Dr W.F. Jacobs-Reitsma
Food Microbiologist
National Institute for Public Health and the Environment (RIVM), Bilthoven

Other members

Prof. Dr D. Machado de Sousa, Wageningen University & Research
Dr M.M.S.M. Wösten, Utrecht University
Dr A. Alvarez-Ordóñez, University of León, León, Spain
Dr E. Franz, National Institute for Public Health and the Environment (RIVM), Bilthoven

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Pjotr S. Middendorf

Thesis

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Pjotr Siebe Middendorf

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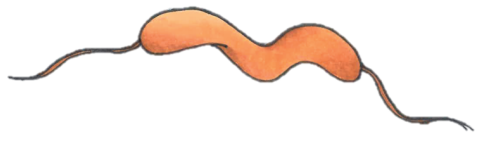

1

General introduction

1

Dr Theodore Escherich was the first to describe a spiral-shaped bacterium back in 1886 when investigating a sample taken from the large intestine of a child suffering from diarrhea, and described the symptoms as "*cholera infantum"* (Escherich, 1886; Vandamme, 2000). It was only in 1906, when two British veterinarians, McFadyean and Stockman, managed to successfully culture this microorganism from aborted bovine fetuses and described it as "*comma shaped"* and "*long spirillar form*" (Skirrow, 2006). In 1927, Smith and Orcutt isolated a group of bacteria from the feces of cattle with diarrhea and named it *Vibrio jejuni (Jones et al., 1931; Silva et al., 2011).* In 1944, a different type of *Vibrio* was isolated from the feces of pigs by Doyle, and was classified as *Vibrio coli* (Doyle, 1944; Epps et al., 2013). In 1963, Sebald and Véron proposed the genus *Campylobacter,* as these bacteria had non-fermentative metabolism and a low DNA base composition, and did not distinguish them as "true" *Vibrio spp* (Silva et al., 2011). In the 1970s proper isolation techniques were developed, by using filtration and selective agar methods, enabling clinical laboratories to easily isolate this pathogen from human feces samples, elucidating *Campylobacter's* significance as a human pathogen and major cause of bacterial diarrhea (Dekeyser et al., 1972; Butzler et al., 1973; Skirrow, 1977). These novel isolation techniques highlighted *Campylobacter's* high incidence in human diarrhea. Currently, there are 57 *Campylobacter* species and 16 subspecies known, including at least 19 species involved in human disease (Parte et al., 2020) (Costa & Iraola, 2019).

Campylobacter has been the leading cause of bacterial gastroenteritis in Europe since 2005 (EFSA, 2021b). *Campylobacter* species information was provided by 20 member states in Europe for 64.7% of the confirmed and reported human disease cases in 2019. Of these, 88.1% were *C. jejuni,* 10.6% *C. coli*, 0.16% *Campylobacter fetus*, 0.11% *Campylobacter upsaliensis,* 0.09% *Campylobacter lari* and 0.94% other *Campylobacter* species (EFSA, 2021b). In 2020, a decrease in cases was observed, most likely due to the COVID-19 pandemic. In 2020, there was an estimated case rate of 40.3 per 100,000 persons, while in 2019 this was approximately 60.6 per 100,000 persons (EFSA, 2021b). Nonetheless, no significant decrease was observed in Europe in the number of reported human cases from 2016-2020. Using data from the Foodborne Diseases Active Surveillance Network (FoodNet), which covers 15% of the United States (US) population, an incidence of 14.4 per 100,000 persons was observed in 2020 and an incidence of 19.5 per 100,000 persons in 2019 (CDC, 2022). Even though a lower

incidence is observed in the US compared to Europe, *Campylobacter* is still the leading cause of gastroenteritis illnesses per year, with no significant decrease observed over the last years. Similar trends were observed by the Public Health England, in England (England, 2020).

Outbreaks of *Campylobacter* occur on a yearly basis, and can develop from many sources. Several studies have shown that there is seasonality involved in *Campylobacter* outbreaks and infections, often with increased cases during the summer months (McCarthy et al., 2012; Nichols et al., 2012; Taylor et al., 2013). Interestingly, next to the more common food source outbreaks, there are many studies regarding waterborne *Campylobacter* outbreaks. Waterborne outbreaks of *Campylobacter* are often linked to spread via rainfall, via farms and via untreated/malfunctioning water supplies of towns, however, also include recreational water and untreated surface water (Lind et al., 1996; Clark et al., 2003b; Auld et al., 2004; Kuusi et al., 2004; O'Reilly et al., 2007). Waterborne *Campylobacter* is also involved in colonization of farm animals (Pearson et al., 1993; Ogden et al., 2007; Pérez-Boto et al., 2010).

Campylobacter transmission occurs via multiple routes including contaminated foods of animal origin, domestic animals, wild birds and water reservoirs. In this thesis we characterized mechanisms contributing to the growth, survival, transmission and virulence of the most important *Campylobacter* species for human health, namely *C. jejuni* and *C. coli*. This introduction chapter will address *Campylobacter*'s physiology, disease burden, virulence, transmission, metabolism and adaption ability to new environments.

Growth characteristics

Generally, *Campylobacter* isolates are able to grow at temperatures between 30-45°C, with optimal growth temperatures between 37 and 42°C (Levin, 2007; Silva et al., 2011). Within a few degrees from the minimum or maximum growth temperature, sudden decrease in growth rates from maximum to near zero was observed, even though electron transfer chain activity and protein synthesis were still detected (Hazeleger et al., 1998). *Campylobacter* lacks cold-shock proteins, which are needed for adaptation

to growth in cold environments (Hazeleger et al., 1998; Park, 2002). Cold-shock proteins can be found in other pathogens, such as *Listeria monocytogenes*, allowing them to grow at low temperatures (below 5°C) (D'Aoust, 1989; Lado & Yousef, 2007). Within the given temperature range, *Campylobacter* is unable to grow in environments with water activity (a_w) levels lower than 0.987, and is sensitive to sodium chloride (NaCl) concentrations of more than 2% w/v (Silva et al., 2011). The optimal a_w condition for growth is 0.997 (appr. 0.5% w/v NaCl) (Silva et al., 2011), however, some *Campylobacter* species are able to survive for weeks in NaCl concentrations higher than 6.5% when cultivated at refrigeration temperatures, indicating their robustness (Fuquay et al., 2011). In addition, *Campylobacter* grows optimally in the pH range 6.5-7.5, while at pH values below 4.9 or above 9.0, *Campylobacter* will not survive (Silva et al., 2011).

Microaerobic growth

Most *Campylobacter* isolates are considered microaerophilic, and grow preferably under microaerobic conditions (~6% O_2). *Campylobacter* isolates use oxygen and several alternative electron acceptors (such as fumarate, nitrate, nitrite, and N- or S-oxides) as a terminal electron acceptor, however, they are very sensitive to the high oxygen concentrations present in the environment (~20% O_2) (Parkhill et al., 2000; Kelly, 2001). Ironically, most *C. jejuni* and *C. coli* species are isolated from samples in atmospheric conditions, such as foods or manure on farms, highlighting their strong survival capabilities (Trimble et al., 2013). Furthermore, (limited) growth of *Campylobacter* in atmospheric conditions produces reactive oxygen species (ROS), which damage the bacteria. Therefore, *Campylobacter* lives in habitats where oxygen levels are low, such as animal intestines and the human gut. Bacteria typically possess redox enzymes that facilitate oxidative phosphorylation using oxygen as an electron carrier, which unavoidably produces ROS, including the superoxide anion (O_2-) and hydrogen peroxide (H_2O_2) (Kim et al., 2015). Aerobic bacteria often have multiple ROS-detoxification enzymes, such as the three different types of superoxide dismutase enzymes: *sodA, sodB* and *sodC* in *E. coli*, while the *C. jejuni* genome only harbors single gene copies of such genes, for example: *sodB* (Parkhill et al., 2000; Atack & Kelly, 2009; Kim et al., 2015). Targeted deletion of these genes in *Campylobacter* showed higher levels of ROS and several morphological changes in which more coccoid shaped cells were observed (Oh et al., 2015).

Campylobacter grows best in an atmosphere of 5% O_2, 10% CO_2 and 85% N_2, while an oxygen concentration between 3-15% is sufficient for growth (Hazeleger et al., 1998). However, when cultivated at high cell densities, growth in aerobic conditions was observed in some isolates (Kaakoush et al., 2007). Rodrigues et al. (2015) described such an isolate, isolate *C. jejuni* Bf, and showed that this isolate is capable of growing at aerobic conditions, unlike the reference isolate *C. jejuni* NCTC11168 (Rodrigues et al., 2015). This study underscores the genetic variety of *C. jejuni* isolates and how they can adapt to survive stresses such as oxidative stress (Rodrigues et al., 2015).

Environmental survival of *Campylobacter*

Although fragile in lab settings, *Campylobacter* developed environmental persistence due to a wide variety of mechanisms, of which many are unknown. Interestingly, when pyruvate is added to liquid medium, concentrations of hydrogen peroxide decrease, allowing some isolates of *C. jejuni* to grow aerobically (Verhoeff-Bakkenes et al., 2008). Furthermore, the combination of ferrous sulfate, sodium metabisulfate and sodium pyruvate allowed *C. jejuni* to maintain spiral-shaped morphology and motility when stored for 20-30 days at 4 °C (Chou et al., 1983). Another study showed that a co-culture of *Pseudomonas* and *C. jejuni* chicken meat and human isolates resulted in prolonged aerobic survival, most likely due to metabolic commensalism with *Pseudomonas* (Hilbert et al., 2010). In another study, when co-cultured with the amoeba *Acanthamoeba polyphaga*, *C. jejuni* showed increased survival against an otherwise lethal dose of gentamicin (Shagieva et al., 2021a). Furthermore, *C. jejuni* is also able to survive in nutrient-poor conditions, like water, where it must cope with starvation and other stresses. Several studies have shown that *Campylobacter* was able to survive in different kinds of water (such as lakes, tap water) for long periods of time (Korhonen & Martikainen, 1991; Thomas et al., 1999; Trigui et al., 2015; Nilsson et al., 2018). There are many genes encoding proteins and enzymes involved in the protection and survival of *Campylobacter* in stressful environments, these stresses include starvation, oxidative, osmatic, heat shock, pH and nitrosative stresses (Andersen et al., 2005; Brøndsted et al., 2005; Candon et al., 2007; Bronowski et al., 2014). For example, at low temperatures (4 and 10 °C) it was shown that the polynucleotide phosphorylase is important in the long-term survival of *Campylobacter*, while having no effect on the survival at higher temperatures (37 and 42 °C) (Haddad et al., 2009). Interestingly, a study regarding waterborne *C. jejuni*

isolates showed that all tested isolates were able to develop aerotolerance, highlighting the ability of *C. jejuni* to adapt and survive in detrimental environmental conditions (Shagieva et al., 2021b).

One of the most common survival strategies for bacteria is biofilm formation. Biofilm formation is a well-studied bacterial mechanism for growth and survival, where bacteria attach to surfaces and produce a protective extracellular matrix (O'Toole et al., 2000; Matz et al., 2005; Chang et al., 2007). This matrix provides protection to the bacteria from harsh environmental conditions. *Campylobacter* is also able to form biofilms, which is increased in stressful conditions, including aerobic- and low nutrients -conditions (Reeser et al., 2007; Reuter et al., 2010; Maal-Bared et al., 2012).

C. jejuni has two main morphologies: spiral-shaped and coccoid-shaped, and the latter is also called the viable but non culturable state (VBNC) (Figure 1A, B). In unfavorable conditions, such as aerobic or low-nutrient environments, the morphology of *C. jejuni* enters the VBNC state (Rollins & Colwell, 1986). During this state, *C. jejuni* lowers its metabolic activity in order to retain viability, and is unable to grow under laboratory conditions (Barer & Harwood, 1999). Interestingly, gene expression can be detected for a prolonged period in VBNC state cells. Patrone at al. (2013) recovered VBNC bacteria from a culture that was incubated from 46-48 days in freshwater at 4°C and measured expression of the fibronectin binding gene *cadF*. The authors found that these cells were still able to adhere and invade human Caco-2 cells (Patrone et al., 2013).

Whether or not *C. jejuni* can revert from its VBNC state to its spiral form remains a topic of controversy. Some reports suggest that *C. jejuni* is not able to revert from a VBNC state, while other studies reported successful reversions (Saha et al., 1991; Beumer et al., 1992; Mederma et al., 1992; Hazeleger et al., 1995; Talibart et al., 2000; Hald et al., 2001; Ziprin et al., 2003; Ziprin & Harvey, 2004; Baffone et al., 2006).

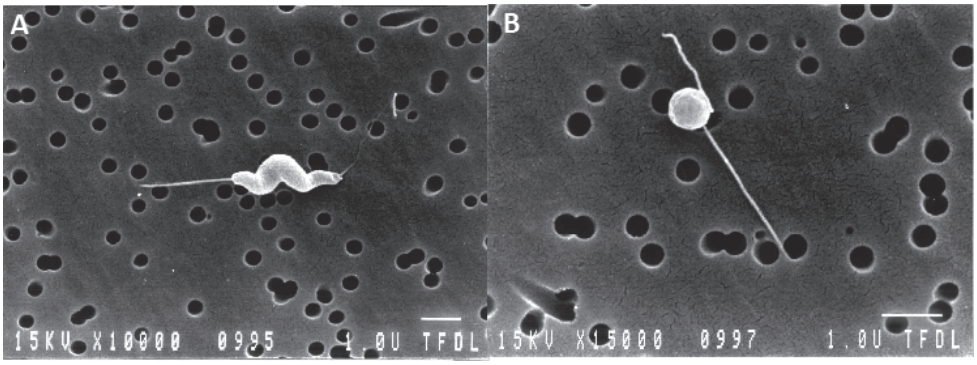

Figure 1 Scanning electron micrographs of: A) spiral and B) coccoid cells of *C. jejuni* (Hazeleger, 1991).

Transmission and reservoirs of *Campylobacter*

In Europe and the USA, most *Campylobacter* infections are linked to the handling and consumption of (undercooked) chicken (EFSA, 2010, 2021a; Williams et al., 2021). However, next to cross-contamination via poultry meat, there are multiple ways of transmission which form significant risk factors for campylobacteriosis (Figure 2). Notably, transmission via the consumption of (poultry) meat only contributes to half of all campylobacteriosis cases, highlighting other transmission routes such as contact with live animals-, contact with pets-, person to person- and environmental water-mediated transmission (Vellinga & Van Loock, 2002; Domingues et al., 2012; Mughini-Gras et al., 2012; Wagenaar et al., 2013; Mughini-Gras et al., 2021). Furthermore, during international travel an increased amount of campylobacteriosis cases was linked to the consumption of foods, such as vegetable salad and drinking water (Ekdahl & Andersson, 2004; Mughini-Gras et al., 2014).

Transmission via farm animals

The gastrointestinal of warm blooded animals is considered a natural habitat for *Campylobacter* spp., due to the presence of many nutrients and an optimal growth temperature. Therefore, the highest risk of campylobacteriosis is linked to contact with live animals and the consumption and handling of contaminated food of animal origin (Kaakoush et al., 2015a; Skarp et al., 2016; Mohammadpour et al., 2018). Colonization of farm animals, such as poultry, often occurs at farm level. Once

introduced, spread of *Campylobacter* throughout the broiler house occurs rapidly. Studies have shown that within a week, *Campylobacter* is able to colonize the majority of broilers within a farm and persist in the intestinal tract until slaughter, despite rarely causing symptoms in chickens (Jacobs-Reitsma et al., 1995; Van Gerwe et al., 2010; Hermans et al., 2011; Hermans et al., 2012). Consumption of raw milk of cattle is also linked to campylobacteriosis as it can be contaminated via fecal matter (Porter & Reid, 1980; Humphrey & Beckett, 1987).

Transmission via surface water

In the Netherlands, contamination via surface water nearly accounts up to 11% of all campylobacteriosis cases (Mughini-Gras et al., 2021). Most *Campylobacter* isolates in surface water could be linked to wild birds and poultry, highlighting the transmission pathways from animal feces and agricultural effluents to surface water (Mughini-Gras et al., 2016; Mughini-Gras et al., 2021). Wild birds in particular have been found to be significant risk factors for transmission, as these often get in contact with rivers and surface waters (Hald et al., 2015). Also in wild animals, such as deer and boars, *Campylobacter* spp. are present. Even though some studies have shown that there is a low prevalence of *Campylobacter* spp. in boars, others have shown a much higher prevalence, indicating that regionality and seasonality also play a role in transmission (Hearnden* et al., 2003; Djennad et al., 2019; Castillo-Contreras et al., 2022; Morita et al., 2022). Furthermore, several case outbreak studies established a clear link between surface water and contaminated drinkable source water, in which there is an intrusion of contaminated surface water into source water (Kuusi et al., 2005; Bartholomew et al., 2014).

Transmission via person-to-person, via pets and via cross-contamination

Another potential transmission route of *Campylobacter* is from person to person, however, due to general hygiene practices, *Campylobacter* does not usually spread from one person to another. One could hypothesize that due to the cross contamination between meat and vegetables during meal preparations, transmission within a family is possible. Furthermore, as humans often share their living environments with their pets, transmission of owners to pets, and pets to owners, may occur. In particularly, ownership of puppies is a significant risk factor for campylobacteriosis (Mughini Gras et al., 2012; Mughini-Gras et al., 2013). It is unclear

what the role of pets in *Campylobacter* transmission is, as pets might acquire *Campylobacter* infections simultaneously next to humans (Gras et al., 2013).

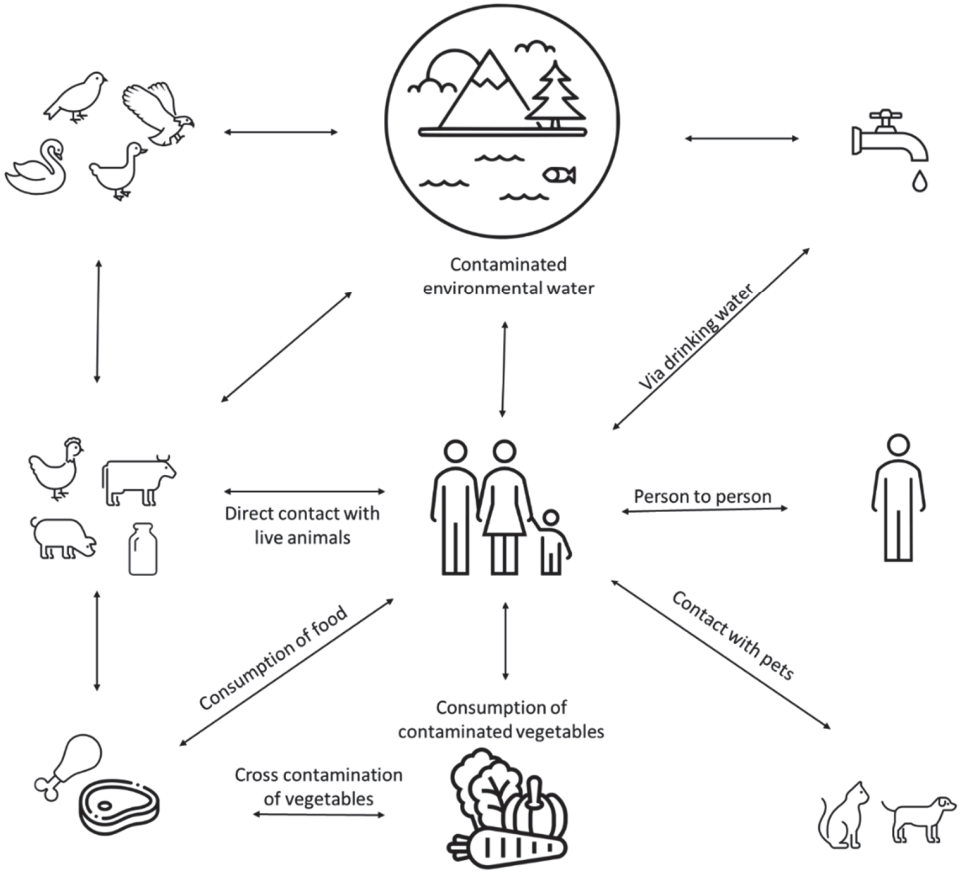

Figure 2 Reservoirs and transmission of *Campylobacter* based on (Domingues et al., 2012; Mughini-Gras et al., 2013; Wagenaar et al., 2013; Mughini-Gras et al., 2021).

1

Pathogenesis of *Campylobacter*

Campylobacteriosis

Human infections with *Campylobacter* are mostly linked to consumption or handling of raw and/or undercooked meat products (predominantly poultry), direct contact with animals, and contact with environmental waters (Clark et al., 2003a; Karagiannis et al., 2010). After ingestion, the incubation time of *Campylobacter* is two to five days. Campylobacteriosis is a collective name of infections with predominantly *C. jejuni* or *C. coli* that leads to a range of symptoms, including profuse diarrhea, abdominal pain, nausea and fever, usually lasting for three to seven days (Scallan et al., 2015). Severity of the symptoms varies per person, resulting in an absence of symptoms for some, while others may have a long-lasting illness (Kuusi et al., 2004; Kuhn et al., 2017; Montgomery et al., 2018; Kenyon et al., 2020). *Campylobacter* can also be involved in several human systemic infections, including bloodstream infections, septic thrombophlebitis, acute colitis of inflammatory bowel disease and acute appendicitis (Morishita et al., 2013; Alnimr, 2014; Lagler et al., 2016). Other significant post-infection diseases include the Guillain-Barré syndrome (GBS), Miller-Fisher syndrome (MFS), and severe demyelinating neuropathy (Scallan et al., 2015; Skarp et al., 2016).

C. jejuni is reportedly able to colonize the human intestine and has a low dose response correlation (Robinson, 1981; Black et al., 1988; Teunis et al., 2018). Infections are most common in age groups below 4 years old and above 75 years old (Levesque et al., 2013). Additional high risk groups of *Campylobacter* infections include patients suffering from inflammatory bowel disease, immunocompromised individuals, and hemoglobinopathies patients (Igwaran & Okoh, 2019).

Pathogenicity

Yearly, *Campylobacter* causes a large economic burden due to the many foodborne illnesses they cause worldwide. In 2020, campylobacteriosis was the most commonly reported zoonosis with over double the amount of reported cases compared to the second largest zoonosis (salmonellosis) (Bolton, 2015; EFSA, 2021b). To gain a better understanding of *Campylobacters* pathogenicity, the genome of isolate *C. jejuni* NCTC11168 was sequenced in 2000 (Parkhill et al., 2000).

Campylobacter pathogenicity strongly depends on the variety of virulence genes that *Campylobacter* species possess (Ketley, 1995; Bravo et al., 2021). These virulence factors do not only play an important role in *Campylobacter's* virulence, but are also involved in many other mechanisms, including survival and resistance to physiological stresses. Virulence-related mechanisms include adhesion, invasion, translocation, colonization, motility, chemotaxis and toxin production (Bhavsar & Kapadnis, 2006; Biswas et al., 2011; Backert et al., 2013). Moreover, biofilm formation is also involved in human pathogenicity, as biofilms are often less susceptible to antibiotics (Nichols, 1991).

Motility

C. jejuni and *C. coli* have amphitrichous flagellation, thus two polar flagella are produced at each pole of the cell (Kaakoush et al., 2015b). The flagella of *C. jejuni* have multiple purposes: they help avoiding hostile environments, reaching the gut and penetrating the viscous mucosa lining of the human epithelial cells. Therefore, the flagella not only promote motility, but also chemotaxis, adhesion and invasion of the human gut (McSweegan & Walker, 1986; Black et al., 1988; Grant et al., 1993; Szymanski et al., 1995; Konkel et al., 2004). Furthermore, the flagella of *Campylobacter* are also involved in biofilm formation and in the secretion of several virulence-associated invasion proteins (Konkel et al., 2004; Christensen et al., 2009; Svensson et al., 2014).

The flagellum of *Campylobacter* consists of three parts, namely, the body, the hook, and the flagellar filament (Figure 3) (Elmi et al., 2021). The role of motility during colonization is often proved via knockout studies of flagellar genes. The filament is composed of the FlaA and FlaB proteins, although only FlaA is essential for motility:

FlaA knockout studies have shown that a mutation in *flaA* leads to non-flagellated and non-motile cells, while mutation of the FlaB protein had no impact on motility (Nuijten et al., 1990; Wassenaar et al., 1991; Wassenaar et al., 1993b). Mutations of the flagellar motor proteins, which are important for the rotation of *Campylobacter*, MotA and MotB, resulted in cells that produced full length flagella but were unable to colonize the chicken gut (Hendrixson & DiRita, 2004). Additionally, studies regarding mutations in flagellar sigma factors (bacterial transcription initiation factors) of *Campylobacter*, σ28 (*fliA*) and σ54 (*rpoN*), resulted in non-motile cells that could not colonize chickens, and were unable to adhere to and invade human epithelial cells (Wassenaar et al., 1991; Fernando et al., 2007).

With the use of chemotaxis, *Campylobacter* is able to sense and move towards certain more favorable environments. Metabolic substrates like L-serine, L-glutamate, L-cysteine and L-aspartate, as well as electron chain donors and acceptors such as D-lactate and fumarate, are important chemoattractants for survival (Hugdahl et al., 1988). Moreover, during colonization, chemotaxis plays a large role in helping *Campylobacter* locate adhesion sites (Chang & Miller, 2006).

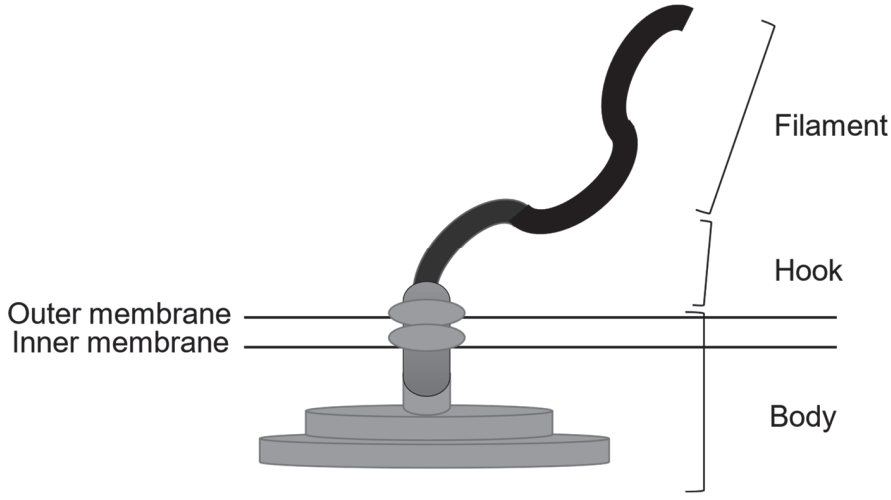

Figure 3 The three parts of the flagella: the body, the hook and the filament (adapted from (Bolton, 2015)).

Toxin production

Campylobacter possesses a wide range of cytotoxins, however, most are not well understood in pathogenesis. Examples of reported toxins include a Shiga-like toxin, a Chinese hamster ovary toxin, a cell-rounding toxin, a hepatoxin, and a porin-LPS toxin (Johnson & Lior, 1986; Wassenaar, 1997; Hänel et al., 1998; Bacon et al., 1999). The most well studied cytotoxin of *Campylobacter* is the cytolethal distending toxin (CDT), which was first discovered in 1988 (Johnson & Lior, 1988). The CDT complex exists of three proteins: CdtA, CdtB and CdtC, in which CdtA and CdtC form a delivery module for the enzymatically-active subunit CdtB (Lara-Tejero & Galán, 2001; Lee et al., 2003). When either of the three proteins was purified, it did not exhibit any toxic activity (Lara-Tejero & Galán, 2001). Furthermore, full inactivation of the CDT complex greatly impairs the cytotoxicity against HeLa cells (Purdy et al., 2000). Studies reported that the toxin of the CDT complex is membrane-bound and blocks the kinase responsible for entry into mitosis, resulting in a G2/M arrest and degradation of the chromosomal DNA (Whitehouse et al., 1998; Purdy et al., 2000). It also created double-stranded breaks in human fibroblasts, and can cause cell arrest in the G1 phase (Lara-Tejero & Galán, 2000). Furthermore, CDT induces interleukin-8 secretion from epithelial cells, leading to DNA damage and fragmentation of the nucleus (Hickey et al., 2000).

Adhesion

One of the most important colonization factors of *Campylobacter* is adhesion to human intestinal cells via adhesins, which are highly specialized surface structures (Kovács et al., 2020). Adhesins contain a single monomeric protein and complex multimeric macromolecules, and they play a key role in bacterial colonization and persistence in the host (Pizarro-Cerdá & Cossart, 2006). Some adhesins have tissue tropism, meaning that a specific adhesin can take on many forms and is able to target different host cells and tissues during infection. Some adhesins are also able to work together, and bind to a specific surface receptor in a cooperative manner. During infection, gene expression of pathogenic bacteria changes and virulence-specific genes, as well as their metabolism, respiration and physiology can change (Stones & Krachler, 2016).

During infection, *Campylobacter* starts by binding to the mucin, followed by attachment to extracellular matrix proteins like laminin and fibronectin, and lastly attaching to the surface of epithelial cells (Kovács et al., 2020). As shown in Figure 3, many proteins are involved in this process, including PEB1, PEB3, PEB4, CapA, CapB, Cj1349, Cj0091, TlyA, P95, JlpA, FlpA and CadF (Kaakoush et al., 2015b). It is clear that, due to the large amount of proteins involved, adhesion is a very complex process in which LOS (lipooligosaccharide), capsule, and O- and N-linked glycosylation are involved. Surprisingly, very little cellular targets are known of *C. jejuni* adhesins. So far, target sites for three *C. jejuni* proteins were identified, specifically, JlpA binds to HSP90 (Heat Shock Protein 90), FlpA binds to fibronectin (FN), and CadF binds to FN (Rubinchik et al., 2012; Lugert et al., 2015; Konkel et al., 2020).

Fibronectin-binding proteins are members of the Microbial Surface Components Recognizing Adhesive Matrix Molecule(s) (MSCRAMMs) family and contribute to the disease process. FN is present in the majority of human organs, tissues and epithelial cells and is synthesized and secreted by many cell types (Sottile & Hocking, 2002; Frantz et al., 2010; To & Midwood, 2011). It plays an important role in cell signaling, cell adhesion, cell migration, extracellular matrix remodeling and tissue regeneration (To & Midwood, 2011). So far, for *C. jejuni*, CadF and FlpA are the only discovered fibronectin-binding proteins. CadF is a 326 amino acid (AA) with a mass of 36,872 Da and is a surface exposed outer membrane protein that binds to insoluble FN (Konkel et al., 1997; Konkel et al., 2020). The FN-binding domain of CadF was localized at the location AA 134-137 (Phe-Arg-Leu-Ser), however, binding sites within FN have not been discovered yet (Konkel et al., 2005; Konkel et al., 2020). The second fibronectin-binding protein of *Campylobacter* is FlpA, which is a 412 AA protein with a mass of 46,124 Da. The FN-binding domain was localized at the location Trp-Arg-Pro-His-Pro-Asp-Phe-Arg-Val (Larson et al., 2013). Within FN, FlpA binds to the gelatin-binding domain which is composed of four FN I repeats and two FN II repeats. Furthermore, there are several indications that FlpA is a lipoprotein that is exposed at the surface (Konkel et al., 2010; Konkel et al., 2020). Both proteins, CadF and FlpA, are very conserved in *Campylobacter's* genome and are present in 99.93% of the sequenced *C. jejuni* isolates. Surprisingly, other pathogenic bacteria are lacking these proteins (Konkel et al., 2020).

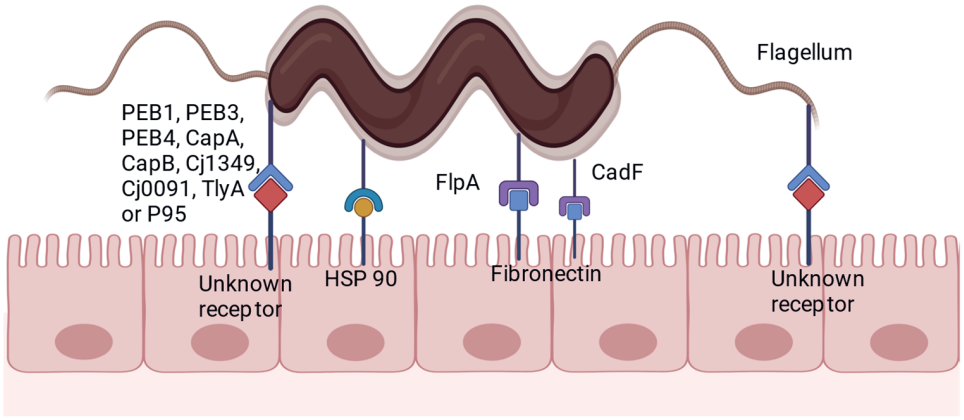

Figure 4 Schematic overview of several proteins that are involved in the adhesion of *Campylobacter* to epithelial cells (Adapted from: (Kaakoush et al., 2015b)). Figure was created using BioRender software.

Invasion

One of the main differences between colonization of the human host and the chicken host with *Campylobacter* is the increased number of bacteria invading epithelial cells in the human, indicating that not only adhesion to epithelial cells, but also invasion of epithelial cells is essential for disease development (Young et al., 2007; Cróinín & Backert, 2012; Kovács et al., 2020). Internalization of *C. jejuni* occurs via a trigger mechanism in a microtubule-dependent and actin-independent manner (Oelschlaeger et al., 1993; Kovács et al., 2020). One of the initial steps of invasion is protrusion of the membrane, which is mediated by Rac1 and Cdc42 (Krause-Gruszczynska et al., 2007). Several proteins are thought important for invasion, such as the flagellum of *Campylobacter*. Several studies have shown that mutations of *fla* and *flg* genes resulted in a reduced invasion of *Campylobacter* (Konkel et al., 2004; Eucker & Konkel, 2012). Furthermore, several proteins are secreted through the T3SS apparatus of the flagellum, including the so-called invasion antigens (Cia) (Eucker & Konkel, 2012). During co-culturing and contact of *Campylobacter* with epithelial cells, genes encoding Cia proteins are upregulated, and 18 putative virulence associated Cia proteins are secreted (Larson et al., 2008). Some Cia proteins are involved in invasion, such as CiaC, which is required for maximal host cell invasion and is involved in

cytoskeletal rearrangements of the host resulting in membrane ruffling (Neal-McKinney & Konkel, 2012). Other proteins, such as CiaI or CiaD, are involved in intracellular survival in human epithelial cells or in the activation of MAP kinase signaling pathways (Buelow et al., 2011; Samuelson et al., 2013). Once internalized, *C. jejuni* is located in a lysosome-free membrane-bound compartment (Watson & Galán, 2008). Current understanding of the different factors involved in these processes is still limited, and how *Campylobacter* is able to maintain functionally during an intracellular lifecycle is one of the many questions that require further research.

Metabolism of *Campylobacter*

Campylobacter encounters many different environments, including the human gut and animal intestine. Therefore, a large repertoire of metabolic features is beneficial, as cells would remain viable, even below suboptimal growth temperatures, for prolonged periods. Interestingly, there is a large genetic variation between *Campylobacter* isolates, i.e. there is an over-representation of genes that are involved in oxygen-independent respiration and the catabolism of amino acids in colonizing isolates, in comparison to poor-colonizing isolates (Hofreuter et al., 2006; Hepworth et al., 2007; Seal et al., 2007; Hiett et al., 2008). *Campylobacter's* metabolism is unique as it lacks to use the ability to metabolize most common carbohydrates (Parkhill et al., 2000). Therefore, until the discovery of the L-fucose utilization cluster in *Campylobacter,* which allows to metabolize L-fucose, *Campylobacter* was thought to be asaccharolytic (Muraoka & Zhang, 2011; Stahl et al., 2011). All of the metabolism pathways that are discussed in the next sections result in the production of pyruvate, fumarate, oxaloacetate, or 2-oxoglutarate, which are directly fed into the citric acid cycle (Stahl et al., 2012). Furthermore, several of these citric acid cycle intermediates can be directly used as nutrient sources.

Amino acid metabolism

Campylobacter's primary nutrient sources are amino acids, which can be acquired in the human gut. Preferred amino acids used by *Campylobacter* are serine, aspartate, asparagine, glutamate and proline (Stahl et al., 2012; Hofreuter, 2014). The utilization of these amino acids occur in sequential phases. Firstly, serine and aspartate are catabolized, facilitating early rapid growth of *Campylobacter*. Next, asparagine and glutamate are catabolized, followed by proline, which is metabolized less rapidly, in comparison to the other amino acids (Leach et al., 1997; Weingarten et al., 2009; Wright et al., 2009; Stahl et al., 2012; Hofreuter, 2014). Interestingly, while serine is the most preferred carbon source of *Campylobacter*, not all isolates are able to grow with it as sole carbon source. Even though the serine metabolism genes (*sdaA and sdaC)* of these isolates possessed no mutations, the serine dehydratase activity was fairly reduced (Hofreuter et al., 2008; Hofreuter et al., 2012). Utilization of the amino acids aspartate, glutamate and proline was a more conserved metabolic trait (Hofreuter et al., 2012).

Organic acids

In the human gastro-intestinal tract, large amounts of organic acids (such as short-chain fatty acids) are produced as by-product of the microbiota. The most significant organic acid that is present is acetate (Duncan et al., 2004). Interestingly, during *Campylobacter's* primary metabolism of compounds such as serine, acetate is produced as by-product. However, as preferable nutrients exhaust, *Campylobacter* will use acetate as carbon source (Stahl et al., 2012). Apart from acetate, also lactate is a by-product that is found in the gut, however, unlike with acetate, *Campylobacter* does not excrete lactate. Once inside the cell, lactate is converted to pyruvate which is directly fed into the citric acid cycle (Thomas et al., 2011). Moreover, even though no pyruvate transporters have been identified, *Campylobacter* is able to utilize pyruvate as primary carbon source from the medium (Velayudhan & Kelly, 2002).

L-fucose and D-glucose utilization

The epithelial cells in the human gastro-intestinal tract have fucose incorporated in glycan structures. Many bacterial inhabitants of the gut, such as *Bacteroides spp.,* possess fucosidases and are able to free L-fucose into the gut, making the gut a fucose-rich environment. Approximately 65% of the sequenced *C. jejuni* isolates and 73% of the sequenced *C. coli* isolates possess the L-fucose utilization cluster (*Cj0480c – Cj0490* in *C. jejeuni* NCTC11168), so called fuc+ isolates (Dwivedi et al., 2016). Isolates possessing this cluster showed increased growth, differences in biofilm formation and increased chemotaxis towards L-fucose (Muraoka & Zhang, 2011; Stahl et al., 2011; Dwivedi et al., 2016). Recently, it was discovered that not only L-fucose could be metabolized via the L-fucose utilization cluster, but also D-arabinose, which is a sugar that is most often found in rosebuds, providing *Campylobacter* with growth benefits (Garber et al., 2020).

Not much longer after the discovery of the L-fucose utilization cluster, the D-glucose utilization cluster was discovered in some *Campylobacter* isolates. Systematic searches for genes of the Entner-Doudoroff (ED) pathway revealed that 1.7% of all *C. jejuni* and *C. coli* isolates possess the genes encoding the complete ED pathway, so called gluc+ isolates. Gluc+ isolates have shown increased growth and biofilm formation on D-glucose (Vorwerk et al., 2015; Vegge et al., 2016). However, due to the large range of isolates from different sources it is currently not clear what the role of the glucose utilization cluster for *Campylobacter* is.

Adaptation of *Campylobacter* to different environments

Campylobacter possesses a large number of phase-variable loci, allowing it to generate genotypic and phenotypic diversity (Burnham & Hendrixson, 2018). *Campylobacter* is able to quickly spread genetic advantageous traits between isolates and can quickly form new combinations of genetic alleles due to its natural competence which allows it to uptake DNA (Boer et al., 2002). Furthermore, several genome-wide association studies that compared genetic variation between human pathogen isolates and poultry isolates, discovered that many mutations within genes occurred, leading to a large genetic and phenotypic variation (Buchanan et al., 2017; Yahara et al., 2017).

Natural transformation and horizontal gene transfer

Even though *C. jejuni* is heterogenous with only a small amount of clonality in population structures, which contributes to intraspecies variation, it can be structured into clusters of related isolates (Burnham & Hendrixson, 2018). Due to this heterogeneity, *Campylobacter* possesses a large gene pool which it can use for genomic recombination, adaptation and evolution, allowing it to evolve during colonization or transmission from host to host. *Campylobacter* is naturally competent, allowing it to evolve via horizontal gene transfer (HGT) and recombination (Wang & Taylor, 1990; Boer et al., 2002). Furthermore, *C. jejuni* does not necessarily need selection pressure for new genomic rearrangements (Boer et al., 2002; Ridley et al., 2008). Interestingly, *Campylobacter* is also able to perform HGT from one species to another, for example from *C. coli* to *C. jejuni* and it highly discriminates the type of foreign DNA, with a very high selectivity for DNA of *Campylobacter* species (Wassenaar et al., 1993a; Guernier-Cambert et al., 2021). To be able to uptake DNA from the environment, *C. jejuni* uses an uptake system for transporting DNA across the membrane barriers. Next, the recombinase protein RecA allows the integration of this foreign DNA into the chromosome (Bacon et al., 2000; Wiesner et al., 2003; Jeon & Zhang, 2007; Jeon et al., 2008; Gaasbeek et al., 2009; Beauchamp et al., 2015). Interestingly, *C. jejuni* does not naturally uptake cloned DNA as studies showed that RAATTY methylation is required on the transformed DNA. When DNA lacks the RAATTY methylation, it is not transported into the cell (Beauchamp et al., 2017).

Genomic differences between *Campylobacter* isolates

In general, typing of *Campylobacter* is done via multilocus sequence typing (MLST) (Dingle et al., 2001). With the use of seven housekeeping genes (*spA*, *atpA*, *glnA*, *gltA*, *glyA*, *pgm*, and *tkt*), unique allelic profiles are assigned to a sequence type (ST) (Miller et al., 2005). As there are many different STs, they can further be clustered into clonal complexes (CCs), which is based on two or more independent isolates that share identical alleles at five or more of the housekeeping genes (Miller et al., 2005). Currently, certain STs are associated with certain host isolates. For example, ST-21, ST-48 and ST-206 are often associated with human disease, while ST-45, ST-42 and ST-353 often represent water/wildlife isolates (Stabler et al., 2013; Mughini-Gras et al., 2021).

Apart from the different CCs where *Campylobacter* isolates belong to, clear signs of environmental adaptations in the genome of *Campylobacter* can be observed, which lead to different phenotypic behaviors. For example, studies showed that waterborne *C. jejuni* isolates are able to survive low temperatures and develop aerotolerance (Shagieva et al., 2021a). Furthermore, sequencing studies of waterborne isolates discovered deletions of genomic regions, which are involved in chicken colonization or in virulence (Hepworth et al., 2011).

Taken together, *Campylobacter* isolates from different sources are genetically different from each other. However, due to *Campylobacters* natural competence, it has the ability to adapt rapidly to new environments, highlighting the importance of Whole Genome Sequencing (WGS) studies in *Campylobacter*.

Outline of thesis

The objective of this thesis was to investigate how specific genetic and metabolic factors may contribute to *Campylobacter's* environmental persistence, and how these factors affect virulence, populations structure and epidemiology of this microorganism.

Chapter 1 provides a general introduction to *Campylobacter*, highlighting its physiology, metabolism, environmental reservoirs and transmission routes, genetic diversity and disease mechanics.

Chapter 2 describes the carbohydrate utilization cluster that allows *C. jejuni* and *C. coli* to utilize L-fucose as an energy source. Long-term culturability studies provided further insight into the importance of this gene cluster for selected *Campylobacter* isolates. The impact of L-fucose metabolism on the general metabolism and morphology of *Campylobacter* isolates was also analyzed.

Chapter 3 provides a continuation of Chapter 2 in which additional analyses on the role of the L-fucose utilization cluster were performed. *In vitro* virulence assays with Caco-2 cells and fibronectin-coated 96-wells plates highlighted the role of L-fucose and D-arabinose metabolism during the pathogenesis of the human *C. jejuni* isolate NCTC11168 that carried the L-fucose utilization cluster. Furthermore, proteomics analyses highlighted several proteins that were linked to the observed virulence phenotypes.

Chapter 4 focuses on one specific *C. jejuni* isolate, namely, *C. jejuni* 18-440. This specific isolate carries an atypical L-fucose utilization cluster, obtained via horizontal gene transfer from *C. coli*, and a glucose utilization cluster. Long-term culturability studies were performed to get insight into the metabolism of L-fucose and D-glucose by this specific isolate. Proteomics analyses were performed to gain new insights into the L-fucose and D-glucose utilization clusters.

Chapter 5 utilizes a bioinformatic approach to investigate the distribution of L-fucose and D-glucose utilization clusters in *C. jejuni* and *C. coli*. Correlation analysis between human isolates and fucose-positive isolates was performed in order to investigate whether fuc+ *C. jejuni* isolates are more likely to survive and proliferate in the human

host. Finally, genetic analysis on L-fucose utilization clusters was performed, leading to new insights in cluster variations and horizontal gene transfer.

Chapter 6 provides a general discussion in which main results of this thesis are discussed and how these traits contribute to the environmental persistence, transmission and virulence of *Campylobacter*. Furthermore, new insights in the lipooligosaccharide synthesis, metal acquisition, metabolism, membrane proteins and virulence proteins of *C. jejuni* are discussed.

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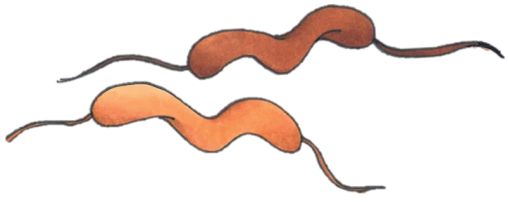

2

Comparative analysis of L-fucose utilization and its impact on growth and survival of *Campylobacter* isolates

Pjotr S. Middendorf[1,2], Wilma F. Jacobs-Reitsma[2]; Aldert L. Zomer[3,4],

Heidy M. W. den Besten[1], and Tjakko Abee[1]

Affiliations:

1. Food Microbiology, Wageningen University, Wageningen, the Netherlands
2. National Institute for Public Health and the Environment, Bilthoven, the Netherlands
3. Faculty of Veterinary Medicine, Department of Infectious Diseases and Immunology, Utrecht University, Utrecht, the Netherlands
4. WHO Collaborating Center for Campylobacter/OIE Reference Laboratory for Campylobacteriosis, Utrecht, the Netherlands.

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Abstract

Campylobacter jejuni and *Campylobacter coli* were previously considered asaccharolytic, but are now known to possess specific saccharides metabolization pathways, including L-fucose. To investigate the influence of the L-fucose utilization cluster on *Campylobacter* growth, survival and metabolism, we performed comparative genotyping and phenotyping of the *C. jejuni* reference isolate NCTC11168 (human isolate), *C. jejuni* Ca1352 (chicken meat isolate), *C. jejuni* Ca2426 (sheep manure isolate), and *C. coli* Ca0121 (pig manure isolate), that all possess the L-fucose utilization cluster.

All isolates showed enhanced survival and prolonged spiral cell morphology in aging cultures up to day seven in L-fucose-enriched MEMα medium (MEMαF) compared to MEMα. HPLC analysis indicated L-fucose utilization linked to acetate, lactate, pyruvate and succinate production, confirming the activation of the L-fucose pathway in these isolates and its impact on general metabolism. Highest consumption of L-fucose by *C. coli* Ca0121, is conceivably linked to its enhanced growth performance up to day 7, reaching 9.3 log CFU/ml compared to approximately 8.3 log CFU/ml for the *C. jejuni* isolates. Genetic analysis of the respective L-fucose clusters revealed several differences, including a 1 bp deletion in the *Cj0489* gene of *C. jejuni* NCTC11168, causing a frameshift in this isolate resulting in two separate genes, *Cj0489* and *Cj0490*, while no apparent phenotype could be linked to the presumed frameshift in the NCTC11168 isolate. Additionally, we found that the L-fucose cluster of *C. coli* Ca0121 was most distant from *C. jejuni* NCTC11168, but confirmation of links to L-fucose metabolism associated phenotypic traits in *C. coli* versus *C. jejuni* isolates requires further studies.

Introduction

Campylobacter is a leading cause of gastroenteritis in humans worldwide (Man, 2011; Devleesschauwer et al., 2017). The incidence and prevalence of campylobacteriosis has increased over the past few years in both developed and developing countries (Kaakoush et al., 2015; Špačková et al., 2019; Tack et al., 2019; EFSA, 2021). Most common *Campylobacter* species causing gastroenteritis are *Campylobacter jejuni* and *Campylobacter coli* , causing 83% and 10% of all human cases, respectively (Blaser & Engberg, 2008; EFSA, 2021). Studies reported that *C. jejuni* can infect and cause diarrhea with a relatively low infection dose, generally causing symptoms like gastroenteritis with acute watery or bloody diarrhea, fever and abdominal pain (Black et al., 1988; Galanis, 2007; Hara-Kudo & Takatori, 2011; Kaakoush et al., 2015). Post-infection complications include the severe Guillain-Barré syndrome and Miller-Fisher syndrome (Rees et al., 1995). Most infections are related to incidents that have been reported due to consumption of meat products predominantly poultry, direct contact with animals and via environmental waters (Vellinga & Van Loock, 2002; Clark et al., 2003; Kuusi et al., 2004; Moore et al., 2005; Karagiannis et al., 2010; Kuhn et al., 2017; Montgomery et al., 2018; Pedati et al., 2019; Kenyon et al., 2020).

Interestingly, *Campylobacter* is generally recognized as being susceptible to a wide variety of environmental stresses (Solomon & Hoover, 1999; Park, 2002; Mihaljevic et al., 2007; Garénaux et al., 2008; Bui et al., 2012). However, outside the animal and human gastro-intestinal tract, *Campylobacter* is able to survive and has therefore a certain degree of environmental robustness which is needed to endure environmental transmission (Sulaeman et al., 2012; Turonova et al., 2015; Rodrigues et al., 2016). Nutrient availability and acquisition support *Campylobacter* transmission between different animal hosts and the human host (Stahl et al., 2012; Gao et al., 2017). Several studies dedicated to the characterization of substrate utilization in *Campylobacter* spp. showed an important role for citric acid cycle intermediates, amino acids and peptides in supporting growth (Parsons, 1984; Hofreuter, 2014; Vorwerk et al., 2014). Preferred amino acids used by *C. jejuni* include serine, aspartate, asparagine, and glutamate (Hofreuter et al., 2008; Wright et al., 2009; Wagley et al., 2014). *Campylobacter* was previously thought to be asaccharolytic, lacking most key enzymes to metabolize sugars. However, more recently, evidence was provided that selected *C.*

coli and *C. jejuni* isolates can metabolize glucose and/or L-fucose (Muraoka & Zhang, 2011; Stahl et al., 2011; Dwivedi et al., 2016; Vegge et al., 2016; van der Hooft et al., 2018; Garber et al., 2020). A systematic search for Entner–Doudoroff (ED) pathway genes encoding glucose utilization enzymes in a wide range of *C. coli* and *C. jejuni* isolates from clinical, environmental and animal sources, and in the *C. jejuni*/*coli* PubMLST database, revealed that 1.7% of the more than 6,000 available genomes encoded a complete ED pathway involved in glucose metabolism (Vegge et al., 2016). Based on additional phenotyping, it was concluded that some glucose-utilizing *C. coli* and *C. jejuni* isolates exhibit specific fitness advantages, including stationary-phase survival and biofilm production, highlighting key physiological benefits of this pathway in addition to energy conservation (Vegge et al., 2016; Vorwerk et al., 2015).

Notably, comparative WGS analysis revealed that approximately 65% of the sequenced *C. jejuni* isolates and 73% of the sequenced *C. coli* isolates possess the L-fucose utilization cluster (designated *Cj0480c – Cj0490* in *C. jejuni* NCTC11168), so called fuc+ isolates. The L-fucose utilization cluster is regulated by *Cj0480c* and contains 2 predicted transporters encoded by *Cj0484* and *Cj0486* (FucP). After L-fucose transport into the cell, L-fucose is further metabolized to the end products pyruvic acid and lactic acid via the metabolic enzymes encoded by C*j0488, Cj0485* (FucX*), Cj0487, Cj0482*/*Cj0483, Cj0481* (DapA) and *Cj0489*/*Cj0490* (Dwivedi et al., 2016; Garber et al., 2020; Stahl et al., 2011). Both, pyruvate and lactate can be further metabolized and support growth of *C. jejuni* and *C. coli* (Stahl et al., 2012; Thomas et al., 2011)

Putative roles for L-fucose in fuc+ *C. jejuni* isolates in growth, biofilm formation and virulence have been reported, however no data are available on L-fucose metabolism in *C. coli* and its impact on growth and survival (Dwivedi et al., 2016; Muraoka & Zhang, 2011; Stahl et al., 2011). The human gastro-intestinal tract is a fucose-rich environment with fucose incorporated in glycan structures found on epithelial cells (Becker & Lowe, 2003; Pickard & Chervonsky, 2015). These fucosylated glycans can be hydrolyzed by fucosidases produced by a range of bacterial inhabitants of the gut such as *Bacteroides* spp.. *C. jejuni* does not possess any obvious fucosidase homologs, however, *C. jejuni* can forage on L-fucose released by *Bacteroides vulgatus* in co-cultures with porcine mucus as a substrate (Garber et al., 2020). Furthermore,

fucosylated glycans are not only commonly used as carbon source but can also serve as adhesion sites or receptors for pathogens like *Helicobacter pylori* and *C. jejuni* (Boren et al., 1993). Previous research in *Campylobacter* has shown that fucose monomers and fucosylated glycans serve as chemoattractant for *C. jejuni*, supporting adherence to epithelial cell surfaces containing such glycans (Hugdahl et al., 1988; Day et al., 2009).

Despite the potential role of L-fucose in the intestinal ecology and infection efficacy of *C. jejuni*, relatively little is known about the activation of L-fucose metabolism and the impact on stationary phase survival in fuc+ *Campylobacter* isolates. Interestingly, in human disease *C. coli* is less prevalent in comparison with *C. jejuni*, however, the L-fucose utilization cluster is more common among *C. coli* isolates (Dwivedi et al., 2016). A comparative analysis of the efficacy and metabolite formation following activation of L-fucose utilization clusters in *C. jejuni* and *C. coli* isolates, combined with impact on growth and survival, has not been reported up to now.

In this study we quantified L-fucose utilization and impact on metabolism of amino acids and short chain (di)carboxylic acids including acetate, lactate and succinate, and long-term culturability of three fuc+ *C. jejuni* isolates and one fuc+ *C. coli* isolate, and correlated this to genetic features of respective L-fucose utilization clusters of the tested *Campylobacter* isolates.

Materials and Methods

Bacterial isolates and culture preparation

The following *Campylobacter* isolates were used during this study*: C. jejuni* NCTC11168 (reference isolate) isolated from human feces, *C. jejuni* Ca1352 isolated from chicken meat, *C. jejuni* Ca2426 isolated from sheep manure and *C. coli* Ca0121 isolated from pig manure. *Campylobacter* stock cultures were prepared using BactoTM Heart Infusion broth (Becton, Dickinson and Company, Vianen, the Netherlands) and were grown for 24 h at 41.5 °C in microaerobic conditions (5% O_2, 10% CO_2, 85% N_2) which was created using an Anoxomat WS9000 (Mart Microbiology, Drachten, the Netherlands). Glycerol stocks were prepared using 30% glycerol and 70% overnight culture and they were stored at -80 °C.

Routinely, prior to growth experiments, the *Campylobacter* freezer stocks were streaked on Columbia Agar Base (CAB (Oxoid, Landsmeer, the Netherlands)) plates supplemented with 5% (v/v) lysed horse blood (BioTrading Benelux B.V. Mijdrecht, the Netherlands) and 0.5% bacteriological agar No.1 (Oxoid) for optimal recovery. The plates were incubated in anaerobic jars for 24 h at 41.5 °C in microaerobic conditions. Colonies were routinely selected and grown overnight in 10 mL GibcoTM MEMα medium (Thermo Fisher Scientific, Bleiswijk, the Netherlands) (see Table. S1 for medium details) supplemented with 20 µM $FeSO_4$ (Merck Schiphol-Rijk, the Netherlands), 10 mL MH2 broth (Merck) or 10 mL MH3 broth (Oxoid). A second overnight culture was made by diluting the *Campylobacter* suspension 1:100 in 10 mL fresh MEMα, MH2 or MH3 medium. The suspension was incubated at 41.5 °C for 24 h in microaerobic conditions to obtain standardized working cultures for use in further experiments.

L-fucose growth experiments

Prior to growth experiments, MEMα medium was supplemented with 21.0 mM L-fucose (MEMαF medium) and filter-sterilized using 0.2-µm pore sized filters. Infusion bottles were closed using a rubber stopper and aluminum cap and next they were sterilized. Sterilized 100 mL infusion bottles were filled with 45 mL filter-sterilized

MEMα medium or MEMαF medium by using a syringe. Filled infusion bottles were stored at 4 °C until further use.

Working cultures were decimally diluted in MEMα medium to a cell concentration of approximately 10^5 CFU/mL. A final dilution step was done by adding 5 mL into the infusion bottles filled with 45 mL MEMα medium or MEMαF medium, resulting in a starting cell concentration of 10^4 CFU/mL. Incubation of the inoculated infusion bottles was done at 37 °C. Daily, approximately 4 mL sample was taken from each infusion bottle, starting at day 0. After each sampling point, the head space of infusion bottles was flushed for 2 min with microaerobic gas (5% O_2, 10% CO_2, 85% N_2) using a home-made gas flushing device using syringes to puncture the rubber stopper.

The samples were used to determine the bacterial concentration and for microscopic analysis. The remainder of each sample was stored at -20 °C for high pressure liquid chromatography (HPLC) analyses. Bacterial concentrations were determined by decimally diluting 1 mL of sample in peptone physiological salt solution (PPS, Tritium Microbiologie, Eindhoven, the Netherlands), followed by surface plating on CAB plates. CAB plates were incubated in jars for 48 h at 41.5 °C in microaerobic conditions. Colonies were counted and expressed in $\log_{10}$ CFU/mL. Each sample was microscopically analyzed using an Olympus BX 41 microscope (lens Ach 100x/1.25, Olympus Nederland, Leiderdorp, the Netherlands) and pictures were captured using CellSens Imaging software (Olympus Corporation). Three biologically independent reproductions were performed per condition, i.e. MEMα medium, and MEMαF medium, on different days.

High pressure liquid chromatography for organic acids

Samples obtained from the growth experiments were centrifugated at 13,000 *g* at 4°C for 5 min. Pellets were removed and the supernatant was treated for protein decontamination with Carrez A ($K_4FeCN)_6.3H_2O$, Merck) and B ($Z_nO_4{\cdot}7H_2O$, Merck). After centrifugation, the supernatant was added to HPLC vials. Quantitative analyses were done using standards with pre-made concentrations for L-fucose, acetate, alpha-ketoglutarate, succinate, pyruvate and lactate. The HPLC was performed on an Ultimate 3000 HPLC (Dionex, Sunnyvile, USA) equipped with an RI-101 refractive index detector (Shodex, Kawasaki, Japan), an autosampler and an ion-exclusion

Aminex HPX – 87H column (7.8 × 300 mm) with a guard column (Bio-Rad, Hercules, CA). As mobile phase, 5 mM H_2SO_4 (Merck) was used at a flow rate of 0.6 mL/min. Column temperature was kept at 40 °C. For each run, the injection volume was 10 µL and the run time 30 min. Chromeleon software (Thermo Fisher Scientific, Waltham, USA) was used for quantification of compound concentrations.

High pressure liquid chromatography for amino acids

Samples obtained from the growth experimented were used in aliquots of 40 µL. These aliquots were kept on ice and were diluted with 50 µL of 0.1 M HCl (containing 250 µM Norvalin as internal standard, Merck). The samples were deproteinized by addition of 10 µL of cold 5-sulphosalicilic acid (SSA, Merck) (300 mg/ml) and centrifuged at 13,000 *g* at 4°C for 10 min. In order to obtain an optimal pH for derivatization (pH between 8.2 to 10.0), approximately 60 to 150 µL of 4N NaOH was added to 5 mL of the AccQ•Tag™ Ultra borate buffer (Borate/NaOH buffer, Waters, Milford, USA). For derivatization 60 µL of Borate/NaOH was added to a total recovery vial. Twenty µL of the supernatant obtained after deproteinization of the plasma was added and mixed. To each of the vials 20 µL of AccQ Tag Ultra derivatization reagent (Waters) dissolved in acetonitrile was added and mixed for 10 s. Each vial was immediately capped. The vials were then heated for 10 min at 55 °C. The vials were stored at -20°C prior to HPLC analysis. Quantitative analyses were done using standards with pre-made concentrations for, histidine, asparagine, serine, glutamine, arginine, glycine, aspartic acid, glutamic acid, threonine, alanine, proline, cysteine, lysine, tyrosine, methionine, and valine. HPLC was performed on an Ultimate 3000 HPLC (Dionex) equipped with an RI-101 refractive index detector (Shodex), an autosampler and an ion-exclusion Aminex HPX – 87H column (7.8 × 300 mm) with a guard column (Bio-Rad). As mobile phase, eluants A and B (Waters) was used at a flow rate of 0.7 mL/min. Column temperature was kept at 55 °C. For each run, the injection volume was 1 µL and the run time 17 min. Chromeleon software (Thermo Fisher Scientific) was used for the determination of compound concentrations. Baseline separation was obtained for all amino acids except glutamine and arginine.

Genomic analyses

The sequence of NCTC11168 was obtained from the public collection of genbank with accession number AL111168. The sequences of the assembled genomes of *C. coli* Ca0121, *C. jejuni* Ca1352 and *C. jejuni* Ca2426 were obtained via the Netherlands Food and Consumer Product Safety Authority (NVWA) and are described in (Mughini-Gras et al., 2021).

Genome alignments were performed using the online Benchling software (www.benchling.com). Each gene was translated into an amino acid sequence which was used for further alignments. Protein interactions were analysed with STRING (https://string-db.org/). Alignments were visualized by using the T-Coffee or clinker (https://github.com/gamcil/clinker). Box shade figures were generated using BOX-SHADE 3.21 (https://embnet.vital-it.ch/software/BOX_form.html) using the RTF_old output format.

Statistical analyses

Differences in $\log_{10}$-counts observed for growth in MEMα medium and growth in MEMαF medium were statistically tested using a two-tailed Student's *t*-test. P values ≤ 0.05 were considered as significant difference.

Results

Diversity in growth performance of *Campylobacter* in the absence and presence of L-fucose

We investigated growth and survival of different *C. jejuni* host isolates and one *C. coli* host isolate; namely *C. jejuni* NCTC11168 (human stool isolate), *C. jejuni* Ca1352 (chicken meat isolate), *C. jejuni* Ca2426 (sheep manure isolate) and *C. coli* Ca0121 (pig manure isolate), in MEMα medium and MEMαF medium up to 7 days. At day 1 and 2, no difference was observed between growth in MEMαF medium and MEMα medium for the tested isolates and at day 2 cell concentrations reached were 8.4, 8.2, 8.3 and 8.6 $\log_{10}$ CFU/mL, for *C. jejuni* NCTC11168, Ca1352, Ca2426 and *C. coli* Ca0121, respectively (Fig. 1A-D). However, from day 3 onwards, cell viability decreased in the absence of L-fucose and significantly lower cell counts were observed from day 3 until day 7, at which final cell counts reached 1.0, 4.4, 5.0 and 5.7 $\log_{10}$ CFU/mL in MEMα medium for isolates *C. jejuni* NCTC11168, Ca2426, Ca1352 and *C. coli* Ca0121, respectively. In MEMαF, cell concentrations did not decrease up to day 4 or 5, after which lower or stable cell concentrations were observed for isolate *C. jejuni* NCTC11168, Ca2426 and Ca1352 (Fig. 1A-C). Notably, *C. coli* Ca0121 showed continuation of growth after day 2, reaching 9.3 $\log_{10}$ CFU/ml at day 7 (Fig. 1D). Linking cell counts to morphology using microscopic images revealed that in MEMα medium, coccoid cells were commonly observed, increasingly over time after day 2, while in MEMαF medium, higher quantities of spiral-shaped cells were observed for all tested isolates (Fig. S1). In line with the observed increase in plate counts, isolate *C. coli* Ca0121 showed almost no increase in the amount of coccoid cells overtime when grown in MEMαF medium (Fig. S1).

HPLC analyses confirmed absence of L-fucose in MEMα medium and enabled quantitative analysis of L-fucose consumption by the tested isolates in MEMαF medium. The L-fucose concentration decreased after day 1 for all four isolates. Interestingly, the *C. coli* isolate showed the highest and fasted L-fucose consumption over time, which was in line with the robust growth performance of this isolate.

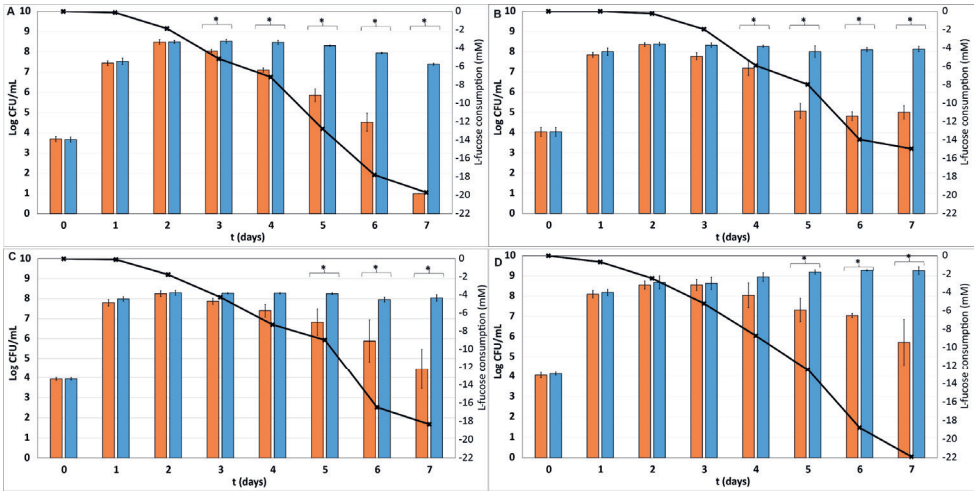

Figure 1 Quantification of planktonic growth for A) isolate *C. jejuni* NCTC11168 (human stool isolate), B) *C. jejuni* Ca1352 (chicken meat isolate) C) *C. jejuni* Ca2426 (sheep manure isolate) and D) *C. coli* Ca0121 (pig manure isolate), in MEMα medium (orange bars) and MEMαF medium (blue bars). The black line shows consumption of L-fucose over time. Each value represents the average of 3 biologically independent replicates, and error bars show the standard deviation. The asterisks indicate significant ($P<0.05$) differences in cell counts after incubation in MEMα medium and MEMαF medium.

Metabolite concentrations in *C. jejuni* and *C. coli* isolates when grown in MEMα or MEMαF medium

We determined the impact of L-fucose on concentrations of metabolites and amino acids during growth in MEMα. Lactate production was not detected in MEMα medium after growth (Fig. 2). In MEMαF medium, up to 1.1 to 1.75 mM lactate was produced by all tested isolates up to day 5 and remained rather stable afterwards (Fig. 2). Starting concentrations of 0.25 mM of pyruvate were found to be depleted at day 2 in MEMα medium, whereas in MEMαF medium pyruvate concentrations remained at these low levels in *C. jejuni* NCTC11168 and *C. coli* Ca0121, and increased up to 0.6 mM and 1.3 mM in *C. jejuni* Ca1352 and Ca2426, respectively (Fig. 2). In MEMα medium low concentrations of acetate (<1 mM) were initially produced up to day 2 by all tested isolates and consumed later on (Fig. 2), indicative of the acetate switch (Wright et al. 2009). In MEMαF medium, higher amounts of acetate were produced and concentrations increased until day 7 in isolates *C. jejuni* NCTC11168, Ca1352 and Ca2426 acetate reaching 4.4 mM, 4.1 mM and 2.9 mM, respectively. Notably, *C. coli*

Ca0121 produced highest levels of acetate at day 5 (6.3 mM), and the lower level at day 7 pointed to acetate consumption, pointing to a delay of the acetate switch, in line with depletion of L-fucose in the medium. We observed a slight increase in succinate concentrations up to day 3, reaching 0.25 mM with *C. jejuni* isolates NCTC11168, Ca1352 and Ca2426, followed by consumption of succinate in both media (Fig. 2). Notably, *C. coli* Ca0121 produced highest levels of succinate up to day 3 in MEMα and up to day 5 in MEMαF, reaching 0.6 mM and 0.8 mM, respectively, after which succinate levels decreased in both media.

Analysis of amino acid metabolism showed rapid depletion of serine and aspartic acid for all tested isolates in MEMα and MEMαF (Fig. 3 and Fig. S3). Glutamic acid and proline were depleted after 3 days of incubation, while asparagine decreased also but remained present at low levels (0.1-0.15 mM). Notably, a strong depletion of the sulphur-containing metabolite cystine in MEMαF medium with all *C. jejuni* isolates was observed in the first 2 or 3 days, whereas no significant consumption was observed in MEMα medium (Fig. 3).

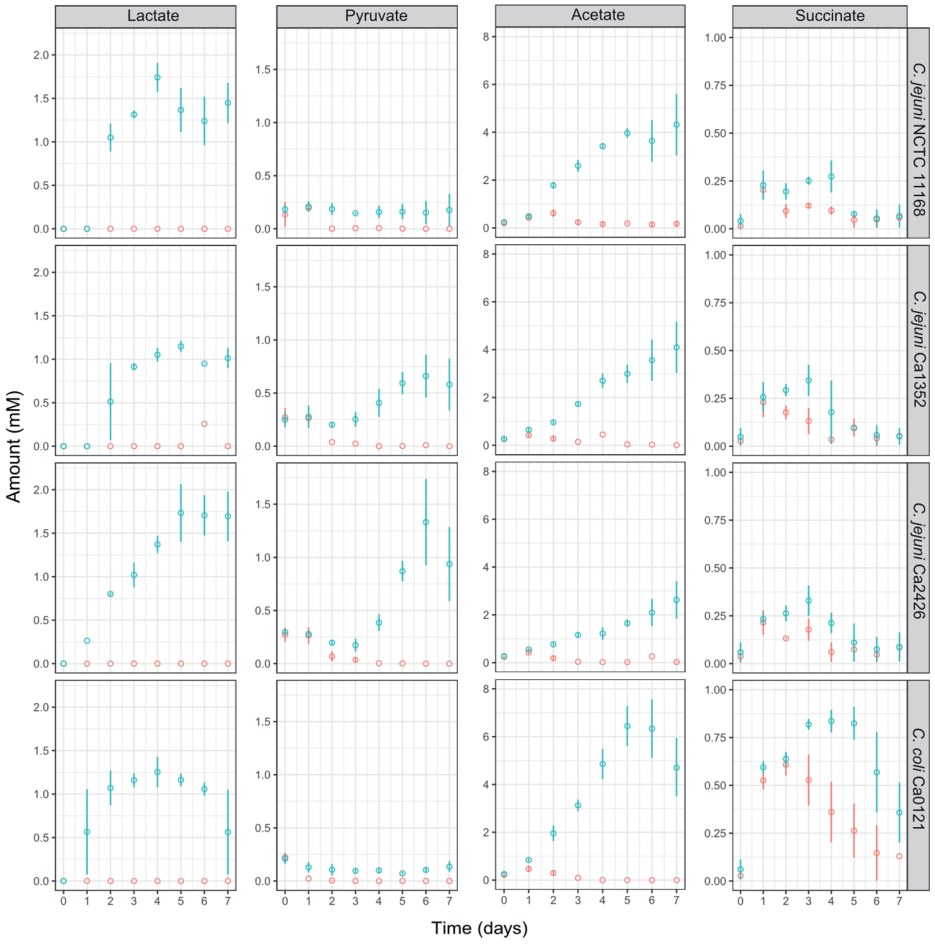

Figure 2 Compound production and consumption of selected *Campylobacter* isolates in MEMαF medium (blue dots) and MEMα medium (red dots) after a 7-day incubation period. Columns correspond to different compounds and each row shows results for a different isolate. Each value represents the average of three biologically independent replicates, and error bars show the standard deviation.

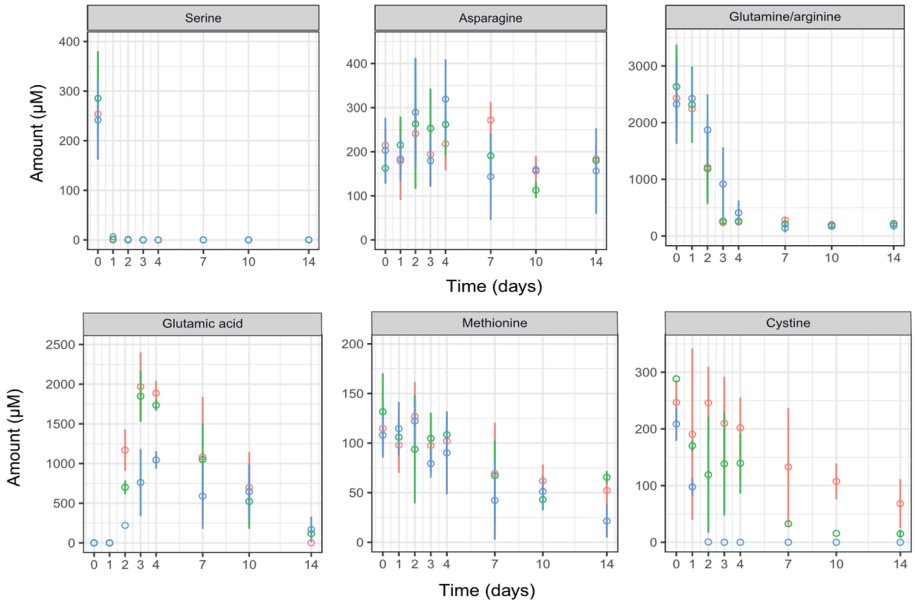

Figure 3 Amino acid production and consumption of selected *Campylobacter* isolates in MEMαF medium (blue dots) and MEMα medium (red dots) after 7-day incubation period. Columns correspond to different amino acids and each row shows results for a different isolate. Each value represents the average of three biologically independent replicates, and error bars show the standard deviation.

Comparative analysis of L-fucose utilization clusters in *C. jejuni* and *C. coli* isolates

To investigate the genomic differences between the L-fucose utilization clusters of the tested isolates, we aligned each of the protein sequences (Cj0480c – Cj0490) of isolates *C. jejuni* Ca1352, Ca2426 and *C. coli* Ca0121 to the respective protein sequences of the reference isolate *C. jejuni* NCTC11168. The corresponding putative functions of the proteins encoded by the genes together constitute the L-fucose degradation pathway (Fig. 4A). Alignments of the gene *Cj0489*, encoding an aldehyde dehydrogenase, confirmed a previously discovered frameshift in the reference isolate NCTC11168 (Muraoka & Zhang, 2011). However, this frameshift was not observed in *C. jejuni* Ca1352, Ca2426 and *C. coli* Ca0121 (Fig. 4B). The *Cj0489*-gene codes for a protein of 479 AA in *C. jejuni* Ca1352, Ca2426 and *C. coli* Ca0121, while the frameshift in the *C. jejuni* NCTC11168 genome, resulted in a shortened *Cj0489* gene, coding for a putative protein composed of 77 AA. Based on the presence of new start codon, a second larger fragment of the *Cj0489* gene, renamed *Cj0490* (Muraoka & Zhang, 2011; Stahl et al., 2011), that is predicted to code for a putative protein of 394 AA. The AA composition of truncated proteins encoded by *Cj0489-Cj0490* of *C. jejuni* NCTC11168 was highly similar to the proteins encoded by the non-truncated *Cj0489* gene of *C. jejuni* Ca1352, Ca2426 and *C. coli* Ca0121, which was 95%, 95% and 97%, respectively. Next, we aligned all encoded proteins of the L-fucose cluster and calculated the percent identities to the reference isolate NCTC11168 (Fig. 4C and Table S2). Only *Cj0482*, which encodes the altronate hydrolase/dehydratase, had 100% amino acid identity in all tested isolates. The altronate hydrolase/dehydratase encoded by *Cj0483* displayed >97% identity in all tested isolates. Clearly, the *C. jejuni* isolates Ca1352 and Ca2426 harbored more genes that displayed 100% protein identity with NCTC11168 than *C. coli* Ca0121. The L-fucose utilization cluster of *C. coli* Ca0121 was genetically the most distant from NCTC11168 with some translated genes having similarities as low as 84% such as the protein encoded by *Cj0487*.

Notably, the proteins encoded by the L-fucose utilization cluster of *C. jejuni* Ca1352 and Ca2426 displayed 100% similarity with each other on amino acid level (Table S2). Several SNPs were found between the 2 isolates, however, these did not result in single amino-acid polymorphisms.

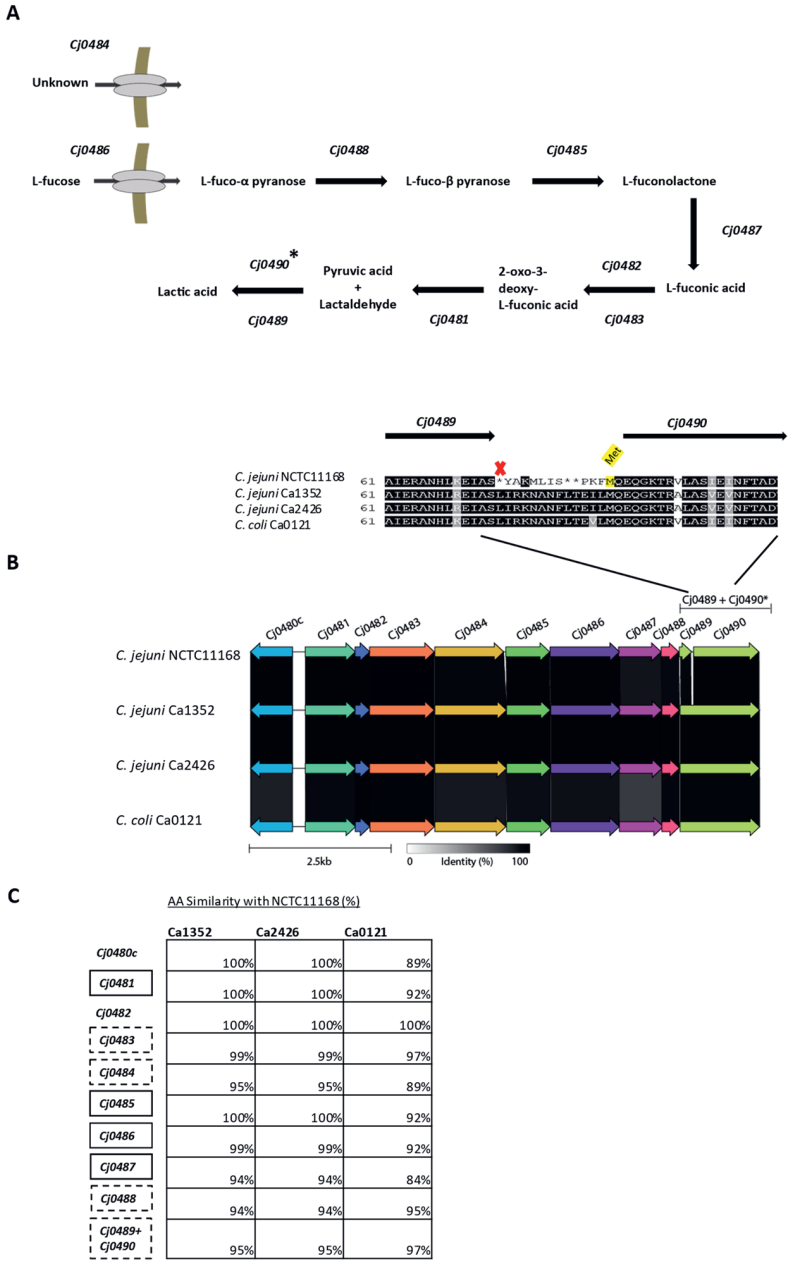

Figure 4 Genomic overview of the L-fucose utilization cluster in the four tested isolates. (A) The predicted L-fucose metabolism cluster based on previously reported research (Stahl et al., 2011; Garber et al., 2020). (B) gene alignments performed with Clinker, AA identity is displayed in grey scale. A zoom-in is shown of

the AA sequence of the Cj0489-Cj0490 gene, indicating a frameshift in isolate NCTC11168, resulting in an early stop codon (marked with an X), splitting Cj0489 (77 AA) and Cj0490 (394 AA). A putative start site (Met) of Cj0490 in C. jejuni NCTC11168, is highlighted in yellow. (C) Amino acid alignments of the genes in the isolates; *C. jejuni* Ca1352 (chicken meat isolate), *C. jejuni* Ca2426 (sheep manure isolate) and *C. coli* Ca0121 (pig manure isolate) with isolate *C. jejuni* NCTC11168 as reference (full alignment in Supplementary Figure 4). The percentages indicate AA similarities between *C. jejuni* NCTC11168 and Ca1352, Ca2426 or *C. coli* Ca0121. Similarity of Cj0489-Cj0490 of isolate *C. jejuni* NCTC11168 is compared to Cj0489 of the other isolates. Black and black dotted - boxes indicate genes to be essential or not for L-fucose metabolism based on mutant studies (34,35,37) (no data available for Cj0482 and Cj0480c).

Discussion

The L-fucose utilization cluster is present in the majority of all *C. jejuni* and *C. coli* isolates, 65% and 73%, respectively, and is predominantly found in livestock-associated *Campylobacter* isolates (Dwivedi et al., 2016). Comparative growth analysis of the four selected fuc+ *C. jejuni* and *C. coli* isolates showed that cell counts reached up to day 2 and 3 were similar in MEMα and MEMαF. This indicates, despite the initial onset of L-fucose consumption, that the MEMα medium contains sufficient energy/carbon sources to support initial growth in the selected conditions. Impact of L-fucose on growth and survival of *C. jejuni* and *C. coli* isolates, became apparent during prolonged incubation up to day 7, concomitant with the significant increase in L-fucose consumption in this period resulting in pyruvate and lactate, that can serve as substrates for growth of *Campylobacter*. In line with these observations, microscopy analysis of cells in MEMαF showed high proportions of spiral shaped cells, while coccoid-shaped cells were overrepresented in samples from non-supplemented MEMα from day 3 to day 7. This points to a nutrient deficiency in the latter medium, in line with previous studies that showed starvation as a stress factor leading to a change from helical shaped to coccoid shaped cells (Hazeleger et al., 1995; Ikeda & Karlyshev, 2012; Frirdich et al., 2017). In addition, enhanced growth of the *C. coli* isolate was reflected in a more prominent fraction of spiral shaped cells morphology for a prolonged duration in MEMαF, pointing to a more robust phenotype for this isolate compared the tested *C. jejuni* isolates. Notably, the number of culturable cells determined on MEMα plates (data not shown) and on CAB plates supplemented with lysed horse blood for optimal recovery, were similar. Similar enhanced performance of the *C. coli* isolate was observed during growth in Mueller Hinton broths, MH2 and MH3 (Fig. S2, containing 0.2% and 0.3% beef extract, respectively. No clear effect of

the measured L-fucose consumption on growth performance in MH2 and MH3 was observed for the tested *C. jejuni* isolates, reaching maximum counts of ~8.0 log CFU/mL, while, *C. coli* Ca0121 reached highest CFU counts in both MH2 and MH3, and with added L-fucose, even ~9.5 log CFU/mL was reached in MH3 (Fig. S2).

HPLC analyses confirmed that the majority of the preferred amino acids were depleted on day 3 in MEMα and MEMαF, in line with previous studies that suggested that amino acids are preferred substrates for growth of *Campylobacter* (Garber et al., 2020). Notably, cystine, the oxidized dimer form of the amino acid cysteine, was only depleted at day 2 or day 3 when L-fucose was present in the medium and fucose consumption had started, indicating that cystine depletion is linked to L-fucose metabolism, while another study concluded that sulfur-containing metabolites including cystine, result from chemical reactions (van der Hooft et al., 2018). The recent identification and characterization of a dedicated cystine transporter (*Cj0025c*) in *C. jejuni*, which is present in all our tested isolates (data not shown), next to the conceivable uptake of cystine via peptide transporters (Vorwerk et al., 2014; Man et al., 2020), may offer support for our observation that cystine depletion is linked to L-fucose metabolism in the tested *C. jejuni* and *C. coli* isolates in the current study.

Metabolite HPLC analyses of cultures grown in MEMα without and with added L-fucose demonstrated that lactate (1-1.5 mM), pyruvate (0.2-1 mM) and acetate (4-6 mM), were mainly detected when *C. jejuni* and *C. coli* strains were grown in MEMαF medium and following depletion of amino acids. Lactate and pyruvate, respective end product and intermediate of the predicted L-fucose metabolism pathway, can be further metabolized by *Campylobacter,* however, their accumulation in the medium has not been reported in previous studies (Mendz et al., 1997; Stahl et al., 2011; Thomas et al., 2011; Garber et al., 2020).

A schematic overview is presented that highlights the impact of L-fucose utilization on (general) metabolism in the tested *C. jejuni* and *C. coli* isolates (Fig. 5). L-fucose is taken up and metabolized in 5 steps to pyruvate and lactaldehyde, with the latter compound converted in step 6 into lactate. This consumption of L-fucose results in efflux and uptake/metabolism of lactate and succinate for all tested isolates, pyruvate and acetate efflux in *C. jejuni* Ca1352 and Ca2426, acetate efflux and uptake/metabolism in *C. jejuni* NCTC11168 and *C. coli* Ca0121 and low pyruvate efflux

in *C. jejuni* NCTC11168 and *C. coli* Ca0121, leading to, in some cases, transiently higher concentrations of lactate, pyruvate, acetate and succinate in the medium, suggesting feedback inhibition during initial phases of fucose utilization. Lastly, cystine consumption was found solely dependent on L-fucose metabolism in all tested isolates.

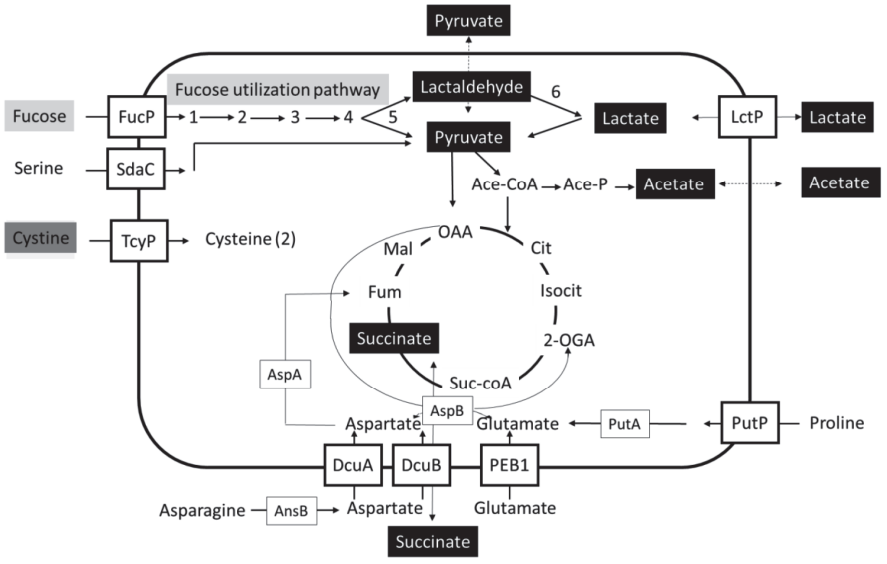

Figure 5 Schematic overview of uptake and metabolism uniquely coupled to fucose utilization in *Campylobacter*. Bidirectional arrows indicate efflux and uptake/metabolism of lactate and succinate for all tested isolates in MEMαF, pyruvate and acetate efflux in *C. jejuni* Ca1352 and Ca2426 in MEMαF, acetate efflux and uptake/metabolism in *C. jejuni* NCTC11168 and *C. coli* Ca0121 in MEMαF and succinate efflux and uptake/metabolism in all tested isolates in MEMα and in MEMαF, with slightly higher extracellular levels. The grey boxes indicate L-fucose transport and metabolism, the black boxes indicate compounds of which the production and metabolism is linked to L-fucose metabolism, and the dark grey box indicates uptake and intracellular reduction resulting in two (2) cysteines, uniquely linked to L-fucose utilization. Uptake and metabolism of serine, asparagine, aspartate, glutamate, proline and cystine with respective putative transporters are indicated. Figure adapted from Hofreuter et al., 2014. (Guccione et al., 2017; Hofreuter, 2014).

The L-fucose utilization cluster consists of 11 genes (*Cj0480c-Cj0490)* for *C. jejuni* NCTC 11168 and 10 genes (*Cj0480c-Cj0489)* for *C. jejuni* Ca1352*,* Ca2426 and *C. coli* Ca0121 and is predicted to metabolize L-fucose into lactic acid (Stahl et al., 2011; Garber et al., 2020). Interestingly, the utilization cluster contains two sets of genes with

putative overlapping functions, *Cj0482* and *Cj0483*, and *Cj0489* and *Cj0490*. Both *Cj0482* (88 AA) and *Cj0483* (389 AA), are two separated genes that are annotated as altronate hydrolases/dehydratases with similar ORFs in all tested isolates (Fig. 4). STRING analyses displayed a possible N-terminus (*Cj0482)* and possible C-terminus (*Cj0483)* of the predicted altronate hydrolase. In *C. jejuni*, only knockout studies of *Cj0483* have been performed, and this gene was found to be not essential for the metabolism of L-fucose (Stahl et al., 2011). The authors of that study also showed, by using microarrays, that both *Cj0482* and *Cj0483* were upregulated in the presence of L-fucose, 1.9 $\log_2$ and 4.4 $\log_2$ fold-change respectively (Stahl et al., 2011). No knockout studies were performed with a *Cj0482* deletion mutant, however, due to the observed activity of this gene we hypothesize that both genes encode enzymes that can perform transformation of L-fuconate into 2-oxo-3-deoxy-L-fuconate. Comparative analysis shows that only in *C. jejuni* NCTC11168, the gene *Cj0489* presents a 1 bp deletion frameshift that results in an early stop, splitting *Cj0489* into *Cj0489* and *Cj0490*, both annotated as aldehyde dehydrogenase (Javed et al., 2010; Muraoka & Zhang, 2011). In *C. jejuni* Ca1352, *C. jejuni* Ca2426 and *C. coli* Ca0121, *Cj0489* does not contain a frameshift and conceivably encodes the intact, 479 amino acid aldehyde dehydrogenase (Fig. 4B). Previous studies in *C. jejuni* NCTC11168 showed that deletion of *Cj0489* or *Cj0490* did not impair growth in the presence of L-fucose (Stahl et al., 2011; Dwivedi et al., 2016; Garber et al., 2020). Our HPLC data confirmed production of lactate in MEMαF, the final product of the L-fucose utilization pathway in all tested isolates, including NCTC11168, suggesting that L-fucose metabolism is not hampered in *C. jejuni* NCTC11168 that contains the frame shift. A recent study performed by Pascoe et al. (2019) reported the outcome of a domestication study analyzing 23 whole genome sequenced *C. jejuni* NCTC11168 isolates collected from a range of research laboratories across the UK (Pascoe et al., 2019). Our analysis of the L-fucose utilization cluster in these 23 *C. jejuni* NCTC11168 isolates showed that the fucose utilization clusters including the frameshift were 100% identical. This points to selection pressure on maintaining this L-fucose cluster with the frameshift in *Cj0489*. Whether the *Cj0489* and *Cj0490* genes in *C. jejuni* NCTC11168 encode (a) functional enzyme(s), or that there is an alternative lactaldehyde dehydrogenase induced in this isolate, remains to be elucidated.

Our comparative genotyping analysis of the four L-fucose utilization clusters, revealed that the cluster of *C. jejuni* NCTC11168 had several additional genomic differences in comparison with *C. jejuni* Ca1352, Ca2426 and *C. coli* Ca0121. Notably, genomic comparison analyses showed that the L-fucose utilization clusters of Ca1352 and Ca2426 were 100% similar, presenting only synonymous SNPs, in line with observed similarity in phenotypic behavior of these two isolates. The L-fucose utilization cluster of *C. coli* Ca0121 was the most distant from the cluster of *C. jejuni* NCTC11168. Our results showed that, in the presence of L-fucose, *C. coli* Ca0121 was able to reach the highest CFU counts, maintained a spiral morphology and completely metabolized available L-fucose in the tested conditions. These results suggest a possible link between the observed *C. coli* Ca0121 phenotype and changes in amino acid composition of enzymes in the L-fucose utilization cluster that could point to altered enzyme levels and/or functionality. However, it should be noted that the housekeeping genes of *C. jejuni* and *C. coli* share 86.5% nucleotide sequence identity and that differences in growth, morphology, survival and metabolism may also be influenced by other genomic differences (Ketley & Konkel, 2005; Sheppard et al., 2008). A recent *in vitro* study showed that locally liberated L-fucose by secreted fucosidases from other species can increase growth and invasion of *fuc* + *C. jejuni* strains at the intestinal epithelial interface (Garber et al., 2020; Luijkx et al., 2020). Similar experiments have not been reported for *C. coli*, and combined with the observation that *C. coli* lacks most of the virulence genes described for *C. jejuni* (Bravo et al., 2021; Elmi et al., 2021), impact of L-fucose utilization on *in vitro* invasion studies and human infection requires further study.

In conclusion, our study demonstrated that possessing the L-fucose cluster is not only beneficial to *C. jejuni* NCTC11168 but also to other tested *C. jejuni* isolates Ca1352 and Ca2426, and *C. coli* isolate Ca0121. All tested isolates originated from different hosts, showed enhanced survival and prolonged spiral shaped morphology in the presence of L-fucose, with the *C. coli* isolate having the most robust phenotype. Further research into *C. jejuni and C. coli* isolates may reveal whether specific L-fucose utilization cluster genotypes link with specific phenotypic behavior including inter-host and environmental transmission, and pathogenesis.

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C. jejuni Ca1352 isolated from chicken meat, *C. jejuni* Ca2426 isolated from sheep manure and *C. jejuni* Ca0121 isolated from pig manure were obtained from Netherlands Food and Consumer Product Safety Authority.

Supplementary files

Table S1 Components of 41061 - MEM alpha, nucleosides, no phenol red.

Components	mM	Components	mM
Amino Acids		**Inorganic Salts**	
Glycine	0.67	Calcium Chloride ($CaCl_2$) (anhyd.)	1.8
L-Alanine	0.28	Magnesium Sulfate ($MgSO_4$) (anhyd.)	0.81
L-Arginine	0.49	Potassium Chloride (KCl)	5.33
L-Asparagine-H_2O	0.33	Sodium Bicarbonate ($NaHCO_3$)	26.19
L-Aspartic acid	0.23	Sodium Chloride (NaCl)	117.24
L-Cysteine hydrochloride-H_2O	0.57	Sodium Phosphate monobasic (NaH_2PO_4-	1.01
L-Cystine 2HCl	0.1	**Ribonucleosides**	
L-Glutamic Acid	0.51	Adenosine	0.037
L-Glutamine	2	Cytidine	0.041
L-Histidine	0.2	Guanosine	0.035
L-Isoleucine	0.4	Uridine	0.04
L-Leucine	0.4	**Deoxyribonucleosides**	
L-Lysine	0.4	2'Deoxyadenosine	0.039
L-Methionine	0.1	2'Deoxycytidine HCl	0.042
L-Phenylalanine	0.19	2'Deoxyguanosine	0.037
L-Proline	0.35	Thymidine	0.041
L-Serine	0.24	**Other Components**	
L-Threonine	0.4	D-Glucose (Dextrose)	5.55
L-Tryptophan	0.05	Lipoic Acid	9.71E-04
L-Tyrosine disodium salt	0.23	Sodium Pyruvate	1
L-Valine	0.39		
Vitamins			
Ascorbic Acid	0.28		
Biotin	4.10E-04		
Choline chloride	0.01		
D-Calcium pantothenate	0.002		
Folic Acid	0.002		
Niacinamide	0.008		
Pyridoxal hydrochloride	0.005		
Riboflavin	2.66E-		
Thiamine hydrochloride	0.003		
Vitamin B12	0.001		
i-Inositol	0.01		

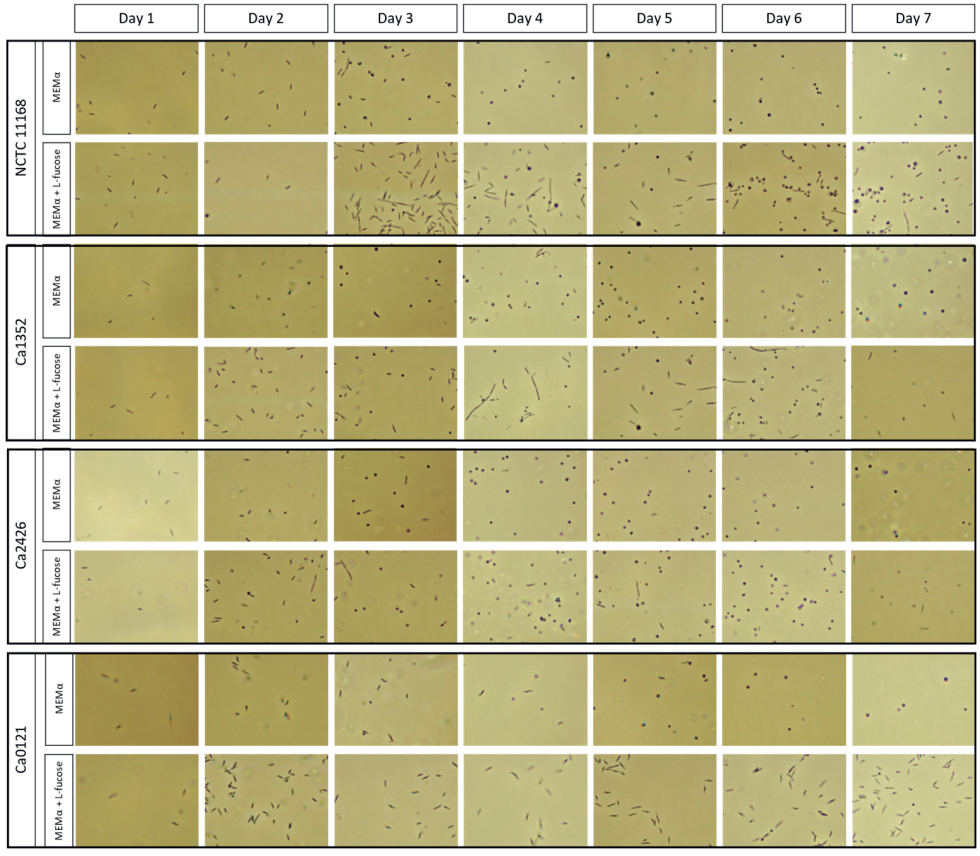

Figure S2 Morphology of *C. jejuni* NCTC11168, Ca1352, Ca2426 and *C. coli* Ca0121 during a 7 day growth experiment in MEMα or MEMαF medium.

Table S2 Overview of genes in the L-fucose utilization cluster.

		Length in AA	AA Similarity with NCTC11168 (%)		
Essential genes	**Function**	**NCTC11168/Ca1352/Ca2426/Ca0121**	**Ca1352**	**Ca2426**	**Ca0121**
Cj0486	Transporter	418/418/418/415	99%	99%	92%
Cj0485	Dehydrogenase	263/263/263/263	100%	100%	92%
Cj0487	Hydrolase	256/256/256/256	94%	94%	84%
Cj0481	DHDPS	301/301/301/301	100%	100%	92%
Non essential genes					
Cj488	Mutarotase	105/105/105/105	94%	94%	95%
Cj0482	Altronate hydrolase/dehydratase	88/88/88/88	100%	100%	100%
Cj0483	Altronate hydrolase/dehydratase	389/389/389/389	99%	99%	97%
Cj0489	Dehydrogenase G	77/479/479/479	14%	14%	15%
Cj0490	Dehydrogenase	129/129/129/129	100%	100%	99%
Extra genes in cluster					
Cj0484	Transporter	411/429/429/424	95%	95%	89%
Cj0480c	L-fucose operon regulator	253/253/253/253	100%	100%	89%

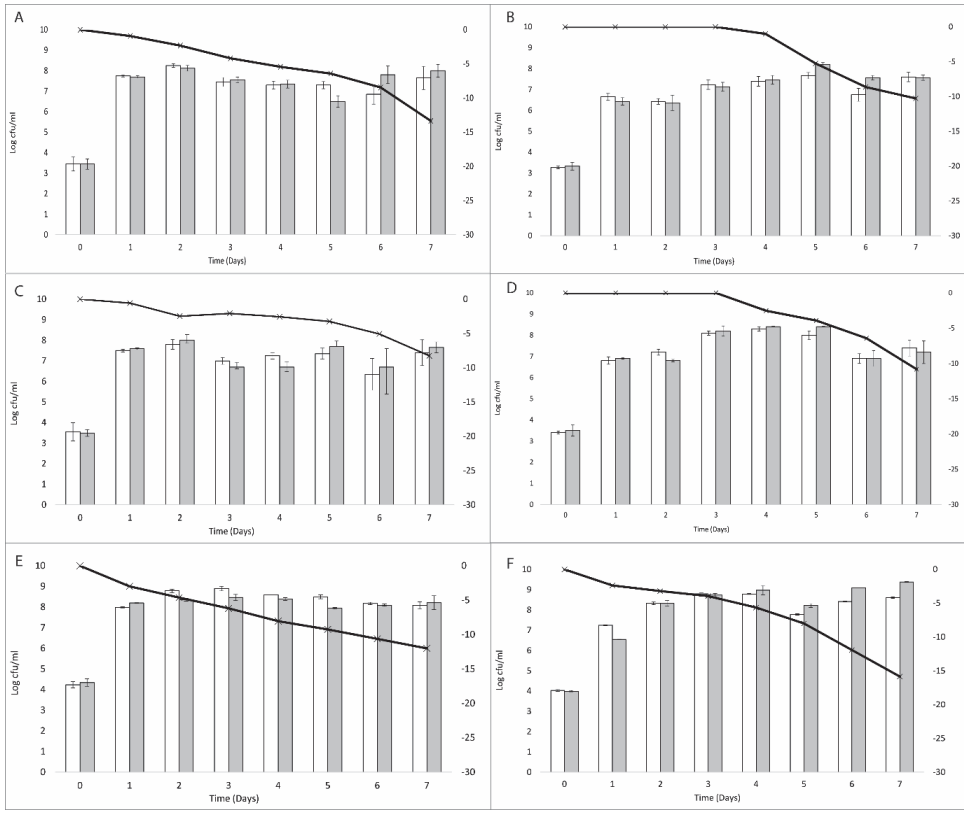

Figure S2 Quantification of planktonic growth for A) isolate *C. jejuni* NCTC11168 in MH2 medium , B) *C. jejuni* NCTC11168 in MH3 medium C) *C. jejuni* Ca2426 in MH2 medium, D) *C. jejuni* Ca2426 in MH2 medium, E) *C. coli* Ca0121 in MH2 medium and F) *C. coli* Ca0121 in MH3 medium. White bars represent MH2 or MH3 medium and grey bars represent MH2 or MH3 medium + L-fucose. The black line shows consumption of L-fucose over time.

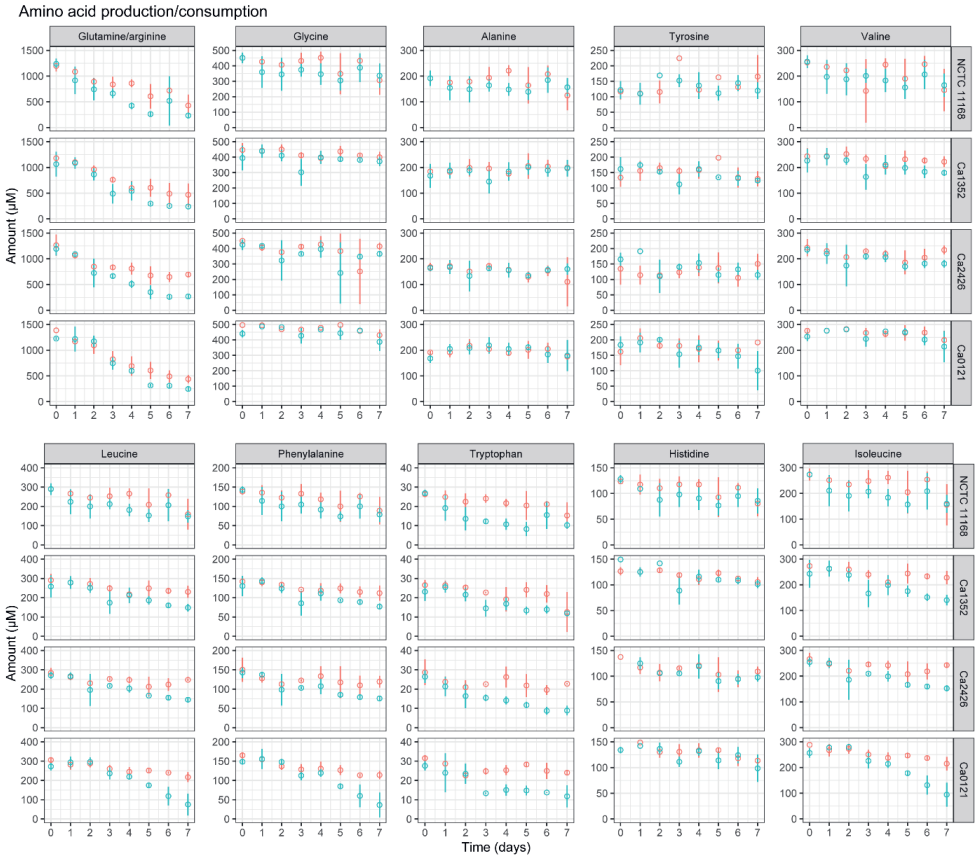

Figure S3 Amino acid production and consumption of selected *Campylobacter* isolates in MEMαF medium (blue dots) and MEMα medium (red dots) after 7-day incubation period. Columns correspond to different amino acids and each row shows results for a different isolate. Each value represents the average of three biologically independent replicates, and error bars show the standard deviation.

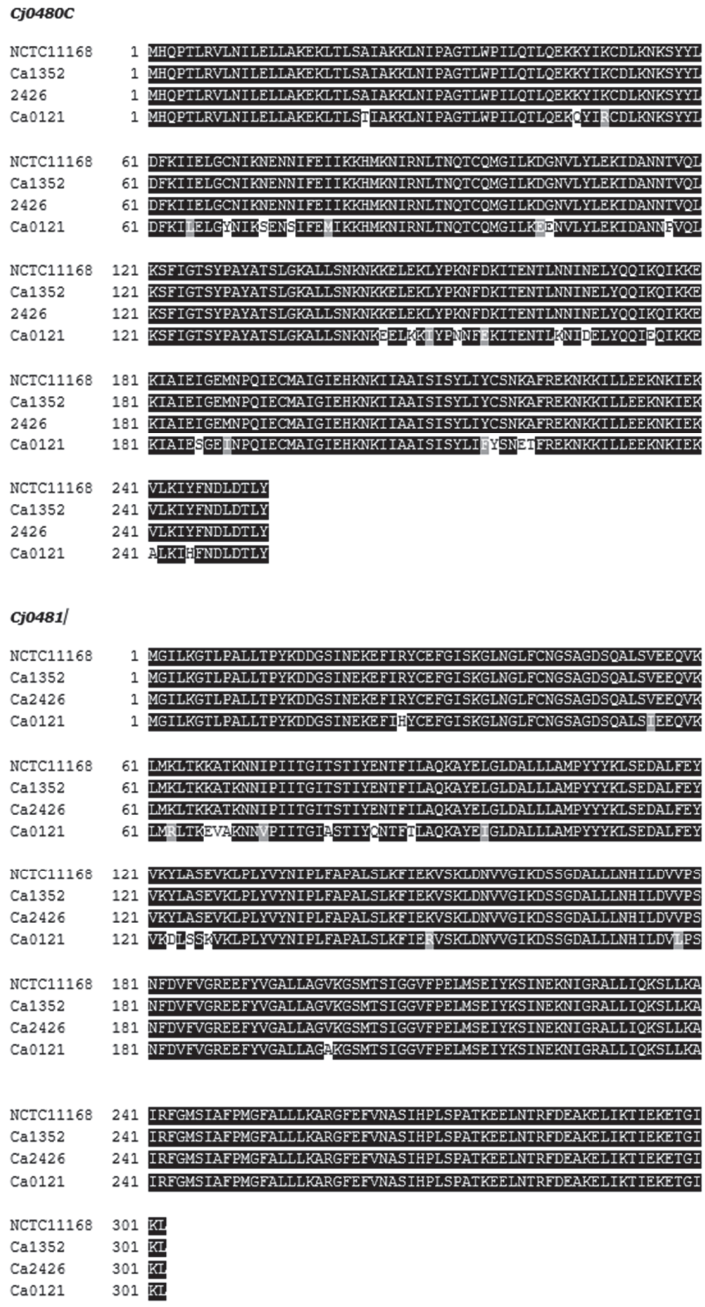

Fig S4 Complete L-fucose gene cluster alignment (continued on next page).

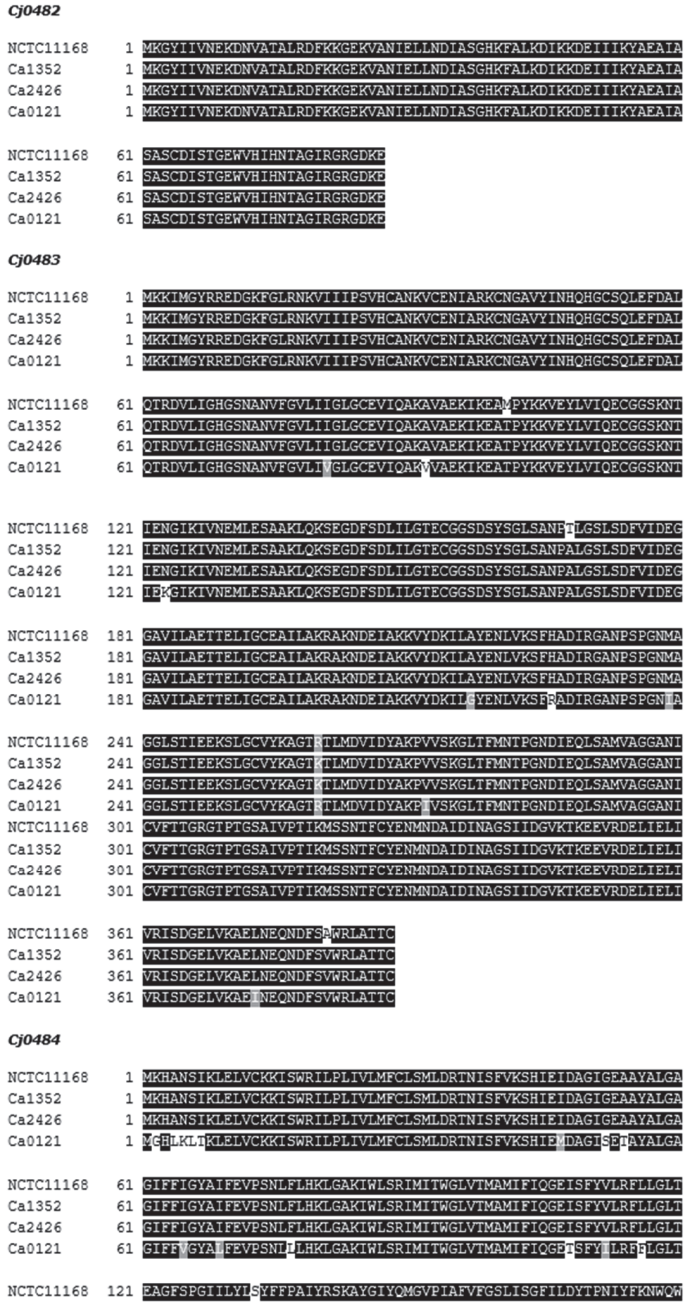

Fig S4 Complete L-fucose gene cluster alignment (continued on next page).

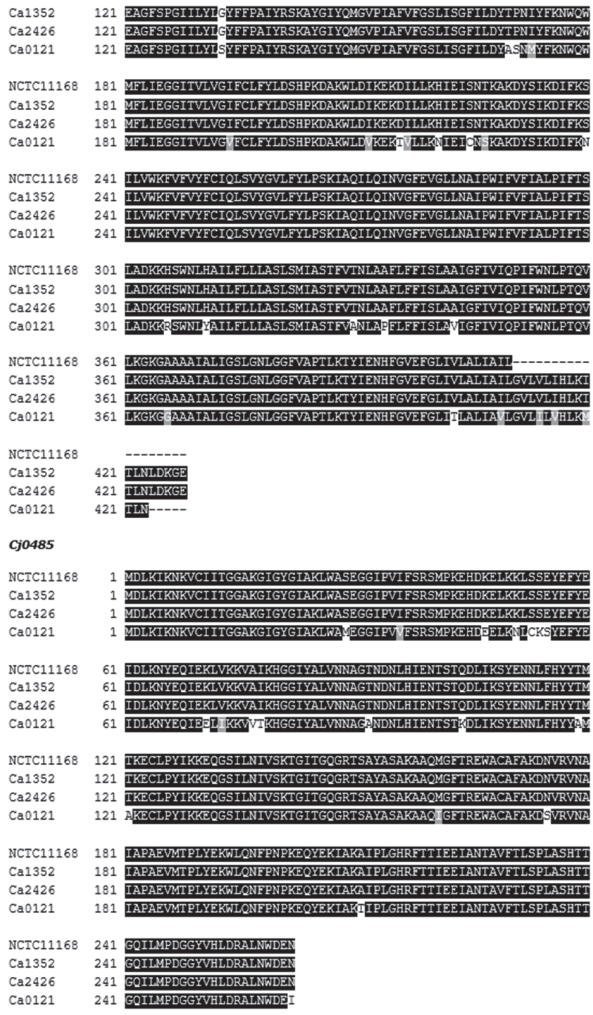

Fig S4 Complete L-fucose gene cluster alignment (continued on next page).

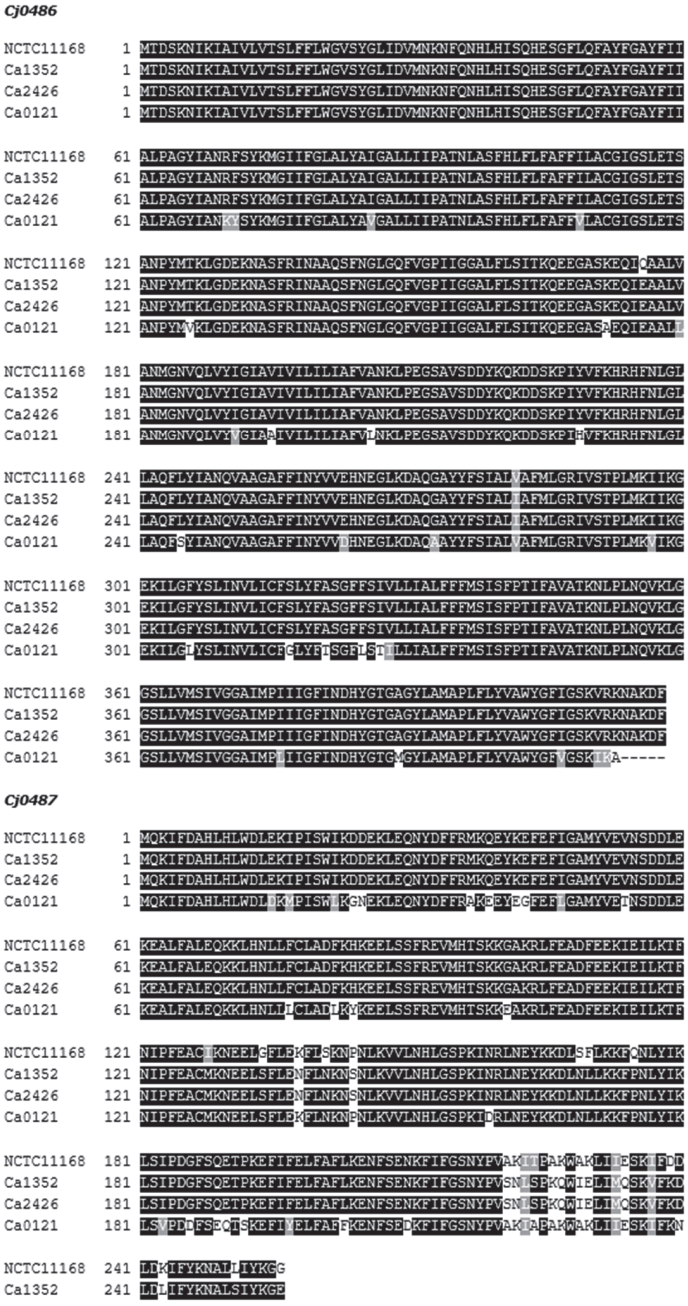

Fig S4 Complete L-fucose gene cluster alignment (continued on next page).

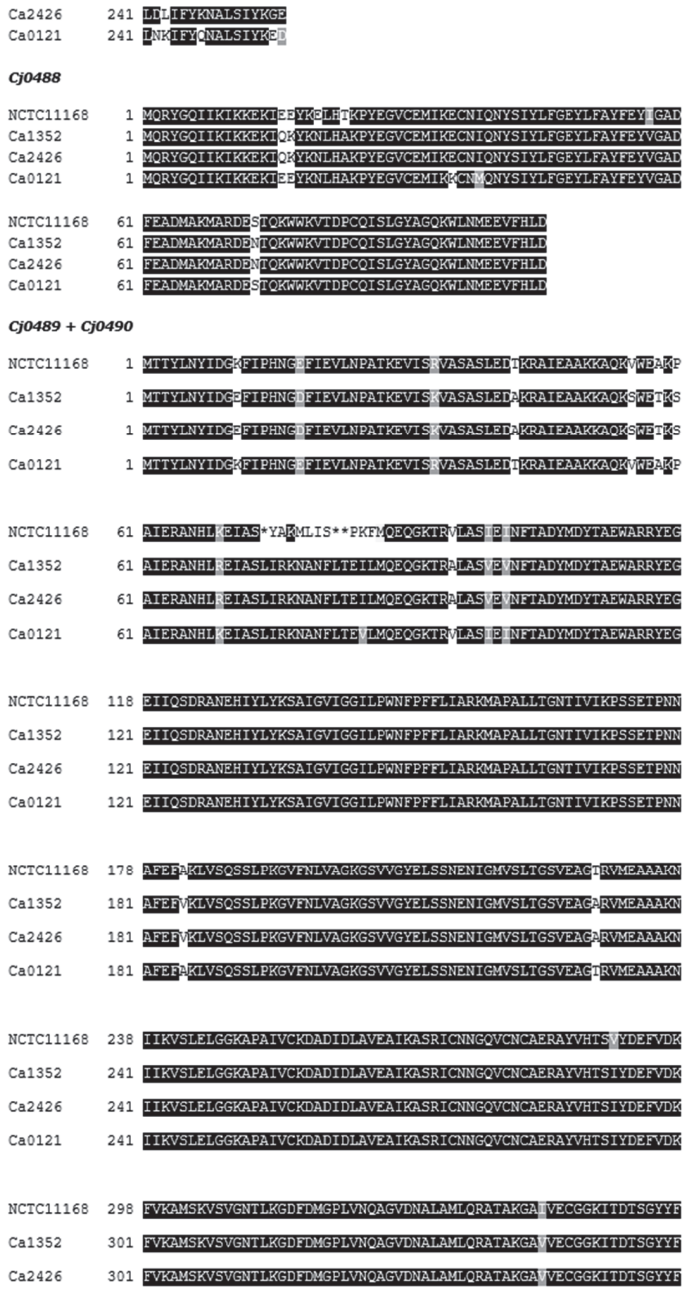

Fig S4 Complete L-fucose gene cluster alignment (continued on next page).

Fig S4 Complete L-fucose gene cluster alignment.

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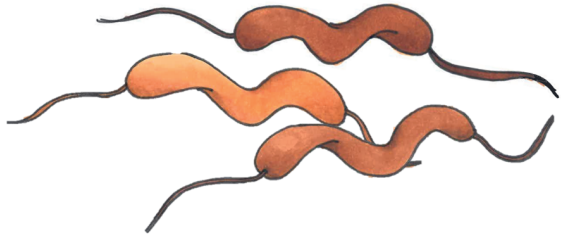

3

Activation of the L-fucose utilization cluster in *Campylobacter jejuni* NCTC11168 induces proteomic changes and enhances Caco-2 cell invasion and fibronectin binding

Pjotr S. Middendorf[1,2], Lucas M. Wijnands[2], Sjef Boeren[3], Aldert L. Zomer[4,5], Wilma F. Jacobs-Reitsma[2], Heidy M. W. den Besten[1], and Tjakko Abee[1]

Affiliations:

1. Food Microbiology, Wageningen University, Wageningen, the Netherlands
2. National Institute for Public Health and the Environment, Bilthoven, the Netherlands
3. Faculty of Veterinary Medicine, Department of Infectious Diseases and Immunology, Utrecht University, Utrecht, the Netherlands
4. WHO Collaborating Center for Campylobacter/OIE Reference Laboratory for Campylobacteriosis, Utrecht, the Netherlands

Abstract

Campylobacter jejuni is known to carry the so-called fucose utilization cluster (*Cj0480c-Cj0489*) that supports the metabolism of L-fucose and D-arabinose. Approximately 39 % of *C. jejuni* isolates, including the model strain NCTC11168, possess a 1 bp deletion frameshift in the gene *Cj0489*, resulting in two open reading frames encoding for putative 77 amino acid N-terminal (Cj0489-S) and 394 amino acid C-terminal (Cj0489-L) proteins, which are both annotated as aldehyde dehydrogenase, similar to the full length Cj0489 protein composed of 480 amino acids. In this study we quantified L-fucose and D-arabinose metabolism and metabolite production, and its impact on Caco-2 cell interaction and binding to fibronectin, using *C. jejuni* NCTC11168 and the closely related human isolate *C. jejuni* strain 286 without a frameshift in the *Cj0489* gene.

Increased survival and changes in metabolism were observed in both tested isolates when cultured with L-fucose and D-arabinose, and resulted in production of acetate, pyruvate and succinate, and the respective signature metabolites lactate and glycolic acid, which was in line with the upregulation of L-fucose cluster proteins. Notably, *C. jejuni* NCTC11168 did not produce the Cj0489-S protein and the Cj0484 protein, a putative transporter with unknown function, while in *C. jejuni* 286 this protein was significantly upregulated in the presence of L-fucose and D-arabinose, though this was not correlated with a faster consumption of the sugars in *C. jejuni* 286.

In vitro Caco-2 cell studies and fibronectin-binding experiments showed significantly higher invasion and fibronectin binding efficacy of *C. jejuni* NCTC11168 cells grown with L-fucose and D-arabinose, compared to cells grown without these substrates, while no significant differences were found with *C. jejuni* 286 between all tested media. Both fibronectin binding proteins, CadF and FlpA, were detected in the two isolates, but they were not significantly differentially expressed in L-fucose or D-arabinose grown cells. However, comparative proteomics analysis linked the *C. jejuni* NCTC11168 phenotypes uniquely to the more than 135-fold upregulated protein Cj0608, putative TolC-like component MacC. This protein is part of the so-called tripartite secretion system MacABC, that is conceivably active in this strain as the Cj0606 and Cj0607 proteins, putative MacA and MacB, were also detected in the proteomics analysis. This system is widely distributed in Gram-negative pathogenic

bacteria and operates in diverse cellular processes, including antibiotic resistance, cell division and lipoprotein trafficking. Combined with the five virulence proteins uniquely produced by *C. jejuni* NCTC11168, FlgB, PseD, PglC, WlaN and Cj1137c, with the latter three putative galactosyl/glycosyltransferases involved in cell wall lipooligosaccharide (LOS) synthesis, it is conceivable that L-fucose and D-arabinose utilization affected cell surface structure, resulting in enhanced fibronectin-mediated invasion of this isolate. Further studies on the MacABC system are required to elucidate its possible role in LOS synthesis and virulence of *C. jejuni*.

Introduction

Campylobacter is the leading cause of bacterial foodborne gastroenteritis in the world and its prevalence has increased over the past years (EFSA, 2021; Kaakoush et al., 2015; Špačková et al., 2019; Tack et al., 2019). Clinically, the most relevant species is *Campylobacter jejuni*, responsible for 80-90% of all diagnosed *Campylobacter* infections (EFSA, 2021). Most infections are linked to consumption of meat products (predominantly poultry), direct contact with animals, and contact with environmental waters (Clark et al., 2003; Karagiannis et al., 2010; Kenyon et al., 2020; Kuhn et al., 2017; Kuusi et al., 2004; Montgomery et al., 2018). *Campylobacter* infection, or campylobacteriosis, generally causes symptoms like watery or bloody diarrhea accompanied by abdominal pain, nausea and fever (Blaser, 1997; Hansson et al., 2018). In a small number of patients, post-infection complications such as the neurological disorder Guillain-Barré syndrome can occur (Nachamkin et al., 1998; Rees et al., 1995; Yuki et al., 1993).

C. jejuni is able to colonize the human intestine and has a relatively low dose-response correlation (Black et al., 1988; Robinson, 1981). In vitro, *C. jejuni* can adhere, invade and survive in human intestinal epithelial cells with a complex array of fitness and virulence factors. The use of genome sequencing techniques has already resulted in identification of several genes encoding putative adhesins, proteases, autotransporters, chemotaxis proteins and cytolethal distending toxin (CDT) (Ashgar et al., 2007; Brøndsted et al., 2005; Konkel et al., 2005; Marchant et al., 2002). Fibronectin is an extracellular glycoprotein on intestinal epithelial cells (IECs) and functions as an adhesion site for *Campylobacter* cells (Konkel et al., 2020). The highly conserved *C. jejuni* fibronectin-like protein A (FlpA) and fibronectin-binding protein (CadF) have been found to be important for adhesion to human IECs and colonization of chicken (Flanagan et al., 2009; Konkel et al., 2010; Konkel et al., 2020). However, due to the genome plasticity of the *Campylobacter* species (Woodcock et al., 2017), the identification of novel key factors that play a role in *Campylobacter* virulence remains a great challenge (Marchant et al., 2002; Sheppard et al., 2013).

C. jejuni may enhance its competitive fitness following rewiring its metabolic repertoire during colonization and infection. Next to utilizing citric acid intermediates, amino acids and peptides, some *C. jejuni* isolates have been described

to utilize L-fucose, D-arabinose and/or D-glucose as carbon and energy sources (Dwivedi et al., 2016; Garber et al., 2020; Hofreuter, 2014; Muraoka & Zhang, 2011; Parsons, 1984; Stahl et al., 2011; van der Hooft et al., 2018; Vegge et al., 2016; Vorwerk et al., 2014). Comparative whole genome sequencing (WGS) analysis revealed that approximately 65% of the sequenced *C. jejuni* isolates possess the so-called L-fucose utilization cluster, designated *Cj0480c – Cj0489*, from here on called fuc+ isolates. Upon consumption of L-fucose, fuc+ isolates have shown increased growth, increased survival, decreased biofilm formation and changes in the general metabolism (Dwivedi et al., 2016; Garber et al., 2020; Middendorf et al., 2022; Stahl et al., 2011). Recently it was discovered that except for L-fucose, also D-arabinose is metabolized by the L-fucose utilization cluster and is able to promote growth (Garber et al., 2020). The L-fucose utilization cluster is regulated by *Cj0480c* and contains two predicted transporters encoded by *Cj0484* and *Cj0486/FucP*. Following transport of L-fucose through the cell membrane, L-fucose is further metabolized to the end products pyruvic acid and lactic acid via metabolic enzymes *Cj0488*, *Cj0485* (FucX), *Cj0487*, *Cj0482/Cj0483*, *Cj0481* (DapA) and *Cj0489/Cj0490*, respectively (Garber et al., 2020). Both pyruvate and lactate can be further metabolized and support growth of *C. jejuni* (Stahl et al., 2011; Thomas et al., 2011). Notably, 61 % of the fuc+ *C. jejuni* isolates contain an intact *Cj0480c – Cj0489* gene cluster, while 39 % including the *C. jejuni* reference strain NCTC11168, possess a 1 bp deletion frameshift in the gene *Cj0489*, resulting in two open reading frames, *Cj0489* and *Cj0490*, encoding for putative 77 AA and 394 AA proteins (from now referred to as *Cj0489-S* and *Cj0489-L*), which are both annotated as aldehyde dehydrogenase (Middendorf et al., 2022; Muraoka & Zhang, 2011). Our recent study has shown that this frameshift does not impair utilization of L-fucose and general metabolism in *C. jejuni* NCTC11168 (Middendorf et al., 2022). However, whether the presumed *Cj0489-S* and *Cj0489-L* proteins are produced and/or functional, and whether additional functions in *C. jejuni* are affected by this frame shift including adhesion and/or invasion of IECs is not known. L-fucose is abundant in host gut mucosal surfaces and incorporated in a variety of fucosylated structures. In Caco-2 cells, type 2 α1,2-fucosylated glycans are evenly distributed over the cell surface, and type 1 α1,2-fucosylated glycans structures were found as membrane-bound mucin patches (Luijkx et al., 2020). Since *C. jejuni* lacks fucosidases, it is not able to release L-fucose from glycosylated host proteins (Elmi et al., 2021; Garber et al., 2020). However, recent co-culture studies with the gastrointestinal bacterium

Bacteroides fragilis provided evidence that an hyperinvasive *C. jejuni* isolate was able to utilize the L-fucose released by *B. fragilis* fucosidases, and show enhanced invasiveness towards human Caco-2 cells (Luijkx et al., 2020).

Despite the influence of L-fucose on growth, survival and its effect on the metabolism of *C. jejuni*, relatively little is known about the role of the L-fucose utilization clusters and the impact on virulence in fuc+ *C. jejuni* isolates. Here we report a comparative analysis of L-fucose and D-arabinose utilization capacity and *in vitro* Caco-2 cell adhesion/invasion and fibronectin binding efficacy of the model strain *C. jejuni* NCTC11168 (with *Cj0489* frameshift) and the closely related human isolate *C. jejuni* 286 (without *Cj0489* frameshift). Correlating observed metabolic and *in vitro* virulence phenotypes of the two *C. jejuni* strains to proteomic profiles will provide insight in strain-specific differences in activation of L-fucose and D-arabinose metabolic enzymes and impact on physiology and *in vitro* virulence.

Methods

Bacterial isolate selection and medium preparation

A phylogenetic tree based on alignments of 128 human *C. jejuni* isolates, obtained via the Dutch National Institute for Public Health and the Environment (RIVM) and described in (Mughini-Gras et al., 2021), was made using Roary (Page et al., 2015), MAFFT (https://mafft.cbrc.jp/alignment/software/) and fasttree software http://www.microbesonline.org/fasttree/). Visualization was done by using ITOL (itol.embl.de). Based on this phylogenetic tree, the reference isolate *C. jejuni* NCTC11168 (possessing a frameshift in *Cj0489*, as described in our previous study (Middendorf et al., 2022)), isolated from human faeces, and the most closely related isolate that did not have a frameshift, namely *C. jejuni* 286, isolated from an hospitalized patient, were selected (Suppl. Fig 1).

Campylobacter cultures were obtained on Columbia Agar Base (CAB, Oxoid, Landsmeer, the Netherlands) plates supplemented with 5% (v/v) lysed horse blood (BioTrading Benelux B.V. Mijdrecht, the Netherlands) and 0.5% (w/w) bacteriological agar No.1 (Oxoid). *Campylobacter* stock cultures were prepared by growing a selected colony in 10 mL BactoTM Heart Infusion broth (Becton, Dickinson and Company, Vianen, the Netherlands) for 24 hours at 41.5 °C in microaerobic conditions (5% O_2, 10% CO_2, 85% N_2), which was created using an Anoxomat WS9000 (Mart Microbiology, Drachten, the Netherlands). Glycerol stocks were prepared using 30% (v/v) glycerol (Fluka) and 70% overnight culture and were stored at -80 °C.

Working cultures were prepared by streaking a loopful of the *Campylobacter* freezer stocks on CAB plates, and plates were incubated for 48 hours at 41.5 °C in microaerobic conditions. A single colony was routinely selected and inoculated in 10 mL MEMα medium (Thermo Fisher Scientific, Bleiswijk, the Netherlands) supplemented with 20 µM $FeSO_4$ (Merck, Schiphol-Rijk, the Netherlands), and the culture was cultured overnight at 41.5 °C microaerobically. A second overnight culture was made by diluting the *Campylobacter* culture 1:100 (v/v) in 10 mL fresh MEMα medium. The culture was incubated at 41.5 °C for 24 hours in microaerobic conditions to obtain standardized working cultures.

Prior to the experiments, MEMα medium was prepared without and with supplementation of 25.0 mM L-fucose (MEMαF medium) or 25.0 mM D-arabinose (MEMαA medium) and filter-sterilized using 0.2-µm pore sized filters. Sterilized infusion bottles, closed using a rubber stopper and aluminium cap, were filled with 45 mL filter-sterilized MEMα medium, MEMαF medium or MEMαA medium by using a syringe. Filled infusion bottles were stored at 4 °C until further use.

L-fucose and D-arabinose growth experiments

As described previously (Middendorf et al., 2022), working cultures were decimally diluted in MEMα medium to a cell concentration of approximately 5.0 $\log_{10}$ CFU/mL. A final dilution step was done by adding 5 mL into the infusion bottles filled with 45 mL MEMα medium, MEMαF medium or MEMαA medium, resulting in a starting cell concentration of 4.0 $\log_{10}$ CFU/mL. Incubation of the inoculated infusion bottles was done at 37 °C microaerobically. At various timepoints, namely, day 0, day 1, day 2, day 3 day, 4 and day 7, approximately 4 mL sample was taken from each infusion bottle. After each sampling, the head space of infusion bottles was flushed for 2 min with microaerobic gas (5% O_2, 10% CO_2, 85% N_2) using a home-made gas flushing device using syringes to puncture the rubber stopper.

The 4 mL-aliquots were used to determine the bacterial concentration. The remainder of each sample (in either MEMα medium, MEMαF medium or MEMαA medium) was stored at -20 °C for high performance liquid chromatography (HPLC) analyses. Bacterial concentrations were determined by decimally diluting 1 mL of sample in peptone physiological salt solution (PPS, Tritium Microbiologie, Eindhoven, the Netherlands), followed by plating on CAB plates. CAB plates were incubated in air-tight jars for 48 hours at 41.5 °C in microaerobic conditions. Colonies were counted and expressed in $\log_{10}$ CFU/mL. Each sample was microscopically analysed using an Olympus BX 41 microscope (lens Ach 100x/1.25, Olympus Nederland, Leiderdorp, the Netherlands) and pictures were captured using CellSens Imaging software (Olympus Corporation). Three biologically independent reproductions were performed per condition, i.e., cells cultured in MEMα medium, MEMαF medium and MEMαA medium, on different days.

High performance liquid chromatography for organic acids and amino acids

The frozen samples stored during the growth experiments were thawed for HPLC analyses using the procedure as previously described by Middendorf et al. (2022). Briefly, For organic acids analysis, collected samples were centrifugated at 13,000 *g* at 4°C for 5 min. Pellets were removed and the supernatant was treated for protein decontamination with Carrez A ($K_4FeCN)6{\cdot}3H_20$, Merck) and B ($ZnO_4{\cdot}7H_20$, Merck). After centrifugation, the supernatant was added to HPLC vials. Quantitative analyses were done using standards with pre-made concentrations for L-fucose, D-arabinose, acetate, alpha-ketoglutarate, succinate, glycolic acid, pyruvate and lactate. The HPLC was performed on an Ultimate 3000 HPLC (Dionex, Sunnyvile, USA) equipped with an RI-101 refractive index detector (Shodex, Kawasaki, Japan), an autosampler and an ion-exclusion Aminex HPX – 87H column (7.8 × 300 mm) with a guard column (Bio-Rad, Hercules, CA, USA). As mobile phase, 5 mM H_2SO_4 (Merck) was used at a flow rate of 0.6 mL/min. Column temperature was kept at 40 °C. For each run, the injection volume was 10 µL and the run time 30 min. Chromeleon software (Thermo Fisher Scientific, Waltham, USA) was used for quantification of compound concentrations. HPLC analyses confirmed the absence of L-fucose and D-arabinose in MEMα medium

For amino acids analyses, samples were used in aliquots of 40 µL. These aliquots were kept on ice and were diluted with 50 µL of 0.1 M HCl (containing 250 µM Norvalin as internal standard, Merck). The samples were deproteinized by addition of 10 µL of cold 5-sulphosalicilic acid (SSA, Merck) (300 mg/mL) and centrifuged at 13,000 *g* at 4°C for 10 min. In order to obtain an optimal pH for derivatization (pH between 8.2 to 10.0), approximately 60 to 150 µL of 4N NaOH was added to 5 mL of the AccQ•Tag™ Ultra borate buffer (Borate/NaOH buffer, Waters, Milford, USA). For derivatization 60 µL of Borate/NaOH was added to a total recovery vial. Twenty µL of the supernatant obtained after deproteinization of the plasma was added and mixed. To each of the vials 20 µL of AccQ Tag Ultra derivatization reagent (Waters) dissolved in acetonitrile was added and mixed for 10 s. Each vial was immediately capped. The vials were then heated for 10 min at 55 °C. Quantitative analyses were done using standards with pre-made concentrations for, histidine, asparagine, serine, glutamine, arginine, glycine, aspartic acid, glutamic acid, threonine, alanine, proline, cysteine, lysine, tyrosine, methionine, and valine. HPLC was performed on an Ultimate 3000 HPLC

(Dionex) equipped with an RI-101 refractive index detector (Shodex), an autosampler and an ion-exclusion Aminex HPX – 87H column (7.8 × 300 mm) with a guard column (Bio-Rad). As mobile phase, eluents A and B (Waters) was used at a flow rate of 0.7 mL/min. Column temperature was kept at 55 °C. For each run, the injection volume was 1 µL and the run time 17 min. Chromeleon software (Thermo Fisher Scientific) was used for the determination of compound concentrations. Baseline separation was obtained for all amino acids used except glutamine and arginine.

C. jejuni adhesion and invasion of Caco-2 cells

To establish a confluent monolayer of Caco-2 cells, production of differentiated Caco-2 cells (human intestinal epithelial cells, ATCC HTB-37) were carried out by growing Caco-2 cells in 12-well plates (Corning Inc. ID 3513) in Dulbecco's Modified Eagle Medium (no glucose) (DMEM) medium (Gibco, Invitrogen, USA). The 12-well plates were incubated at 37 °C for 12-14 days with the DMEM medium being refreshed every 2 days. For more detail see (Wijnands et al., 2017)

Prior to adhesion and invasion of the Caco-2 cells, the standardized working culture of *C. jejuni* was inoculated in MEMα, MEMαF or MEMαA medium aiming for an initial concentration of 3-4 $\log_{10}$ CFU/mL, and cells were grown microaerobically for 48 hours to reach CFU counts of approximately 8.0 $\log_{10}$ CFU/mL to induce L-fucose or D-arabinose utilization, respectively. Next, 40-mL aliquots cultures were spun down for 5 minutes at 10,000 *g* and were three times washed in Phosphate-buffered saline (PBS). Then the *C. jejuni* cultures were concentrated to a volume of 4 mL reaching 9.0 $\log_{10}$ CFU/mL.

For adhesion and invasion experiments, differentiated Caco-2 cells were inoculated to reach a total cell count of 1.6×10^5 cells/well into the 12-well tissue culture plates containing either DMEM, DMEM supplemented with 25 mM L-fucose or DMEM supplemented with 25 mM D-arabinose. Fifty µl of the *C. jejuni* cultures grown in MEMα or in MEMαF or MEMαA medium were inoculated in the 12-well tissue culture plates containing Caco-2 cells, reaching approximately 8.0 $\log_{10}$ CFU per well. The 12 well plates were then centrifuged for 1 min at 175 *g* to create a proximity between the Caco-2 and *C. jejuni* cells, after which the 12-wells plates were incubated microaerobically at 37 °C for 3.5 hours.

After incubation, the wells were washed three times with PBS using a pipette to remove non-adhered *C. jejuni* cells. Half of the plate was lysed with 1 mL of 1% (v/v) Triton X-100 in PBS and serially diluted in PBS for quantification of the number of adhered and invaded *C. jejuni* cells. The other half of the plate was subsequently incubated microaerobically for 1 hour with 0.3% gentamicin (Gibco, Invitrogen, USA) to eliminate all extracellular *C. jejuni* cells. Then each well was washed with PBS buffer three times to remove all gentamicin, after which the Caco-2 cells were lysed with 1 mL of 1% (v/v) Triton X-100 in PBS and serially diluted in PBS for quantification of the number of invaded *C. jejuni* cells. Three biologically independent reproductions were performed per condition.

Fibronectin binding assay

Fibronectin (Fn) binding assays were performed using a previously described method (Konkel et al., 2010). Flat-bottom 96-well plates (Costar, Corning, NY, USA) were coated with a 1-mg/mL solution of Fn in 0.05 M Tris-buffered saline at pH 7.5 (Sigma, Munich, Germany) and incubated overnight at 4°C. The *C. jejuni* cultures were grown for 48 hours in MEMα, MEMαF or MEMαA medium and the cultures were washed three times using PBS and were resuspended in PBS to reach a final concentration of approximately 8.0 $\log_{10}$ CFU/mL. The Fn-coated 96 wells plate was rinsed three times with PBS, and 100 µl of bacterial suspensions were added to each well and incubated in microaerobic conditions at 37 °C for 1 hour After incubation, the wells were washed three times with 100 µl PBS per well, and adhered bacteria were removed by the addition of 100 µL Tryple™ Express (Gibco, Invitrogen). To enumerate the number of adhered bacteria, serial dilutions of the Tryple suspension were made in MEMα medium, plated on CAB plates and colonies were counted after 48 hours incubation at 41.5°C in microaerobic conditions. Three biologically independent reproductions were performed per condition.

Proteomics

The working cultures of *C. jejuni* were used to inoculate in triplicate infusion bottles to a concentration of 5.0 $\log_{10}$ CFU/mL that were filled with 45 mL of MEMα, MEMαF or MEMαA medium, and cultures were grown for 48 hours at 37 °C in microaerobic conditions. Thirty mL of each culture was centrifugated and washed three times in

TRIS buffer (pH 8.0) and resuspended in a final volume of 100 µl. The cultures were then sonicated with a sonication probe for 30 s. Protein concentrations were determined by the bicinchoninic acid (BCA) assay.

The protein aggregation capture (PAC) method as described by (Batth et al., 2019) and Liu et al. (2019) was used in a slightly modified way for sample preparation for proteomics analysis. Briefly, each sample, containing 60 µg of protein, was reduced with 15 mM DTT at 45°C for 30 min, unfolded in 6 M urea and alkylated with 20 mM acrylamide at room temperature for 30 min. The pH of the protein solution was adjusted to seven using 10% (v/v) trifluoro-acetic acid (TFA). SpeedBeads (magnetic carboxylate modified particles, GE Healthcare, Chicago, USA) of products 45152105050250 and 65152105050250 were mixed with 1:1 ratio at 50 µg/µl, and 8 µl of SpeedBeads was added to each protein sample. Acetonitrile was added up to 71% (v/v) to the protein beads mixture, incubated at room temperature with gentle shaking for 20 min. A magnet was used to separate the SpeedBeads from the supernatant for 30 s, and the supernatant was removed. The SpeedBeads were then washed with 1 mL of 70% ethanol and 1 mL of 100% acetonitrile successively, resuspended in 100 µl of 5 ng/µl sequencing grade trypsin solution in 50 mM ammonium bicarbonate and incubated overnight at room temperature with gentle shaking. The pH of SpeedBeads suspension was adjusted to 3 using 10% TFA, and the SpeedBeads were separated from the supernatant by using a magnet. The supernatant was filtered using C8 Empore disk filters. To improve yield, 0.1% formic acid was used to wash the beads and a 1:1 (v/v) mixture of acetonitrile and 0.1% formic acid was used to wash the filter. All eluents were combined and dried to 10–15 µl, then topped up to 50 µl with 0.1% formic acid.

For the LC-MS/MS analysis, 5 µl of prepared sample was injected into the system, and the analysis was performed as described in Liu et al. (2019). The MaxQuant quantitative proteomics software package was used to analyse LC-MS data with all MS/MS spectra as described by Cox et al. (2014) and the proteome of *C. jejuni* NCTC11168 (UniProt ID UP000000799) was used as the protein database. Next, Perseus was used for filtering and further bioinformatics and statistical analysis of the MaxQuant ProteinGroups files (Tyanova et al., 2016). Reverse hits were removed; identified protein groups contained minimally two peptides, of which at least one is

unique and one unmodified. The normalised label-free quantification (LFQ) intensity values as calculated by MaxQuant of each tested condition measured in biological triplicates were used.

The lower detection limit was set at 5.6 $\log_{10}$ (LFQ protein abundance), just below the lowest measured LFQ intensity by imputation NaN's by 5.6 after logarithmization. T-tests were performed on triplicates of each tested condition and were corrected with a false-discovery rate of 0.05. Protein fold-changes are displayed as fold-change, as example: a fold-change of 2 indicates that a two times higher protein concentration was measured in the tested condition versus the reference condition; and a fold-change of -2 indicates that a two times lower protein concentration was observed in the tested condition versus the reference condition. Proteins are only considered to be differentially expressed when a significant fold-change of over 1.5 or below -1.5 was calculated.

Results

Effect of L-fucose and D-arabinose on the growth of *C. jejuni* NCTC11168 and *C. jejuni* 286 and survival in MEMα medium

Growth and survival of *C. jejuni* NCTC11168 and *C. jejuni* 286 was investigated in MEMα, MEMαF and MEMαA medium up to 7 days (Figure 1). At day 1, 2 and 3, no significant differences in growth were observed in the tested media, with CFU counts reaching up to 8.7 (+/- 0.1) and 8.2 (+/- 0.1) $\log_{10}$ CFU/mL for *C. jejuni* NCTC11168 and *C. jejuni* 286, respectively. From day 4 onwards, cell counts in MEMα medium decreased, reaching 4.2 and 4.6 $\log_{10}$ CFU/mL at day 7 for isolates *C. jejuni* NCTC11168 and *C. jejuni* 286, respectively. In MEMαF medium, CFU counts were more stable overtime, slightly decreasing to final cell concentrations of 8.1 and 7.5 $\log_{10}$ CFU/mL for strain *C. jejuni* NCTC11168 and *C. jejuni* 286, respectively. In MEMαA medium, CFU counts decreased after day 4 for isolate *C. jejuni* NCTC11168, while *C. jejuni* 286 CFU counts remained more stable, reaching 5.5 and 7.5 $\log_{10}$ CFU/mL at day 7, respectively.

Incubation of the two *C. jejuni* strains in MEMαF and MEMαA medium showed that L-fucose and D-arabinose concentrations started to decrease at day 2, pointing to activation of metabolism. For *C. jejuni* NCTC11168 similar L-fucose and D-arabinose consumption patterns were observed and final consumed concentrations were approximately 12.5 mM at day 7, while CFU counts at day 7 were highest in MEMαF medium. For isolate *C. jejuni* 286, slightly lower consumption of L-fucose and D-arabinose compared to *C. jejuni* NCTC11168 was observed, with an approximate 6.0 mM and 7.5 mM consumption at day 7, respectively.

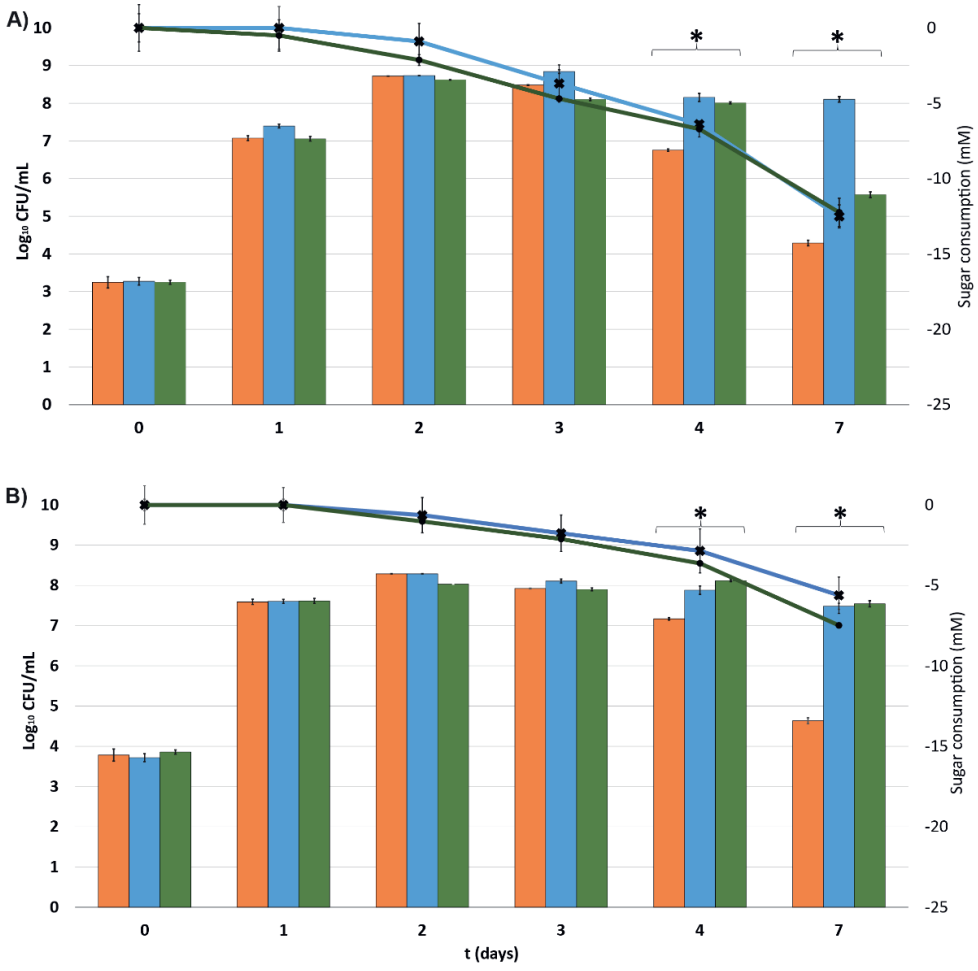

Figure 1 Growth of two human *C. jejuni* isolates in MEMα medium (orange bars), MEMαF medium (blue bars) and MEMαA medium (green bars). A) Growth of *C. jejuni* NCTC11168. B) Growth of *C. jejuni* 286. The blue line indicates L-fucose consumption and the green line D-arabinose consumption. Asterisks indicate a significant difference of the MEMαF medium or MEMαA medium, in comparison with $\log_{10}$ CFU counts/mL in MEMα medium.

Effect of L-fucose and D-arabinose on the metabolism of *C. jejuni* NCTC11168 and *C. jejuni* 286

We determined the concentrations of selected compounds which were previously found to be utilized or produced during *C. jejuni's* L-fucose metabolism (Middendorf

et al., 2022) (Figure 2). A schematic overview of uptake and metabolism uniquely coupled to L-fucose or D-arabinose is presented in Suppl. Fig 2. Activation of the L-fucose pathway via L-fucose or D-arabinose metabolism was confirmed by measuring the respective signature products, lactic acid and glycolic acid (Figure 2, Suppl. Fig. 3).

Acetate production was observed for *C. jejuni* NCTC11168 and *C. jejuni* 286 in all tested media and was highest in the presence of L-fucose (5.22 and 3.25 mM), followed by D-arabinose (3.58 and 2.23 mM), in comparison with MEMα medium (0.72 and 0.41 mM) (Fig. 2). For lactic acid, final concentrations were 1.59 mM and 1.74 mM in MEMαF medium at day 7 for isolate *C. jejuni* NCTC11168 and *C. jejuni* 286, respectively. Pyruvate was first consumed by *C. jejuni* NCTC11168, while concentrations increased at a later stage reaching 0.70 mM and 0.26 mM at day 7 in MEMαF and MEMαA medium, respectively. For isolate *C. jejuni* 286, pyruvate was produced in all tested media, reaching a final concentration of 0.54 mM, 0.92 mM and 1.41 mM in MEMα, MEMαF and MEMαA medium, respectively. No changes in succinate concentrations were observed for *C. jejuni* NCTC11168 and *C. jejuni* 286, when grown in MEMα and MEMαF medium, while succinate concentration increased when grown in MEMαA medium, reaching final concentrations of 1.19 mM and 0.96 mM for *C. jejuni* NCTC11168 and *C. jejuni* 286, respectively.

As expected, a strong preference was observed for the substrate serine, which was fully depleted in all tested media in two days by isolate NCTC11168 and in one day by isolate *C. jejuni* 286 (Figure 2B). Both isolates also fully depleted proline by day 3 in MEMα medium and by day 7 in MEMαF medium. No full depletion was observed in MEMαA medium, where proline was consumed until day 7, reaching a final concentration of 48.15 µM and 48.60 µM for isolate *C. jejuni* NCTC11168 and *C. jejuni* 286, respectively. Aspartic and glutamic acid were fully depleted on day 2 in all tested conditions, whereas asparagine concentrations decreased the first 4 days and remained constant at low levels of approximately 0.15 mM in all tested media. In MEMαF and MEMαA media cystine was fully depleted on day 2 in both tested isolates, while no depletion (isolate *C. jejuni* NCTC11168) or delayed depletion (isolate *C. jejuni* 286) was observed in MEMα medium.

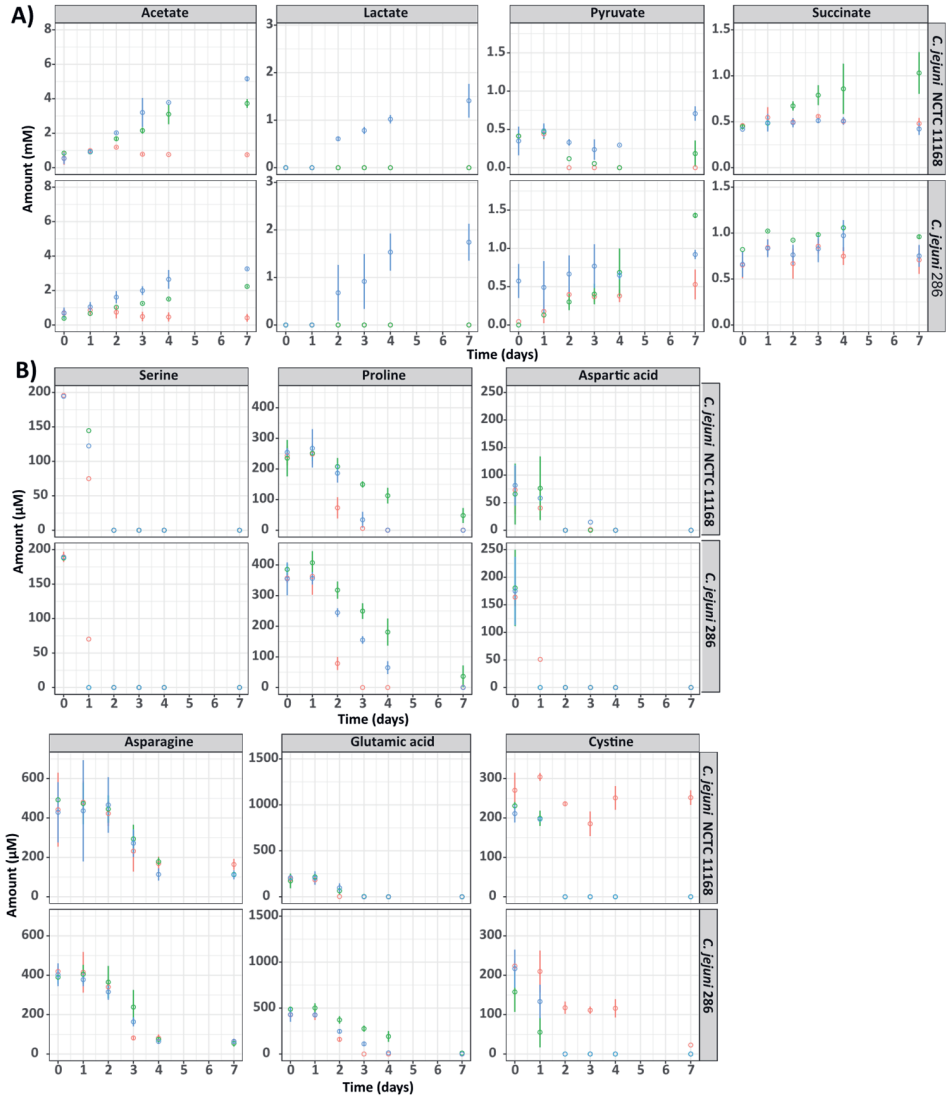

Figure 2 HPLC quantifications of *C. jejuni* NCTC11168 and *C. jejuni* 286 in MEMα medium (red dots), MEMαF medium (blue dots) or MEMαA medium (green dots). A) Organic acids. B) Amino acids. Each value represents the average of three biologically independent replicates, and error bars show the standard deviation.

Adhesion and invasion of Caco-2 cells and binding to fibronectin with L-fucose and D-arabinose grown cells

The impact of pre-activation of the L-fucose utilization cluster on adhesion and invasion of Caco-2 cells was assessed in DMEM medium, DMEM-F medium and DMEM-A medium, as this medium is commonly used during invasion assays. L-fucose and D-arabinose preculturing did not affect the adhesion efficacy for both isolates, neither the supplementation of the adhesion medium with L-fucose or D-arabinose (Figure 3A and 3C). On the other hand, the invasion capacity of *C. jejuni* NCTC11168 was increased upon pre-activation of the L-fucose cluster by L-fucose or D-arabinose preculturing, while supplementation of the invasion medium with L-fucose or D-arabinose did not significantly affect invasion efficacy (Figure 3B). Notably, *C. jejuni* 286 showed no significant differences in invasion capacity when precultured with L-fucose or D-arabinose (Figure 3D).

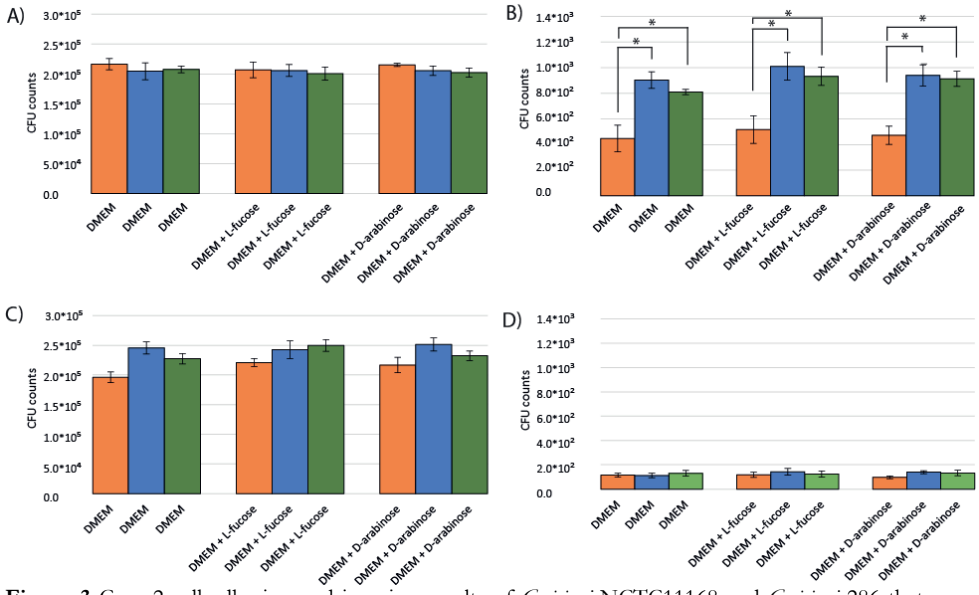

Figure 3 Caco-2 cell adhesion and invasion results of *C. jejuni* NCTC11168 and *C. jejuni* 286 that were precultured for two days in MEMα medium (orange bars), MEMαF medium (blue bars) or MEMαA medium (green bars) and incubated in in DMEM, DMEM+ L-fucose or DMEM + D-arabinose. Adhesion (A) and invasion (B) of *C. jejuni* NCTC11168, and adhesion (C) and invasion (D) of *C. jejuni* 286. Asterisks indicate a significant difference with cells precultured in the absence of L-fucose or D-arabinose. Due to the large biological variance observed within the biological replicates (see Supp. Fig. 4), the results described in this section correspond to one biological replicate, with statistical analyses performed using the three technical replicates.

Next, the effect of L-fucose and D-arabinose pre-culturing on binding to fibronectin was investigated. Following an 1 hour incubation of isolate *C. jejuni* NCTC11168, a significantly higher number of cells were bound when cells were precultured with L-fucose or D-arabinose compared to the control cells (Figure 4A). *C. jejuni* 286 showed low fibronectin binding capacity and no significant differences were observed in the tested conditions (Figure 4B). In all cases similar results were obtained after extending the incubation time up to 3.5 hours (data not shown).

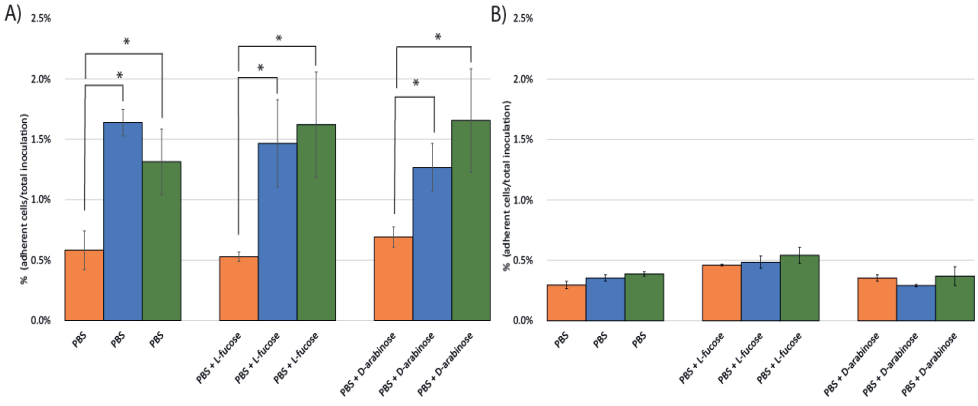

Figure 4 Fibronectin binding of *C. jejuni* NCTC11168 and *C. jejuni* 286 that were precultured for two days in MEMα medium (orange bars), MEMαF medium (blue bars) or MEMαA medium (green bars). and incubated in PBS, PBS supplemented with L-fucose, and PBS supplemented with D-arabinose. A) fibronectin binding of *C. jejuni* NCTC11168. B) Fibronectin binding of *C. jejuni* 286. Data from three biological replicates; asterisks indicate significant difference with the cells cultured in non-supplemented medium.

Significant up and down regulation of proteins after activation of the L-fucose utilization cluster

In total, 88 proteins were significantly differentially expressed in the two strains when cultured in MEMαF and MEMαA compared to MEMα medium (Fig. 5, Supp. Table 1). Eight of these proteins were upregulated in all tested conditions; seven of them belonged to the L-fucose utilization cluster (Cj0481, Cj0482, Cj0483, Cj0485, Cj0486, Cj0487, Cj0488), and one was a membrane related protein (Cj0771c). Furthermore, two proteins were downregulated in all tested conditions, namely, the bifunctional protein PutA that is involved in proline oxidation, and putative periplasmic protein

Cj0200c, a member of the two-component system DccRS (Cj1223c-Cj1222c) regulon (Wösten et al., 2010).

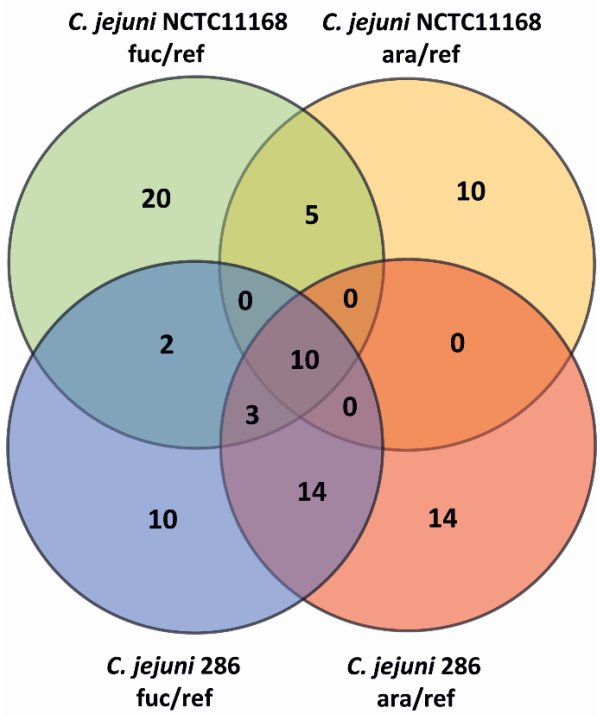

Figure 5 VENN-diagram of all significantly up-/down-regulated proteins in *C. jejuni* NCTC11168 and *C. jejuni* cultured in MEMαF and MEMαA medium compared to MEMα medium. The condition fuc/ref and ara/ref indicate the proteome comparisons of cells grown in MEMαF versus MEMα medium and in MEMαA versus MEMα medium, respectively.

Proteome analysis revealed several differences in expression of enzymes of the L-fucose utilization pathway between the two tested isolates. In the presence of L-fucose, higher fold-changes were observed for Cj0486 (L-fucose transporter), Cj0487 (conversion of L-fuconolactone to L-fuconic acid), Cj0482 and Cj0483 (conversion of L-fuconic acid to 2-oxo-3-deoxy-L-fuconic acid) for isolate *C. jejuni* NCTC11168, in comparison with *C. jejuni* 286 (Figure 6A). The proteins Cj0488 (conversion L-fuco-α pyronose to L-fuco β pyranose) had lower fold-changes for isolate *C. jejuni* NCTC11168 in comparison with *C. jejuni* 286. Furthermore, in the presence of L-fucose or D-arabinose, for isolate *C. jejuni* NCTC11168, the putative 394 amino acid Cj0489-L protein was detected and showed 3.0-fold and 2.0-fold upregulation in MEMαF and

MEMαA grown cells, respectively, while the putative 77 amino acid Cj0489-S protein was not detected (Figure 6A, 6B). For isolate *C. jejuni* 286, the putative 480 amino acid Cj0489 (Cj0489-S + Cj0489-L) protein was detected and upregulated 26.6-fold and 18.1-fold in MEMαF and MEMαA, respectively. Notably, the putative transporter Cj0484 with unknown function, and previously shown not to transport L-fucose in *C. jejuni* NCTC1168, was not detected. However, this protein was detected in *C. jejuni* 286 and significantly upregulated 102.3-fold in MEMαF and 69.2-fold in MEMαA grown cells compared to MEMα grown cells. Since no apparent phenotype could be linked to the strain-specific activation of this putative transporter in the tested conditions, its role in *C. jejuni* remains to be elucidated.

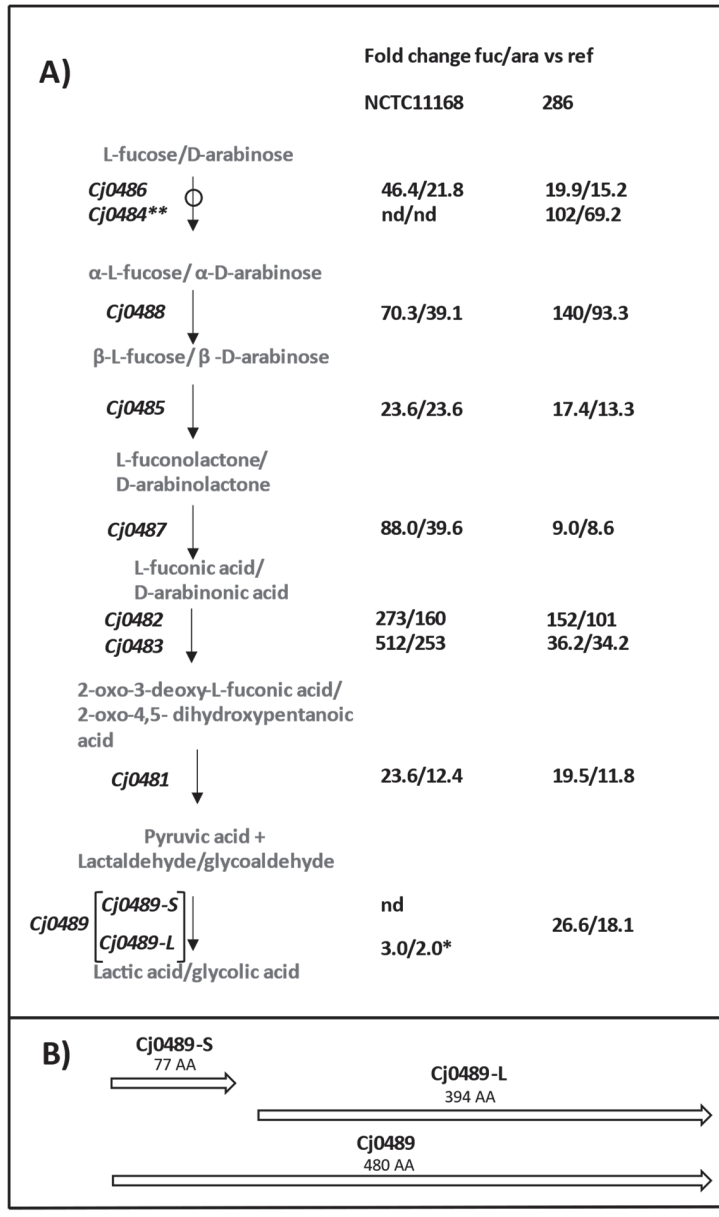

Figure 6 Protein fold-change of the L-fucose utilization cluster pathways. A) Fold-changes upon L-fucose and D-arabinose utilization. nd = not detected,* Indicates a non-significant fold-change. ** indicates the non-characterized transporter Cj0484. B) Schematic presentation of the frameshift proteins Cj0489-S and Cj0489-L present in *C. jejuni* NCTC11168, and the intact Cj0489 protein is present in *C. jejuni* 286.

Effect of L-fucose and D-arabinose on metabolic, ribosomal and periplasmic/membrane proteins

In both tested isolates, only one differentially expressed protein could be directly associated with the observed metabolic profiles of cells when cultured in MEMαF and MEMαA medium, namely, PutA. Downregulation of PutA (Suppl. Table. 1), a proline dehydrogenase which oxidizes L-proline to L-glutamate, is conceivably linked to the observed reduced proline consumption in MEMαF and MEMαA medium compared to MEMα medium.

Additionally, several significant differences in protein expression were observed that could not directly be linked to our metabolic data and were only affected in either one of the isolates. In *C. jejuni* NCTC11168 the following proteins were affected when grown in MEMαF and MEMαA medium (Suppl. Table. 1), namely, TrpF (involved in synthesizing L-tryptophan), PurQ (involved in the conversion of glutamine to glutamate) and TpiA (which is involved in gluconeogenesis). Furthermore, in *C. jejuni* 286 the proteins AcsA (which catalyses the conversion of acetate into acetyl-CoA), MetA and MetE (both involved in L-methionine biosynthesis) were differentially expressed when grown in MEMαF and MEMαA medium.

Furthermore, a higher number of ribosomal proteins was upregulated in the MEMαF grown NCTC11168 isolate (Suppl. Table 1), which is conceivably linked to continuation of growth in this medium at day 3 versus day 2 at which the proteomics samples were taken. Furthermore, high quantities of membrane, periplasmic and capsular proteins were significantly differentially expressed upon L-fucose or D-arabinose activation in both tested isolated. However, as most of these proteins were putative proteins, the functions remain to be further elucidated.

Putative virulence associated proteins

The analysis of presence and significantly differentially expressed putative virulence factors in *C. jejuni* NCTC11168 and 286 was based on the list of 236 putative *C. jejuni* virulence proteins presented in Suppl. Table 2, which was extracted from the *virulence factors of pathogenic bacteria* database (http://www.mgc.ac.cn/VFs/) and extended with information from selected publications on *Campylobacter* virulence (Bravo et al.,

2021; Cooper et al., 2013; Dasti et al., 2010; Liu et al., 2019; Panzenhagen et al., 2021; Redondo et al., 2019). Notably, in total 177 and 166 of these 236 putative virulence factors were detected in our proteomics analysis (Suppl. Table 2), and of these, 8 and 9 were significantly up or down regulated in the presence of L-fucose and/or D-arabinose in *C. jejuni* NCTC11168 and *C. jejuni* 286, respectively (Table 1).

From these proteins, only one putative virulence protein, Cj0608, was uniquely upregulated in MEMαF and MEMαA grown cells of *C. jejuni* NCTC11168, and therefore conceivably linked to the observed enhanced Caco-2 invasion and fibronectin binding phenotypes of these cells. Cj0608 is a putative outer membrane protein, part of the MacABC complex (*Cj0606-Cj0608*), a putative Type 1 secretion system, linked to the DccSR regulon. Previous studies showed that while *C. jejuni* mutants defective for the DccRS system grew and survived identically to wild type under a variety of *in vitro* growth conditions, they exhibited markedly reduced colonization in three animal models of infection including chicken, as well as for their ability to induce intestinal inflammation in a SCID-LF mouse model (Allos, 1997; Flint et al., 2010; Wösten et al., 2010). Furthermore, it often has a co-occurrence and co-expression with *Cj1478c*, encoding CadF (fibronectin binding protein) (Guccione et al., 2017; Guérin et al., 2020).

Additionally, several significant differences in protein expression were observed that could not directly be linked to our observed invasion and fibronectin binding phenotype. Of these virulence proteins, three were downregulated in isolate *C. jejuni* 286, but not in isolate *C. jejuni* NCTC11168, namely, Peb3 (adhesin), FlgD (basal-body rod modification protein) and Cj1169c (putative periplasmic protein) (Konkel et al., 2020; Sałamaszyńska-Guz et al., 2022; Taheri et al., 2018). Furthermore, the upregulation of Cj0371 in isolate *C. jejuni* 286, but not in isolate *C. jejuni* NCTC11168, potentially influences the virulence of this isolate, as activation of this protein has a negative role on *Campylobacters* chemotaxis (Du et al., 2018).

Notably, CadF and FlpA, involved in binding to fibronectin, were detected in both isolates, but not significantly differentially expressed in the presence of L-fucose or D-arabinose. Interestingly, the protein WlaN/Cj1139c, Beta-1,3 galactosyltransferase, which is only produced in *C. jejuni* NCTC11168, is co-expressed with the fibronectin binding protein CadF (string-db.org), suggesting a possible link towards the increased

fibronectin binding in this isolate. Four other virulence proteins were present in *C. jejuni* NCTC11168, that were absent in *C. jejuni* 286, namely, FlgB, PseD, PglC and Cj1337c. Furthermore, one protein was absent in *C. jejuni* NCTC11168 and present in *C. jejuni* 286, namely, CipA.

Moreover, in the presence of either L-fucose or D-arabinose, several proteins were uniquely significantly differentially expressed in isolate *C. jejuni* NCTC11168. In the presence of L-fucose, two proteins were uniquely upregulated, catalase KatA, involved in H_2O_2 detoxification (survival), and capsular glycosyltransferase PglC, involved in cell wall synthesis, while another capsular protein, Cj1417c (labelled as immune modulation) was downregulated. In the presence of D-arabinose, CdtB, an exotoxin protein, and Cj0125c, encoding a DksA-like protein, were uniquely upregulated, while Cj0358, a putative cytochrome peroxidase protein, was downregulated. Additionally, a number of putative virulence proteins were uniquely upregulated in the presence of L-fucose in *C. jejuni* 286, including exotoxin CdtC and the enterochelin uptake periplasmic binding protein CeuE. In the presence of D-arabinose, CmeA, a subunit of multidrug efflux system CmeABC, was upregulated, while Peb1A, a major host cell-binding factor protein, was downregulated.

Table 1 Presence and up or down regulation of potential virulence proteins. Values indicate a fold-change in comparison with MEMα medium. Present indicates that the protein is detected in all tested conditions, but not significantly changed. Nd indicates that the protein is not detected in any of the tested conditions.

		L-fucose	D-arabinose	L-fucose	D-arabinose
Uniprot	**Protein**	*C. jejuni* NCTC11168	*C. jejuni* NCTC11168	*C. jejuni* 286	*C. jejuni* 286
Linked to observed Caco-2 invasion and fibronectin binding phenotypes					
Q0PAQ9	**Cj0608**	136	138	present	present
Q0PBL7	**Peb3/Cj0289c**	present	present	-1.6	-1.8
Q0PC84	**FlgD/Cj0042**	present	present	-2.0	-1.7
Q0P987	**Cj1169c**	present	present	-3.9	-2.5
Q9PID1	**Cj0371**	present	present	1.9	1.5
Fibronectin binding proteins					
Q0P8D9	**CadF/Cj1478c**	present	present	present	present
Q0P8X7	**FlpA/Cj1279c**	present	present	present	present
Presence and absence of virulence proteins					
Q0P9B5	**WlaN/Cj1139c**	present	present	nd	nd
Q0PAY8	**FlgB/Cj0528c**	present	present	nd	nd
Q0P8S5	**PseD/Cj1333**	present	present	nd	nd
Q0P9B7	**Cj1137c**	present	present	nd	nd
Q0PAJ4	**CipA/Cj0685c**	nd	nd	present	present
Uniquely up or downregulated virulence proteins					
Q59296	**KatA/Cj1385**	21.1	present	present	present
Q0P9D0	**PglC/Cj1124c**	10.2	present	nd	nd
Q0P8J7	**Cj1417c**	-67.3	present	present	present
Q0PC12	**Cj0125c**	present	11.1	present	present
Q0PC57	**CdtB/Cj0078c**	present	1.7	present	present
Q0PBF1	**Cj0358**	present	-1.6	present	present
Q0PC58	**CdtC/Cj0077c**	present	present	28.4	present
Q0P8Q4	**CeuE/Cj1355**	present	present	1.5	present
Q0PBE3	**CmeA/Cj0367c**	present	present	present	1.6
Q0P9X8	**Peb1A/Cj0921c**	present	present	present	-2.1

Discussion

The L-fucose utilization cluster is present in the majority of *C. jejuni* and *C. coli* isolates and is involved in growth, biofilm formation, survival and virulence of *Campylobacter* (Dwivedi et al., 2016; Luijkx et al., 2020; Muraoka & Zhang, 2011; Stahl et al., 2011). Via the L-fucose utilization cluster, both L-fucose and D-arabinose can be metabolized, however, L-fucose is the preferred carbon source over D-arabinose (Garber et al., 2020). In the current study we provided novel insights in the L-fucose utilization cluster on protein level and linked this to phenotypic observations. Our proteomic analyses of two selected fuc+ isolates; reference isolate *C. jejuni* NCTC11168 (human stool isolate; *Cj0480c* – truncated *Cj0489*) and isolate *C. jejuni* 286 (hospitalized patient isolate; *Cj0480c* – intact *Cj0489*), showed significant upregulation of proteins in the L-fucose utilization cluster when cells were grown in the presence of L-fucose or D-arabinose compared to the reference condition. Previous studies have shown that the frameshift in *Cj0489* (resulting in the formation of *Cj0489-S* and *Cj0489*-L) did not impair growth or influence its metabolism differently than an intact L-fucose cluster (Middendorf et al., 2022; Muraoka & Zhang, 2011; Stahl et al., 2011). Our metabolic and proteomic analyses provided evidence that the Cj0489-S (77 AA) was not detected and conceivably not functional in *C. jejuni* NCTC11168, while Cj489-L (394 AA), was detected and upregulated in the presence of L-fucose or D-arabinose, supporting efficient metabolism of both substrates. Furthermore, the previously described putative transporter Cj0484 (Dwivedi et al., 2016; Stahl et al., 2011), was not detected in *C. jejuni* NCTC11168, indicating that it is not functional in this strain in the tested conditions. Interestingly, Cj0484 was detected and highly upregulated in *C. jejuni* 286, suggesting a function in this isolate. Subsequent protein alignment analysis of *Cj0484* between *C. jejuni* NCTC11168 and *C. jejuni* 286 showed two differences, a point mutation on AA location 145, resulting in the amino acid substitution of an alanine for a valine, and a slightly reduced size of 411 AA in ACTC11168 compared to 429 AA in *C. jejuni* 286 (Suppl. Fig. 5). Whether these differences influence respective Cj0484 protein levels and/or activity, requires further study on additional *C. jejuni* isolates.

In the current study, comparative growth analysis of the two isolates showed comparable growth in all tested media up to day 3, with *C. jejuni* NCTC11168 and *C. jejuni* 286 reaching maximum CFU/mL of 8.7 $\log_{10}$ and 8.2 $\log_{10}$, respectively. The L-

fucose metabolism and growth data of isolate *C. jejuni* NCTC11168 are in line with previous growth performance and increased survival observed for this isolate (Middendorf et al., 2022). HPLC analyses showed a higher L-fucose consumption for isolate *C. jejuni* NCTC11168, in comparison with isolate *C. jejuni* 286, which could be linked to the higher fold expression- of several proteins in the L-fucose utilization cluster, including the transporter Cj0486, the amidohydrolase Cj0487 and the altronate hydrolases Cj0482 and Cj0483. Further HPLC analyses confirmed that the amino acids serine, aspartic and glutamic acid were depleted at day 3 in MEMα, MEMαF and MEMαA medium, in line with previous studies that suggested that these amino acids are preferred substrates for growth of *Campylobacter* (Garber et al., 2020; Middendorf et al., 2022). In addition, both tested isolates depleted proline more rapidly in the absence of L-fucose or D-arabinose, in line with the observed downregulation of PutA, previously described to play an essential role in proline metabolism (Gao et al., 2017).

We next investigated whether activation of the fucose utilization clusters in the selected *C. jejuni* NCTC11168 and 286 strains affected their interaction with Caco-2 cells and their binding to fibronectin. Using preactivated cells harvested at day 2 from cultures grown in MEMα without and with L-fucose or D-arabinose, an approximate ten-fold higher invasion efficacy of Caco-2 cells was observed for *C. jejuni* NCTC11168 when grown with added substrates. Additionally, these preactivated *C. jejuni* 11168 cells showed significantly higher fibronectin binding efficacy compared to control cells. For *C. jejuni* 286 neither enhanced Caco-2 cells invasion nor enhanced binding to fibronectin was observed for pre-activated cells. Our data obtained with pre-activated *C. jejuni* NCTC11168 provide further support for the results presented by Luickx et al. (2020), who suggested that *C. jejuni* fuc+ isolates scavenge and metabolise L-fucose and alter their invasive properties. Using non-preactivated cells of hyper virulent *C. jejuni* 108 (intestinal isolate from patient with recurrent infections), that carries a L-fucose utilization cluster with a frameshift in *Cj0489* (similarly as *C. jejuni* NCTC11168), the authors showed that exogenous L-fucose supplied by action of fucosidases from *B. fragilis*, promoted growth and invasion of Caco-2 cells. Invasion counts of isolate *C. jejuni* 108 were 6000 CFU in DMEM medium and 8000 CFU when L-fucose was added (Luijkx et al., 2020). Our reference isolate, *C. jejuni* NCTC11168, a model strain that was initially isolated from human stool in 1977,

showed Caco-2 invasion counts ranging from 400-1,000 CFU, and as expected, significantly lower than the hyper virulent isolate *C. jejuni* 108 (Luijkx et al., 2020; Pascoe et al., 2019). Interestingly, in our study with non-preactivated *C. jejuni* NCTC11168 cells, addition of L-fucose or D-arabinose to the Caco-2 assay did not enhance invasion capacity of the cells. These results suggest that the incubation time of 3.5 hours was not sufficient to enable activation of the L-fucose utilization cluster in this strain, which is in line with our current and previously published data (Middendorf et al. 2022), that showed onset of L-fucose and D-arabinose metabolism at day 2, after depletion of preferred amino acids including L-serine from the medium. Alignment of the L-fucose cluster of *C. jejuni* NCTC11168 to that of hyper virulent *C. jejuni* 108 showed that both clusters are nearly identical (>99%), including the frameshift in *Cj0489* that is observed for both isolates (data not shown). Possible mechanisms underlying differences between *C. jejuni* isolates in transcriptional activation of L-fucose utilization and/or modulation by amino acid metabolism remain to be elucidated.

The current study presented evidence that preactivated cells of *C. jejuni* NCTC11168 grown in MEMαF and MEMαA medium, showed significant higher Caco-2 invasion and fibronectin binding capacity compared to MEMα grown cells, while *C. jejuni* 286 showed low invasion and binding capacity in all tested conditions. Linking phenotypes to proteomics data, several virulence proteins were affected in cell cultured in the presence of L-fucose or D-arabinose, summarized in Figure 7. In isolate NCTC11168, the protein Cj0608, which is annotated as putative MacC, was 136.1 and 137.7 fold-change upregulated in the presence of L-fucose and D-arabinose, respectively, while no change was observed in isolate *C. jejuni* 286. Authors of previous studies made a clear link between Cj0608, the putative type 1 secretion system MacABC (Cj0606-Cj0608) and the DccSR regulon, which is involved in adaptation to new environments and host colonization of chicken intestine (Flint et al., 2010; Wösten et al., 2010). The MacABC system is understudied in *Campylobacter*, however, it is a system that is well conserved and is linked to diverse processes in Gram-negative pathogenic bacteria (Greene et al., 2018). In *Salmonella,* the MacAB system affected oral *Salmonella* infections in animal models (Bogomolnaya et al., 2013; Nishino et al., 2006). Furthermore, in *Helicobacter pylori,* a tripartite efflux system was recently found, which affected flagellum stability (Gibson et al., 2022). In *Serratia marcescens*

and *Acinetobacter baumannii,* deletion of the MacAB efflux system decreased the ability to form biofilms (Robin et al., 2022; Shirshikova et al., 2021). Interestingly, studies in *C. jejuni* NCTC11168 showed that this isolate produced less biofilm in the presence of L-fucose (Dwivedi et al., 2016), and whether the upregulation of Cj0608 (MacC) and the conceivable MacABC activity is involved in this process remains to be elucidated in future studies. The putative MacABC system in *Campylobacter* is highly similar to another tripartite efflux pump, the CmeABC system (Greene et al., 2018). Notably, the respective intact MacABC and CmeABC membrane protein complexes were recently extracted from *C. jejuni* cells and characterized (Guérin et al., 2020). For example, both CmeA and MacA belong to the HlyD superfamily and CmeC and MacC are phylogenetically very closely related (Guérin et al., 2020). In isolate *C. jejuni* 286, the protein CmeA is significantly upregulated with a fold-change of 1.4 (data not shown) and 1.5 in the presence of L-fucose or D-arabinose (100x lower fold-change than the MacC protein of the MacABC system in *C. jejuni* NCTC11168), respectively, while no significant change was observed in *C. jejuni* NCTC11168 (Figure 7). Future studies are required to investigate both efflux systems and what processes they affect upon L-fucose utilization cluster activation.

In isolate *C. jejuni* 286, three proteins were uniquely significantly downregulated and one significantly upregulated, Peb3 (adhesin), FlgD (basal-body rod modification protein) and Cj1169c (putative periplasmic protein), and Cj0371 (involved in chemotaxis), respectively. Interestingly, Cj0371 is considered an "anti-virulence gene", as it plays a negative role on *Campylobacters* chemotaxis. In the study of Du et al. (2018), the authors hypothesized that functions encoded by such genes may support colonization and coexistence of *Campylobacter* in animal hosts, including chickens (Du et al., 2018). However, these findings do not directly provide a link with *in vitro* virulence data obtained with *C. jejuni* 286, as cells precultured in MEMα, MEMαF and MEMαA medium showed comparable low Caco-2 invasion and fibronectin binding capacity. Moreover, *C. jejuni* NCTC11168 did not produce CipA, while it produced five virulent proteins that *C. jejuni* 286 did not produce, namely, FlgB, PseD, WlaN, PglC and Cj1137c, with the latter three having roles in lipooligosaccharide (LOS) synthesis. LOS represent an integral component of the *Campylobacter* cell membrane with a structure of core oligosaccharides forming inner and outer core regions and a lipid A

moiety, and has been shown to play roles in *Campylobacter* physiology and virulence (Burnham & Hendrixson, 2018; Hameed et al., 2020; Szymanski, 2022).

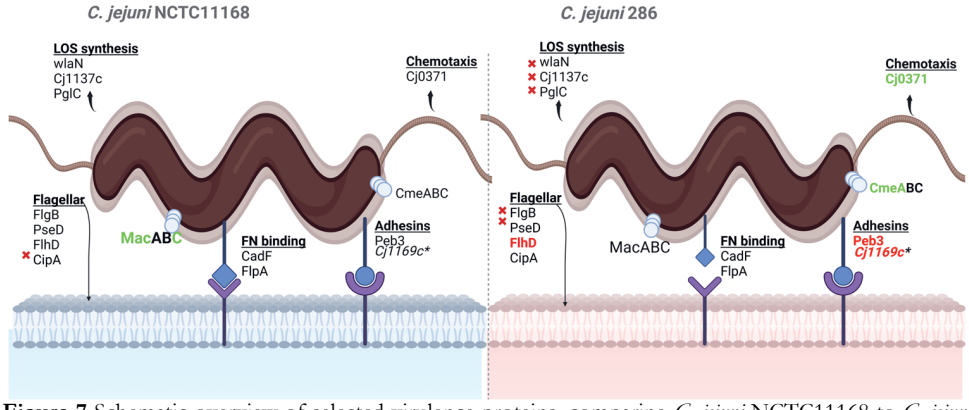

Figure 7 Schematic overview of selected virulence proteins, comparing *C. jejuni* NCTC11168 to *C. jejuni* 286. Red crosses indicate the absence of the protein in that specific isolate. Green text indicates significantly upregulated protein, red text indicates a significantly downregulated protein and black text indicates that the protein is not significantly differentially expressed. Asterisk indicates an hypothetical virulence protein (Cj1169c). LOS stands for lipoologosaccharides and FN stands for fibronectin. See text for details. Figure was created using BioRender software.

In conclusion, our study demonstrated that possessing the L-fucose utilization cluster allows *Campylobacter* isolates *C. jejuni* NCTC11168 and *C. jejuni* 286 to metabolize L-fucose and D-arabinose, resulting in enhanced survival for both isolates. Increased invasion of Caco-2 cells and binding to fibronectin after pre-activation with L-fucose or D-arabinose was only found in isolate *C. jejuni* NCTC11168 in the tested conditions. We were able to link the protein Cj0608, the putative TolC-like component MacC, to this observed phenotype. Cj0608 forms together with the detected Cj0606 (MacA) and Cj0607 (MacB) proteins, the tripartite secretion system MacABC, previously shown to operate in diverse cellular processes, including antibiotic resistance, cell division and lipoprotein trafficking in a range of Gram-negative pathogenic bacteria (Greene et al., 2018). L-fucose and D-arabinose utilization induced-upregulation of this system, in combination with five virulence proteins uniquely produced by *C. jejuni* NCTC11168, including three enzymes involved in LOS synthesis, WlaN (Beta-1,3 galactosyltransferase), PglC (glycosyltransferase) and Cj1137c (putative glycosyltransferase) (Hameed et al., 2020; Szymanski, 2022), conceivably affected cell

surface structure, resulting in enhanced fibronectin-mediated invasion of this isolate. Further research is necessary to verify this hypothesis and to clarify the underlying mechanisms.

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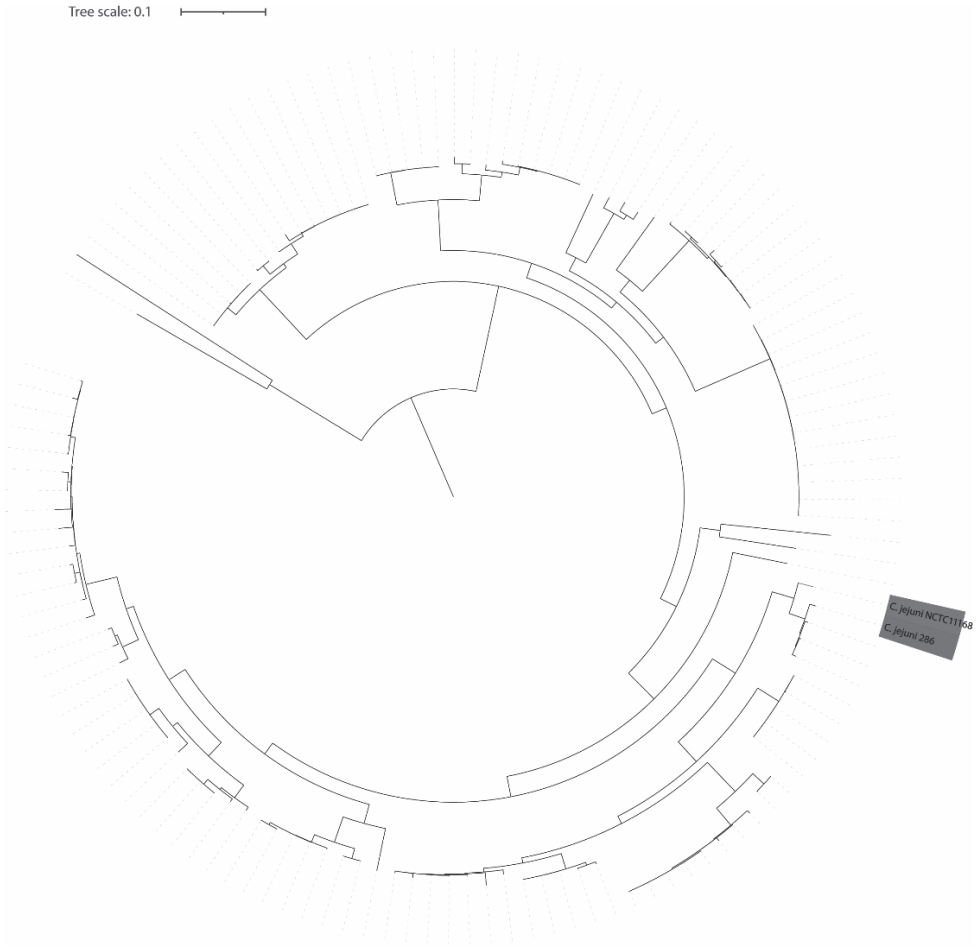
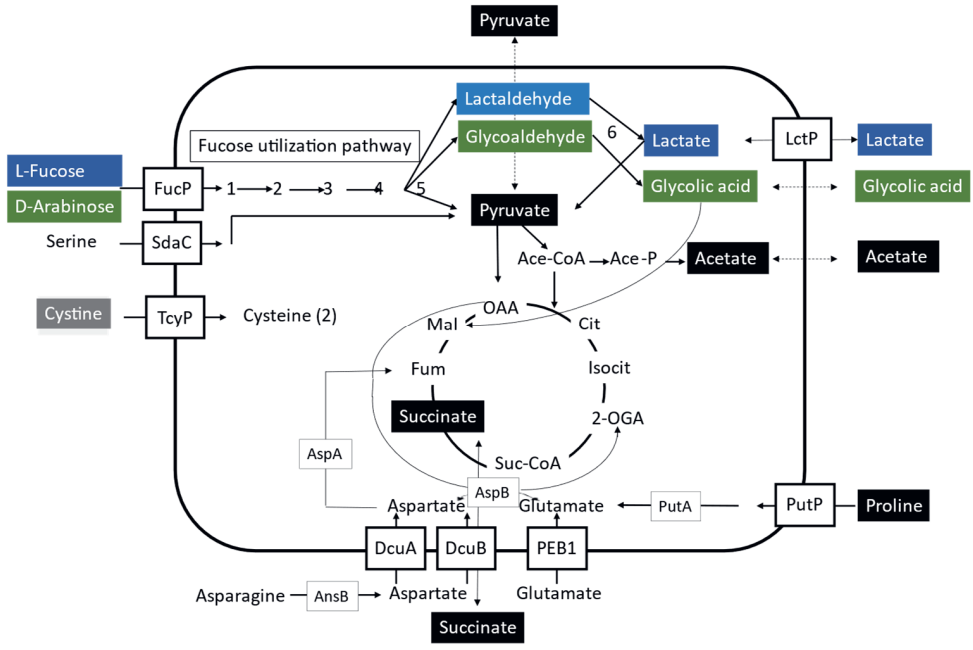
C. jejuni 286 isolated from a hospitalized patient was obtained via the Dutch National Institute for Public Health and the Environment (RIVM).

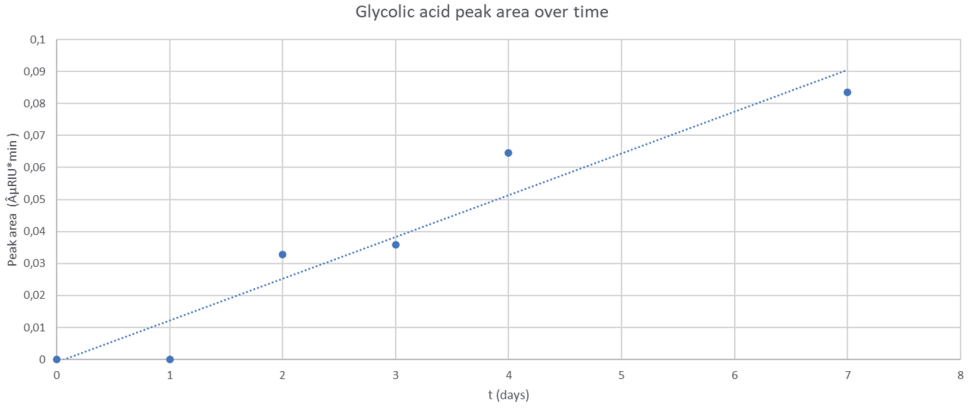
Supplementary files



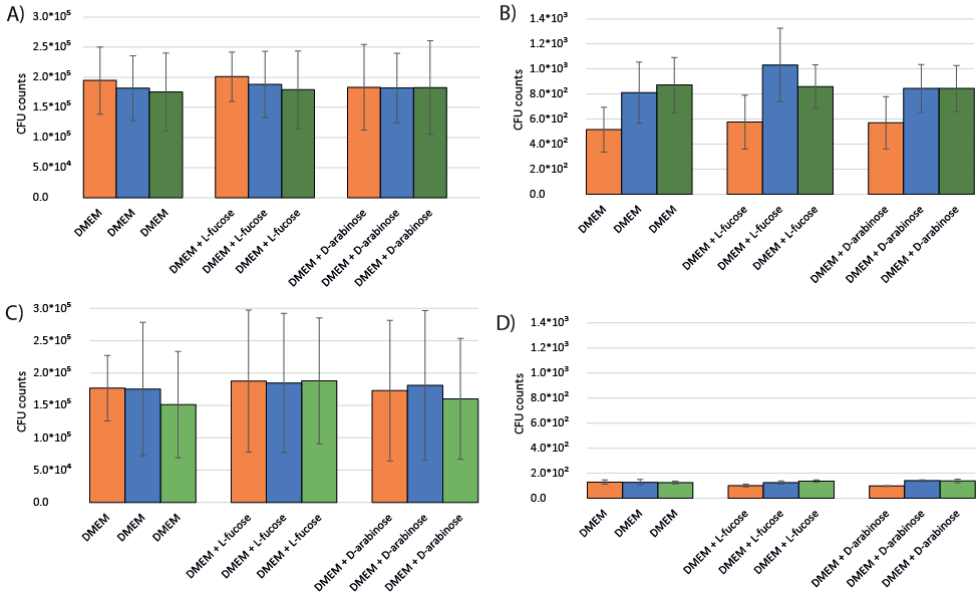
Suppl. Fig. 1 Phylogenetic tree of genomes of human *C. jejuni* isolates. *C. jejuni* NCTC11168 and *C. jejuni* 286 are highlighted in red and blue, respectively.



Suppl. Fig. 2 Schematic overview of uptake and metabolism uniquely coupled to fucose (blue) or arabinose (green) utilization in *Campylobacter*. Bidirectional arrows indicate efflux and uptake/metabolism of lactate in MEMαF, glycolic acid in MEMαA and acetate, pyruvate and succinate in MEMαF and MEMαA. The blue boxes indicate L-fucose transport and metabolism, the green boxes represent D-arabinose transport and metabolism, the black boxes show compounds of which the production and metabolism is linked to L-fucose and D-arabinose metabolism, and the dark grey box displays uptake and intracellular reduction resulting in two (2) cysteines, uniquely linked to L-fucose utilization. Uptake and metabolism of serine, asparagine, aspartate, glutamate, proline and cystine with respective putative transporters are indicated. Figure adapted from Middendorf et al. (2022).



Suppl. Fig. 3 Glycolic acid production in the presence of D-arabinose. Peak area increased over time, indicating a larger amount of glycolic acid in the medium.



Suppl. Fig. 4 Caco-2 cell adhesion and invasion results of *C. jejuni* NCTC11168 and *C. jejuni* 286 that were precultured for two days in MEMα medium (orange bars), MEMαF medium (blue bars) or MEMαA medium (green bars) and incubated in in DMEM, DMEM+ L-fucose or DMEM + D-arabinose. Adhesion (A) and invasion (B) of *C. jejuni* NCTC11168, and adhesion (C) and invasion (D) of *C. jejuni* 286. Error bars indicate the standard deviation of the biological replicates.

Suppl. Table 1 Full list of significant up and down regulated proteins (>1.5-fold-change).

Protein	Function	NCTC11168 fuc vs ref	NCTC11168 ara vs ref	286 fuc vs ref	286 ara vs ref
Cj0481|Q0PB32	Dihydrodipicolinate synthase	23.6	12.4	19.5	11.8
Cj0482|Q0PB31	Altronate hydrolase/dehydratase	273	160	152	101
Cj0483|Q0PB30	Altronate hydrolase/dehydratase	512	253	36.2	34.2
Cj0484|Q0PB29	MFS transporter			102	69.2
Cj0485|Q0PB28	Short chain dehydrogenase	23.6	23.6	17.4	13.3
Cj0486|Q0PB27	MFS transporter	46.4	21.8	19.9	15.2
Cj0487|Q0PB26	Amidohydrolase	88.0	39.6	9.0	8.6
Cj0488|Q0PB25	Epimerase	70.3	39.1	140	93.3
Cj0489-S|Q0PB24	Aldehyde dehydrogenase			26.6	18.1
Cj0489-L|Q0PB23	Aldehyde dehydrogenase	3.0		26.6	18.1
KatA|Q59296	H_2O_2 detoxification	21.1			
PglC|Q0P9D0	Galactosyltransferase	10.2			
Cj1658|Q0P7X1	ferri-rhodotorulic acid	7.6			
RpmB|Q9PI58	50S ribosomal protein L28	4.1			
RpsT|Q9PM64	30S ribosomal protein S20	3.7			
Cj0771c|Q0PAB9	Membrane	3.5	54.4	5.6	5.8
RpmF|Q9PIG9	50S ribosomal protein L32	3.4			
RpsS|Q9PLX5	30S ribosomal protein S19	2.4			
RpmA|Q9PJ31	50S ribosomal protein L27	2.3		-2.3	
RpsI|Q9PMI3	30S ribosomal protein S9	2.1			
Rp10|Q0P7U2	50S ribosomal protein L15	2.0			
DsbA|Q0PA24	Thiol:disulfide interchange protein DsbA	1.9	1.6		
RplV|Q9PLX6	50S ribosomal protein L22	1.9			
RpmG|Q9PI38	50S ribosomal protein L33	1.8			
RplP|Q9PLX8	50S ribosomal protein L16	1.8			
RpmC|Q9PLX9	50S ribosomal protein L29	1.7			
Cj0185c|Q0PBV4	Putative phnA domain protein	1.7	1.7		
RpsU|Q9PID2	30S ribosomal protein S2	1.6			
TrpF|Q9PIF3	N-(5'-phosphoribosyl)anthranilate isomerase	1.6			
RplU|Q0PC43	50S ribosomal protein L21	1.6	1.7		
Cj0414|Q0PB96	Xidoreductase subunit	1.6		2.5	2.2
Cj0539|Q0PAX7	Uncharacterized protein	-1.5			
Cj0413|Q0PB97	Beta-lactamase	-1.6		-2.4	
KdsB|Q0PA77	3-deoxy-manno-octulosonate cytidylyltransferase	-1.6			
PutA|Q0P8B5	Oxidizes proline to glutamate	-1.7	-1.6	-1.6	-2.2
PurQ|Q9PHZ7	Phosphoribosylformylglycinamidine synthase subunit	-1.7			
MoaE|Q0P8A1	MPT synthase subunit 2	-2.0	-1.7		

Cj0375|Q0PBD5	putative lipoprotein	-2.1			
CstA|Q0P9Y2	carbon starvation protein A	-3.2		-1.9	-2.5
TpiA|Q9PMQ6	Putative triosephosphate isomerase	-35.8	-14.3		
Cj0200c|Q0PBT9	putative periplasmic protein	-262	-99.0	-115	-115
Cj0519|Q0PAZ6	Putative rhodanese-like domain protein ?		15.5		
CeuE|Q0P8Q4	Enterochelin uptake periplasmic binding protein			1.5	
Cbf2|Q0PAS1	peptidyl-prolyl cis-trans isomerase			2.0	
Cj1417c|Q0P8J7	Gamma-glutamyl-CDP-amidate hydrolase	-67.3			
Cj0415|Q0PB95	GMC oxidoreductase subunit			2.5	2.2
Cj0915|Q0P9Y4	Putative hydrolase			1.6	1.8
AcsA|Q9PMD2	Acetyl-coenzyme A synthetase			-1.6	-2.7
FabD|Q0PC21	Malonyl CoA-acyl carrier protein transacylase				-1.6
MetAA|Q9PLV2	Homoserine O-acetyltransferase			1.7	1.5
MetE|Q9PN94	homocysteine methyltransferase			1.7	1.8
Cj0653c|Q0PAL5	Putative aminopeptidase			-3.2	-4.5
CfbpC|Q0PBW6	Putative iron-uptake ABC transport system			-1.6	
HydA|Q0P8Y9	Ni/Fe-hydrogenase small chain		1.6		
HydC|Q0P8Z1	Ni/Fe-hydrogenase B-type cytochrome subunit		-40.0		
SdhC|Q0PB71	Putative succinate dehydrogenase subunit C		-1.8		
LeuB|Q9PLW0	3-isopropylmalate dehydrogenase				1.6
Cj0264c|Q0PBP1	Trimethylamine-N-oxide reductase				1.5
Cj0075c|Q0PC60	Putative oxidoreductase iron-sulfur subunit				-1.6
LctP|Q0PC21	Malonyl CoA-acyl carrier protein transacylase				-1.6
Cj1515c|Q0P8A4	Carboxynorspermidine/carboxyspermidine decarboxylase				-1.6
Cj0073c|Q0PC62	LUD_dom domain-containing protein				-1.5
Cj1653c|Q0P7X5	Putative lipoprotein		1.8		
Cj0599|Q0PAR8	Putative OmpA family membrane protein		-1.5		
Peb3|Q0PBL7	Major antigenic peptide			-1.6	-1.8
Cj0093|Q0PC44	Putative periplasmic protein				1.5
Cj0455c|Q0PB58	Putative membrane protein			-40.1	
FlgD|Q0PC84	Basal-body rod modification protein FlgD			-2.0	-1.7
PrmA|Q9PNH7	Ribosomal protein L11 methyltransferase			-30.4	
Cj0722c|Q0PAF7	Putative DNA methylase				-78.5
Cj0358|Q0PBF1	Putative cytochrome C551 peroxidase		-1.6		
Cj1289|Q0P8W8	Possible periplasmic protein				1.6

Cj0761|Q0PAC9	possibly membrane related	54.4		
CdtB|Q0PC57	Cytolethal distending toxin B	1.7		
RplQ|Q0P832	50S ribosomal protein L17		1.8	
Cj0144|Q0PBZ3	Methyl-accepting chemotaxis signal transduction protein		-10.6	-10.6
Cj0834c|Q0PA56	Ankyrin repeat-containing putative periplasmic protein			-1.5
Peb1A|Q0P9X8	Major cell-binding factor ABC-transporter			-2.1
CmeA|Q0PBE3	Periplasmic fusion protein CmeA			1.6
CJ1232|Q0P924	Uncharacterized protein	15.0		
Cdtc|Q0PC58	Cytolethal distending toxin C		28.4	
Cj0092|Q0PC45	Putative periplasmic protein		3.8	2.5
Cj1666c|Q0P7W3	periplasmic protein		2.3	
Cj0371|Q9PID1	lipoprotein		1.9	1.5
Cj0998c|Q0P9Q4	utative periplasmic protein		1.5	
Cj1169c|Q0P987	Putative periplasmic protein		-3.9	-2.5
Cj1650|Q0P7X8	Uncharacterized protein		-1.6	
Cj1623|Q0P805	Putative membrane protein			-8.6

Suppl. Table 2 Full presence/absence list of 236 potential virulence proteins in *C. jejuni* NCTC11168 and 286. Presence and up or down regulation of potential virulence proteins in MEMαF and MEMαA grown cells is indicated, with fold-change in comparison to cells in MEMα. Present, indicates that the protein is detected in tested conditions, but not significantly changed; nd indicates that the protein is not detected in tested conditions.

		L-fucose	D-arabinose	L-fucose	D-arabinose
Uniprot	**Protein**	***C. jejuni* NCTC11168**	***C. jejuni* NCTC11682**	*C. jejuni* 286	***C. jejuni* 2862**
Q0P8D9	**CadF/Cj1478c**	present	present	present	present
Q0P8X7	**FlpA/Cj1279c**	-1.3	present	present	present
Q0PAY8	**FlgB/Cj0528c**	present	present	nd	nd
Q9PHP0	**Cj0627/capA**	present	present	nd	nd
Q0P8S5	**PseD/Cj1333**	present	present	nd	nd
Q0P9B5	**WlaN/Cj1139c**	present	present	nd	nd
Q0P9B7	**Cj1137c**	present	present	nd	nd
Q0P9D0	**PglC/Cj1124c**	10.2	present	nd	nd
Q0PAJ4	**CipA/Cj0685c**	nd	nd	present	present
Q0P9Y2	**CstA/Cj0917c**	-3.2	present	-1.9	-2.5
Q0PC12	**Cj0125c**	present	11.1	present	present
Q0P8J9	**Cj1415c**	-1.2	present	present	present
Q0P8W3	**PseC/Cj1294**	1.2	present	present	present
Q0P863	**PlfA/Cj1565c**	-1.1	present	present	present
Q0P8Z5	**RacR/Cj1261**	1.2	present	present	1.4
Q0P8X7	**CJE1415/Cj1279c**	-1.3	present	present	present
Q59296	**KatA/Cj1385**	21.1	present	present	present
Q0P8J7	**Cj1417c**	-67.3	present	present	present
Q9PN97	**LuxS/Cj1198**	present	-1.3	present	present
Q0P8I5	**Cj1429c**	present	1.2	present	present
Q0P9C9	**PglA/Cj1125c**	present	-1.3	present	present
Q0P8U1	**PseA/Cj1316c**	present	present	1.1	present
Q0P8H1	**Cj1443c**	present	-1.2	present	present
Q0P8R0	**CjE1538**	present	-1.3	present	present
Q0PBF1	**Cj0358**	present	-1.6	present	present
Q0PC57	**CdtB/Cj0078c**	present	1.7	present	present
Q0PC58	**CdtC/Cj0077c**	present	present	28.4	present
Q0P9X8	**Peb1A/Cj0921c**	present	present	present	-2.1
Q0PBL7	**Peb3/Cj0289c**	present	present	-1.6	-1.8
P96747	**FlaC/Cj0720c**	present	present	present	1.4
Q0P7V5	**MqnD/Cj1674**	present	present	present	1.4
Q0P8T3	**Cj1324**	present	present	-1.5	present
Q0PBE3	**cmeA/Cj0367c**	present	present	1.4	1.6
Q9PID1	**Cj0371**	present	present	1.9	1.5
Q0P8Q4	**CeuE/Cj1355**	present	present	1.5	present
Q0P8H7	**Cj1437c**	present	present	-1.3	present
Q0PC84	**FlgD/Cj0042**	present	present	-2.0	-1.7
Q0P7Q2	**FlgE/Cj0043**	present	present	-1.3	-1.3
Q0PBU4	**FliI/Cj0195**	present	present	present	1.3
P96747	**FlaC/Cj0720c**	present	present	present	1.4

Q0P7Q2	**FlgE2/Cj1729c**	present	present	-1.3	-1.3
Q0P987	**Cj1169c**	present	present	-3.9	-2.5
Q0PBH4	**FlhB/Cj0335**	present	present	present	present
Q0PAY9	**FlgC/Cj0527c**	present	present	present	present
Q9PHW6	**FliD/Cj0548**	present	present	present	present
Q0PAI1	**FlgG/Cj0698**	present	present	present	present
Q0P7Y2	**IamB/Cj1646**	present	present	present	present
Q0PC56	**CdtA/Cj0079c**	present	present	present	present
O85213	**DnaJ/Cj1260c**	present	present	present	present
Q0P9Y5	**CiaB/Cj0914c**	present	present	present	present
Q0P8D9	**CadF/To0461**	present	present	present	present
Q0PB65	**AcfB/Cj0448c**	present	present	present	present
Q0P966	**CetA/Cj1190c**	present	present	present	present
Q0P9X5	**CheB/Cj0924c**	present	present	present	present
Q0P9X6	**CheR/Cj0923c**	present	present	present	present
Q0PBM1	**CheV/Cj0285c**	present	present	present	present
Q0PBM3	**cheW/Cj0283c**	present	present	present	present
P0C635	**CheY/Cj1118c**	present	present	present	present
Q0P8B2	**Tlp1/Cj1506c**	present	present	present	present
Q0PCA6	**Tlp10/Cj0019c**	present	present	present	present
Q0PA15	**FlhA/Cj0882c**	present	present	present	present
Q0PA70	**FliP/Cj0820c**	present	present	present	present
Q0P928	**HtrA/Cj1228c**	present	present	present	present
Q0P8T2	**Cj1325**	present	present	present	present
Q9PMJ8	**FlgI/Cj1462**	present	present	present	present
Q0PC72	**FliA/Cj0061c**	present	present	present	present
Q0PBJ1	**FliF/Cj0318**	present	present	present	present
Q0PC73	**FliM/Cj0060c**	present	present	present	present
Q0PC74	**FliY/Cj0059c**	present	present	present	present
Q0PAK4	**RpoN/Cj0670**	present	present	present	present
Q0PBE4	**CmeB/Cj0366c**	present	present	present	present
Q0PBE5	**CmeC/Cj0365c**	present	present	present	present
Q0PBE2	**CmeR/Cj0368c**	present	present	present	present
Q0PBH5	**AhpC/Cj0334**	present	present	present	present
Q0PCB3	**Rrc/Cj0012c**	present	present	present	present
Q0PCA5	**Cj0020c**	present	present	present	present
Q0P8Y4	**SpoT/Cj1272c**	present	present	present	present
Q9PPE0	**Tpx/Cj0779**	present	present	present	present
Q0PB65	**Cj0448c**	present	present	present	present
P45492	**JlpA**	present	present	present	present
P80672	**PorA/Cjj81176**	present	present	present	present
Q0P8K1	**Cj1413c**	present	present	present	present
Q0P8K0	**Cj1414c**	present	present	present	present
Q0P8J8	**Cj1416c**	present	present	present	present
Q0P8J6	**Cj1418c**	present	present	present	present
Q0P8J5	**Cj1419c**	present	present	present	present
Q0P8J4	**Cj1420c**	present	present	present	present

Q0P8J3	**Cj1421c**	present	present	present	present
Q0P8J2	**Cj1422c**	present	present	present	present
Q0P8J1	**Cj1423c**	present	present	present	present
Q9PMN3	**Cj1424c**	present	present	present	present
Q0P8I9	**Cj1425c**	present	present	present	present
Q0P8I8	**Cj1426c**	present	present	present	present
Q0P8I7	**Cj1427c**	present	present	present	present
Q0P8I6	**Cj1428c**	present	present	present	present
Q0P8I4	**Cj1430c**	present	present	present	present
Q0P8I3	**Cj1431c**	present	present	present	present
Q0P8I2	**Cj1432c**	present	present	present	present
Q0P8I1	**Cj1433c**	present	present	present	present
Q0P8I0	**Cj1434c**	present	present	present	present
Q0P8H9	**Cj1435c**	present	present	present	present
Q0P8H8	**Cj1436c**	present	present	present	present
Q0P8H6	**Cj1438c**	present	present	present	present
Q0P8H5	**Cj1439c**	present	present	present	present
Q0P8H4	**Cj1440c**	present	present	present	present
Q0P8H3	**Cj1441c**	present	present	present	present
Q0P8H2	**Cj1442c**	present	present	present	present
Q0P8H1	**Cj1444c**	present	present	present	present
Q0P8H0	**Cj1445c**	present	present	present	present
Q0P8G8	**Cj1447c**	present	present	present	present
Q0P9D5	**PglG/Cj1119c**	present	present	present	present
Q0P9D4	**PglF/Cj1120c**	present	present	present	present
Q0P9D3	**PglE/Cj1121c**	present	present	present	present
Q0P9D1	**PglD/Cj1123c**	present	present	present	present
Q0P9C8	**PglB/Cj1126c**	present	present	present	present
Q0P9C7	**PglJ/Cj1127c**	present	present	present	present
Q0P9C6	**PglI/Cj1128c**	present	present	present	present
Q0P9C5	**PglH/Cj1129c**	present	present	present	present
Q0P8W4	**PseB/Cj1293**	present	present	present	present
Q0P8U6	**PseF/Cj1311**	present	present	present	present
Q0P8U5	**PseG/Cj1312**	present	present	present	present
Q0P8U4	**PseH/Cj1313**	present	present	present	present
Q0P8U0	**PseI/Cj1317**	present	present	present	present
Q0P8T1	**NeuB2/Cj1327**	present	present	present	present
Q0P8T0	**NeuC2/Cj1328**	present	present	present	present
Q0P8S7	**PtmB/Cj1331**	present	present	present	present
Q0P8S6	**PtmA/Cj1332**	present	present	present	present
Q0P8S2	**PseE/Cj1337**	present	present	present	present
Q0P8S3	**Maf1/Cj1318**	present	present	present	present
Q0P8S3	**Maf4/Cj1336**	present	present	present	present
Q0P8R9	**Maf6/Cj1340c**	present	present	present	present
Q0P8R8	**Maf7/Cj1341c**	present	present	present	present
Q0PC70	**FlhG/Cj0063c**	present	present	present	present
O52908	**FlhF/Cj0064c**	present	present	present	present

Q0PBJ0	**FliG/Cj0319**	present	present	present	present
Q0PBI9	**FliH/Cj0320**	present	present	present	present
Q0PBH3	**MotB/Cj0336c**	present	present	present	present
Q0PBH2	**MotA/Cj0337c**	present	present	present	present
Q0PBF8	**FliN/Cj0351**	present	present	present	present
Q9PHY8	**FliE/Cj0526c**	present	present	present	present
Q0PAW9	**FlaG/Cj0547**	present	present	present	present
Q0P9M8	**FlgR/Cj1024c**	present	present	present	present
P56964	**FlaB/Cj1338c**	present	present	present	present
P56963	**FlaA/Cj1339c**	present	present	present	present
Q0P8K6	**FliL/Cj1408**	present	present	present	present
Q0P8E9	**FlgK/Cj1466**	present	present	present	present
Q0P8Z4	**RacS/Cj1262**	present	present	present	present
Q0PC46	**Cj0091**	present	present	present	present
Q0PAS9	**TylA/cj0588**	present	present	present	present
Q0P9Y5	**CiaB/Cj0914c**	present	present	present	present
Q0P967	**CetB/Cj1189c**	present	present	present	present
Q0PBM2	**CheA/Cj0284c**	present	present	present	present
Q0P8S4	**Maf3/Cj1334**	present	present	present	present
Q0P9C1	**C8J/1073/WaaC**	present	present	present	present
Q0P9A9	**C8J/1091/WaaV**	present	present	present	present
Q0P9A8	**C8J/1092/WaaF**	present	present	present	present
Q9PNE6	**C8J/1095/GmhA1**	present	present	present	present
Q6TG09	**C8J/1096/WaaE**	present	present	present	present
Q0PA08	**CJE0969/Cj0890c**	present	present	present	present
Q0P934	**CJE1357/DccS**	present	present	present	present
Q0P933	**CJE1358/DccR**	present	present	present	present
Q0P930	**CJE1361/Cj1226c**	present	present	present	present
Q0P929	**CJE1362/Cj1227c**	present	present	present	present
Q0P8C5	**CjE1665/Cj1492c**	present	present	present	present
Q0P914	**Cj1242/CiaC**	present	present	present	present
Q0P9I5	**Cj1069**	present	present	present	present
Q0P870	**Cj1556**	present	present	present	present
Q0P8K4	**Cj1410c**	present	present	present	present
Q0P9S5	**Cj0977**	present	present	present	present
Q9PM85	**InfA/Cj1590**	present	present	present	present
Q0P8N8	**Cj1371**	present	present	present	present
Q0P9L0	**Cj1042c**	present	present	present	present
Q0PC83	**FlgE**	nd	nd	nd	nd
Q0PAW7	**FliS/Cj0549**	nd	nd	nd	nd
Q0PA11	**FlaD/Cj0887c**	nd	nd	nd	nd
Q0P8Q8	**PldA**	nd	nd	nd	nd
Q0P7V4	**FliQ/Cj1675**	nd	nd	nd	nd
Q0P977	**FliR/Cj1179c**	nd	nd	nd	nd
Q0PAD4	**CfrA/Cj0755**	nd	nd	nd	nd
Q0P814	**ChuA/Cj1614**	nd	nd	nd	nd
Q0PBW1	**Cj0178**	nd	nd	nd	nd

Q0P8T6	**Cj1321**	nd	nd	nd	nd
Q0P8T4	**Cj1323**	nd	nd	nd	nd
Q9PPM0	**FgH/Cj0687c**	nd	nd	nd	nd
Q0PC85	**FliK/Cj0041**	nd	nd	nd	nd
Q0P8G8	**TagH/CCC13826**	nd	nd	nd	nd
Q0P8T6	**Cj1321**	nd	nd	nd	nd
Q0P8G7	**Cj1448c**	nd	nd	nd	nd
Q0P9C0	**C8J/1074/HtrB**	nd	nd	nd	nd
Q0PC85	**Cj0041**	nd	nd	nd	nd
Q0PAI2	**FlgG2/Cj0697**	nd	nd	nd	nd
Q0P8C6	**CjE1664/Cj1491c**	nd	nd	nd	nd
Q0P7V2	**Cj1677/CapB**	nd	nd	nd	nd

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                    1  ........10........20........30........40........50........60
NCTC11168             MKHANSIKLELVCKKISWRILPLIVLMFCLSMLDRTNISFVKSHIEIDAGIGEAAYALGA
286                   MKHANSIKLELVCKKISWRILPLIVLMFCLSMLDRTNISFVKSHIEIDAGIGEAAYALGA

                    61........70........80........90.......100.......110.......120
NCTC11168             GIFFIGYAIFEVPSNLFLHKLGAKIWLSRIMITWGLVTMAMIFIQGEISFYVLRFLLGLT
286                   GIFFIGYAIFEVPSNLFLHKLGAKIWLSRIMITWGLVTMAMIFIQGEISFYVLRFLLGLT

                    121......130.......140.......150.......160.......170.......180
NCTC11168             EAGFSPGIILYLSYFFPAIYRSKAYGIYQMGVPIAFVFGSLISGFILDYTPNIYFKNWQW
286                   EAGFSPGIILYLSYFFPAIYRSKVYGIYQMGVPIAFVFGSLISGFILDYTPNIYFKNWQW

                    181......190.......200.......210.......220.......230.......240
NCTC11168             MFLIEGGITVLVGIFCLFYLDSHPKDAKWLDIKEKDILLKHIEISNTKAKDYSIKDIFKS
286                   MFLIEGGITVLVGIFCLFYLDSHPKDAKWLDIKEKDILLKHIEISNTKAKDYSIKDIFKS

                    241.....250.......260.......270.......280.......290.......300
NCTC11168             ILVWKFVFVYFCIQLSVYGVLFYLPSKIAQILQINVGFEVGLLNAIPWIFVFIALPIFTS
286                   ILVWKFVFVYFCIQLSVYGVLFYLPSKIAQILQINVGFEVGLLNAIPWIFVFIALPIFTS

                    301......310.......320.......330.......340.......350.......360
NCTC11168             LADKKHSWNLHAILFLLLASLSMIASTFVTNLAAFLFFISLAAIGFIVIQPIFWNLPTQV
286                   LADKKHSWNLHAILFLLLASLSMIASTFVTNLAAFLFFISLAAIGFIVIQPIFWNLPTQV

                    361......370.......380.......390.......400.......410.......420
NCTC11168             LKGKGAAAAIALIGSLGNLGGFVAPTLKTYIENHFGVEFGLIVLALIAIL*
286                   LKGKGAAAAIALIGSLGNLGGFVAPTLKTYIENHFGVEFGLIVLALIAILGVLVLIHLKI

                    421.........
NCTC11168
286                   TLNLDKGE*
```

Suppl. Fig. 5 Cj0484 protein alignment between *C. jejuni* NCTC11168 and *C. jejuni* 286.

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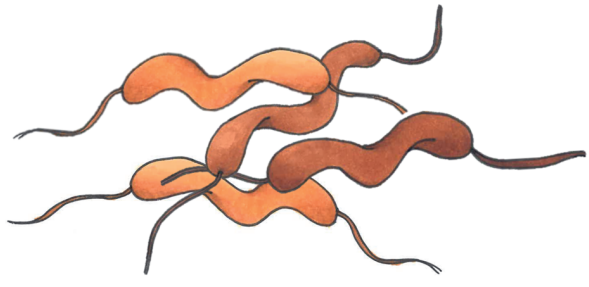


4

Impact of L-fucose and D-glucose metabolism on growth performance and proteomics changes in *Campylobacter jejuni* strain 18-440 that contains the L-fucose and D-glucose utilization clusters

Pjotr S. Middendorf[1,2], Lenja M. Stok[1], Aldert Zomer[3,4] , Sjef Boeren[5], Wilma F. Jacobs-Reitsma[2], Heidy M. W. den Besten[1], and Tjakko Abee[1]

Affiliations:

1. Food Microbiology, Wageningen University & Research, Wageningen, the Netherlands
2. National Institute for Public Health and the Environment, Bilthoven, the Netherlands
3. Faculty of Veterinary Medicine, Department of Infectious Diseases and Immunology, Utrecht University, Utrecht, Netherlands
4. WHO Collaborating Center for Campylobacter/OIE Reference Laboratory for Campylobacteriosis, Utrecht, Netherlands.
5. Laboratory of Biochemistry, Wageningen University & Research, Wageningen, the Netherlands.

Abstract

Campylobacter was previously considered asaccharolytic, but it is now known that some isolates possess specific saccharide metabolization pathways, including the L-fucose utilization pathway (*Cj0480c – Cj0489)* and the D-glucose utilization pathway (*glcP, pgi2, glk, pgl, zwf, edd* and *eda)*. In this study, we characterized the whole genome-sequenced water isolate *C. jejuni* 18-440, which carries both pathways. The impact of L-fucose and D-glucose on metabolism, growth, and survival was quantified, and complemented with proteomics data of cells incubated in DMEM medium without and with added saccharides.

Both L-fucose and D-glucose consumption stimulated growth and prolonged survival of *C. jejuni* up to 14 days, and was linked to the production of respective signature end-products, lactate and pyruvate. Proteomics analysis showed that 75 and 22 proteins were significantly differentially expressed in the presence of L-fucose and D-glucose, respectively, compared to DMEM-grown cells. Proteins in the L-fucose utilization cluster were 5.5-52.5-fold higher expressed, while only the GlcP glucose transporter protein was higher expressed in the D-glucose cluster. In addition, significant differential expression of membrane and periplasmic located proteins with functions in lipooligosaccharide (LOS) synthesis, metal (heme) acquisition (including multicomponent transport systems), redox reactions and metabolism, were observed in L-fucose grown cells. Notably, in these cells, expression of several proteins in the D-glucose utilization cluster was either strongly repressed (Glk, Pgi2, Pgl) or completely absent (GlcP), conceivably to prevent metabolism of glucose-6-phosphate derived from glyceraldehyde-3-phosphate via gluconeogenesis. In D-glucose grown cells, significant differential expression of membrane and periplasmic proteins involved in redox reactions and metabolism was also observed; albeit on much smaller scale than observed with L-fucose. The results of the current study suggest that L-fucose and D-glucose metabolism provides a fitness advantage by improving growth and survival of this *C. jejuni* 18-440 isolate. The extensive L-fucose-induced rewiring of *C. jejuni*'s metabolism including proteins involved in LOS production, may additionally result in capsular changes. Whether these combined effects provide a competitive advantage in L-fucose-rich environments such as encountered in animal and human hosts remains to be elucidated.

Introduction

Campylobacter is a Gram-negative zoonotic bacterium and is the principal cause of bacterial foodborne gastroenteritis in the world (Kaakoush et al., 2015; Špačková et al., 2019; Tack et al., 2019; EFSA, 2021). *Campylobacter jejuni* and *Campylobacter coli* are the most relevant species and account for nearly all diagnosed *Campylobacter* infections, of which most are linked to handling and consumption of (poultry) meat products, direct contact with animals and contact with environmental waters (Clark et al., 2003; Kuusi et al., 2004; Karagiannis et al., 2010; Kuhn et al., 2017; Montgomery et al., 2018; Kenyon et al., 2020; EFSA, 2021). Infection with *C. jejuni* is characterized by fever, watery or bloody diarrhea, abdominal pain and can lead in rare cases to complications such as the neurological disorder Guillain-Barré syndrome (Yuki et al., 1993; Rees et al.; Blaser, 1997; Nachamkin et al., 1998; Hansson et al., 2018).

It is well known that *Campylobacter* utilizes citric-acid intermediates, amino acids and peptides, however, studies have shown that some *Campylobacter* isolates are also able to utilize L-fucose, D-arabinose and/or D-glucose as carbon/energy sources (Parsons, 1984; Muraoka & Zhang, 2011; Stahl et al., 2011; Hofreuter, 2014; Vorwerk et al., 2015; Dwivedi et al., 2016; Vegge et al., 2016; van der Hooft et al., 2018; Garber et al., 2020; Middendorf et al., 2022). To be able to metabolize these sugars, two carbon utilization clusters were identified, the L-fucose utilization cluster, responsible for utilization of L-fucose and D-arabinose, and the D-glucose utilization cluster, responsible for D-glucose utilization (Muraoka & Zhang, 2011; Stahl et al., 2011; Vorwerk et al., 2015; Vegge et al., 2016). *Campylobacter* is naturally competent, allowing the microorganism to evolve via horizontal gene transfer (HGT) and recombination (Wang & Taylor, 1990; Boer et al., 2002).

Comparative whole genome sequence (WGS) analysis of *C. jejuni* and *C. coli* isolates, isolated from different sources in the Netherlands, revealed that approximately 51% of the sequenced *C. jejuni* isolates and 47% of the sequenced *C. coli* isolates possess the L-fucose utilization cluster (*Cj0480c – Cj0489*), from here on called fuc+ isolates, and approximately 0.2% of the sequenced *C. jejuni* isolates and 10% of the sequenced *C. coli* isolates possess the D-glucose utilization cluster (*glcP*, *pgi2*, *glk*, *pgl*, *zwf*, *edd* and *eda*), from here on called gluc+ isolates (Chapter 5). Interestingly, in this representative genomic dataset of Dutch *C. jejuni* and *C. coli* isolates, 100% of the gluc+

C. jejuni isolates and 83% of the gluc+ *C. coli* isolates also carried the L-fucose utilization cluster. Furthermore, the phylogenetic study of the Dutch genomic dataset identified several isolates that carried an L-fucose utilization cluster that was obtained via HGT from another *Campylobacter* species (*C. jejuni* or *C. coli*) (Chapter 5). Upon consumption of L-fucose, fuc+ isolates have shown increased growth, increased survival, decreased biofilm formation, changes in the general metabolism and increased virulence (Stahl et al., 2011; Dwivedi et al., 2016; Garber et al., 2020; Middendorf et al., 2022; Middendorf et al., 2023). In fuc+ isolates, L-fucose is transported by the transporter Cj0486/*FucP* (and potentially *Cj0484*) and further metabolized via the metabolic enzymes *Cj0488*, *Cj0485* (*FucX*), *Cj0487*, *Cj0482*/*Cj0483*, *Cj0481* (DapA) and *Cj0489*/*Cj0490*, respectively (Garber et al., 2020; Stahl et al., 2011).

The glucose utilization cluster is much rarer and therefore less studied in *Campylobacter*. So far, the effect of D-glucose has only been studied in *C. coli*, where gluc+ isolates showed increased growth, survival and an enhanced biofilm formation in the presence of D-glucose (Vorwerk et al., 2015; Vegge et al., 2016). Glucose is metabolized via the Entner-Doudoroff (ED) pathway in which glucose is transported into the cell via GlcP and further metabolized to pyruvate via *glk, pgi2, zwf, pgl, edd* and *eda,* respectively. The end products of L-fucose metabolism (lactate and pyruvate) and of the D-glucose metabolism (pyruvate) can be further metabolized via the general metabolism of *Campylobacter* (Stahl et al., 2012; Hofreuter, 2014). Both L-fucose and D-glucose can be found in environments like the human gut and the intestine of several animals including chicken and pigs, potentially contributing to *Campylobacter*'s fitness (Rath et al., 2022).

In chapter 5, the identification of the *C. jejuni* 18-440 isolate is described , which carries an atypical L-fucose cluster with high similarity to that of *C. coli*. Moreover, this isolate also carries the rare D-glucose utilization cluster. Here we provide an analysis of the activation of these clusters in this strain grown in DMEM medium without and with added L-fucose and D-glucose, and quantified the impact on growth, survival, metabolism, and proteome expression.

Methods

Bacterial isolate selection and medium preparation

The water isolate *C. jejuni* 18-440 was used is this study. This isolate carries a *C. coli* L-fucose utilization cluster and a D-glucose utilization cluster, as previously described in Chapter 5. Isolate *C. jejuni* 18-440 was obtained via the National Institute for Public Health and the Environment (RIVM, Bilthoven, the Netherlands) and was described in (Mughini-Gras et al., 2021).

C. jejuni cultures were obtained on CAB plates, consisting of Columbia Agar Base (Oxoid, Landsmeer, the Netherlands) supplemented with 5% (v/v) lysed horse blood (BioTrading Benelux B.V. Mijdrecht, the Netherlands) and 0.5% (w/w) bacteriological agar No.1 (Oxoid). *Campylobacter* stock cultures were prepared by inoculating a colony in BactoTM Heart Infusion broth (Becton, Dickinson and Company, Vianen, the Netherlands) followed by incubation for 24 hours at 41.5 °C in microaerobic conditions (5% O_2, 10% CO_2, 85% N_2), which was created using an Anoxomat WS9000 (Mart Microbiology, Drachten, the Netherlands). Glycerol stocks were prepared using 30% glycerol (Sigma) and a 70% 24-hours-culture and the stocks were stored at -80 °C.

Working cultures were prepared by streaking the *C. jejuni* freezer stocks on CAB plates which were incubated for 48 hours at 41.5 °C in microaerobic conditions. Single colonies were routinely selected and inoculated in 10 mL Dulbecco's Modified Eagle Medium (DMEM medium) (Thermo Fisher Scientific, Bleiswijk, the Netherlands) supplemented with 20 µM $FeSO_4$ (Merck, Schiphol-Rijk, the Netherlands) and microaerobically cultured overnight at 41.5 °C. A second overnight culture was made by diluting the *C. jejuni* suspension 1:100 (v/v) in 10 mL fresh DMEM and the suspension was incubated at 41.5 °C for 24 hours in microaerobic conditions to obtain standardized working cultures.

Prior to experiments, DMEM medium and DMEM medium supplemented with 25.0 mM L-fucose (DMEM-F medium) or supplemented with 25.0 mM D-glucose (DMEM-G medium) were prepared and filter-sterilized using 0.2-µm pore-sized filters. Sterilized infusion bottles that were closed using a rubber stopper and an aluminum cap were filled with 45 mL filter-sterilized DMEM medium, DMEM-F medium or

DMEM-G medium using a syringe. Filled infusion bottles were stored at 4 °C until further use.

L-fucose utilization cluster alignments and SMASH++ genome comparisons

L-fucose utilization cluster alignments were performed by the online tool Benchling (www.benchling.com). L-fucose utilization clusters of *C. jejuni* 18-440, *C. jejuni* Ca2426 and *C. coli* Ca0121, were aligned against the cluster of the reference isolate *C. jejuni* NCTC11168. Isolates *C. jejuni* Ca2426 and *C. coli* Ca0121 were previously described in (Middendorf et al., 2022).

SMASH++ genome comparisons were performed as described previously (Hosseini et al., 2020; Dieckmann et al., 2021). Briefly, the genomes of *C. jejuni* NCTC11168, *C. jejuni* 18-440 and the *C. coli* reference genome *C. coli* ASM973039v1 were divided into non-overlapping segments of 1000 bp each, using the linux tool splitter. SMASH++ was used to match these segments against the genomes of *C. jejuni* NCTC11168 and *C. jejuni* 18-440. Using the build-in visualization tool of SMASH++, images were created displaying overlapping regions.

L-fucose and D-glucose growth experiments

As described previously (Middendorf et al., 2022), working cultures were decimally diluted in DMEM medium to a cell concentration of approximately 5.0 $\log_{10}$ CFU/mL. A final dilution step was done by adding 5 mL into the infusion bottles filled with 45 mL DMEM medium, DMEM-F medium or DMEM-G medium, resulting in a starting cell concentration of 4.0 $\log_{10}$ CFU/mL. Incubation of the inoculated infusion bottles was done at 37 °C. At various timepoints, namely, day 0, day 1, day 2, day 3, day 4, day 7, day 10 and day 14, approximately 4 mL sample was taken from each infusion bottle and the head space of infusion bottles was flushed for 2 minutes with microaerobic gas (5% O_2, 10% CO_2, 85% N_2) using a home-made gas flushing device and syringes to puncture the rubber stopper and aluminum cap of the infusion bottles.

The 4 mL-aliquots were used to determine the bacterial concentration. The remainder of each sample (in either DMEM medium, DMEM-F medium or DMEM-G medium) was stored at -20 °C for high pressure liquid chromatography (HPLC) analyses. Bacterial concentrations were determined by decimally diluting 1 mL of sample in

peptone physiological salt solution (PPS, Tritium Microbiologie, Eindhoven, the Netherlands), followed by surface plating on CAB plates. CAB plates were incubated in jars for 48 hours at 41.5 °C in microaerobic conditions. Colonies were counted and expressed in $\log_{10}$ CFU/mL. Each sample was microscopically analysed using an Olympus BX 41 microscope (lens Ach 100x/1.25, Olympus Nederland, Leiderdorp, the Netherlands) and pictures were captured using CellSens Imaging software (Olympus Corporation). Three biologically independent reproductions were performed per condition, i.e., DMEM medium, DMEM-F medium and DMEM-G medium, on different days.

High pressure liquid chromatography for organic acids and amino acids

The frozen samples stored during the growth experiments were thawed for HPLC analyses using the procedure as previously described by Middendorf et al. (2022). Briefly, for organic acids analysis, 250 µL of the collected samples were centrifugated at 13,000 *g* at 4 °C for 5 minutes. Pellets were removed and the supernatant was treated for protein decontamination with Carrez A ($K_4FeCN)6{\cdot}3H_20$, Merck) and B ($ZnO_4{\cdot}7H_20$, Merck). After centrifugation, the supernatant was added to HPLC vials. Quantitative analyses were done using standards with pre-made concentrations for L-fucose, D-glucose, acetate, succinate, pyruvate and lactate. The HPLC was performed on an Ultimate 3000 HPLC (Dionex, Sunnyvile, USA) equipped with an RI-101 refractive index detector (Shodex, Kawasaki, Japan), an autosampler and an ion-exclusion Aminex HPX – 87H column (7.8 × 300 mm) with a guard column (Bio-Rad, Hercules, CA, USA). As mobile phase, 5 mM H_2SO_4 (Merck) was used at a flow rate of 0.6 mL/min. Column temperature was kept at 40 °C. For each run, the injection volume was 10 µL and the run time 30 minutes. Chromeleon software (Thermo Fisher Scientific, Waltham, USA) was used for quantification of compound concentrations.

For amino acids analyses, samples were used in aliquots of 40 µL. These aliquots were kept on ice and were diluted with 50 µL of 0.1 M HCl (containing 250 µM Norvalin as internal standard, Merck). The samples were deproteinized by addition of 10 µL of cold 5-sulphosalicilic acid (SSA, Merck) (300 mg/mL) and centrifuged at 13,000 *g* at 4°C for 10 minutes. In order to obtain an optimal pH for derivatization (pH between 8.2 to 10.0), approximately 60 to 150 µL of 4N NaOH was added to 5 mL of the AccQ•Tag™ Ultra borate buffer (Borate/NaOH buffer, Waters, Milford, USA). For

derivatization 60 µL of Borate/NaOH was added to a total recovery vial. Twenty µL of the supernatant obtained after deproteinization of the plasma was added and mixed. To each of the vials 20 µL of AccQ Tag Ultra derivatization reagent (Waters) dissolved in acetonitrile was added and mixed for 10 s. Each vial was immediately capped. The vials were then heated for 10 minutes at 55 °C. The vials were stored at -20°C prior to HPLC analysis. Quantitative analyses were done using standards with pre-made concentrations for histidine, asparagine, serine, glutamine, arginine, glycine, aspartic acid, glutamic acid, threonine, alanine, proline, cystine, lysine, tyrosine, methionine, and valine. HPLC was performed on an Ultimate 3000 HPLC (Dionex) equipped with an RI-101 refractive index detector (Shodex), an autosampler and an ion-exclusion Aminex HPX – 87H column (7.8 × 300 mm) with a guard column (Bio-Rad). As mobile phase, eluants A and B (Waters) was used at a flow rate of 0.7 mL/min. Column temperature was kept at 55 °C. For each run, the injection volume was 1 µL and the run time 17 minutes. Chromeleon software (Thermo Fisher Scientific) was used for the determination of compound concentrations. Baseline separation was obtained for all amino acids except glutamine and arginine.

Proteomics

The working cultures of *C. jejuni* 18-440 were used to inoculate in triplicate infusion bottles to a concentration of 5.0 $\log_{10}$ CFU/mL that were filled with 45 mL of DMEM, DMEM-F or DMEM-G medium, and cultures were grown for 48 hours at 37 °C in microaerobic conditions. Thirty mL of each culture was centrifugated and washed three times in TRIS buffer (pH 8.0) and resuspended in a final volume of 100 µl. The cultures were then sonicated with a sonication probe for 30 seconds. Protein concentrations were determined by the bicinchoninic acid (BCA) assay.

The protein aggregation capture (PAC) method as described by (Batth et al., 2019) and (Liu et al., 2021) was used in a slightly modified way for sample preparation for proteomics analysis. Briefly, each sample, containing 60 µg of protein, was reduced with 15 mM DTT at 45 °C for 30 minutes, unfolded in 6 M urea and alkylated with 20 mM acrylamide at room temperature for 30 minutes. The pH of the protein solution was adjusted to 7.0 using 10% (v/v) trifluoro-acetic acid (TFA). SpeedBeads (magnetic carboxylate modified particles, GE Healthcare, Chicago, USA) of products 45152105050250 and 65152105050250 were mixed with 1:1 (v/v) ratio at 50 µg/µl, and 8

µl of SpeedBeads was added to each protein sample. Acetonitrile was added up to 71% (v/v) to the protein beads mixture, incubated at room temperature with gentle shaking for 20 minutes. A magnet was used to separate the SpeedBeads from the supernatant for 30 seconds, and the supernatant was removed. The SpeedBeads were then washed with 1 mL of 70% ethanol and 1 mL of 100% acetonitrile successively, resuspended in 100 µl of 5 ng/µl sequencing grade trypsin solution in 50 mM ammonium bicarbonate and incubated overnight at room temperature with gentle shaking. The pH of SpeedBeads suspension was adjusted to 3 using 10% TFA, and the SpeedBeads were separated from the supernatant by using a magnet. The supernatant was filtered using C8 Empore disk filters. To improve yield, 0.1% formic acid was used to wash the beads and a 1:1 (v/v) mixture of acetonitrile and 0.1% formic acid was used to wash the filter. All eluents were combined and dried to 10–15 µL, then topped up to 50 µl with 0.1% formic acid.

For the LC-MS/MS analysis, 5 µl of prepared sample was injected into the system, and the analysis was performed as described in Liu et al. (2021). The MaxQuant quantitative proteomics software package was used to analyse LC-MS data with all MS/MS spectra as described by (Cox et al., 2014) and the proteome of *C. jejuni* NCTC11168 (UniProt ID UP000000799) was used as the protein database. Next, Perseus was used for filtering and further bioinformatics and statistical analysis of the MaxQuant ProteinGroups files (Tyanova et al., 2016). Reverse hits were removed; identified protein groups contained minimally two peptides, of which at least one is unique and one unmodified. The normalised label-free quantification (LFQ) intensity values as calculated by MaxQuant of each tested condition measured in biological triplicates were used.

The lower detection limit was set at 5.6 $\log_{10}$ (LFQ protein abundance), just below the lowest measured LFQ intensity by imputation NaN's by 5.6 after logarithmization. T-tests were performed on triplicates of each tested condition and were corrected with a false-discovery rate of 0.05. Protein fold-changes were displayed as fold-change, as example: a fold-change of 2 indicates that a two times higher protein concentration was measured in the tested condition versus the reference condition; and a fold-change of -2 indicates that a two times lower protein concentration was observed in the tested condition versus the reference condition. Proteins are only considered to

be differentially expressed significantly when a fold-change of over 1.5 or below -1.5 was calculated with a p value of less than 0.05.

Results

Genomic characterization of the L-fucose cluster in *C. jejuni* 18-440

Here we examined whether the *C. jejuni* 18-440 L-fucose utilization cluster differs from clusters in other isolates. Cluster alignments of *C. jejuni* 18-440, and two isolates that were previously used in L-fucose metabolism of *C. jejuni* (Middendorf et al., 2022), namely *C. jejuni* Ca2426 and *C. coli* Ca0121, were performed against the L-fucose utilization cluster of the reference isolate *C. jejuni* NCTC11168 (Figure 1). Results clearly indicate a high similarity between the L-fucose utilization cluster of *C. jejuni* NCTC11168 and *C. jejuni* Ca2426, while isolate *C. jejuni* 18-440 carries many point mutations when compared to *C. jejuni* NCTC11168. Interestingly, only the gene *Cj0484* of *C. jejuni* 18-440 displayed high similarities to *Cj0484* of isolate *C. jejuni* NCTC11168. Furthermore, the alignment highlighted that the genes *Cj0485 (*partly*), Cj0486, Cj0487 and Cj0488* of isolate *C. jejuni* 18-440 had similar point mutations as *C. coli* Ca0121, compared to the reference *C. jejuni* NCTC11168 cluster (Figure 1), highlighting that these genes are more similar to that of *C. coli* Ca0121.

Dieckmann et al. (2021) described several hybrid *C. coli*/*C. jejuni*, with large quantities of both *C. coli* and *C. jejuni* DNA. To investigate whether isolate *C. jejuni* 18-440 is a hybrid *C. coli*/*C. jejuni* isolate, we investigated whether this isolate carried large amounts of *C. coli* DNA. For this, the novel alignment tool SMASH++ was used to compare the genomes of *C. coli* ASM973039v1, *C. jejuni* 18-440 and *C. jejuni* NCTC11168 with each other. Genomes were divided in 1000 bp fragments. Results showed a high similarity between most *C. jejuni* 18-440 and *C. jejuni* NCTC11168 fragments (Suppl. Fig. 1A), while a comparison of the *C. jejuni* 18-440 genome to the *C. coli* ASM973039v1 genome highlighted small amounts of genomic rearrangements (Suppl. Fig. 1C). Similar results were obtained when comparing the reference genome *C. jejuni* NCTC11168 to the *C. coli* ASM973039v1 genome (Suppl. Fig. 1B), suggesting that the tested *C. jejuni* 18-440 is not a hybrid isolate that carries large amounts of *C. coli* DNA, but that the fucose cluster does show a HGT signature.

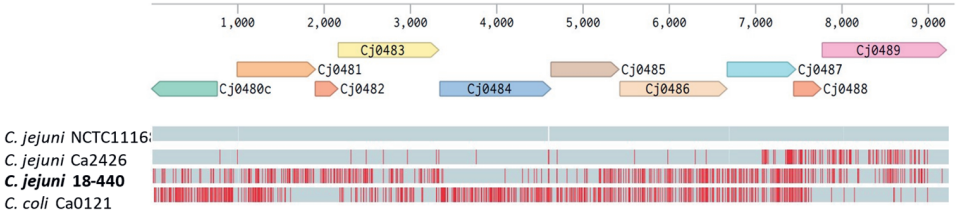


Figure 1 Overview of a genomic alignment of the L-fucose utilization cluster against the L-fucose utilization cluster of the reference genome *C. jejuni* NCTC11168. Red lines indicate point mutations in comparison with isolate *C. jejuni* NCTC11168.

Growth of *C. jejuni* 18-440 in DMEM supplemented with L-fucose and D-glucose

We investigated growth and survival of isolate *C. jejuni* 18-440 for up to 14 days in DMEM, DMEM-F and DMEM-G medium (Figure 2A). At day 1, no significant growth differences were observed, with cell counts reaching 7.4, 7.6 and 7.4 $\log_{10}$ CFU/mL in DMEM, DMEM-F and DMEM-G medium, respectively. At day 2, significantly higher cell counts were observed when grown in DMEM-F, compared to DMEM. CFU counts reached 7.3, 7.8 and 7.8 $\log_{10}$ CFU/mL when grown in DMEM, DMEM-F and DMEM-G, respectively. At day 3 and 4, significantly higher cells were observed when grown in either DMEM-F or DMEM-G medium, compared to DMEM, reaching 7.3, 7.9 and 7.8 log10 CFU/mL at day 3 and 7.4, 8.0 and 7.7 at day 4 when grown in DMEM, DMEM-F and DMEM-G, respectively. From here on, no significant differences were observed until day 10 between the CFU counts (7.7 +/- 0.4 $\log_{10}$ CFU/mL) in all tested media. At day 14, CFU counts significantly decreased in DMEM medium, while CFU counts in DMEM-F and DMEM-G remained at higher concentrations, reaching 5.5, 7.0 and 6.7 $\log_{10}$ CFU/mL, respectively.

HPLC analyses confirmed the absence of L-fucose and D-glucose in DMEM medium. Incubation in DMEM-F medium resulted in L-fucose consumption and lactate and pyruvate production from day 2 onwards (Figure 2A,B). In DMEM-G medium, consumption of D-glucose was measured starting from day 1, with a simultaneous production of pyruvate (Figure 2A,B). On day 14, total consumption of L-fucose and D-glucose was 16.6 mM and 3.7 mM, respectively. Morphologically, the earliest

apparent differences between the tested media were observed at day 4 (Figure 2C). When grown in DMEM medium, more coccoid shaped cells were observed than when grown in either DMEM-F or DMEM-G media.

Amino acid metabolism of *C. jejuni* 18-440-1 in DMEM +/- L-fucose/D-glucose

Quantification of selected amino acids, including cystine in cultures grown in DMEM, DMEM-F and DMEM-G medium, showed a strong preference for serine utilization, reflected in its depletion at day 1 in all tested media (Figure 3). The amino acid asparagine was first produced until day 4 and was after consumed in all tested media, reaching final concentrations of 183.9 µM, 156.4 µM and 179.7 µM in DMEM, DMEM-F and DMEM-G medium, respectively. Glutamine/arginine concentration decreased and was nearly fully depleted on day 4 in all tested media. Levels of glutamic acid increased at day 2 and reached its highest concentrations of 1885.8 µM, 1045.0 µM and 1735.4 µM at day 4 for DMEM, DMEM-F and DMEM-G, respectively, when glutamine was fully depleted. After day 4, glutamic acid was slowly consumed and was fully consumed by day 14 in all tested media. Methionine was slowly consumed, reaching final concentrations of 52.31 µM, 21.4 µM and 65.54 µM in DMEM, DMEM-F and DMEM-G, respectively. Lastly, cystine depletion was observed at day 3 in DMEM-F medium and at day 7 in DMEM-G medium, while it was slowly consumed until day 14 in DMEM medium.

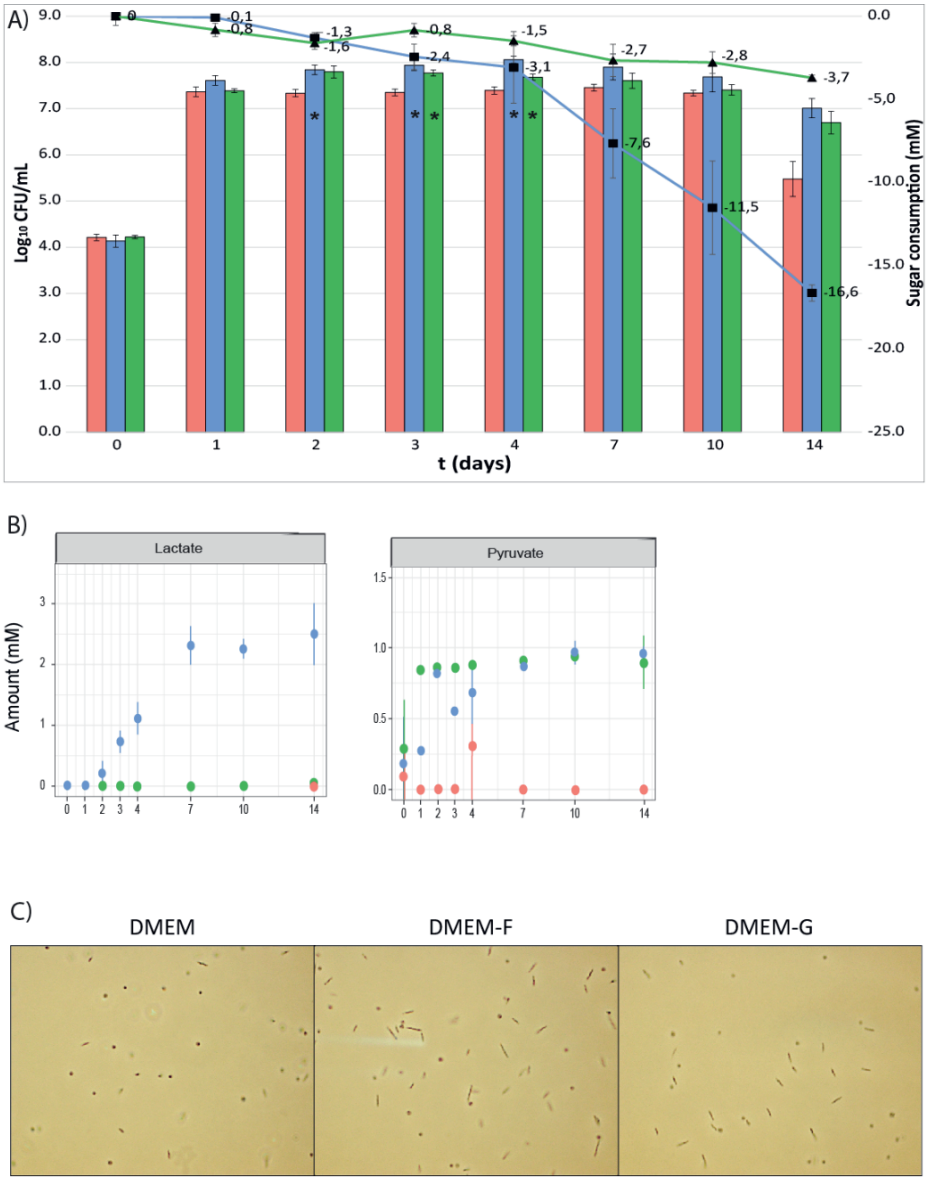


Figure 2 Growth, metabolism and morphology of isolate *C. jejuni* 18-440 in DMEM (red), DMEM-F (blue) and DMEM-G (green). A) Growth of *C. jejuni* 18-440. Asterisk indicates a significant difference compared with log10 CFU counts/mL in DMEM medium (red bar). The blue line (square) indicates L-fucose consumption and the green line (triangle) D-glucose consumption. B) HPLC quantification of the organic acids lactate and pyruvate when grown in DMEM (red dots), DMEM-F (blue dots) and DMEM-G (green dots) medium. C) Microscopic images of *C. jejuni* 18-440 on day 4.

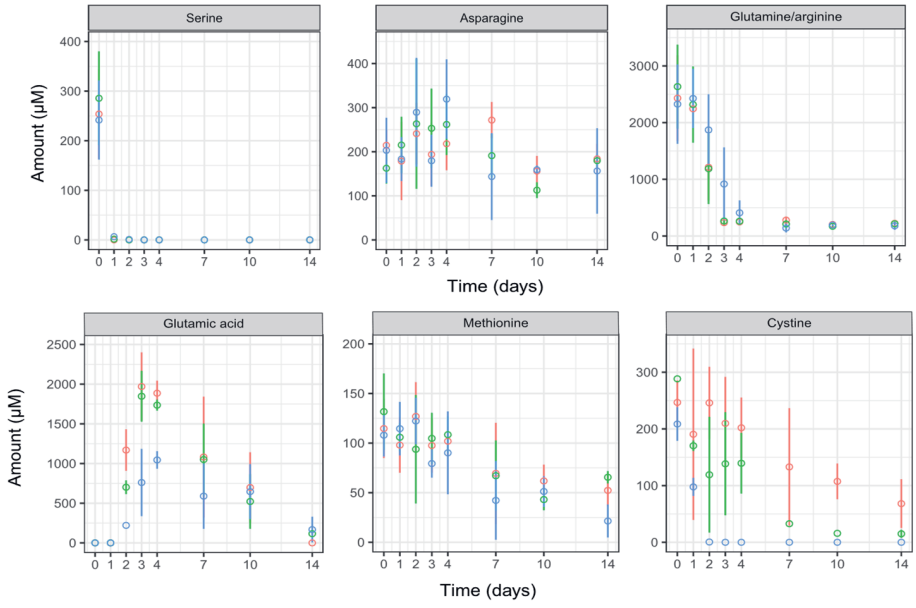


Figure 3 Amino acid HPLC quantifications of *C. jejuni* 18-440. Red dots indicate that the cells were grown in DMEM medium, blue dots in DMEM + L-fucose and green dots in DMEM + D-glucose. Each value represents the average of three biologically independent replicates, and error bars show the standard deviation.

Activation of the L-fucose and D-glucose utilization clusters

Samples were taken for proteome analyses at day 2 of growth in DMEM, DMEM-F and DMEM-G, and proteome analyses of proteins in the L-fucose utilization cluster revealed the upregulation of several proteins when grown in DMEM-F, in comparison to DMEM (Table 1). Firstly, the transporter protein Cj0486, which is essential for the transport of L-fucose into the cell, was upregulated 5.5 fold-change. Cj0484, which is also annotated as a transporter, was not detected, similarly as the regulator Cj0480c. Furthermore, all other proteins of the L-fucose utilization cluster, which are responsible for the conversion of L-fuco-α pyronose to pyruvate and lactic acid, namely, Cj0488, Cj0485, Cj0487, Cj0482, Cj0483, Cj0481 and Cj0489, were all upregulated.

Similar analyses was performed for the D-glucose utilization cluster in which 2-days-grown cultures in DMEM-G medium were compared to DMEM medium (Table 2). Surprisingly, only one protein of this cluster was significantly upregulated, namely, the glucose transporter GlcP, which was upregulated 54.4 fold-change. The other proteins of this cluster, involved in the conversion from glucose to pyruvate, were all produced in the tested conditions, however, did not significantly increase in DMEM-G compared to DMEM.

Proteome response of L-fucose or D-glucose grown cells

In total, 93 proteins were significantly differentially expressed (up or down regulated with +/- 1.5-fold-change or more) in either DMEM-F or DMEM-G, in comparison to DMEM medium (Figure 4A-C). From the 93 proteins, four proteins were similarly differentially affected in both conditions, namely, the proteins Cj1410c (hypothetical) and Cj0906c/Pgp2 (Peptidoglycan L,D-Carboxypeptidase) were upregulated, while the proteins: PutA (proline metabolism) and HrcA (heat-inducible transcription repressor) were downregulated (Suppl. Table 1 and Table 2). Notably, Cj0906c is involved in helical shape maintenance and is conceivably linked to our observed increased spiral shaped morphology in the presence of L-fucose and D-glucose (Figure 2C) (Sycuro et al., 2012; Frirdich et al., 2014; Min et al., 2020; Lin et al., 2021).

Furthermore, three proteins were upregulated in the presence of L-fucose, but downregulated in the presence of D-glucose, namely, NapL (nitrate reductase), NapA (nitrate reductase) and Cj0185c (hypothetical protein). Moreover, of the 92 proteins, 71 were uniquely up- or down-regulated in DMEM-F, while only 17 were up-or down-regulated in DMEM-G (Figure 4A-C)

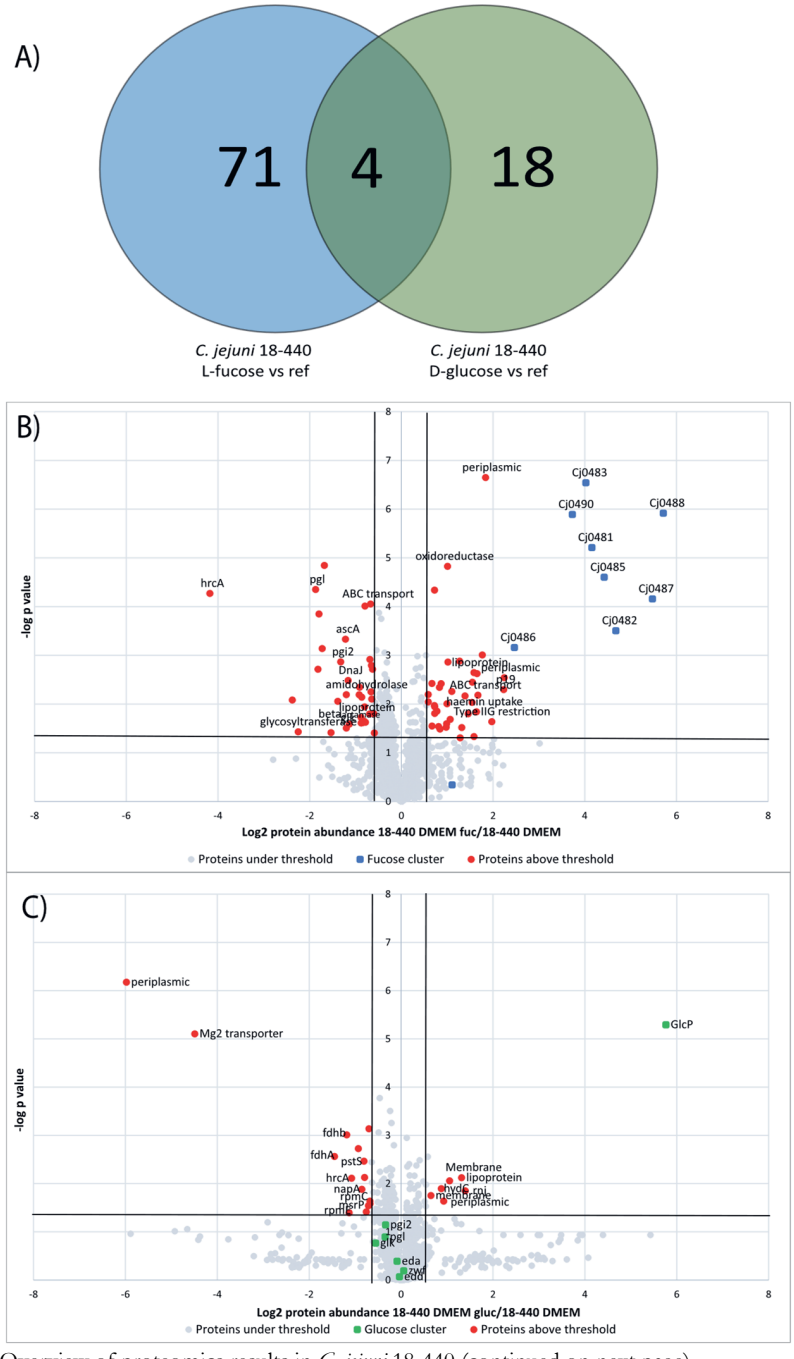


Figure 4 Overview of proteomics results in *C. jejuni* 18-440 (continued on next page).

A) VENN diagram of significantly up or down regulated proteins > -1.5/1.5 fold change. B) Vulcano plot of up and down regulated proteins of isolate *C. jejuni* 18-440 when grown in DMEM-F, in comparison with DMEM. Red dots indicate proteins with a significant fold change of > -/+ 1.5 and blue dots indicate all proteins of the L-fucose utilization cluster of which Cj0481-Cj0490 were significantly upregulated. C) Vulcano plot of up and down regulated proteins of isolate *C. jejuni* 18-440 when grown in DMEM-G, in comparison with DMEM. Red dots indicate proteins with a significant fold change of > -/+ 1.5 and green dots indicate proteins of the D-glucose utilization cluster of which GlcP was significantly upregulated.

We further investigated differentially expressed proteins in L-fucose grown cells that may impact cell physiology. In cells grown in DMEM-F compared to DMEM medium, 35 proteins were significantly upregulated (excluding proteins of the L-fucose utilization cluster) and 32 were significantly downregulated (Table 1 and Suppl. Table 1 for the full list). In total, twelve proteins are labeled as (putative) membrane or (putative) periplasmic proteins, pointing to possible cell surface modulations. Nine of these proteins were significantly upregulated (Cj1666c, Cj0092, Cj1626c, Cj1653c, Cj0649, Cj1410c, Cj0633, Cj0906c/Ppg2 and Cj0736), while three were significantly downregulated (Cj0879c, Cj0413 and Cj1136). Of these proteins, several have been mentioned in studies, which highlight their potential roles. The upregulated protein Cj1626c was linked to the DccR-regulon, which is linked to multiple biological functions, including colonization (Wösten et al., 2010). The upregulated protein Cj0649 encodes a beta-barrel LptD-like protein and is not studied in *Campylobacter*. However, in *Pseudomonas aeruginosa* this protein functions in the assembly of lipopolysaccharides (LPS) in the outer leaflet of the outer membrane (Hoang et al., 2012), suggesting similar functions for the tested *C. jejuni* isolate. Also the upregulated Cj0633 protein is not studied in *Campylobacter*, however, Pfam analyses suggest that this protein is a glycosidase and possibly involved in N-linked glycoprotein biosynthesis. Lastly, the downregulated protein Cj1136 is a glycosyltransferase and was previously shown to have an important role in lipooligossacharides (LOS) production, invasion and colonization of *C. jejuni* (Javed et al., 2012).

Furthermore, sixteen proteins are involved in metal acquisition and redox reactions, of which fourteen were upregulated and two were downregulated. The upregulated proteins included: Cj1658, Cj1659/p19, Cj1661, Cj1663, ChuD, ChuC, CeuD, CeuE, Cj0173c, HugZ, Cj0414, Cj0415, MoaD and CfbpC. Notably, a review study highlighted that most of these proteins are part of multicomponent transport systems; Cj1658,

Cj1659/p19, Cj1661 and Cj1663 are part of the rhodotorulic acid uptake system (Cj1658-Cj1663), ChuD and ChuC are part of a heme uptake system (ChuA,B,C,D,Z), CeuD and CeuE are part of the Enterochelin uptake system (CeuBCDE), and Cj0173c is part of the ferritransferrins uptake system (Cj0173c-Cj0178) (Miller et al., 2009). Furthermore, the proteins HugZ, Cj0414, Cj0415, MoaD and CfbpC are annotated as heme oxygenase, oxidoreductase, oxidoreductase, putative molybdopterin converting factor and putative iron-uptake ABC transport system, respectively. The downregulated proteins included Cj0991c and FdxA, and function as glycolate oxidase iron-sulfur subunit and ferredoxin, respectively. Activation of these iron and heme transporters, next to redox proteins and enzymes, is conceivably linked to the observed extended growth up to day 4, in which cells acquire iron, heme and other metal ions to support production of proteins and enzymes, enabling metabolic shifts and energy production. This metabolic shift is further supported by the downregulation of several metabolic pathway proteins, namely, LeuD (L-leucine metabolism) Cj0920c and Peb1C (involved in aspartate and glutamate metabolism), Mqo (involved in malate metabolism), Cj0546 (involved in vitamin K2 synthesis), AscA (involved in acetate metabolism), PutA (involved in proline metabolism) and three D-glucose metabolism proteins; Glk, Pgi2 and Pgl.

We also investigated the effect of D-glucose on the proteome of *C. jejuni* 18-440. In contrast to the effect of L-fucose on the proteome, the effect of D-glucose on the proteome was much smaller (Figure 4C).

In total, excluding the glucose transporter (GlcP), 21 proteins were significantly affected by the presence of D-glucose (Table 2). Of these 21 proteins, six proteins were up- and 15 down-regulated. The most notable proteins, apart from GlcP, were Cj1011 (putative CorA-like Mg2+ transporter protein) and Cj1406c (putative periplasmic protein), which were -22.5 and -63.0 fold-change downregulated, respectively. Fold-changes of other proteins varied between 2.63 and -2.73 and were mostly putative proteins that were linked to the membrane or periplasm.

Table 1 Up- and down-regulated proteins of *C. jejuni* 18-440 grown in DMEM-F, compared to DMEM. All L-fucose utilization cluster proteins and significant differentially expressed proteins (>1.5 fold-change) that are grouped into: L-fucose utilization cluster proteins, membrane or periplasmic proteins, metal acquisition and redox reactions and metabolic pathway proteins are displayed.Full list of all significant differentially expressed proteins is found in Suppl. Table. 1. Nd indicates not detected.

Uniprot/protein	Function	L-fucose
L-fucose utilization cluster proteins		
Q0PB33/Cj0480c	Putative transcriptional regulator	nd
Q0PB32/Cj0481	Putative dihydrodipicolinate synthase	17.8
Q0PB31/Cj0482	Putative altronate hydrolase	25.6
Q0PB30/Cj0483	Putative altronate hydrolase	16.3
Q0PB29/Cj0484	Putative transport protein	nd
Q0PB28/Cj0485	Putative oxidoreductase	21.5
Q0PB27/Cj0486	L-fucose transporter	5.5
Q0PB26/Cj0487	Putative amidohydrolase	44.5
Q0PB25/Cj0488	Putative amidohydrolase	52.5
Q0PB23/Cj0489	Putative aldehyde dehydrogenase	13.3
Membrane or periplasmic proteins		
Q0P7W3/Cj1666c	Putative periplasmic protein	4.7
Q0PC45/Cj0092	Putative periplasmic protein	3.6
Q0P802/Cj1626c	Putative periplasmic protein	3.0
Q0P7X5/Cj1653c	Putative lipoprotein	3.0
Q0PAL9/Cj0649	(Hypothetical) Putative OstA family protein	2.5
Q0P8K4/Cj1410c	Putative membrane protein	2.1
Q0PAN5/Cj0633	Putative periplasmic protein	2.0
Q0P9Z2/Cj0906c/Ppg2	Putative periplasmic protein	3.4
Q0PAE3/Cj0736	Periplasmic protein	1.8
Q0PA18/Cj0879c	Putative periplasmic protein	-1.8
Q0PB97/Cj0413	Beta-lactamase/putative periplasmic protein	-2.2
Q0P9B8/Cj1136	Glycosyltransferase	-4.8
Metal acquisition and redox reactions		
Q0P7X1/Cj1658	Putative iron permease	2.9
Q0P7X0/Cj1659/P19	Periplasmic protein	4.7
Q0P7W8/Cj1661	Possible ABC transport system permease	3.2
Q0P7W6/Cj1663	Putative ABC transport system ATP-binding protein	2.0
Q0P811/ChuD	Putative haemin uptake system	3.1
Q0P812/ChuC	Putative haemin uptake system ATP-binding protein	2.4
Q0P8Q5/CeuD	Enterochelin uptake ATP-binding protein	2.7
Q0P8Q4/CeuE	Enterochelin uptake periplasmic binding protein	2.1
Q9PID1/Cj0371c	Ferritransferrins uptake system	

A0A3X8NE32/HugZ	(Hypothetical) family heme oxygenase	2.6
Q0PB96/Cj0414	Putative oxidoreductase subuni	2.0
Q0PB95/Cj0415	Putative GMC oxidoreductase subunit	1.5
Q0P8A2/MoaD	Putative molybdopterin converting factor,subunit 1	1.6
Q0PBW6/CfbpC	Putative iron-uptake ABC transport system	1.8
Q0P9R1/Cj0991c	Glycolate oxidase iron-sulfur subunit	-1.6
Q0PBH6/FdxA	Ferredoxin	-3.5
Metabolic pathway proteins		
Q9PLW2/LeuD	3-isopropylmalate dehydratase small subunit	-1.6
Q0P9X9/Cj0920c	Putative ABC-type amino-acid transporter permease	-1.5
Q0P9X7/Peb1C	Probable ABC transporter ATP-binding protein PEB1C	-1.6
P56954/Mqo	Probable malate:quinone oxidoreductase	-1.6
Q0PAX0/Cj0546	Menaquinone biosynthesis decarboxylase	-1.7
Q9PMD2/AcsA	Acetyl-coenzyme A synthetase	-2.3
Q0P8B5/PutA	Bifunctional protein PutA	-3.2
Glk	D-glucose utilization cluster	-2.3
Pgi2	D-glucose utilization cluster	-2.5
Pgl	D-glucose utilization cluster	-3.7

Table 2 Up- and down-regulated proteins of *C. jejuni* 18-440 grown in DMEM-G, compared to DMEM. All D-glucose utilization cluster proteins and significant differentially expressed proteins (>1.5 fold-change) that are grouped in D-glucose utilization cluster proteins and other proteins. Ns indicates that no significantly differentially expressed protein was measured.

Uniprot/protein	Function	D-glucose
D-glucose utilization cluster proteins		
GlcP	Glucose transporter	54.4
Pgi2	Phosphoglucose isomerases	ns
Glk	Glucokinase	ns
Pgl	6-phosphogluconolactonase	ns
Zwf	Glucose-6-phosphate dehydrogenase	ns
Edd	6-phosphogluconate dehydratase	ns
Eda	2-keto-3-deoxy-6-phosphogluconate aldolase	ns
Other proteins		
Q0P7S1/Cj1710c	Ribonuclease J	2.6
Q0P7X5/Cj1653c	Putative lipoprotein	2.5
Q0P8K4/Cj1410c	Putative membrane protein	2.1
Q0P9Z2/Cj0906c/Ppg2	Putative periplasmic protein	1.9
Q0P8Z1/hydC	Ni/Fe-hydrogenase B-type cytochrome subunit	1.8
Q0P8B8/Cj1500	Putative integral membrane protein	1.6
Q0PAA6/napL	Putative periplasmic protein	-1.6
Q9PLX9/rpmC	50S ribosomal protein L29	-1.6
Q0P8B5/putA	Bifunctional protein PutA	-1.6
Q9PIC3/msrP	MsrPQ system (oxidative stress)	-1.6
Q9PPD9/napA	Periplasmic nitrate reductase	-1.7
Q0P956/Cj1200	Putative NLPA family lipoprotein	-1.7
Q0PAQ4/Cj0613	Putative periplasmic phosphate binding protein	-1.7
Q0PBV4/Cj0185c	Putative phnA domain	-1.8
Q0PAG2	Putative ArsC family protein	-1.9
Q9PPG2/hrcA	Heat-inducible transcription repressor	-2.1
Q9PIX2/rpmE	50S ribosomal protein L31	-2.2
Q0P8A8/fdhB	Putative formate dehydrogenase iron-sulfur subunit	-2.3
Q0P8A7/fdhA	Putative formate dehydrogenase large subunit	-2.7
Q0P9P1/Cj1011	Putative CorA-like Mg2+ transporter protein	-22.5
Q0P8K8/Cj1406c	Putative periplasmic protein	-63.0

Discussion

Campylobacter mainly metabolizes amino acids, however, some isolates are also able to metabolize L-fucose and/or D-glucose (Muraoka & Zhang, 2011; Stahl et al., 2011; Vorwerk et al., 2015; Dwivedi et al., 2016; Vegge et al., 2016; Luijkx et al., 2020). The L-fucose utilization cluster allows fuc+ *Campylobacter* isolates to metabolize L-fucose, while the D-glucose utilization cluster allows gluc+ *Campylobacter* isolates to metabolize D-glucose via the ED pathway (Vegge et al., 2016; van der Hooft et al., 2018; Middendorf et al., 2022). In the current study we characterized an unique *C. jejuni* isolate from water, *C. jejuni* 18-440, that carries an atypical L-fucose utilization cluster and a D-glucose utilization cluster. The L-fucose utilization cluster of this isolate was identified as a L-fucose cluster which was obtained from *C. coli* isolates via HGT, as described in Chapter 5. The D-glucose utilization cluster is comprised of seven genes, namely, *glcP, pgi2, glk, pgl, zwf, edd* and *eda,* and has only been studied in *C. coli* isolates (Vorwerk et al., 2015; Vegge et al., 2016). In the current study we performed growth and metabolism analyses of *C. jejuni* 18-440 when grown with L-fucose and D-glucose and highlighted observed proteomic changes. We observed an improved survival and an improved growth performance of isolate *C. jejuni* 18-440 when grown in DMEM medium with L-fucose. Proteomics analyses were performed to investigate the role of L-fucose activation in *C. jejuni* 18-440 when grown in DMEM-F compared to DMEM. Of the proteins in the L-fucose utilization cluster, two were not detected, namely, Cj0480c and Cj0484. This finding was in line with our previous study in which Cj0480c and Cj0484 were not detected in the isolate *C. jejuni* NCTC11168 (Middendorf et al., 2023).

Campylobacter has developed a wide variety of mechanisms for iron acquisition and several studies highlighted important connections between iron limitation and *C. jejuni* metabolism (Stintzi et al., 2008). In the current study, sixteen proteins related to metal acquisition and redox reactions were significantly differentially expressed in the presence of L-fucose. Notably, most of the upregulated proteins are detrimental to the uptake of metals, as they are part of multicomponent transport systems (Miller et al., 2009). The proteins Cj1658, Cj1659/p19, Cj1661 and Cj1663 are part of the rhodotorulic acid uptake system (Cj1658-Cj1663), ChuD and ChuC are part of a heme uptake system (ChuA,B,C,D,Z), CeuD and CeuE are part of the Enterochelin uptake

system (CeuBCDE), and Cj0173c is part of the ferritransferrins uptake system (Cj0173c-Cj0178) (Miller et al., 2009). A study by Liu et al. (2018) highlighted that several of these iron uptake systems, namely, CfbpA, CeuB, ChuC and the cluster Cj1658/P19-Cj1664, were higher expressed in human fecal extracts compared to chicken cecal extracts (Liu et al., 2018). Furthermore, in iron restricted medium, the Cj1658-Cj1664 genes were required for optimal growth when using human fecal extracts as an iron source (Liu et al., 2018). Knockout mutant studies of the same cluster highlighted an iron-sensitive phenotype with reduced growth in acidic medium, increased sensitivity to streptomycin and higher resistance to H_2O_2 stress (Chan et al., 2010; Liu et al., 2018). These studies combined with our findings highlight the link between L-fucose consumption and the upregulation of proteins involved in iron scavenging, stress survival and growth in the human intestine environment.

Furthermore, growth of *C. jejuni* 18-440 in DMEM-F medium compared to DMEM medium, resulted in the downregulation of numerous metabolic proteins. These proteins included LeuD (L-leucine metabolism) Cj0920c and Peb1C (involved in aspartate and glutamate metabolism), Mqo (involved in malate metabolism), Cj0546 (involved in vitamin K2 synthesis), AcsA (involved in acetate metabolism), and PutA (involved in proline metabolism). Notably, PutA was also downregulated in the presence of L-fucose and D-arabinose when grown in MEMα medium (Middendorf et al., 2023). These findings highlight the metabolic shift *C. jejuni* undergoes upon exposure and metabolism of L-fucose, likely due to environmental changes towards L-fucose rich environments such as the human gut environment.

Apart from iron/metal acquisition and metabolism, also several membrane and LOS producing proteins are significantly differentially expressed. However, as most of these proteins are putative proteins, we were only able to describe four of these proteins, namely, Cj0649, Cj0633, Cj1136 and Cj1626. Firstly, the upregulated protein Cj0649 (which encodes a beta-barrel LptD-like protein) functions in the assembly of LPS in *Pseudomonas aeruginosa* (Hoang et al., 2012), and although not studied in *Campylobacter*, it is likely that it functions in the development of the outer membrane and LOS production of *C. jejuni*. Next, the upregulated protein Cj0633 is not described in literature, however, Pfam analysis highlight its involvement in the development of the outer membrane via its N-linked glycoprotein biosynthesis. Furthermore, the

protein Cj1136 was downregulated and functions as glycosyltransferase. Cj1136 is part of the LOS biosynthesis gene cluster (*Cj1132c – Cj1153*), which is involved in multiple biological functions in *C. jejuni*, such as antibiotic resistance, colonization, invasion, preserving cell membrane structure and transport of molecules (Moran, 1997; Karlyshev et al., 2005; Javed et al., 2012; Hameed et al., 2020). Lastly, the protein Cj1626 was linked to the DccR-regulon (Wösten et al., 2010), similarly as the proteins MacC and Cj0200 of *C. jejuni* NCTC11168, which were previously found significantly differentially expressed in the presence of L-fucose and D-arabinose (Middendorf et al., 2023). Based on these significantly expressed membrane associated proteins (i.e. LOS, capsule polysaccharide locus (CPS) and glycan related proteins), we investigated the role of L-fucose on these proteins using a schematic overview (Figure 5). In L-fucose grown cells, expression of the L-fucose utilization pathway also affects expression of proteins in the D-glucose utilization and gluconeogenesis pathways. The proteins Pgi2, Glk, Zwf (-1.2 fold-change) and Pgl were all significantly downregulated and the protein GlcP (glucose transporter) was absent in cells grown in DMEM-F compared to DMEM, conceivably to prevent cycling towards the ED pathway from glucose-6-phosphate that is derived from glyceraldehyde-3-phosphate via gluconeogenesis. Although the protein Pgi2 was also downregulated, we hypothesize that, upon L-fucose metabolism, pyruvate enters the tricarboxylic acid cycle (TCA), forms Acetyl-CoA or enters the gluconeogenesis pathway and forms glucose-6-phosphate, ultimately leading to several significantly differentially expressed membrane associated proteins (Cj1626c, Cj0649, Cj0633 and Cj1136).

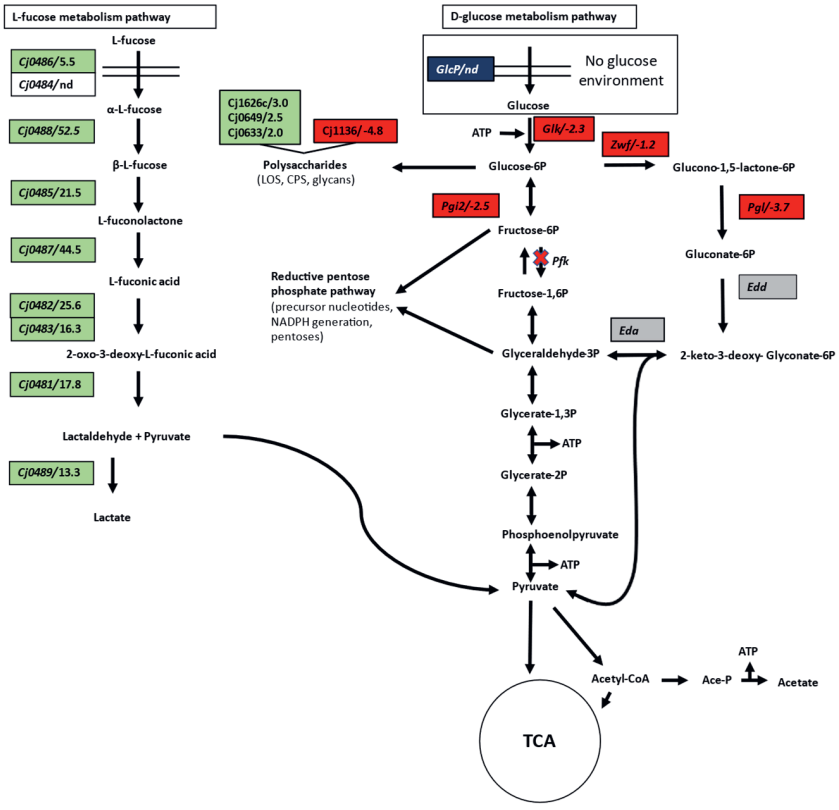


Figure 5 Schematic overview of the effect of L-fucose metabolism on polysaccharides production via the gluconeogenesis pathway. L-fucose metabolism results in the production of pyruvate, which enters the tricarboxylic acid cycle (TCA), forms Acetyl-CoA or enters the gluconeogenesis pathway to ultimately significantly alter the production of polysaccharides. Green blocks indicate upregulated proteins when grown in DMEM-F compared to DMEM, red blocks indicate downregulated proteins when grown in DMEM-F compared to DMEM, grey blocks indicate no significantly differentially expressed proteins when grown in DMEM-F compared to DMEM and the blue block indicate that the protein GlcP is not present when grown in DMEM-F medium. Furthermore, the red cross in the reaction from fructose-6P to fructose-1,6P indicates that *Campylobacter* does not possess phosphofructokinase (Pfk) and is therefore not able to perform that reaction. Notably, *Campylobacter* carries the protein fructose- 1,5-bisphosphatase (Fbp), allowing the reaction from fructose-1,6P to fructose-6P. Figure is adapted from Vegge et al. (2016).

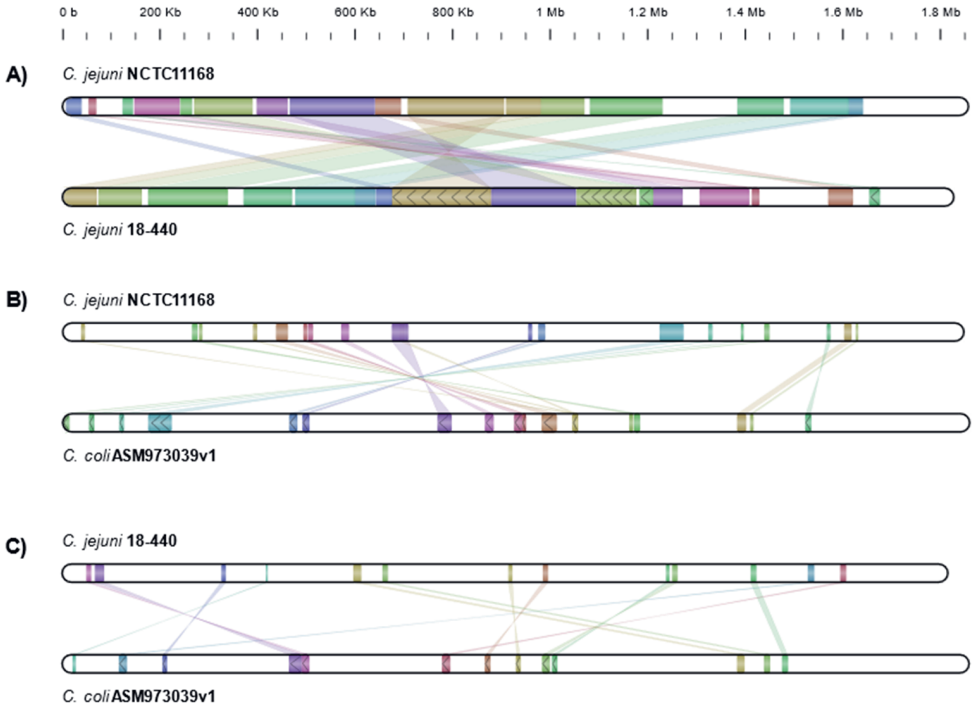
In the current study we provided insight in the ability of *C. jejuni* to metabolize glucose, extending previous studies that only focused on *C. coli*. In those studies, gluc+ *C. coli* isolates showed improved growth, survival and biofilm formation upon D-glucose metabolism (Vorwerk et al., 2015; Vegge et al., 2016). Furthermore, upon D-glucose metabolism, gluc+ *C. coli* isolates displayed higher concentrations of citric-acid cycle compounds (such as pyruvate) and showed increased depletion of the amino acids isoleucine, phenylalanine and methionine (Vorwerk et al., 2015; Vegge et al., 2016). In the current study, improved growth and extended survival were observed for isolate *C. jejuni* 18-440 in the presence of D-glucose, which was linked to an increased pyruvate (linked to the ED pathway) and decreased cystine concentration in DMEM-G medium, compared to normal DMEM medium. Cystine, the oxidized dimer form of the amino acid cysteine, was previously also linked to L-fucose metabolism in MEMα medium (Middendorf et al., 2022; van der Hooft et al., 2018). Comparative proteomics analyses of DMEM and DMEM-G grown *C. jejuni* 18-440 cells, revealed that from the proteins of the D-glucose utilization cluster, only the glucose transporter GlcP, which mediates uptake of D-glucose into the cell, was upregulated in the presence of D-glucose. Based on the schematic overview that is displayed in Figure 5, it is conceivable that the upregulation of GlcP allows D-glucose to enter the cell as carbon source. However, with use of gluconeogenesis, and intermediates of the pathway being interchangeable, proteins of the ED pathway remain active in the presence of pyruvate. Therefore, an increase of D-glucose into the cell apparently does not significantly increase the production of proteins involved in the ED pathway. Other proteins that were significantly differentially affected by D-glucose were mainly annotated as putative membrane or periplasmic proteins, with some related to metal acquisition proteins. However, on a much smaller scale than observed with L-fucose.

Three proteins were similarly affected in the presence of L-fucose and D-glucose, two were upregulated, namely, PutA and Cj0906/Pgp2 and one was downregulated, namely, HrcA. As earlier discussed, PutA is involved in proline metabolism and was previously found to be significantly downregulated in the presence of L-fucose and D-arabinose (Middendorf et al., 2023). The protein Pgp2 may be associated with the observed morphological phenotype, in which higher quantities of spiral shaped cells were observed in the presence of L-fucose and D-glucose, compared to normal

medium. Pgp2 is responsible for cell morphology and was linked previously to helical cell shapes of *C. jejuni* and *Helicobacter pylori* (Sycuro et al., 2012; Frirdich et al., 2014; Min et al., 2020; Lin et al., 2021). Lastly, the protein HrcA is a heat shock response repressor and produces products that repress the transcription of major heat shock proteins in *H. pylori* involved in protein quality homeostasis (Stintzi et al., 2005; Holmes et al., 2010). As during the current study only one temperature setting was used (37 °C), further investigation regarding this heat shock repressor and how it is influenced by L-fucose and D-glucose metabolism and other stresses such as pH, is needed.

In conclusion, our study characterized the water isolate *C. jejuni* 18-440, which carries an atypical L-fucose utilization cluster and a D-glucose utilization cluster. The L-fucose utilization cluster and the D-glucose utilization cluster allowed *C. jejuni* to metabolize L-fucose and D-glucose, respectively, which resulted in increased growth and prolonged survival. L-fucose metabolism subsequently led to the up and down regulation of a wide range of LOS, iron/metal acquisition/redox and metabolism proteins, while D-glucose metabolism induced a limited response. The results of the current study suggest that isolates upon L-fucose and D-glucose metabolism gain a fitness advantage by improving their growth and survival. Furthermore, especially upon L-fucose metabolism, *C. jejuni*'s metabolism is rewired, possibly resulting in capsular changes that may provide a competitive advantage in fucose-rich environments such as encountered in animal and human hosts.

Supplements



Suppl. Fig. 1 SMASH++ genomic comparison. A) comparison between the reference isolate *C. jejuni* NCTC11168 and *C. jejuni* 18-440. B) Comparison between the reference isolate *C. jejuni* NCTC11168 and the reference isolate *C. coli* ASM973039v1. C) Comparison between *C. jejuni* 18-440 and the reference isolate C. coli ASM973039v1.

Suppl. Table 1 All Up- and down-regulated proteins of *C. jejuni* 18-440 grown in DMEM-F, compared to DMEM. All L-fucose utilization cluster proteins and significant differentially expressed proteins (>1.5 fold-change).

Uniprot/protein	Function	L-fucose
Q0PB33/Cj0480c	L-fucose utilization cluster	
Q0PB32/Cj0481	L-fucose utilization cluster	17.8
Q0PB31/Cj0482	L-fucose utilization cluster	25.6
Q0PB30/Cj0483	L-fucose utilization cluster	16.3
Q0PB29/Cj0484	L-fucose utilization cluster	
Q0PB28/Cj0485	L-fucose utilization cluster	21.5
Q0PB27/Cj0486	L-fucose utilization cluster	5.5
Q0PB26/Cj0487	L-fucose utilization cluster	44.5
Q0PB25/Cj0488	L-fucose utilization cluster	52.5
Q0PB23/Cj0489	L-fucose utilization cluster	13.3
Q0P7W3/Cj1666c	Putative periplasmic protein	4.7
Q0P7X0/p19	Periplasmic protein	4.7
Q0PAI9/Cj0690c	Site-specific DNA-methyltransferase (adenine-specific)	3.9
Q0PC45/Cj0092	Putative periplasmic protein	3.6
Q0P9Z2/Cj0906c	Putative periplasmic protein	3.4
Q0P7W8/Cj1661	Possible ABC transport system permease	3.2
A0A695HS74/DSN88_02505	Iron permease	3.1
Q0P811/chuD	Putative haemin uptake system	3.1
Q0P802/Cj1626c	Putative periplasmic protein	3.0
Q0P7X5/Cj1653c	Putative lipoprotein	3.0
Q0P7X1/Cj1658	Putative iron permease	2.9
Q0P8Q5/ceuD	Enterochelin uptake ATP-binding protein	2.7
A0A3X8NE32/hugZ	(Hypothetical) family heme oxygenase	2.6
Q0PAL9/Cj0649	(Hypothetical) Putative OstA family protein	2.5
Q0P812/chuC	Putative haemin uptake system ATP-binding protein	2.4
Q0PAS1/cbf2	Putative peptidyl-prolyl cis-trans isomerase	2.4
Q0P8Q4/ceuE	Enterochelin uptake periplasmic binding protein	2.1
Q0P8K4/Cj1410c	Putative membrane protein	2.1
Q0PAN5/Cj0633	Putative periplasmic protein	2.0
Q0PB96/Cj0414	Putative oxidoreductase subuni	2.0
Q9PID1/Cj0371	lipoprotein	2.0
Q0P7W6/Cj1663	Putative ABC transport system ATP-binding protein	2.0
Q0PBW6/cfbpC	Putative iron-uptake ABC transport system	1.8
Q9PPD9/napA	Periplasmic nitrate reductase	1.8
Q0PAE3/Cj0736	Hypothetical protein	1.8
A0A695HSH0/DSN88_03195	Hypothetical protein	1.8
Q0PAA6/napL	Putative periplasmic protein	1.7

Q0PBV4/Cj0185c	Putative phnA domain	1.7
Q0P832/rplQ	50S ribosomal protein L17	1.7
Q9PLX8/rplP	ribosome	1.7
Q0PAC0/Cj0770c	Putative NLPA family lipoprotein	1.6
Q0P9G0/yajC	Sec translocon accessory complex subunit YajC	1.6
Q0P8A2/moaD	Putative molybdopterin converting factor,subunit 1	1.6
Q0PB95/Cj0415	Putative GMC oxidoreductase subunit	1.5
Q0P9L1/Cj1041c	Putative periplasmic ATP/GTP-binding protein	1.5
Q9PNJ2/truB	tRNA pseudouridine synthase B	-1.5
Q0P9X9/Cj0920c	Putative ABC-type amino-acid transporter permease	-1.5
Q9PP92/trmA	tRNA/tmRNA (uracil-C(5))-methyltransferase	-1.5
Q9PLW2/leuD	3-isopropylmalate dehydratase small subunit	-1.6
Q0PAR5/Cj0602c	(Hypothetical) MOSC-domain containing protein	-1.6
Q0P9R1/Cj0991c	Glycolate oxidase iron-sulfur subunit	-1.6
Q0P9X7/peb1C	Probable ABC transporter ATP-binding protein PEB1C	-1.6
P56954/mqo	Probable malate:quinone oxidoreductase	-1.6
Q0PBX4/Cj0163c	Hypothetical protein	-1.6
Q0PAS3/Cj0594c	Putative DNA/RNA non-specific endonuclease	-1.7
A0A695HSI6/rlmH	ribosome	-1.7
Q0PAX0/Cj0546	Menaquinone biosynthesis decarboxylase	-1.7
Q0PBY4/Cj0153c	Putative rRNA methylase	-1.8
Q0P887/tupC	Tungstate uptake system ATP-binding protein	-1.8
Q0PA18/Cj0879c	Putative periplasmic protein	-1.8
Q0PAW7/fliS	Flagellar secretion chaperone	-1.8
Q0PBD5/Cj0375	Putative lipoprotein	-1.8
Q0P8M2/Cj1387c	Helix-turn-helix containing protein	-1.9
Q0PAW0/Cj0556	(Hypothetical) Putative amidohydrolase family protein	-1.9
Q0PB97/Cj0413	Beta-lactamase/putative periplasmic protein	-2.2
Q0P9L8/Cj4034c	Putative DnaJ-like protein	-2.2
glk	D-glucose utilization cluster	-2.3
Q9PMD2/acsA	Acetyl-coenzyme A synthetase	-2.3
pgi2	D-glucose utilization cluster	-2.5
Q0P992/Cj1164c	zf-TFIIB domain-containing protein	-2.9
Q0P8B5/putA	Bifunctional protein PutA	-3.2
Q0P9Y2/cstA	Peptide transporter CstA	-3.3
Q0PBH6/fdxA	Ferredoxin	-3.5
pgl	D-glucose utilization cluster	-3.7
Q0P9B8/Cj1136	Putative glycosyltransferase	-4.8
A0A695HTM/mqnE2	Aminodeoxyfutalosine synthase	-5.2
Q9PPG2/hrcA	Heat-inducible transcription repressor	-18.1

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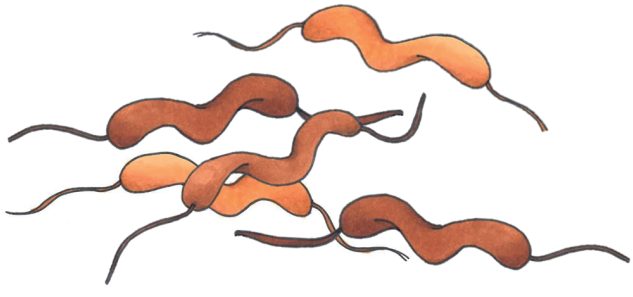

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Host associations of *Campylobacter jejuni* and *Campylobacter coli* isolates carrying the L-fucose or D-glucose utilization cluster

Pjotr S. Middendorf[1,2], Aldert L. Zomer[3,4], Indra L. Bergval[2], Wilma F. Jacobs-Reitsma[2], Heidy M. W. den Besten[1], and Tjakko Abee[1]

Affiliations:

1. Food Microbiology, Wageningen University and Research, Wageningen, the Netherlands
2. National Institute for Public Health and the Environment, Bilthoven, the Netherlands
3. Faculty of Veterinary Medicine, Department of Infectious Diseases and Immunology, Utrecht University, Utrecht, Netherlands
4. WHO Collaborating Center for Campylobacter/OIE Reference Laboratory for Campylobacteriosis, Utrecht, Netherlands.

Abstract

Campylobacter was considered asaccharolytic, but is now known to carry saccharide metabolization pathways for L-fucose and D-glucose. We hypothesized that these clusters are beneficial for *Campylobacter* niche adaptation and may help establish human infection.

We investigated the distribution of D-glucose and L-fucose clusters among ~9,600 *C. jejuni* and *C. coli* genomes of different isolation sources in the Netherlands, the United Kingdom, the United States of America and Finland. The L-fucose utilization cluster was integrated at the same location in all *C. jejuni* and *C. coli* genomes, and was flanked by the genes *rpoB*, *rpoC*, *rspL*, *repsG* and *fusA,* which are associated with functions in transcription as well as translation and in acquired drug resistance. In contrast, the flanking regions of the D-glucose utilization cluster were variable amongst the isolates, and integration sites were located within one of the three different 16S-23S ribosomal RNA areas of the *C. jejuni* and *C. coli* genomes. In addition, we investigated whether acquisition of the L-fucose utilization cluster could be due to horizontal gene transfer between the two species and found three isolates which carried atypical L-fucose clusters: one *C. jejuni* isolate carrying a *C. coli* L-fucose cluster, and two *C. coli* isolates which carried a *C. jejuni* L-fucose cluster. Furthermore, L-fucose utilization cluster alignments revealed multiple frameshift mutations, most of which were commonly found in the non-essential genes for L-fucose metabolism, namely, *Cj0484* and *Cj0489*. These findings support our hypothesis that the L-fucose cluster was integrated multiple times across the *C. coli*/*C. jejuni* phylogeny.

Notably, association analysis using the *C. jejuni* isolates from the Netherlands showed a significant correlation between human *C. jejuni* isolates and *C. jejuni* isolates carrying the L-fucose utilization cluster. This correlation was even stronger when the Dutch isolates were combined with the isolates from the UK, the USA and Finland, suggesting a selective advantage for the L-fucose cluster in human infection. No such correlations were observed for *C. coli* or for the D-glucose cluster for both species. This research provides insight into the spread and host associations of the L-fucose and D-glucose utilization clusters in *C. jejuni* and *C. coli*, and the potential benefits in human infection and/or survival and proliferation in humans, conceivably after transmission from any reservoir.

Introduction

Campylobacter is a zoonotic bacterium and is the main cause of bacterial foodborne gastroenteritis worldwide, with most human campylobacteriosis cases being the result of infection with *Campylobacter jejuni* or *Campylobacter coli* (Kaakoush et al., 2015; Tack et al., 2019; EFSA, 2021). Campylobacteriosis generally involves symptoms like watery or bloody diarrhea accompanied by abdominal pain, nausea and fever, and in rare occasions infection with *Campylobacter* can lead to the development of serious illnesses like the Guillain-Barré syndrome or irritable bowel syndrome (Rees et al., 1995; Allos, 1997; Blaser, 1997). All animals and environmental waters are a potential source for *Campylobacter* (Hepworth et al., 2011; Wagenaar et al., 2015; Nilsson et al., 2018; Mughini-Gras et al., 2021). However, in most countries, poultry and cattle have been identified as the main sources for *Campylobacter*, which is also reflected in multiple case-control studies that identify the consumption of raw and/or undercooked meat as risk factors for human campylobacteriosis (Doorduyn et al., 2010; EFSA, 2010; Mughini-Gras et al., 2012; Mughini-Gras et al., 2021).

Interestingly, *Campylobacter* isolates are considered fragile organisms, as they grow microaerobically at temperatures between 30-45°C, yet they are able to withstand many stresses during transmission from host to host (Levin, 2007; Silva et al., 2011). *Campylobacter* carries many genes of which their products are involved in the protection and survival of *Campylobacter* in stressful environments, such as starvation and oxidative, osmatic, heat shock, pH and nitrosative stresses (Andersen et al., 2005; Brøndsted et al., 2005; Candon et al., 2007; Bronowski et al., 2014). Due to its natural competence, one way of *Campylobacter* to adapt to new environments is through horizontal gene transfer (HGT) (Golz & Stingl, 2021). DNA-rich environments, such as the animal and human gastro-intestinal tracts, are ideal for the uptake of new DNA, and can result in strain variety of *Campylobacter* isolates (Sheppard & Maiden, 2015; Golz & Stingl, 2021). However, in a study that estimated the molecular clock rate of *Campylobacter*, the authors highlighted that multiple lineages are maintained, implying that large-scale clonal sweeps (such as uptake of resistance genes) may take hundreds of years or more in these species (Calland et al., 2021).

For a long time *Campylobacter* was thought to be asaccharolytic, lacking most key enzymes to metabolize sugars. However, the recent discovery of the L-fucose and D-

glucose utilization clusters indicates that most *Campylobacter* isolates are able to metabolize L-fucose, D-arabinose and/or D-glucose (Muraoka & Zhang, 2011; Stahl et al., 2011; Vorwerk et al., 2015; Vegge et al., 2016; Garber et al., 2020). The human gut is an L-fucose rich environment, and intestinal epithelial cells produce fucosylated mucins, making L-fucose metabolism beneficial for *Campylobacter* residing in the human gut. Furthermore, *Campylobacter* lacks fucosidases and it has been shown that in the presence of fucosidase-producing bacteria, like *B. fragilis*, *Campylobacter* uses cross-feeding by exchanging nutrients with *B. fragilis*, resulting in higher invasiveness of epithelial cells (Garber et al., 2020; Luijkx et al., 2020). Not only the human gut, but also the intestine of pigs and chicken are heavily fucosylated. Interestingly, unlike during pig colonization, no competitive advantage was observed for isolates carrying the L-fucose utilization cluster during the colonization of poultry, possibly due to a decreased fucosidase activity in poultry, as chicken fucosylated O-glycan mucin structures are more sulfated and therefore resistant to enzymatic processing (Stahl et al., 2011).

The L-fucose utilization cluster is a genomic element that comprises nine to ten genes, depending on a frameshift in *Cj0489* that is observed in some isolates (*Cj0480c – Cj0489* or *Cj0480c – Cj0489-S + Cj0489-L*), and has been identified in approximately 60% of the investigated *C. jejuni* and *C. coli* isolates, from here on referred to as fuc+ isolates. Several studies have shown that L-fucose metabolism affects growth, survival and virulence of *Campylobacter* isolates (Stahl et al., 2011; Dwivedi et al., 2016; Garber et al., 2020; Luijkx et al., 2020; Middendorf et al., 2022). L-fucose is metabolized to pyruvate and lactic acid, which can be further metabolized by *Campylobacter* (Middendorf et al., 2022).

Another, but less commonly encountered sugar metabolic cluster is the *Campylobacter* D-glucose utilization cluster, which comprises of seven genes (*glcP*, *pgi2*, *glk*, *pgl*, *zwf*, *edd* and *eda*) (Vorwerk et al., 2015; Vegge et al., 2016), from here on referred to as gluc+ isolates. It supports growth and enhances survival and biofilm formation in *Campylobacter* (Vorwerk et al., 2015; Vegge et al., 2016). Similar to L-fucose metabolism, D-glucose is metabolized to the end product pyruvate, which can be further metabolized (Vegge et al., 2016). Both the L-fucose utilization cluster and

the D-glucose utilization cluster were partly discovered due to the availability of online deposited genomic sequences.

Whole-genome sequencing (WGS) is rapidly becoming the standard for genotyping a wide variety of pathogens. It is a powerful tool to investigate genomic associations with the epidemiology of the microorganism, as it can additionally be used to identify potential important survival and virulence factors (Franz et al., 2016; Besser et al., 2018). Several public databases contain deposited genomic sequences and the PubMLST database is the largest *Campylobacter* genome database, currently harboring over 60,000 *C. jejuni* and *C. coli* genomes (Jolley et al., 2018).

In this study, we analyzed *C. jejuni* and *C. coli* isolate genomes from four different countries, namely, the Netherlands, the United Kingdom (UK), the United States of America (USA) and Finland. The Dutch dataset provides a balanced snapshot of the *C. coli* and *C. jejuni* population in the Netherlands (Mughini-Gras et al., 2021), while the UK, the USA and Finland datasets reflect the highest deposited genomic sequences counts in PubMLST. We studied the organization and origin of the L-fucose and D-glucose utilization clusters, including their flanking regions and position in the genomes, and we investigated the possibility of horizontal gene transfer. By extrapolating the phylogeny, we studied the presence of L-fucose and D-glucose utilization clusters and investigated the possible correlation of fuc+ isolates and human origin. Results give further insight in the diversity of these clusters and their putative contribution to survival, transmission and virulence in the animal and human host.

Methods

Isolate collection

Isolate collections were used from four different countries, namely, the Netherlands, the UK, the USA and Finland. Isolates from the UK, the USA and Finland were obtained from the PubMLST database (https://pubmlst.org/) accessed in June 2021.

All *C. jejuni* and *C. coli* WGS data from the PubMLST database were downloaded and accompanied by metadata, namely, isolate ID, isolate name, country of isolation, year of isolation, clonal complex, and species.

The Netherlands

The WGS of the Dutch dataset were provided by the National Institute for Public Health and the Environment (RIVM) and are described in (Mughini-Gras et al., 2021) and show a balanced snapshot of the *C. coli* and *C. jejuni* isolates from difference sources. In the current study, 1,057 *C. jejuni* and 349 *C. coli* whole-genome sequenced isolates from the Netherlands were used that were collected in 2014–2019. This set included isolates from human cases (n=280), water (n=251), sheep/goat (n=110), chicken (n=256), turkey (n=37), cattle (n=207), swine (n=110), wild birds (n=61) and pets (n=94). The isolates from human cases were collected from 13 different medical microbiology laboratories in the Netherlands. Isolates from livestock animals were collected by Wageningen Bioveterinary Research (WBVR) and Wageningen Food Safety Research (WFSR), in collaboration with the RIVM and the Netherlands Food and Consumer Product Safety Authority (NVWA), within the framework of established surveillance programs for zoonotic agents in food-producing animals in the Netherlands during 2014–2019. Isolates of pets were collected from veterinary clinics all across the Netherlands by The Veterinary Microbiological Diagnostic Centre (VMDC) of Utrecht University. Wild bird isolates were collected in June and December 2018 by Wageningen Ecological Research (WER). The water isolates were collected from six different geographic areas of comparable size in the Netherlands.

United Kingdom (UK)

In total, 21,690 *C. jejuni* isolates were deposited in the PubMLST database, however, only a randomly selected subset of 3,150 *C. jejuni* isolates was used in the current study, as the total number was too high for the creation of phylogenetic trees. Random selection was performed using R. These isolates included isolates from human cases (n=2,407), water (n=1), sheep/goat (n=47), chicken (n=531), turkey (n=18), cattle (n=73), wild bird (n=4), goose/duck (n=12) and unknown source (n=57). Furthermore, all 2,786 *C. coli* isolates from the PubMLST database were used for the current study. These isolates included isolates from human cases (n=1,401), water (n=38), sheep/goat (n=148), chicken (n=564), turkey (n=11), cattle (n=67), swine (n=182), wild bird (n=21), goose/duck (n=39), soil (n=35) and unknown source (n=280). The isolates from the UK were collected between 1980 and 2018.

United States of America (USA)

In total, 16,772 *C. jejuni* isolates were deposited in the PubMLST database, however, due to a large number of isolates being of unknown source, a sub selection was made of 1,251 isolates. These isolates included isolates from human cases (n=619), water (n=41), sheep/goat (n=22), chicken (n=207), turkey (n=6), cattle (n=160), wild bird (n=167) and goose/duck (n=29). The *C. jejuni* isolates from the USA were collected between 1979 and 2020, however, for the majority of the isolates no isolation year was reported. For *C. coli,* all 7,400 isolates were selected. However, similarly as for the *C. jejuni* isolates, a large number of isolates were isolated from unknown sources. Therefore, more than 95% the unknown sources isolates were removed (for a collection over 300 isolates), leaving 335 *C. coli* isolates. These isolates included isolates from human cases (n=26), chicken (n=108), turkey (n=24), cattle (n=31), swine (n=90), goose/duck (n=11) and unknown source (n=45). The *C. coli* isolates from the USA were collected between 1979 and 2019, however, for the majority of the isolates no isolation year was reported.

Finland

All 634 *C. jejuni* isolates deposited in the PubMLST database were used. These isolates included isolates from human cases (n=104), water (n=4), chicken (n=124), cattle (n=6), wild bird (n=359), goose/duck (n=21) and unknown source (n=16). Isolates from Finland were collected between 1998 and 2018 and concerned only *C. jejuni* isolates.

Integration sites of the L-fucose and D-glucose utilization cluster

Integration sites of the L-fucose utilization cluster were studied in all fuc+ isolates of the Dutch dataset. The L-fucose utilization cluster and 10 Kb flanking regions were selected and annotated using Prokka v1.13 (Seemann, 2014). Annotated flanking regions were visualized using the Benchling software (www.benchling.com).

Since Illumina paired end sequencing did not cover the flanking regions of the D-glucose utilization cluster in the Dutch dataset, four randomly selected isolates were used for long-read sequencing, namely, *C. jejuni* 103292-005-103, *C. coli* 18-556, *C. coli* 8230 and *C. jejuni* 18-440 (here named *C. jejuni* 4). Up to 50 Kb flanking regions were selected and annotated using Prokka v1.13, which were visualized using the Benchling software.

Next, all gluc+ isolates deposited in the PubMLST database (UK, USA and Finland) were screened for the presence of these flanking regions and the integration sites of the D-glucose utilization cluster.

For long-read sequencing, DNA was isolated using the Qiagen UltraClean Microbial DNA isolation kit (Qiagen, Venlo, the Netherlands) and sequenced using Oxford Nanopore technology to fully resolve the genome. This was performed as outlined in the genomic DNA ligation protocol (SQK-LSK109), with sequencing on a MinION device using flow cell type R9.4.1 (FLO-MIN106D) (Oxford Nanopore, Oxford, United Kingdom) using "super accurate" basecalling. Reads were filtered with options minimal length of 5,000 and keep percentage of 90 % using Filtlong v0.2.1 (https://github.com/rrwick/Filtlong). Reads were assembled using Flye v2.720 using options -nano hq -min overlap 1000 -meta, into a single scaffold and used the option "existing long read assembly" in Unicycler v. 0.4.721. Gene alignments and visualization were done using the Clinker alignment tool (Gilchrist & Chooi, 2021).

HGT and frameshift analyses of the L-fucose utilization cluster

In silico HGT analyses were performed on all L-fucose utilization cluster of the *C. jejuni* and *C. coli* isolates of the Dutch dataset. The L-fucose utilization cluster were selected in these genomes and aligned using Mafft (Katoh et al., 2002). Phylogenetic trees were built using Fasttree (Price et al., 2009). Branch lengths of the trees were square root transformed in R using ape 5.4.1 (Paradis & Schliep, 2019) to improve visualization of strain differences in phylogenetic trees. Frameshifts were visualized by viewing all L-fucose utilization clusters using the Benchling software. For the outgroup of the tree 8 fuc+ *Campylobacter* isolates from the NCBI database were selected, namely, *C. jejuni* doylei FDAARGOS 295, NCTC11924 and NCTC11951, *Campylobacter insulaenigrae* NCTC12927 and NCTC12928, *Campylobacter upsaliensis* NCTC11540 and NCTC11541, *Campylobacter canadensis* LMG24001.

Phylogeny & statistical procedures

Genome phylogenetic trees were constructed using Mashtree (Katz et al., 2019). Clades in the phylogenetic tree were partitioned into clusters using Treestruct (Volz et al., 2020), and MLST (Multi-Locus Sequence Typing) types were obtained from PubMLST. The percentage of fuc+ isolates and percentage of human derived isolates was determined per cluster and the correlation between the values per cluster were correlated using Spearman rank and Pearson correlations. By condensing the data to one datapoint per cluster, the effect of oversampled lineages was removed.

Results

Integration sites of the L-fucose and D-glucose utilization clusters in *C. jejuni* and *C. coli*

To get a better understanding of the distribution and transfer of the L-fucose and D-glucose utilization clusters between the *C. jejuni* and *C. coli* isolates, we investigated the integration location of these clusters in the genome. For the L-fucose utilization cluster, we analyzed up to 10 Kb flanking regions of this operon in both *C. jejuni* and *C. coli* isolates. In all flanking regions the same genes were observed, namely, *rpoB*, *rpoC* upstream and *rspL*, *repsG*, *fusA* downstream of the cluster (Figure 1). Protein analysis with Protein blast, uniprot and STRINGdb showed that these genes encode proteins with functions in transcription and translation.

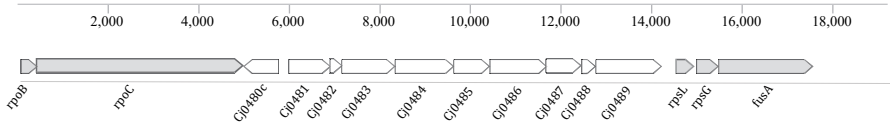

Figure 1 Flanking regions of the L-fucose utilization cluster (Cj0480c-Cj0489) in *C. jejuni* and *C. coli*.

Next, we investigated the flanking regions of the D-glucose cluster (*glcP*, *pgi2*, *glk*, *pgl*, *zwf*, *edd* and *eda*). In the Dutch dataset, which consisted out of 37 gluc+ isolates, the D-glucose utilization cluster was always present on small contigs of the genome sequences, making it difficult to accurately acquire large proportions of the flanking regions. Therefore, four isolates of the Dutch dataset were selected for long-read sequencing, for a more in-depth analysis of the flanking regions. Furthermore, of all gluc+ isolates present in the PubMLST database (from the UK, the USA and Finland), 27 isolates carried large enough contigs to investigate proportions of the flanking regions of the D-glucose utilization cluster. Combined with the Dutch isolates, in total 31 isolates were used for this analysis. Homology analysis of the D-glucose utilization clusters and the 10 Kb flanking regions using the alignment tool Clinker (Gilchrist & Chooi, 2021), selected *C. jejuni and C. coli* isolates were divided in six groups in total based on flanking region similarity (Figure 2).

Groups 1 and 2 carried, next to the D-glucose utilization cluster (*glcP*, *pgi2*, *glk*, *pgl*, *zwf*, *edd* and *eda*), also a galactose cluster (*galK_1, galK_2, galT_1, galT_2* and *galT_3*). Further analyses of the galactose cluster showed that it was only present in three *C. jejuni* isolates and one *C. coli* isolate. For group 1, the integration sites could be identified, and here the cluster was flanked by *Cj0756* and *Cj0734c*. Further analyses highlights that this region is in the internal transcribed spacer (ITS) domain which is between the 23S and 16S ribosomal RNA region (Suppl. Fig. 1A). Group 3 was integrated in the same region as group 1 and was flanked by *Cj0755* or *Cj0753c* and by *Cj0734c*. From group 4, only genes upstream of the cluster could be identified with certainty, as genes downstream were encoding hypothetical proteins. The upstream part of the cluster was formed by *Cj0029*, *Cj0028* and *Cj0027*, which is even more upstream in the 23S and 16S ribosomal RNA region than groups 1 and 3 (Suppl. Fig 1B). In the 10 Kb flanking regions of group 5, only hypothetical genes were observed, therefore, 50 Kb flanking regions were analyzed. From these isolates, three different flanking regions were observed around the genomic locations: *Cj0734c*, *Cj0031* and *Cj0431* (Suppl. Fig. 2). Lastly, group 6, was flanked by *Cj0432c* and *Cj0431*, which is a 23S and 16S ribosomal RNA region that is upstream of what was found in groups 1 and 3, and downstream of what was found in group 4 (Suppl. Fig. 1C).

Taken together, there are three genomic areas where the glucose cluster was integrated, which was either in the neighborhood region of *Cj0734c,* of *Cj0029* or of *Cj0431*. Notably, the cluster that was found in these three genomic regions were all inserted within one of the three copies of 23S and 16S ribosomal RNA in *C. jejuni* and *C. coli* genomes (Suppl. Fig. 1 and 3).

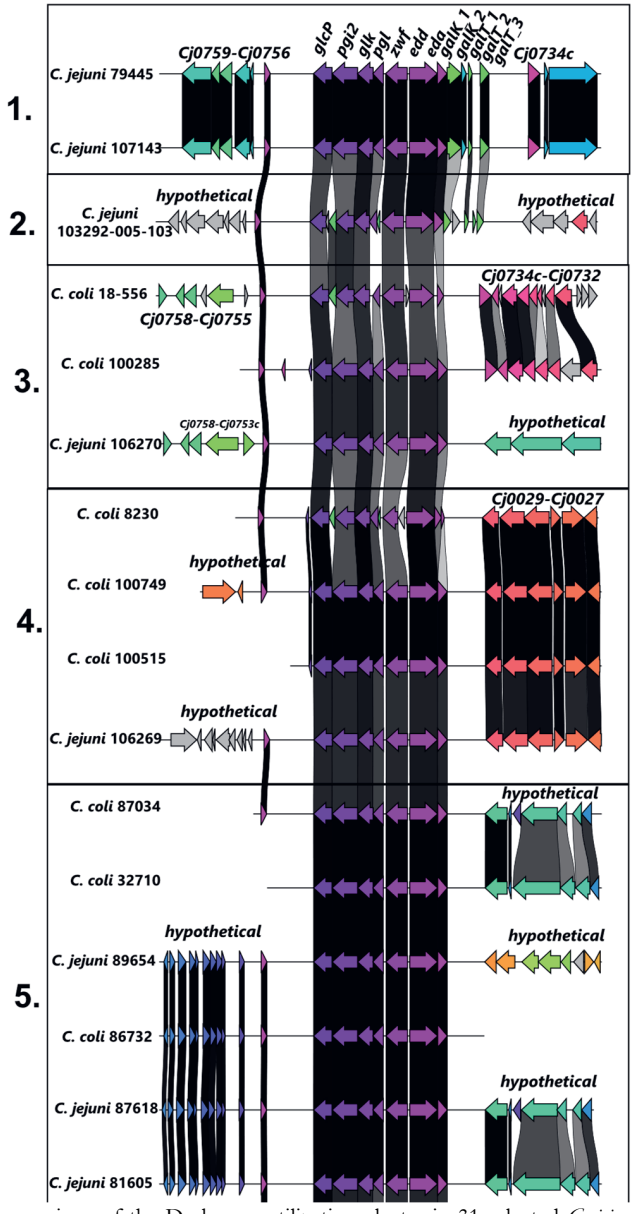

Figure 2 Flanking regions of the D-glucose utilization cluster in 31 selected *C. jejuni* and *C. coli* isolates. Based on analysis of 10 Kb flanking regions, Isolates were divided into 6 groups based on homology. Colors signify different gene groups based on genetic difference. For *C. jejuni*/*coli* isolates number 1 to 4, we performed long-read sequencing and for the other isolates, numbers indicate the PubMLST isolate ID, as described in materials and methods (continued on next page).

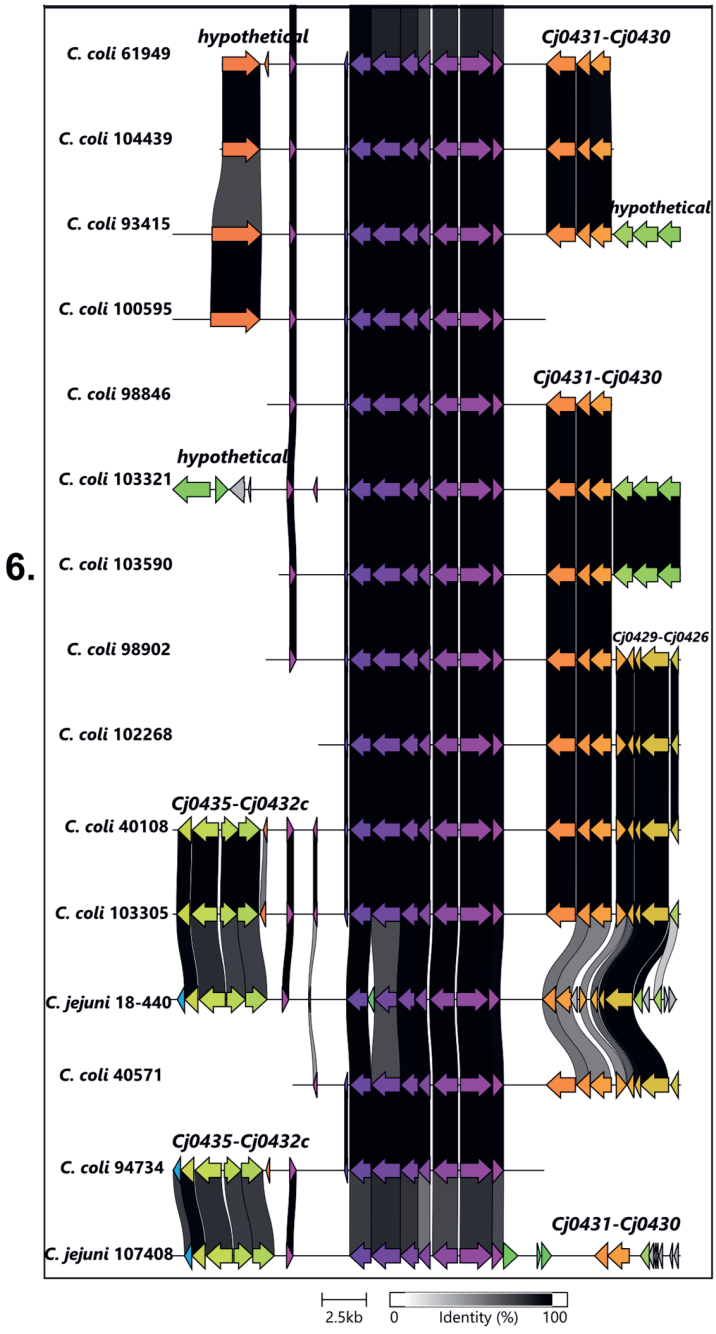

Figure 2 continued.

Phylogeny of *C. jejuni* and *C. coli* isolates and horizontal gene transfer between the species

As similar integration sites of either the L-fucose or the D-glucose utilization clusters were observed in both tested species, we investigated the phylogeny of *C. jejuni* and *C. coli* isolates and analyzed whether HGT of the utilization clusters occurred between both species. For these analyses, the Dutch collection was used, as it provided a balanced snapshot of the *C. coli* and *C. jejuni* population in the Netherlands, which is well described in literature (Mughini-Gras et al., 2021), unlike the collections that were obtained from PubMLST. In the Dutch collection, we first investigated the presence of the L-fucose and D-glucose utilization clusters using a phylogenetic tree based on the whole genome sequences of both species. The phylogenetic tree showed a clear distinction between *C. jejuni* and *C. coli* isolates (Figure 3). For *C. coli*, four different clades were observed, while this was less clear for *C. jejuni* isolates. Clonal clusters of fuc+ isolates were observed in especially *C. jejuni*, but large sections within these clonal clusters were also lacking the L-fucose cluster. The D-glucose utilization cluster was sporadically present in the *C. jejuni* isolates (0.2% of all tested isolates), but more prevalent in *C. coli* isolates (10% of all tested isolates). Furthermore, despite conceivable introduction at multiple stages in the phylogeny, subsequent clonal spread of the D-glucose utilization cluster, as observed for the L-fucose utilization cluster, was not observed.

Due to the large number of fuc+ isolates, we were able to investigate HGT of the L-fucose utilization cluster between these *C. jejuni* and *C. coli* isolates, using a phylogenetic tree based on the L-fucose utilization cluster (~9Kb per isolate) of all Dutch *C. jejuni* and *C. coli* isolates. Other *Campylobacter* (non-*jejuni* and non-*coli*) species were included as outgroup for the rooting of the tree (Figure 4). Clear indications were found of two HGT events within the dataset, namely, one *C. jejuni* isolate carried an L-fucose utilization cluster that was highly identical to the L-fucose utilization clusters typically observed in *C. coli,* and two *C. coli* isolates carried an utilization cluster that was highly identical to the L-fucose utilization clusters typically found in *C. jejuni* (marked in red).

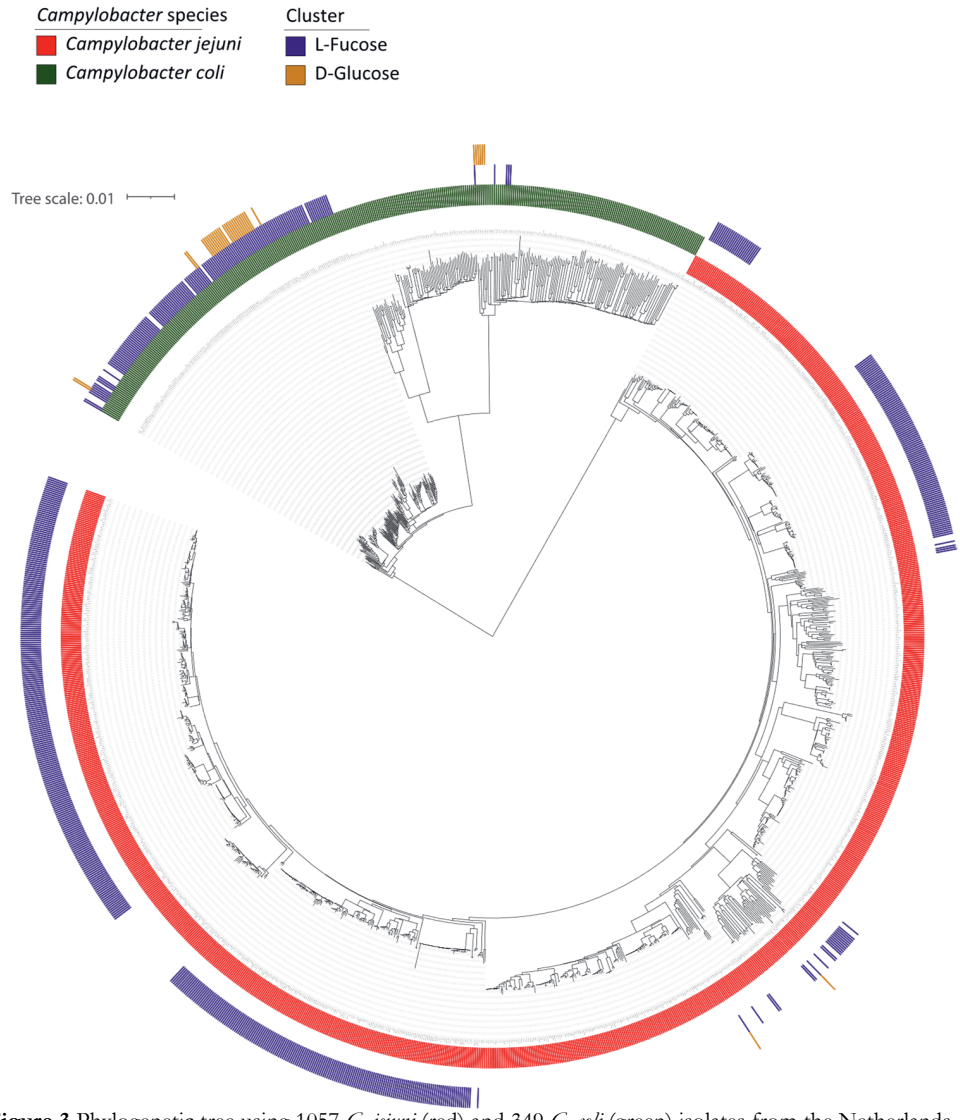

Figure 3 Phylogenetic tree using 1057 *C. jejuni* (red) and 349 *C. coli* (green) isolates from the Netherlands, based on the whole genome. Isolates were collected between 2014-2019 and consisted of multiple sources, including human, water, sheep/goat, chicken, turkey, cattle, swine, wild bird and pet isolates (Mughini-Gras et al., 2021).

Distribution of *Campylobacter* spp. based on isolation source and L-fucose/D-glucose utilization clusters

We further analyzed the presence of the L-fucose and D-glucose utilization clusters using larger databases which we grouped on the basis of country of isolation: the Netherlands, the United Kingdom (UK), the United States of America (USA) and Finland. We included in the analysis 1,057 *C. jejuni* and 349 *C. coli* isolates from the Netherlands, 3,150 *C. jejuni* and 2,786 *C. coli* isolates from the UK, 1,251 *C. jejuni* and 335 *C. coli* isolates from the USA and only 634 *C. jejuni* isolates from Finland because *C. coli* isolates from this country were not present in the PubMLST database (Tables 1 and 2). Together, these isolates consisted of 4,837 human isolates, 335 water isolates, 327 sheep/goat isolates, 1,790 chicken isolates, 96 turkey isolates, 544 cattle isolates, 382 swine isolates, 612 wild bird isolates, 94 pet isolates, 112 goose/duck isolates, 35 soil isolates and 398 isolates of unknown isolation source.

The L-fucose cluster prevalence in *C. jejuni* was for the Netherlands 51%, for the UK 65%, for the USA 44% and for Finland 15%. For *C. coli*, the L-fucose prevalence was 43%, 77% and 91% for the Netherlands, the UK and the USA, respectively. To investigate the cluster prevalence over time, we split the *C. jejuni* and *C. coli* collection of the UK per year, as this was the largest dataset that included metadata such as year of isolation (Suppl. Fig. 4). For *C. jejuni,* no notable changes in the percentage of fuc+ isolates were observed throughout the years 2001-2018. For *C. coli* an increasing trend in the prevalence of the L-fucose cluster was observed in later years (2011-2018) and was not linked to clonal spread.

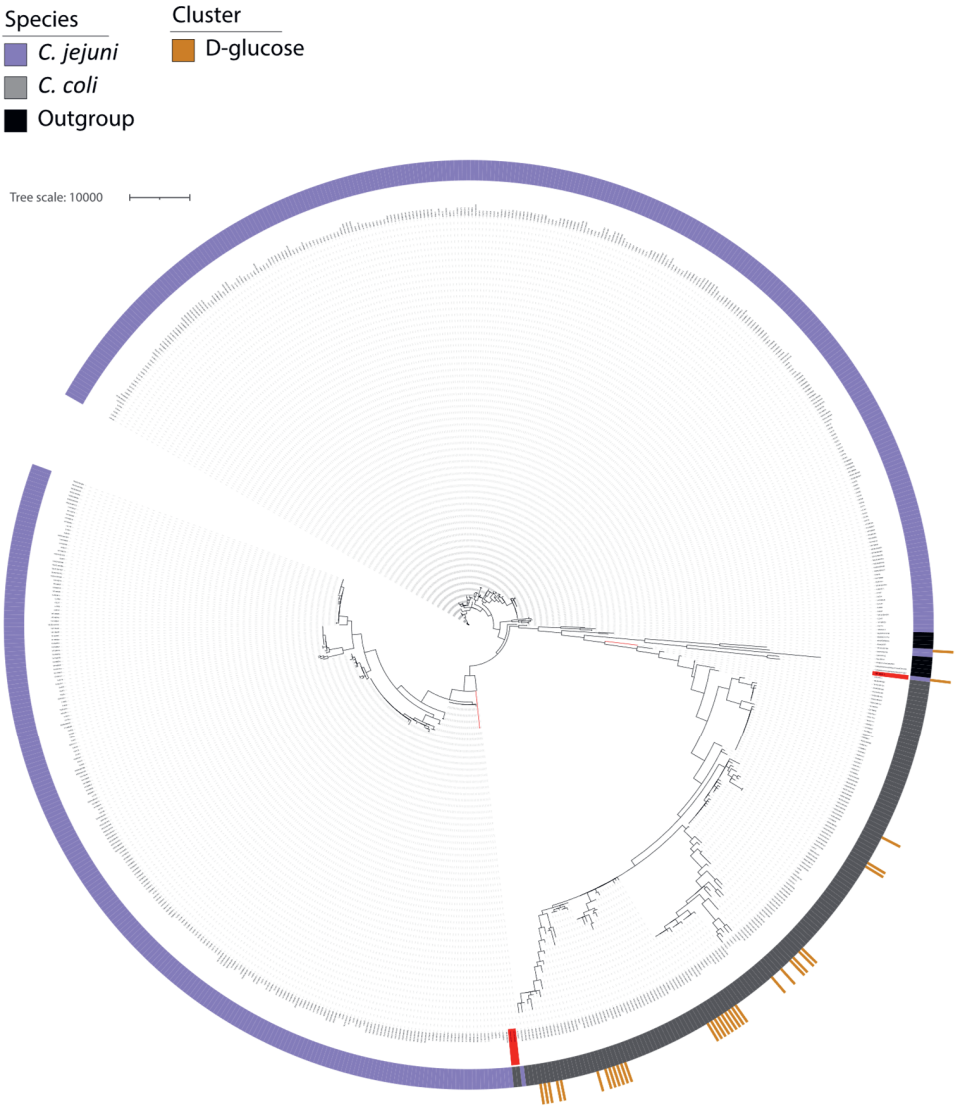

Figure 4 Phylogenetic tree of *C. jejuni* and *C. coli* isolates from the Netherlands, based on 685 sequences of the L-fucose utilization cluster (all fuc+ isolates). Two species were included, namely *C. jejuni* (purple) and *C. coli* (grey). The outgroup (black, fuc+) consisted of 8 isolates: *C. jejuni doylei* FDAARGOS 295, NCTC11924 and NCTC11951, *C. insulaenigrae* NCTC12927 and NCTC12928, *C. upsaliensis* NCTC11540 and NCTC11541, and *C. canadensis* LMG24001. Isolates marked in red highlight events of HGT.

The D-glucose cluster prevalence for *C. jejuni* per country was for the Netherlands 0.2%, for the UK 0.6%, for the USA 0.7% and for Finland 9.9%. For *C. coli,* the D-

glucose cluster prevalence was 10%, 4.3% and 6.6% for the Netherlands, the UK and the USA, respectively.

Interestingly, isolates carrying the D-glucose utilization cluster often carried the L-fucose utilization cluster. For *C. jejuni* this was observed in 2/2 isolates (100%), 16/19 isolates (84%), 2/9 isolates (22%) and 8/63 isolates (13%), for the Netherlands, the UK, the USA and Finland, respectively, and for *C. coli*, 29/35 isolates (83%), and 120/121 isolates (99%), and 22/22 (100%) isolates, for the Netherlands, the UK and the USA, respectively.

Table 1 *C. jejuni* isolate collection used in the current study. Total number of isolates and percentages of fuc+ isolates, gluc+ isolates and fuc+/gluc+ isolates per source are displayed.

	Netherlands	**UK***	**USA***	**Finland**
Source	**Nr. *C. jejuni* total (%fuc+/%gluc+/%both**	**Nr. *C. jejuni* total (%fuc+/%gluc+/%both**	**Nr. *C. jejuni* total (%fuc+/%gluc+/%both**	**Nr. *C. jejuni* total (%fuc+/%gluc+/%both**
Human	272 (67%/0%/0%)	2407 (68%/0.2%/0.1%)	619 (52%/0%/0%)	104 (19%/0%/0%)
Water	76 (16%/1.3%/1.3%)	1 (0%/0%/0%)	41 (41%/2.4%/0%)	4 (0%/0%/0%)
Sheep/Goat	85 (49%/0%0%)	47 (40%/2%/0%)	22 (100%/0%/0%)	-
Chicken	241 (56%/0%/0%)	531 (56%/0%/0%)	207 (40%/0%/0%)	124 (1.6%/0%/0%)
Turkey	37 (49%/0%/0%)	18 (78%/0%/0%)	6 (17%/0%/0%)	
Cattle	196 (53%/0%/0%)	73 (44%/0%/0%)	160 (47%/0%/0%)	6 (67%/0%/0%)
Swine	10 (40%/0%/0%)	-		
Wild Bird	46 (17%/6.7%/6.7%)	4 (100%/0%/0%)	167 (22%/4.8%/1.2%)	359 (18%/18%/2.2%)
Pet	94 (32%/0%/0%)	-		
Goose/duck	-	12 (8%/0%/0%)	29 (0%/0%/0%)	21 (9.5%/0%/0%)
Soil	-	-	-	
Unknown	-	57 (68%/23%/23%)	-	16 (0%/0%/0%)
Total	**1057 (51%/0.2%/0.2%)**	**3150 (65%/0.6%/0.5%)**	**1251 (44%/0.7%/0.2%)**	**634 (15%/9.9%/1.3%)**

Table 2 *C. coli* isolate collection used in the current study. Total number of isolates and precentages of fuc+ isolates, gluc+ isolates and isolates with both clusters per source are displayed.

	Netherlands	UK*	USA*
	Nr. *C. coli*	Nr. *C. coli*	Nr. *C. coli*
Source	total (%fuc+/%gluc+/%both)	total (%fuc+/%gluc+/%both)	total (%fuc+/%gluc+/%both)
Human	8 (100%/13%/13%)	1401(81%/1.9%/1.9%)	26 (77%/0%/0%)
Water	175 (2.9%/2.9%/0%)	38 (7.9%/0%/0%)	-
Sheep/Goat	25 (88%/20%20%)	148 (99%/7.4%/7.4%)	-
Chicken	15 (47%/0%/0%)	564 (56%/1.2%/1.2%)	108 (100%/1.9%/1.9%)
Turkey	-	11 (55%/0%/0%)	24 (92%/0%/0%)
Cattle	11 (100%/0%/0%)	67 (99%/0%/0%)	31 (97%/0%/0%)
Swine	100 (95%/23%/22%)	182 (92%/36%/36%)	90 (80%/18/18%)
Wild Bird	15 (13%/2.2%/2.2%)	21(19%/0%/0%)	-
Pet	-	-	-
Goose/duck	-	39 (18%/0%/0%)	11 (100%/0%/0%)
Soil	-	35 (89%/17%/17%)	-
Unknown	-	280 (89%/1.8%/1.8%)	45 (91%/8.9%/8.9%)
Total	**349 (43%/10%/8.3%)**	**2786 (77%/4.3%/4.3%)**	**335 (91%/6.6%/6.6%)**

Phylogenetic trees were created per country to further investigate the distribution of the L-fucose and D-glucose utilization clusters and possible host associations. For the generation of the country-specific phylogenetic *C. jejuni* trees, 1,057 (the Netherlands), 3,150 (UK), 1,289 (USA) and 634 (Finland) *C. jejuni* isolates were used (Figure 5, Suppl. Fig. 5-7). Isolates within the trees were clustered into groups using the classification methods Treestruct and MLST. MLST subclassification is based on the sequences (alleles) of seven household genes, whereas Treestruct is based on the branch lengths in the phylogenetic trees using the whole genome. Note that there are no universal Treestruct clusters between different phylogenetic trees, as the branch lengths in each tree are different (Volz et al., 2020), therefore, Treestruct groups are tree-specific. Only the Dutch dataset was the result of a surveillance study in a defined period of two years and therefore little to no sampling bias expected, we mainly focused on the Dutch dataset, as in that dataset there was no unknown sampling bias.

In the Dutch dataset, large clusters of fuc+ isolates (> 40 isolates per cluster) were observed, with apparent clonal expansion, while only two isolates carried the D-glucose utilization cluster. Similarly for the PubMLST datasets of the UK (> 100

isolates) and the USA (> 50 isolates per cluster), also large fuc+ isolate clusters were observed, while the D-glucose utilization cluster was much more rare. Interestingly, in the Finland dataset, large clusters of either fuc+ or gluc+ isolates (>12 isolates per cluster) were present, indicating clonal expansion. Notably, the Finland dataset consisted of 56% wild bird isolates, while the other countries only had between 0.1% and 13% wild bird isolates. In all tested countries, gluc+ isolates were mostly wild bird isolates, highlighting a clear link between gluc+ isolates and wild bird isolates. However, the implications of this link requires further studies.

Correlation between fuc+ isolates and human isolates in *C. jejuni*

Studies have shown that fuc+ isolates show increased survival and growth in the presence of L-fucose, however, no link to source specific isolates was made (Stahl et al., 2011; Garber et al., 2020; Middendorf et al., 2022; Middendorf et al., 2023). In the current study when using the Dutch dataset, large quantities of human fuc+ isolates (67%) were observed. Therefore, we hypothesized that fuc+ isolates are more likely to survive and proliferate in the human host after transmission from an animal or environmental reservoir. This was investigated by dividing the phylogenetic trees in groups using the classification methods Treestruct and MLST, and calculating the correlation between the percentage of fuc+ isolates and the percentage of human isolates over all clusters to alleviate oversampling biases for specific clones. For the Dutch dataset, isolates were partitioned into 22 different Treestruct groups and in 16 MLST groups. In this dataset a significant correlation was observed between human isolates and fuc+ isolates, with the Treestruct (Spearman p-value=0.0072 and Pearson p-value = 0.0177) and MLST (Spearman p-value = 0.0400 and Pearson p-value = 0.0384) classification methods (Suppl. Table 1), suggesting that fuc+ isolates have advantages to survive and proliferate in the human hosts.

Although the PubMLST datasets (UK, USA and Finland) potentially had unknown sampling biases, we screened whether a correlation was also observed in these countries. For the UK 40 Treestruct groups (>35 isolates per group) and 15 MLST groups (>35 isolates per group), for the USA 18 Treestruct groups (>25 isolates per group) and 12 MLST groups (>25 isolates per group) and for Finland 18 Treestruct groups and 7 MLST groups (>25 isolates per group). However, no significant correlations were observed.

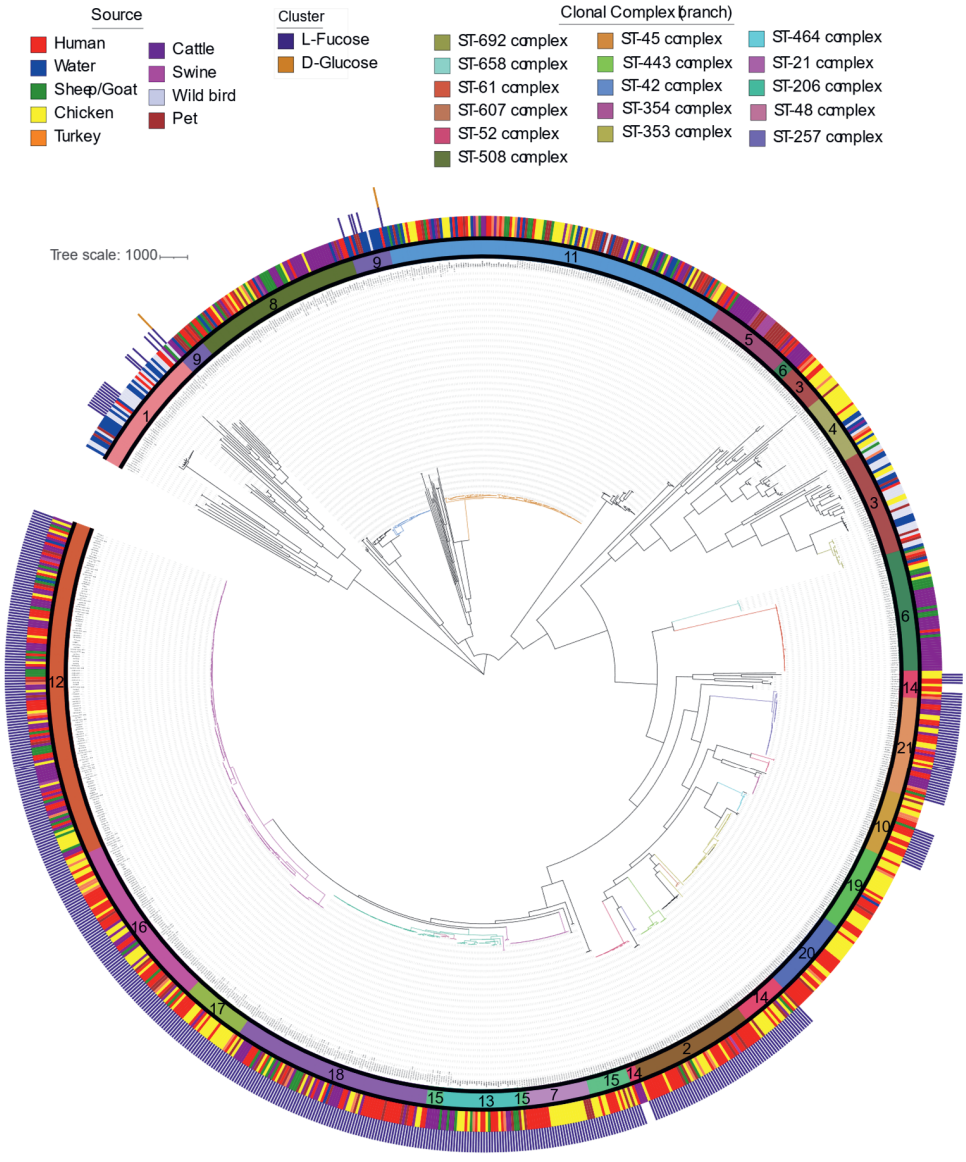

Figure 5 Phylogenetic tree using 1057 full genomes of *C. jejuni* isolates from the Netherlands. From outside to inside: the outer (orange) ring indicate gluc+ isolates, the purple ring indicates fuc+ isolates, the multicolored ring indicates the isolation source per isolate and, lastly, the inner ring (multicolored with numbers) indicates the different Treestruct groups. Branch colors indicate the different clonal complexes (MLST groups).

Notably, after combining the *C. jejuni* datasets of the four countries, which resulted in 6092 *C. jejuni* isolates, a significant correlation with both the Treestruct (Spearman p-value = 0.0017 and Pearson p-value <0.0001) and MLST classification (Spearman p-value = 0.034 and Pearson p-value = ns) methods was observed between human isolates and the presence of the L-fucose utilization cluster.

L-fucose and D-glucose cluster distribution in *C. coli*

Similar analyses were performed with the phylogenetic trees of *C. coli*, for which 349, 2,786 and 335 *C. coli* isolates were used for the Netherlands, the UK and the USA, respectively (Figure 6 and Suppl. Fig. 8-9).

In the Dutch dataset, large clusters of fuc+ isolates (> 15 isolates per cluster) were observed in livestock associated isolates, with apparent clonal expansion, while the D-glucose utilization cluster was only sporadically present. Interestingly, the Dutch dataset included a high number (n=175, 50%) of water isolates, which were present in multiple clades, and these water isolates rarely carried the L-fucose utilization cluster (n=3). However, unlike what was observed in animal host environments, none of these integrations resulted in clonal fuc+ isolate clusters, suggesting that there was no benefit for carrying this cluster in a water environment or, alternatively, that it is challenging to isolate these isolates in a water environment. In the *C. coli* PubMLST dataset (the UK and the USA), large clusters of fuc+ isolates (> 100 isolates per cluster for the UK and > 15 isolates per cluster for the USA) were observed, with apparent clonal expansion. In this dataset, also several clusters of gluc+ isolates were observed, however, on a much smaller (< 14 isolates per cluster) scale than compared to the fuc+ clusters.

Next, similarly as for *C. jejuni*, we investigated whether the isolates carrying the L-fucose utilization clusters are more likely to proliferate in the human host after transmission from an animal or environmental reservoir. For the *C. coli* phylogenetic trees, isolates were only divided into different Treestruct complexes, as MLST complexes are very limited for *C. coli*. Based on branch lengths, each phylogenetic group was divided into 7 Treestruct groups, which were used for the statistical correlation analysis. However, no significant correlations were observed in any of the *C. coli* datasets (Suppl. Table 1).

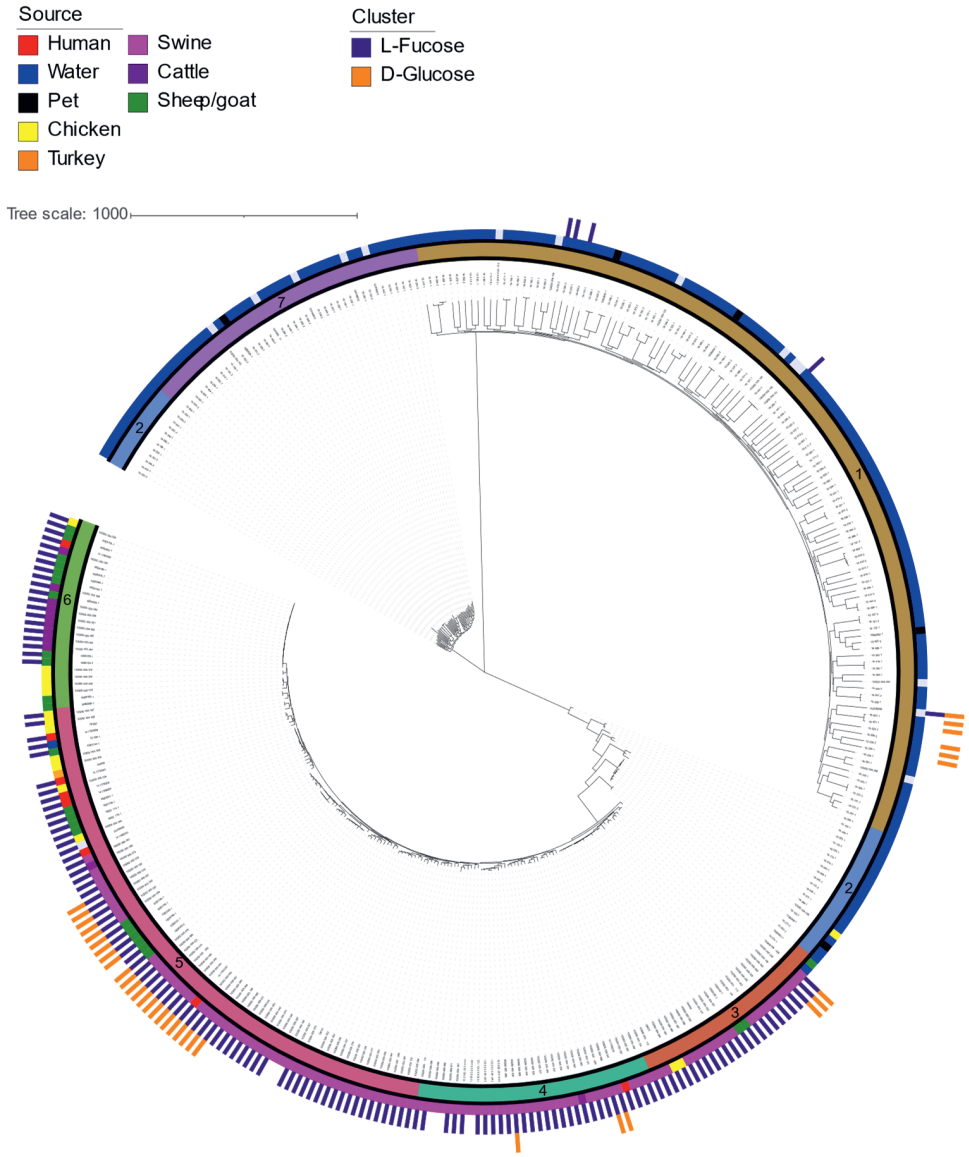

Figure 6 Phylogenetic tree using 349 full genomes of *C. coli* isolates from the Netherlands. From outside to inside: the outer (orange) ring indicate gluc+ isolates, the purple ring indicate fuc+ isolates, The multicolored ring indicate the isolation source per isolate and, lastly, the inner ring (multicolored with numbers) indicates the different Treestruct groups.

Discussion

We investigated two carbohydrate utilization clusters that have previously been associated with growth, survival and biofilm formation of *Campylobacter*; the L-fucose and the D-glucose utilization clusters (Muraoka & Zhang, 2011; Stahl et al., 2011; Vorwerk et al., 2015; Dwivedi et al., 2016; Vegge et al., 2016). The sugars L-fucose and D-glucose are present in many environments, including the human and animal gastro-intestinal tracts. Previous studies have shown a role for fucose utilization in colonization of chicken and piglets, and in adhesion and invasion efficacy of Caco-2 cells (Middendorf et al., 2023). To date, several studies have been performed to investigate the transmission of *Campylobacter* between animals and humans (Kaakoush et al., 2015; Mughini-Gras et al., 2021), however, attributes that promote this transmission were rarely studied. In the current study we performed an *in silico* analysis to investigate the distribution of *C. jejuni* and *C. coli* isolates carrying the L-fucose and D-glucose utilization cluster and correlated the presence of the L-fucose utilization cluster in *Campylobacter* isolates to increased survival and proliferation in the human host.

Previous studies highlighted that there are at least two versions of the L-fucose utilization cluster, one with an intact cluster (*Cj0480c – Cj0489)* and one with a frameshift in the *Cj0489* gene (*Cj0480c – Cj0489-L)* (Muraoka & Zhang, 2011; Middendorf et al., 2022). Furthermore, proteomics analyses of cells with an activated L-fucose utilization cluster highlighted that not all proteins were produced, namely, proteins Cj0484 and Cj0489-S for isolate *C. jejuni* NCTC11168, while cells still showed increased invasion to Caco-2 cells and an increased binding to fibronectin (Middendorf et al., 2023). Here we found that all L-fucose utilization clusters, including the mutated versions, were integrated in the exact same location of the genome in both *C. jejuni* and *C. coli* isolates (Figure 1). The L-fucose utilization cluster was flanked upstream by the genes *rpoB* and *rpoC* while downstream by *rspL*, *repsG*, and *fusA*. This is a genomic area that is typically targeted by antibiotics such as rifampicin and streptomycin (Huang et al., 2003; Goldstein, 2014), and resistance is almost exclusively acquired by point mutations or indels (Comas et al., 2012; Lo et al., 2018; Godfroid et al., 2020). To gain a better understanding about the L-fucose cluster and how it evolved, we subsequently aligned all *C. jejuni* and *C. coli* fucose utilization

clusters of the dataset from the Netherlands to identify mutations within the cluster (Suppl. Fig. 10). Interestingly, only 36% of the fuc+ *C. jejuni* isolates did not carry any frameshift mutation in the cluster; in the remaining strains common frameshift mutations were found in the aldehyde dehydrogenase *Cj0489* (39%), the unknown transporter *Cj0484* (14%) and the amidohydrolase *Cj0487* (8%) (Suppl. Fig. 10). Studies where knockout assays of each gene of the L-fucose utilization clusters were performed showed that knockouts of *Cj0489* and *Cj0484* did not result in a loss of function, because these knockouts showed the same phenotype as those with a fully intact cluster. In contrast, *Cj0487* knockouts showed no growth stimulation in the presence of L-fucose, highlighting that *Cj0487* is an essential gene for L-fucose metabolism (Stahl et al., 2011; Dwivedi et al., 2016; Garber et al., 2020). Notably, these knockout isolates did still show chemotaxis towards L-fucose (Stahl et al., 2011; Dwivedi et al., 2016). In the current study, all isolates that carried the *Cj0487* mutation also carried a mutation in Cj0489. Furthermore, all these isolates were either chicken or human isolates and belonged to the same clonal cluster (Suppl. Fig. 11). Since information on performance of such *C. jejuni* double mutants is not available, additional studies with selected isolates are required to determine the effect of L-fucose on metabolism, growth, chemotaxis and virulence. Unlike *C. jejuni*, *C. coli* isolates did not carry any mutation in the L-fucose utilization cluster. Comparing the source of fuc+ *C. jejuni* and *C. coli* isolates, much higher quantities of fuc+ isolates were found in livestock associated *C. coli* isolates, with over 80% of the *C. coli* swine isolates carrying the L-fucose cluster. These results highlight a selection pressure of carrying an intact L-fucose utilization cluster in *C. coli*, possibly for the colonization and/or long-term survival in the animal host.

The integration of the D-glucose utilization cluster was much more diverse. We found evidence that the cluster was integrated in at least three different genomic locations, all within the three different 16S-23S ribosomal RNA regions, which we highlighted by viewing the genome of *C. jejuni* NCTC11168 (accession: NC_002163) (Suppl. Fig. 1 and 3). There could be several reasons for this observation, such as the finding that rearrangement can take place in well conserved regions like the 16S-23S areas (Page et al., 2020). Alternatively, due to faulty assemblies, possible rearrangements within the 16S-23S areas are observed in genome sequences, yet are not actually existing (Page et al., 2020). On the other hand, the high sequence identity of the flanking regions of the

D-glucose utilization cluster allows for efficient integration of DNA from the environment, as *Campylobacter* is genetically competent.

Interestingly, some gluc+ *C. jejuni* and *C. coli* isolates carried several galactose utilization genes next to the D-glucose utilization cluster. Although the number of gluc+ *C. jejuni* and *C. coli* isolates in which the flanking regions could be identified was low (10 *C. jejuni* and 21 *C. coli* isolates), this suggested that this version of the D-glucose utilization cluster with neighboring the galactose cluster, has a different origin than the other analyzed D-glucose utilization clusters and was most likely introduced from other bacterial species to *Campylobacter*. However, as the current dataset of glucose integration sites is very limited, more long-read sequencing procedures have to be performed to obtain a better insight in the origin of the galactose cluster. The function of the observed *gal* genes are putative, however, studies in different kinds of bacteria have shown that several of the genes were linked to the LPS synthesis, galactose metabolism (via the Leloir pathway) or synthesis of polysaccharides (Houng et al., 1990; Maskell et al., 1991; Kanipes et al., 2008).

The influence of L-fucose on the virulence and colonization of *Campylobacter* has been studied in piglets and chicken models, and in human cell lines, using wildtype (wt) and selected mutants in the L-fucose utilization cluster (Stahl et al., 2011; Luijkx et al., 2020). In addition, L-fucose pre-activated *C. jejuni* NCTC11168 cells recently showed increased invasion of Caco-2 epithelial cells and binding to fibronectin, highlighting a possible advantage of fuc+ strains in transmission across fucose-rich rich environments, such as from the animal intestine to the human gut (Middendorf et al., 2023). In the current study we investigated whether isolates carrying the L-fucose utilization cluster are more prevalent in human isolates by using correlation analyses on selected datasets from four countries. The dataset from the Netherlands was obtained from a country-wide study that was performed over a time period of two years. In the study design, elimination of issues related to differential recall bias, selection bias and misclassification were performed (Mughini-Gras et al., 2021). The other *C. jejuni* collections were obtained from the PubMLST database, in which the exact origin of isolates and type of sampling plans are unknown. Furthermore, possible selection bias in these datasets, for example towards disease symptoms or outbreaks, cannot be excluded. It was therefore not surprising that only a significant correlation

was found between the percentage of human isolates and the percentage of fuc+ isolates in the *C. jejuni* dataset from the Netherlands. The significant correlation in this dataset, together with the previous studies that found prolonged survival and increased virulence (Garber et al., 2020; Luijkx et al., 2020; Middendorf et al., 2022), suggest that fuc+ *C. jejuni* have enhanced or extended proliferation in the human gut after the transmission from an animal or environmental reservoir. In the intestine of poultry, wt isolates do not have an apparent competitive colonization advantage over *fucP* mutant isolates, possibly due to the fucosylated O-glycan mucin structures from chicken being more sulfated compared to the human and pig counterparts (Stahl et al., 2011; Luis et al., 2022). This was further supported by adding an excess of free L-fucose during chick colonization, resulting in a competitive advantage of wt *C. jejuni* over *fucP* mutants (Stahl et al., 2011). Interestingly, as 80% of the campylobacteriosis cases in the Netherlands are linked to the poultry reservoir as a whole (Doorduyn et al., 2010; Mughini-Gras et al., 2012; EFSA, 2021; Mughini-Gras et al., 2021), it is likely that *C. jejuni* isolates carrying the L-fucose utilization cluster have an advantage after transmission from poultry towards humans by increased survival and proliferation in the human gut. Not only was a correlation between fuc+ isolates and human isolates observed in the Dutch *C. jejuni* dataset, but also in the combined dataset, suggesting improved survival and/or proliferation of fuc+ *C. jejuni* in the human gut after transmission from animal or environmental reservoirs world-wide.

In conclusion, our study provided new insights into *C. jejuni* and *C. coli* isolates carrying the L-fucose and D-glucose utilization cluster. Our study identified one integration site of the L-fucose utilization cluster and at least three integration sites for the D-glucose utilization cluster in the genomes of *C. jejuni* and *C. coli*. A correlation between human *C. jejuni* isolates and the presence of the L-fucose cluster was observed in the Dutch dataset and when combining all datasets of *C. jejuni*. This correlation highlights a potential selective advantage for fuc+ *C. jejuni* isolates in human infection and/or during survival and proliferation within the human host, conceivably after transmission from any reservoir.

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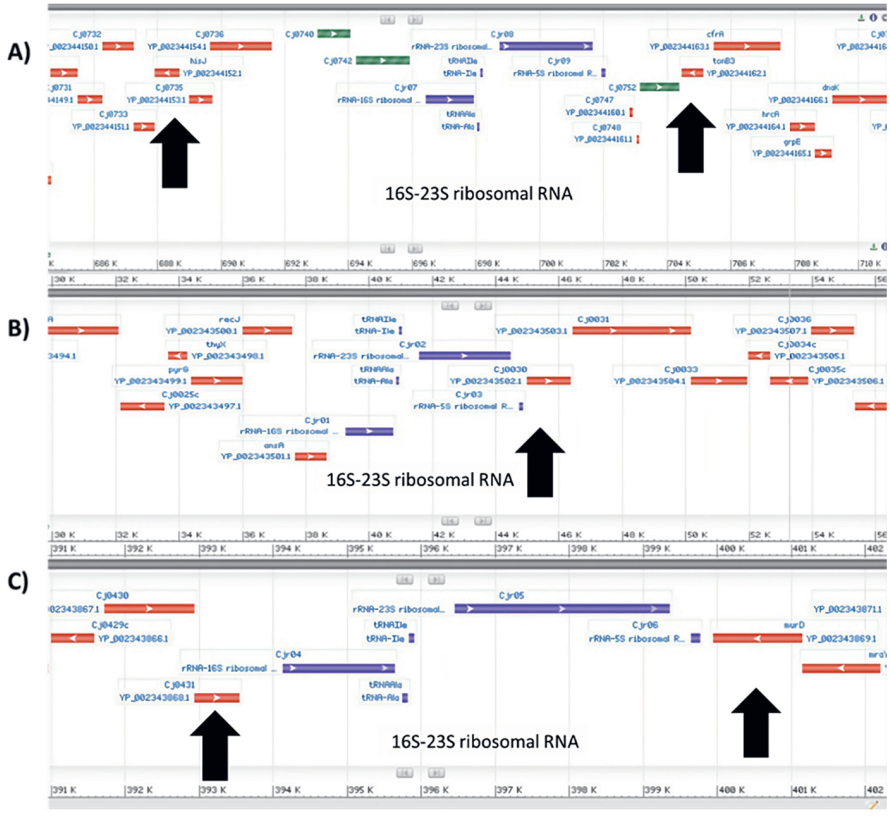
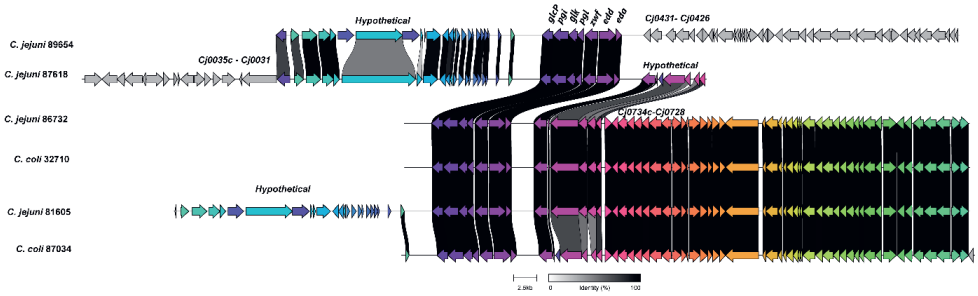
All genomes of the Dutch dataset were obtained via the Dutch National Institute for Public Health and the Environment (RIVM).

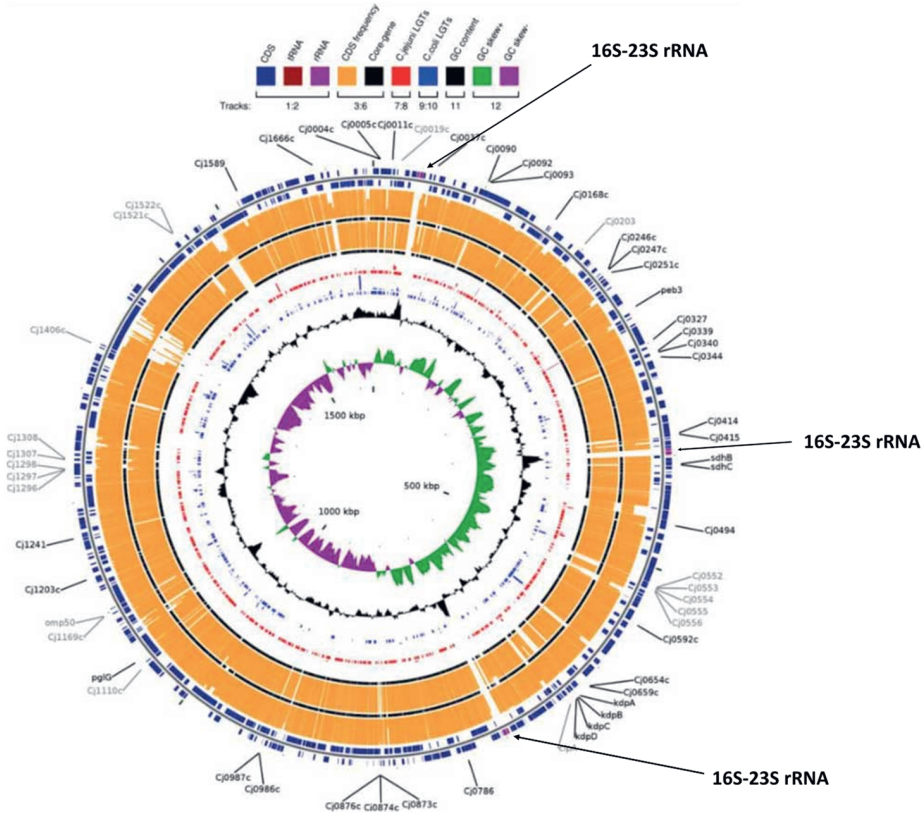
Supplements



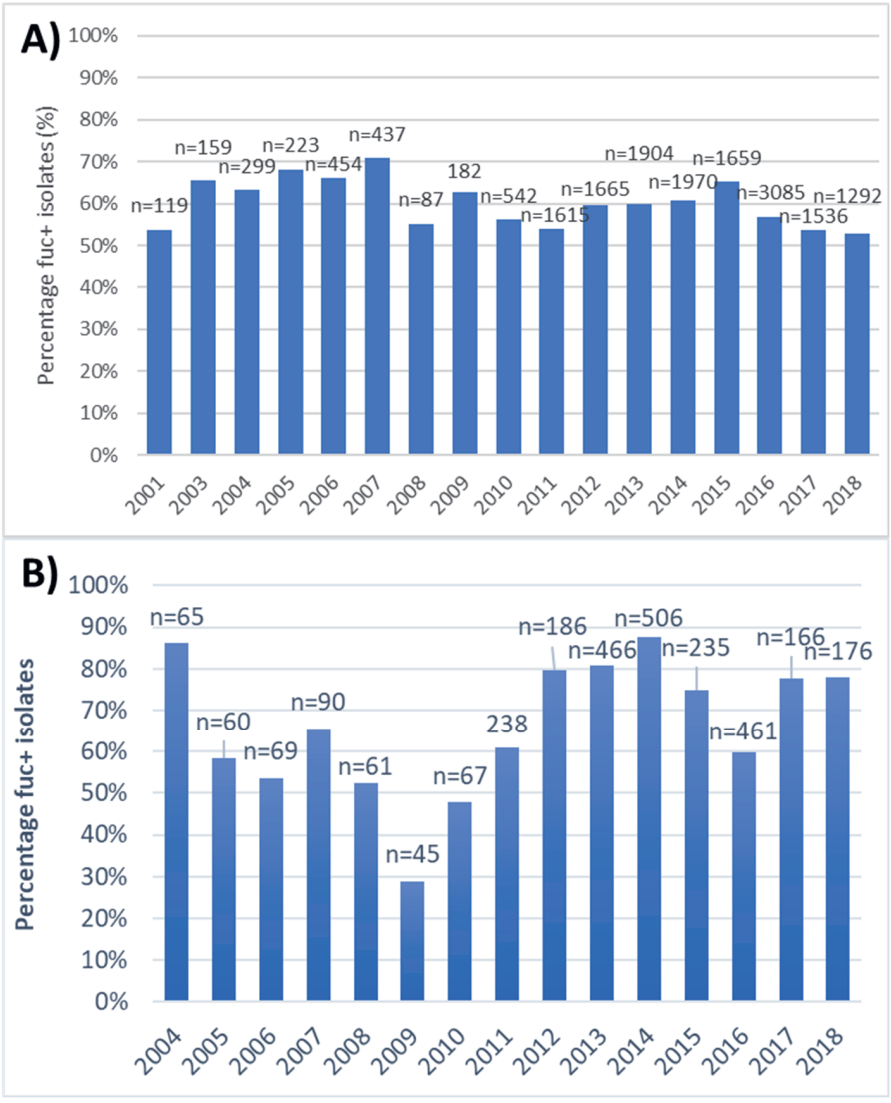
Suppl. Fig. 1 The three 16S-23S regions in *C. jejuni* NCTC11168. Images were made using de NCBI genome viewer. A) Flanking regions (Cj0756 and Cj0734c) of groups 1, 2 and 5 (partly). B) Flanking regions (Cj0029, Cj0028 and Cj0027) of group 4 and 5 (partly). C) Flanking regions (Cj0432c and Cj0431) of groups 6 and 5 (partly). Black arrows indicate the flanking genes of the L-fucose utilization cluster. 16S-23S ribosomal RNA regions are highlighted in purple.



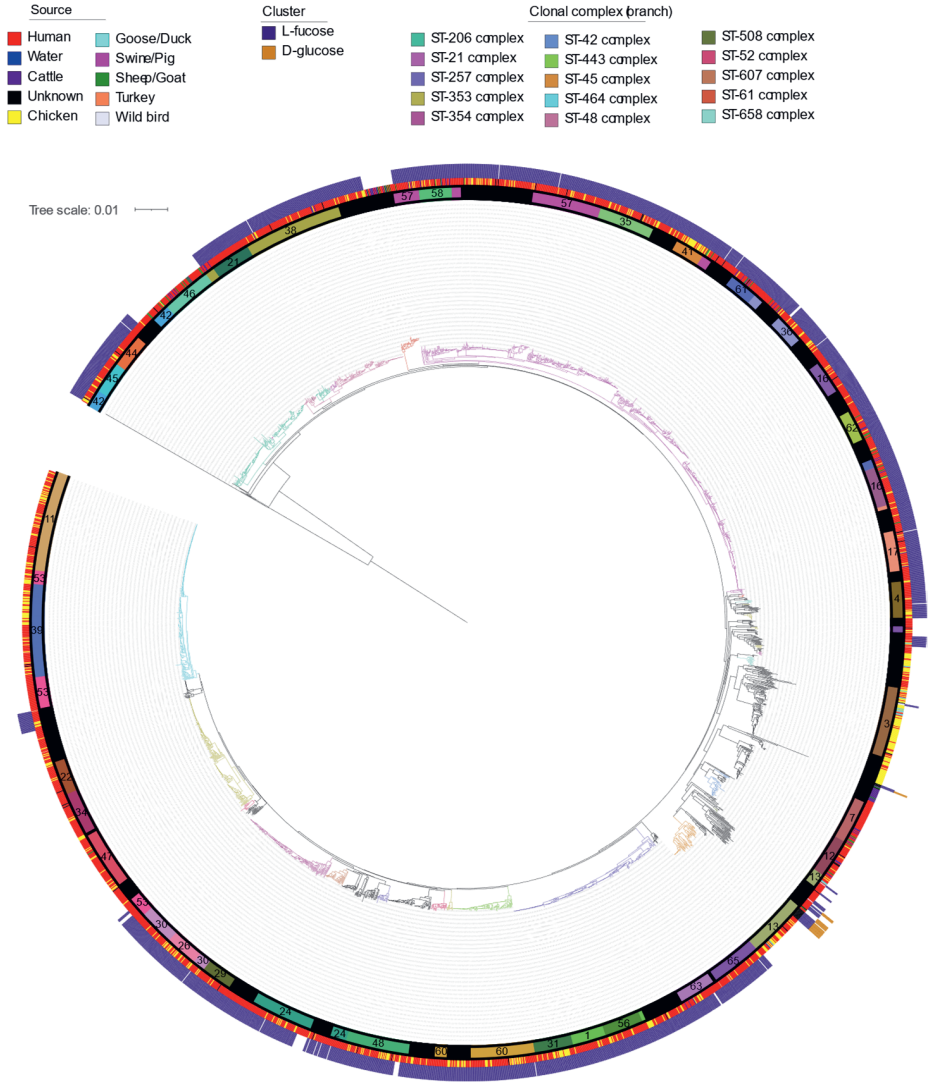
Suppl. Fig. 2 Flanking regions of the D-glucose utilization cluster of group 5. 50 Kb flanking regions were selected. Isolate numbers indicate the PubMLST isolate ID, as described in the Method section.



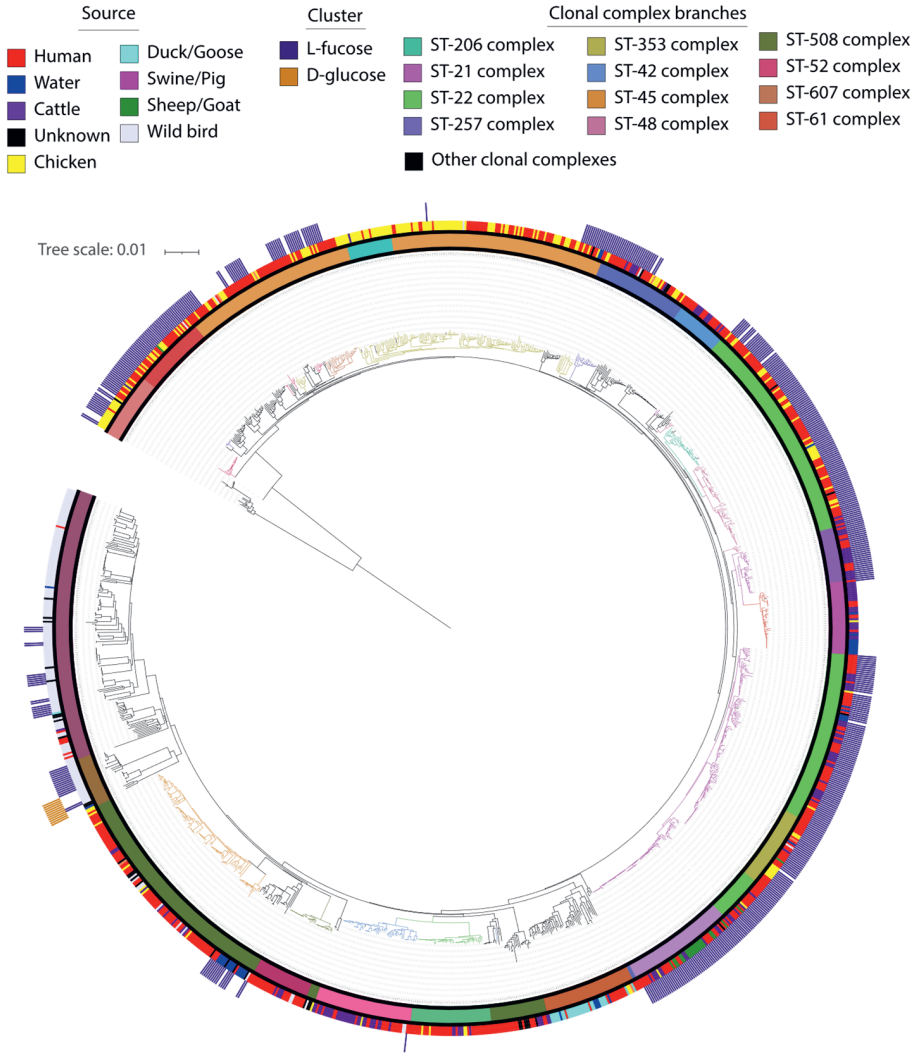
Suppl. Fig. 3 *C. jejuni* NCTC11168 genome map with genes. The three different 16S-23S rRNA regions are highlighted in purple. Figure adapted from (Lefebure et al., 2010).



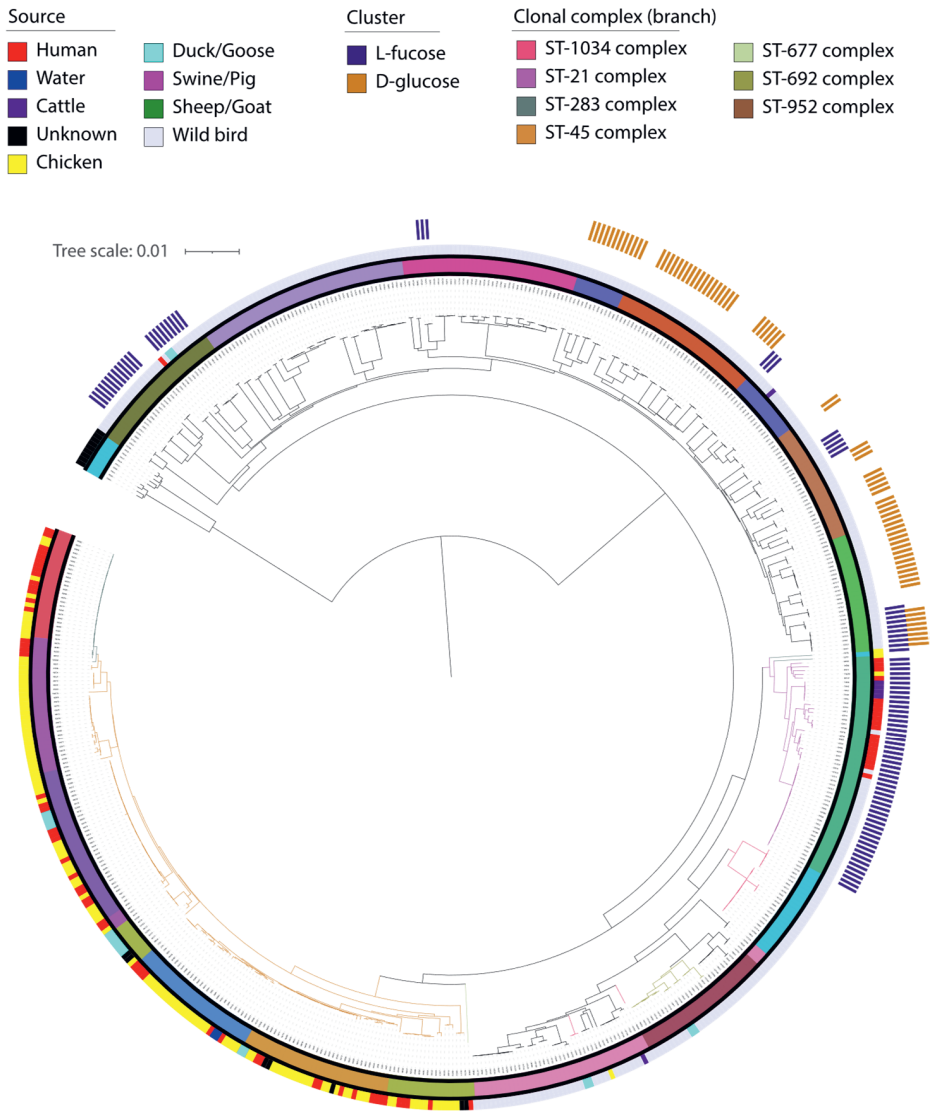
Suppl. Fig. 4 Percentage of fuc+ isolates per year (2001-2018) in *C. jejuni* (A) and *C. coli* (B) isolates collected in the UK (PubMLST database). Fraction of fuc+ isolates is expressed in percentage, number of fuc+ isolates (n) is indicated above each value.



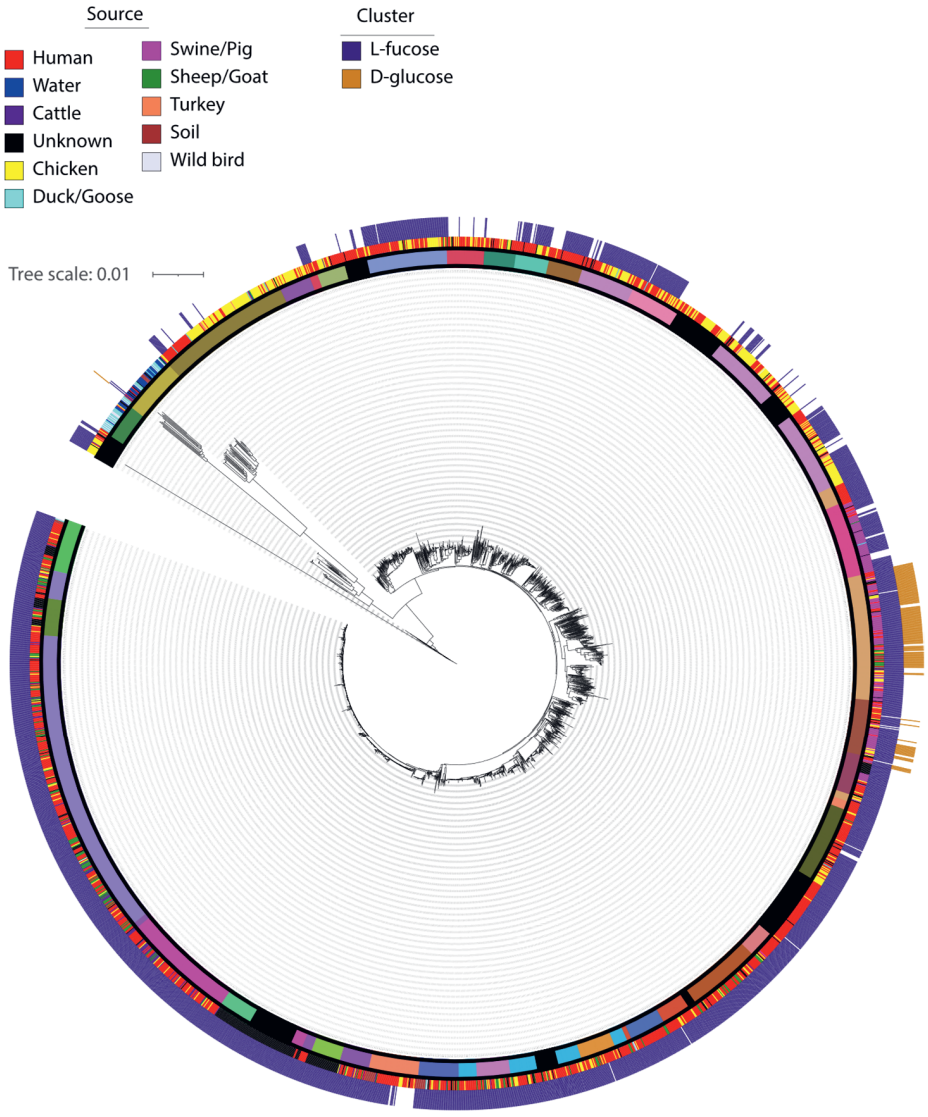
Suppl. Fig. 5 Phylogenetic tree using 3,150 full genomes of *C. jejuni* isolates from the UK. From outside to inside: the outer (orange) ring indicate gluc+ isolates, the purple ring indicate fuc+ isolates, The multicolored ring indicate the isolation source per isolate, and the inner ring (multicolored with numbers) indicates the different Treestruct groups. Branch coloring indicates the different clonal complexes (MLST groups).



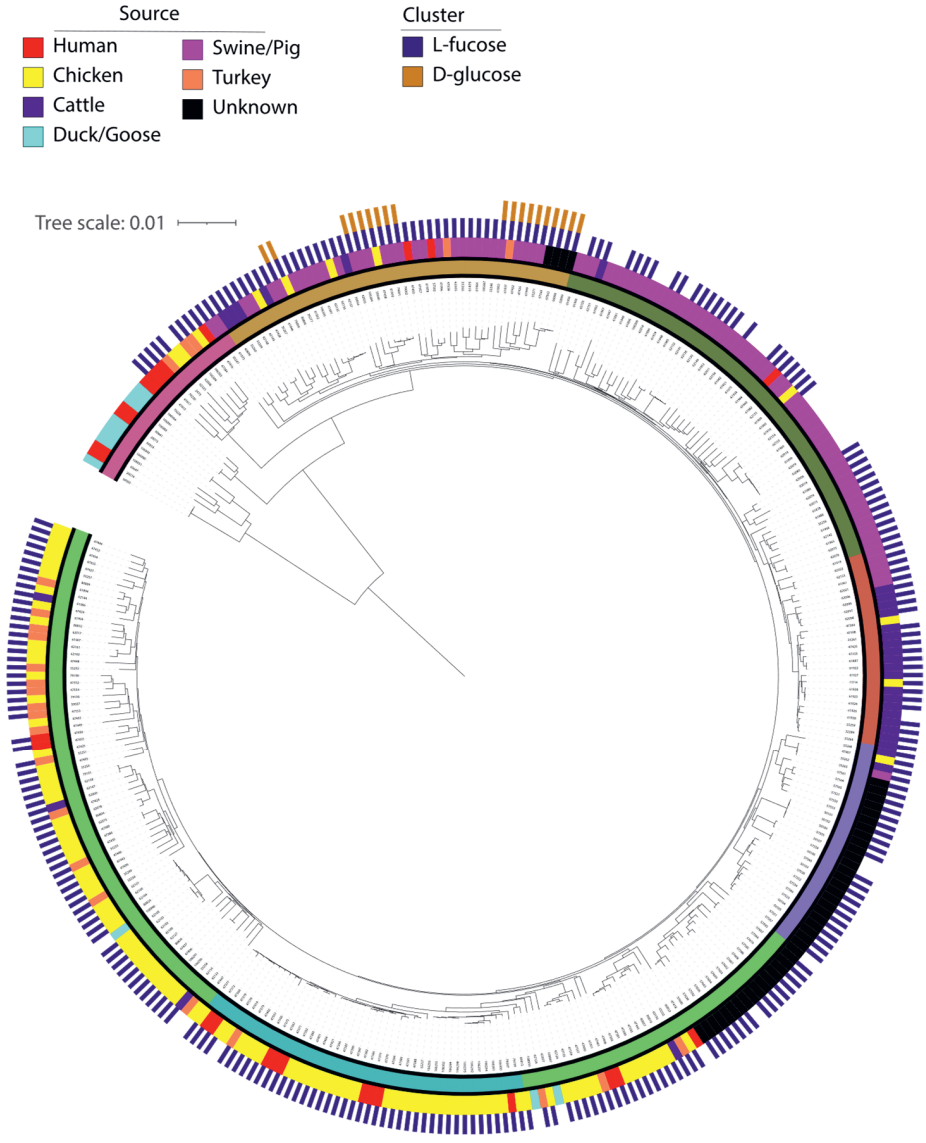
Suppl. Fig. 6 Phylogenetic tree using 1,251 full genomes of *C. jejuni* isolates from the USA. From outside to inside: the outer (orange) ring indicate gluc+ isolates, the purple ring indicate fuc+ isolates, The multicolored ring indicate the isolation source per isolate, and the inner ring (multicolored with numbers) indicates the different Treestruct groups. Branch coloring indicates the different clonal complexes (MLST groups).



Suppl. Fig. 7 Phylogenetic tree using 634 full genomes of *C. jejuni* isolates from Finland. From outside to inside: the outer (orange) ring indicate gluc+ isolates, the purple ring indicate fuc+ isolates, The multicolored ring indicate the isolation source per isolate, and the inner ring (multicolored with numbers) indicates the different Treestruct groups. Branch coloring indicates the different clonal complexes (MLST groups).



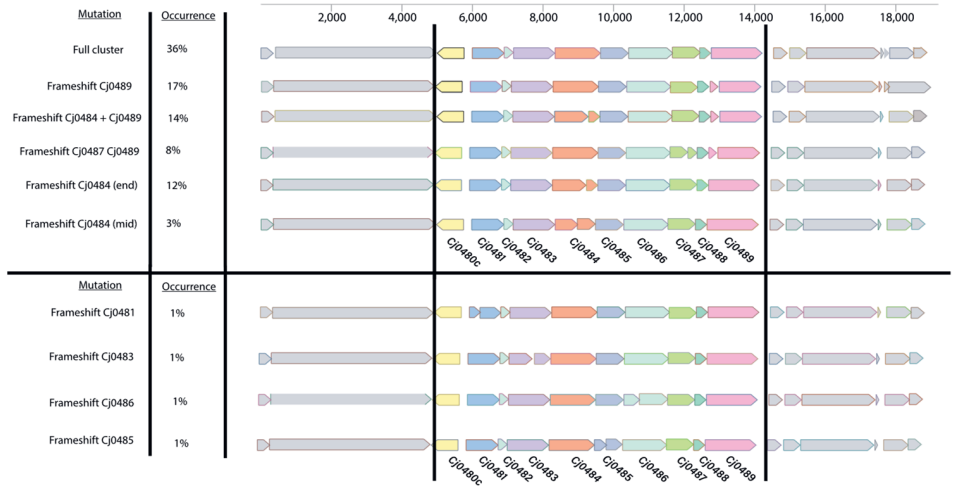
Suppl. Fig. 8 Phylogenetic tree using 2,786 full genomes of *C. coli* isolates from the UK. From outside to inside: the outer (orange) ring indicate gluc+ isolates, the purple ring indicate fuc+ isolates, The multicolored ring indicate the isolation source per isolate, the inner ring (multicolored with numbers) indicates the different Treestruct groups.



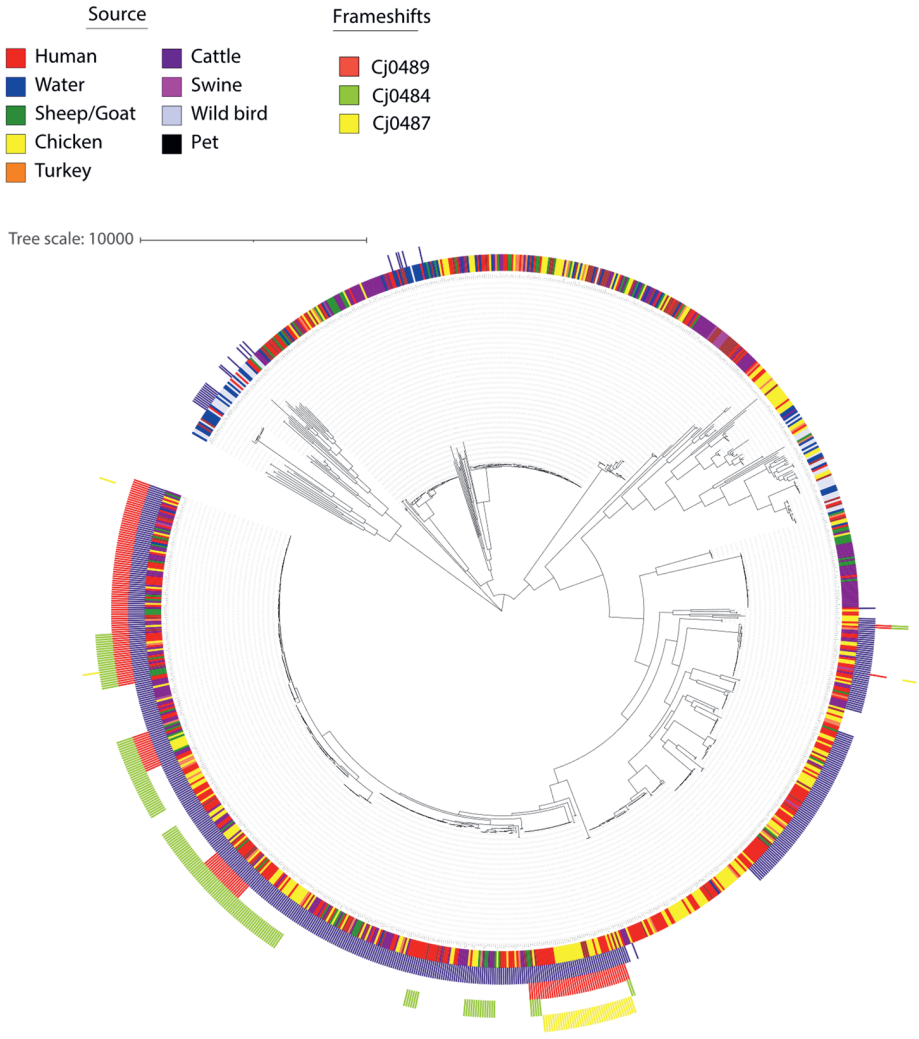
Suppl. Fig. 9 Phylogenetic tree using 335 full genomes of *C. coli* isolates from the USA. From outside to inside: the outer (orange) ring indicate gluc+ isolates, the purple ring indicate fuc+ isolates, The multicolored ring indicate the isolation source per isolate, the inner ring (multicolored with numbers) indicates the different Treestruct groups.

Suppl. Table 1 Correlation percentage fuc+ and percentage human disease isolates. P ≤0.05 is considered significant. Ns = not significant. Combined indicates the combined dataset of all tested countries.

	Treestruct Spearman	Treestruct Pearson	MLST Spearman	MLST Pearson
Combined data *C. jejuni*	0.0017	P<0.0001	0.034	ns
Combined data *C. coli*	ns	ns	ns	ns
NL *C. jejuni*	0.0072	0.0177	0.0400	0.0384
NL *C. coli*	ns	ns	ns	ns
UK *C. jejuni*	ns	ns	ns	ns
UK *C. coli*	ns	ns	ns	ns
USA *C. jejuni*	ns	ns	ns	ns
USA *C. coli*	ns	ns	ns	ns
Finland *C. jejuni*	ns	ns	ns	ns



Suppl. Fig. 10 L-fucose utilization cluster frameshift analyses. Analyses is based on 535 fuc+ *C. jejuni* isolates from the Dutch dataset.



Suppl. Fig. 11 Phylogenetic tree based on 1057 *C. jejuni* isolates from the Netherlands, based on the whole genome sequence. The outer (yellow) ring indicates isolates carrying the Cj0487 frameshift, the green ring indicates isolates carrying the Cj0484 frameshift and the red ring indicates isolates carrying the Cj0489 frameshift. Furthermore, the purple ring indicates fuc+ isolates. The inner ring (multicolored) indicates the isolation source per isolate.

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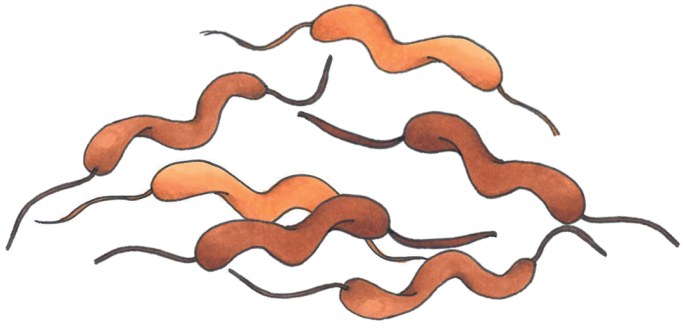

6

Discussion

Campylobacter has been the leading cause of foodborne bacterial gastroenteritis in the world for many years and despite decades of research, numbers of cases have decreased over the years.

The intestinal tract of warm blooded animals is considered a natural habitat for *Campylobacter spp.,* due to the presence of many nutrients and an optimal growth temperature. Therefore, the highest risk of campylobacteriosis is linked to contact with live animals and the consumption and handling of contaminated food of animal origin (Kaakoush et al., 2015; Skarp et al., 2016; Mohammadpour et al., 2018). Transmission from livestock farms to surface water, wild birds and retail products are all linked to human campylobacteriosis cases (Figure 1). The ability of *Campylobacter* to survive in optimal and non-optimal conditions varies amongst different isolates that originate from different animal reservoirs and environmental niches constituting different populations with large genetic diversity (Teh et al., 2014). Factors such as metabolism, lipooligosaccharide (LOS) production and colonization play key roles in the persistence of *Campylobacter* in the environment and in the intestine animals and humans.

In the human gut and animal intestine large quantities of compounds such as L-fucose and D-glucose are available, however, very limited information is available about how these compounds affect the transmission to the human host and pathogenicity of *Campylobacter*. Therefore, the objective of this thesis was to elucidate the effect of L-fucose and D-glucose metabolism on *Campylobacter's* environmental persistence, transmission, population structure, epidemiology, and virulence, and to investigate the spread and host associations of *C. jejuni* and *C. coli* isolates carrying the L-fucose and D-glucose utilization clusters.

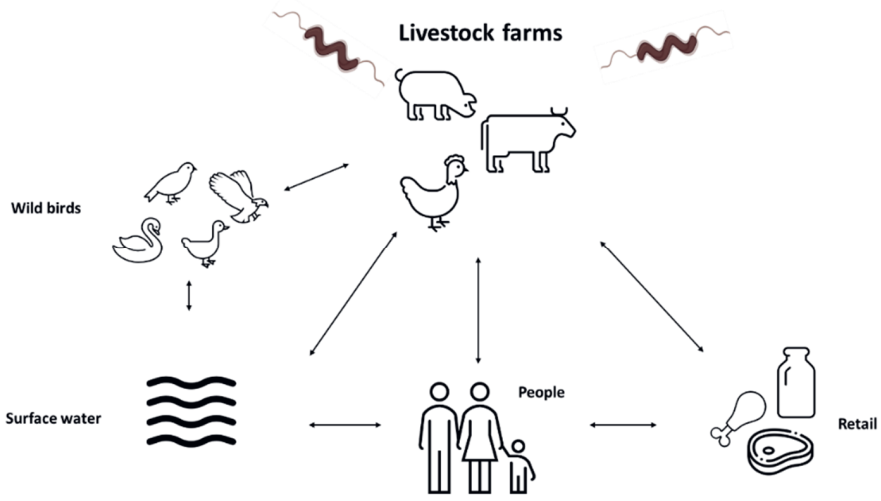

Figure 1 Most important reservoirs and transmission routes of *Campylobacter*. Transmission of *Campylobacter* often occurs from livestock farms and wild birds and most campylobacteriosis cases are linked to farm animals, specifically chickens (Mughini-Gras et al., 2021).

Following the general introduction in **Chapter 1**, the observations and results of **Chapter 2 to 5** provided a better understanding of the capability of *Campylobacter* to proliferate and adapt to different environments. Figure 2 displays a schematic overview of the topics that will discussed with a special focus on fuc+ *C. jejuni* isolates. In the presence of L-fucose (blue squares), the membrance-associated systems MacABC and CmeABC were significantly upregulated in *C. jejuni* NCTC11168 and 286, respectively, possibly affecting the virulent phenotype of *C. jejuni*. These complexes are glycosylated by PglC, which was also significantly upregulated in the presence of L-fucose. Furthermore, the presence of L-fucose significantly affected proteins involved in the LOS production of *C. jejuni* (1), possibly significantly impacting the survival, colonization, proliferation and virulence of *Campylobacter*, metal acquisition (2) and metabolic proteins (3), pointing to a shift in metabolism upon exposure to L-fucose, and the putative membrane and periplasm space proteins (4), the flagella/chemotaxis proteins (5) and toxin production proteins (6), which point towards a more virulent phenotype of *C. jejuni.* Observations regarding the D-glucose utilization will also be discussed with the focus on proliferation, colonization, LOS production and the capsular polysaccharides of *C. jejuni*.

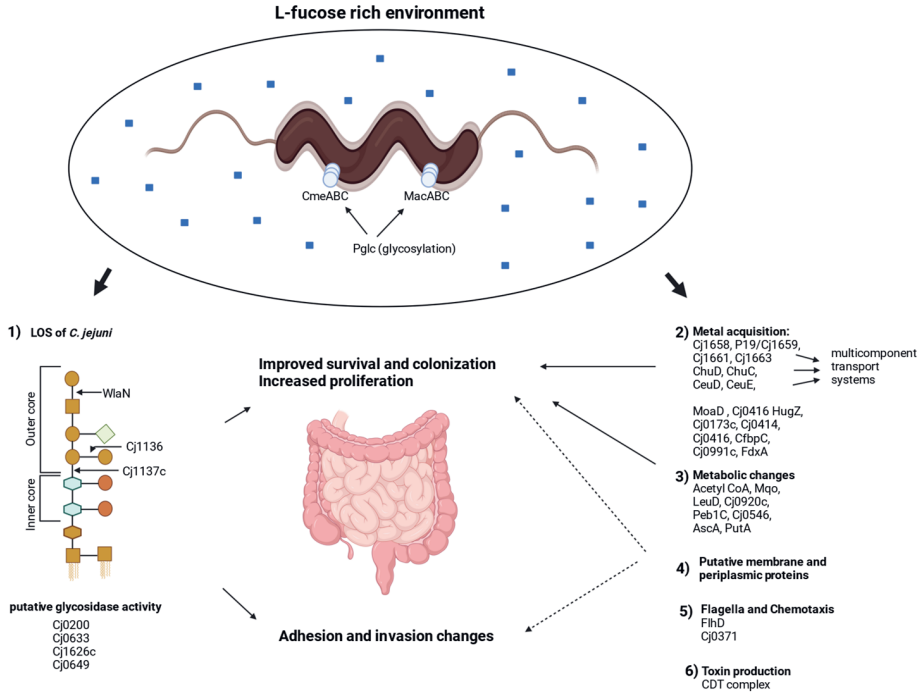

Figure 2 Schematic overview of the different cellular processes that L-fucose (blue squares) affects in different *C. jejuni* isolates. In the presence of L-fucose, the protein complexes MacABC and CmeABC are affected, which are glycosylated by PglC. Several other processes are affected and will be discussed in this chapter; 1) LOS production, 2) Metal acquisition, 3) Metabolic changes, 4) Putative membrane and periplasmic proteins, 5) Flagella and chemotaxis and 6) Toxin production. Arrows indicate the roles in *C. jejuni* and the dotted arrows indicate the putative role in the human intestine. Figure was created using BioRender software.

Campylobacter jejuni and *Campylobacter coli*

Two *Campylobacter* species were studied in this thesis, namely, *C. jejuni* and *C. coli*, both accounting for 88.1% and 10.6% of all reported *Campylobacter* human disease cases in Europe, respectively (EFSA, 2021). As *Campylobacter* is microaerophilic, it grows best in an atmosphere of 5% O_2, 10% CO_2 and 85% N_2, (Hazeleger et al., 1995), such as in parts of the animal intestines and the human gut. Most diseases in humans are linked to handling or consumption of contaminated poultry or porcine products for *C. jejuni* and *C. coli*, respectively (Hepworth et al., 2011). *Campylobacter* can survive

outside of the intestinal tract of both animals and humans, exhibiting a degree of environmental robustness that enables it to endure environmental transmission (Turonova et al., 2015; Rodrigues et al., 2016). Studies have shown that *Campylobacter* carries genes that encode proteins and enzymes that protect the cells against starvation, oxidative, heat shock, and pH stresses (Andersen et al., 2005; Brøndsted et al., 2005; Candon et al., 2007; Bronowski et al., 2014). Eventually, *Campylobacter* colonizes differently in animal and human hosts, which is largely dependent on the nutrient availability in the intestine (Stahl et al., 2012; Gao et al., 2017). Multiple studies focused on the metabolic capacity of *Campylobacter* at growth-supporting temperatures, showing that citric acid cycle intermediates, amino acids and peptides are crucial for Campylobacter's growth and survival (Wright et al., 2009; Stahl et al., 2012; Hofreuter, 2014; Wagley et al., 2014; Lanzl et al., 2022). Furthermore, at growth-supporting temperatures, compound preferences were identified for *Campylobacter*, highlighting a preference for serine, aspartate, asparagine and glutamate in *C. jejuni* (Wright et al., 2009; Hofreuter, 2014). Of these compounds serine, aspartate, glutamate, and proline make up some of the most common amino acids found in chick excreta, perhaps explaining why these compounds play such a central role in *C. jejuni* metabolism (Parsons, 1984; Stahl et al., 2012).

Pathogenicity of *Campylobacter* strongly depends on the variety of virulence genes that *Campylobacter* species possess (Ketley, 1995; Bravo et al., 2021). These virulence factors do not only play an important role in *Campylobacter*'s virulence, but are also involved in many other mechanisms that ultimately lead to virulence, including survival and resistance to physiological stresses such as gastric acid, virulence-related mechanisms include adhesion, invasion, translocation, colonization, motility, chemotaxis and toxin production (Biswas et al., 2011; Backert et al., 2013).

Phenotypic effect of L-fucose on the proliferation, survival and virulence of *C. jejuni* and *C. coli*

The dogma of *Campylobacter* being asaccharolytic was disproven in 2011, when two studies highlighted the capability of the majority of *C. jejuni* and *C. coli* isolates to metabolize L-fucose (Muraoka & Zhang, 2011; Stahl et al., 2011). L-fucose is a common carbohydrate in the gastrointestinal tract and is incorporated in mucins, human milk oligosaccharides, and in capsules and glycoproteins of other microbial species

(Kornfeld & Kornfeld, 1985; Coyne et al., 2000; Newburg & Grave, 2014). Interestingly, *Campylobacter* does not possess any fucosidases and it relies on other gut bacteria, such as *Bacteroides vulgatus*, to free L-fucose from mucins in the gut (Garber et al., 2020), as displayed in Figure 3. Many of these gut bacteria, such as *Faecalibacterium prausnitzii* and *Akkermansia muciniphila* are anaerobic, while *Campylobacter* is microaerobic, suggesting that these bacteria are in different locations of the gut. However, although these bacteria are labeled anaerobic, previous studies showed that *Faecalibacterium prausnitzii* can use a so-called extracellular electron shuttle in to grow at oxic-anoxic interphases (Khan et al., 2012). Furthermore, the anaerobic bacterium *Akkermansia muciniphila* highly expressed several respiration genes upon exposure of oxygen, suggesting some aerotolerance in this bacterium (Ouwerkerk et al., 2016). Epithelial cells are excreting small amounts of oxygen and this creates an oxygen gradient along the mucus layer (Van den Abbeele et al., 2011), allowing the microaerophilic *Campylobacter* to locate itself near the mucus layer in more anaerobic parts of the gut. The genome of *C. jejuni* NCTC11168 carries a wide range of alternative electron acceptors next to oxygen, including fumarate, nitrate, nitrite, and *N*- or *S*-oxides, suggesting that alternative respiratory pathways can contribute significantly to energy conservation under oxygen-limited conditions (Sellars et al., 2002).

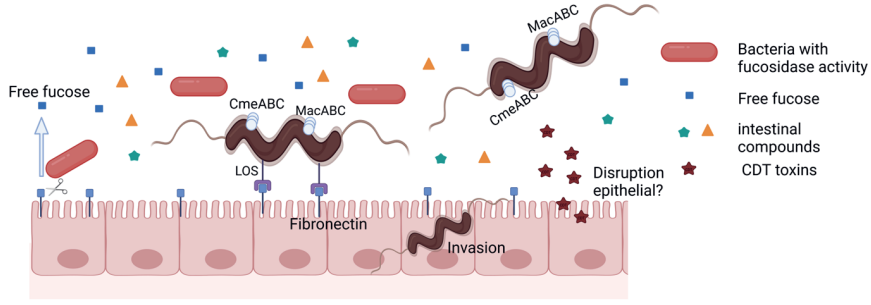

Figure 3 Schematic overview of pathogenesis of *C. jejuni* in the human intestine. Fucosidase-positive bacteria free L-fucose from the epithelial cells. L-fucose can then be used by *C. jejuni* to improve proliferation, binding to fibronectin and invasion of epithelial cells. Furthermore, upregulation of toxin-related proteins suggest a potential epithelial disruption. The exact role of the MacABC and CmeABC complexes requires further investigation. Figure was created using BioRender software.

Previous research showed that L-fucose affected the growth, biofilm formation and virulence in *C. jejuni* isolates that carried a L-fucose utilization cluster (*Cj0480c* –

Cj0489), so called fuc+ isolates (Muraoka & Zhang, 2011; Stahl et al., 2012; Dwivedi et al., 2016; Garber et al., 2020; Luijkx et al., 2020). We used this as stepping stone for our research in **Chapter 2, 3 and 4** where we investigated the effect of L-fucose on the growth, survival and metabolism of fuc+ *C. jejuni* and *C. coli* isolates. In chapter 2 and 3, cells were grown microaerobically in MEMα medium at 37 °C, similarly to what was done in previous research (Stahl et al., 2011). Here, we observed that all tested isolates showed enhanced survival and prolonged spiral-shaped morphology when cultured in the presence of L-fucose, with the *C. coli* isolate having the most robust phenotype. Our results highlighted that in laboratory conditions *Campylobacter* is able to remain its culturable spiral-shaped morphology, instead of entering the viable but nonculturable (VBNC) state in which low metabolic activity is observed (Rollins & Colwell, 1986; Barer & Harwood, 1999). In the natural habitats of *Campylobacter*, such as the human gut, there is an overflow of compounds, including L-fucose that is freed from fucosylated mucin structures by other gut habitants (Figure 3). Therefore, with the excess of L-fucose in the human gut, it is likely *C. jejuni* cells remain viable spiral-shaped cells for a prolonged duration in this environment. In **Chapter 4** we investigated the effects of L-fucose in *C. jejuni* strain 18-440, a water isolate, that also carried the glucose utilization cluster. Here we used DMEM medium, a minimal medium that lacks proline, aspartic acid, glutamic acid and glucose, compared to MEMα medium. This enabled us to investigate the effects of added L-fucose and D-glucose, which both improved *Campylobacters* proliferation and survival in DMEM.

Next to improved proliferation and survival of fuc+ *Campylobacter* isolates in the presence of L-fucose, also virulence factors were affected. In piglet models, fuc+ isolates showed a competitive advantage during colonization compared to fuc- isolates, while this was not paralleled during the colonization of chickens (Stahl et al., 2011). The authors of that study observed that the fucosylated O-glycan mucin structures of chicken were more sulfated than the pig counterparts and hypothesized that this could be the reason for the difference in colonization between pigs and chicken (Stahl et al., 2011). However, in the addition of free L-fucose to the chicken gut, a competitive advantage during colonization was observed when fuc+ isolates were compared to fuc- isolates (Stahl et al., 2011). We hypothesize that due to the complexity of the chicken gut microbiota, it seem unlikely that L-fucose is not freed from the fucosylated O-glycan mucin of chickens, suggesting that a similar

competitive advantage of fuc+ isolates compared to fuc- isolates is observed during the colonization of chickens as observed with pigs. Another study highlighted that during human virulence assays, a hypervirulent *C. jejuni* isolate was more invasive towards Caco-2 cells in the presence of L-fucose or in the presence of *B. fragilis*, a bacterium that possesses fucosidases to free L-fucose from the Caco-2 cells (Luijkx et al., 2020). In **Chapter 3** we further investigated the virulence of *Campylobacter* by testing Caco-2 invasion and binding to fibronectin of two closely related human *C. jejuni* isolates. Interestingly, only one of the isolates showed more invasion of Caco-2 cells and more binding to fibronectin in the presence of L-fucose. Although these findings highlight that the effect of L-fucose on the invasion of Caco-2 cells and the binding of fibronectin is a strain-specific phenotype in the tested conditions, our findings in **Chapter 5** highlighted a positive correlation between fuc+ *C. jejuni* isolates and human isolates. These findings suggest a selective advantage for fuc+ *C. jejuni* isolates in human infection and/or during survival and proliferation within the human host.

Phenotypic effect of D-arabinose metabolism on the proliferation of *C. jejuni*

A recent study provided evidence that fuc+ *C. jejuni* isolates were not only able to metabolize L-fucose via the L-fucose utilization cluster, but also D-arabinose, a sugar that is rare in nature and was identified in rose buds (Tang et al., 2018; Garber et al., 2020). Fuc+ isolates displayed an enhanced growth in the presence of D-arabinose and could swim to this attractant (Garber et al., 2020). Authors of the same paper suggested that the main observed difference between L-fucose and D-arabinose metabolism is that fucose metabolism results in the production of lactate and that glycolic acid is a signature product of D-arabinose metabolism (Figure 4) (Garber et al., 2020), which was confirmed in **Chapter 3**. Furthermore, we observed no large differences in growth, survival and virulence of fuc+ *C. jejuni* isolates in the presence of L-fucose or D-arabinose.

Although it is unlikely that D-arabinose is a true carbon source of *Campylobacter*, as it is a rare sugar that is not present in the natural habitats of *Campylobacter*, the finding of D-arabinose being metabolized via the L-fucose utilization cluster highlights the possibilities of alternative sugars and intermediates to be metabolized via different metabolism clusters. As the human gut is full of different bacterial

species, which use and excrete intermediates of metabolic pathways, it is likely that *Campylobacter* is able to utilize more carbon sources than currently known.

Phenotypic effect of D-glucose metabolism on the proliferation of *C. jejuni*

Glycolysis via the Embden-Meyerhof-Parnas Glycolytic Pathway (EMP pathway), in which glucose is converted into two molecules of pyruvate, is one of the most studied metabolism pathways in bacteria. However, due to the lack of phosphofructokinase (pfk), the reaction from fructose-6P to fructose- 1,6P is not possible in *Campylobacter* (Figure 4) (Vegge et al., 2016). Instead, gluc+ *Campylobacter* isolates carry the genes needed to metabolize glucose via the Etner-Doudoroff (ED) pathway, resulting in the ability to produce pyruvate, which in turn can be used for gluconeogenesis to produce compounds for the reductive pentose phosphate pathway or for polysaccharides (Velayudhan & Kelly, 2002; Vegge et al., 2016). The D-glucose utilization cluster (*glcP*, *pgi2*, *glk, pgl*, zwf, *edd* and *eda*) supports increased growth, survival and a promoted biofilm formation in *C. coli* in the presence of D-glucose (Vorwerk et al., 2015; Vegge et al., 2016). In **Chapter 4** a similar observation was made with a gluc+ *C. jejuni* isolate, in which longer survival and increased growth was observed in the presence of D-glucose. Furthermore, consumption of D-glucose was linked to an increased pyruvate production, which is the end product of the D-glucose utilization pathway and points to overflow metabolism, as pyruvate can be further metabolized via the general metabolism or used for the LOS synthesis of *Campylobacter* (Stahl et al., 2012; Hofreuter, 2014; Burnham & Hendrixson, 2018). In **Chapter 5** we highlighted that this cluster is more common in *C. coli* compared to *C. jejuni* and the cluster was found in 10% and 0.2% of the *C. coli* and *C. jejuni* isolates, respectively. Furthermore, it was evident in the current and in previous studies that most of the gluc+ *C. coli* isolates have been isolated from swine (Vegge et al., 2016), while most gluc+ *C. jejuni* isolates were most commonly observed in wild birds and rats (Vegge et al., 2016).

L-fucose and D-glucose utilization cluster activation

Based on the proteomic observations in the **Chapters 3 and 4**, we made a schematic overview of the general metabolism in all tested isolates (Figure 4). The schematic overview represents proteins of the L-fucose and D-glucose metabolism pathways and highlights which proteins were significantly upregulated in all tested isolates (green

block), only in *C. jejuni* 286 (yellow block) and only in *C. jejuni* 18-440 (blue block), when cultured in the presence of L-fucose, D-arabinose or D-glucose. Each pathway produces pyruvate, which can be used for LOS synthesis, energy via the TCA cycle or can form Acetyl-CoA. Notably, in the presence of L-fucose, the proteins Pgi2, Glk, Zwf and Pgl were downregulated, possibly pointing towards increased changes in the LOS and capsule synthesis, however, the downregulation of these proteins which is discussed in **Chapter 4** is not further discussed in the schematic figure.

Although **Chapter 4** highlighted the downregulation of Pgi2, Glk, Zwf and Pgl upon activation of the L-fucose utilization cluster, which possibly links towards increased changes in the LOS and capsule synthesis, here we focus on the upregulated metabolic changes.

In the presence of L-fucose the proteins Cj0486, Cj0488, Cj0485, Cj0487, Cj0482, Cj0483, Cj0481 and Cj0489 were upregulated in all tested isolates. During knockout studies, several genes of the L-fucose utilization cluster were labeled as essential genes as no growth enhancement was determined in mutants of these genes, namely, Cj0486, Cj0485, Cj0487, Cj0483 and Cj0481 (Dwivedi et al., 2016; Garber et al., 2020). These findings highlight that the proteins Cj0488, Cj0482 and Cj0489, although upregulated in all our tested isolates, are not essential for enhanced growth on L-fucose. Interestingly, in these tested isolates, no other proteins with similar functions as Cj0488 and Cj0489 were found upregulated in the proteome of *C. jejuni*. For the protein Cj0482 however, one similarly annotated protein was significantly upregulated, namely, Cj0483, suggesting that Cj0483 is able to carry out the function of Cj0482. Furthermore, in **Chapter 5** a frameshift analysis was performed on the L-fucose utilization cluster, which highlighted that no frameshifts were observed in *Cj0488* and *Cj0482*, while frameshifts were observed in *Cj0489*. These findings suggest that, although not essential for growth on L-fucose metabolism, *Cj0488* and *Cj0482* are essential for different processes (such as survival and colonization of the human gut), as there is an evolutionary selection pressure in maintaining these genes. Interestingly, during the frameshift analysis in **Chapter 5**, frameshifts in *Cj0484* were found in a high number of fuc+ isolates. Furthermore, previous research has shown that *Cj0484* is not essential for L-fucose metabolism (Dwivedi et al., 2016). However, in **Chapter 3**, one isolate produced and upregulated the hypothetical transporter

Cj0484, namely, *C. jejuni* 286 (yellow block). In this isolate, Cj0484 was over 100-fold upregulated in the presence of L-fucose and D-arabinose, and was much higher upregulated than the L-fucose transporter Cj0486 (20-fold change). These findings highlight strain diversity between fuc+ *C. jejuni* isolates and that multiple isolates should be tested in order to define a gene as essential gene for this metabolism pathways. Mutant studies of both transporters in isolate *C. jejuni* 286 will provide evidence whether *Cj0484* is a true fucose transporter in some isolates and will further elucidate differences between isolates.

Of the D-glucose utilization cluster, only the glucose transporter GlcP was upregulated in the gluc+ isolate *C. jejuni* 18-440 in the presence of D-glucose. Since *C. jejuni* is able to use gluconeogenesis, and intermediates of the pathway being interchangeable, the other proteins of the ED pathway, namely, Zwf, Pgl, Edd, Eda and Pgi2, conceivably remain present and active in DMEM grown cells.

L-fucose and D-glucose influence the LOS, CPS and virulence of *C. jejuni*

LOS is an integral component of the *Campylobacter* cell membrane with a structure of core oligosaccharides forming inner and outer core regions and a lipid A moiety (Figure 2) (Moran, 1997). In addition to LOS, *Campylobacter* exhibits other cell-surface structures such as capsular polysaccharides (CPS), O-linked glycosylated flagellum, and N-linked glycoproteins. Among these structures, LOS, CPS, and O-linked glycans (primarily flagellar glycans) show variability across various strains, whereas N-linked glycoproteins remain unchanged (Szymanski et al., 2003; Karlyshev et al., 2005; Day et al., 2012). Interestingly, LOS mutants exhibited growth attenuation and a loss of ability to perform natural transformation and to invade cultured host cells (Marsden et al., 2009). The reduced adhesion and invasion phenotype might have occurred due to the reduced interaction between altered LOS structures and host epithelial cell receptors (Fry et al., 2000; Javed et al., 2012; Hameed et al., 2020). Furthermore, *C. jejuni* strains that displayed sialylated LOS exhibited greater capability for adhesion, invasion, and translocation compared to those with non-sialylated LOS (Louwen et al., 2012). Not only are cell-surface LOS structures

important for the invasion of epithelial cells, they are also important in colonization of for example chickens (Iwata et al., 2013).

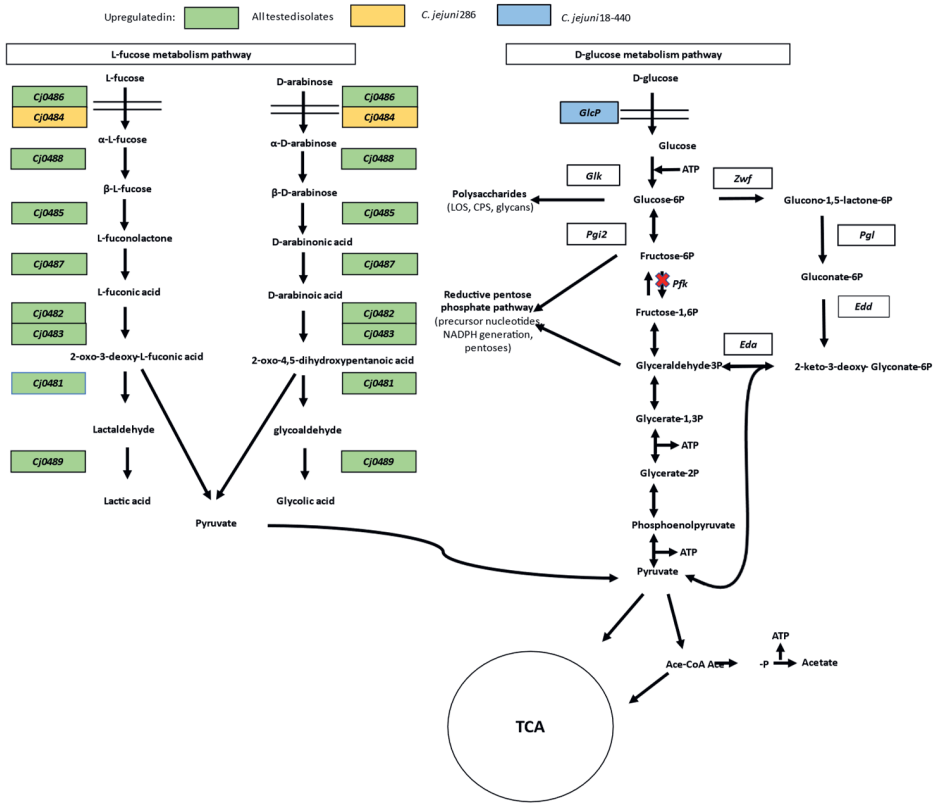

Figure 4 Figure 4 Schematic overview of L-fucose, D-arabinose and D-glucose metabolism. Metabolism of these substrates results in the production of pyruvate, which can enter the TCA cycle, be converted to acetate, or enter the gluconeogenesis pathway and via glucose-6P affects the production of polysaccharides. Green blocks indicate significantly upregulated proteins in all tested isolates in the presence of L-fucose (Chapters 3 and 4), yellow blocks indicate significantly upregulated proteins in *C. jejuni* 286 in the presence of L-fucose and D-arabinose (Chapter 3) and the blue block indicates the only significantly upregulated protein GlcP in *C. jejuni* 18-440 in the presence of D-glucose, while other cluster proteins in white blocks are not differentially expressed (Chapter 4). Furthermore, the red cross in the reaction from fructose-6P to fructose-1,6P indicates that *Campylobacter* does not possess phosphofructokinase (Pfk) and is therefore not able to perform that reaction. Notably, *Campylobacter* carries the protein fructose- 1,5-bisphosphatase (Fbp), allowing the reaction from fructose-1,6P to fructose-6P. Figure is adapted from Vegge et al. (2016).

The most commonly found sialic acid biosynthesis genes are *cgtB* and *wlaN*, which were often found in highly invasive *C. jejuni* isolates (Müller et al., 2007). In **Chapter 3** we observed that *C. jejuni* NCTC11168 displayed higher invasion counts than *C. jejuni* 286. It is unknown why *C. jejuni* NCTC11168 displayed a higher invasive phenotype compared to *C. jejuni* 286, as *C. jejuni* 286 was recently isolated from an hospitalized patient. Whether the WlaN protein, which was only produced in *C. jejuni* NCTC11168, was involved in these higher invasion counts and whether the presence of L-fucose indirectly affected this by affecting other parts of the LOS synthesis of *C. jejuni,* requires further studies. Also the LOS biosynthesis can have affected the higher invasion counts that were observed for isolate *C. jejuni* NCTC11168. LOS biosynthesis is generally produced by a specific gene cluster (Cj1132c – Cj1153) that is involved in either monosaccharide biosynthesis or addition of a particular monosaccharide to the LOS structure (Karlyshev et al., 2005; Parker et al., 2005; Parker et al., 2008; Iwata et al., 2013; Hameed et al., 2020). LOS is divided into two cores, namely, the inner core and the outer core, in which the outer core is very diverse among *C. jejuni* isolates, and is involved in the invasion of epithelial cells (Kanipes et al., 2008; Javed et al., 2012). The outer core is synthesized by the glycosyltransferases *Cj1136, Cj1137* and *Cj1138*, the N-acetyl galactosaminyl transferase *neuA1*, the sialyltransferase *cst-III* and the galactosyltransferase *wlaN* (Karlyshev et al., 2005; Javed et al., 2012; Hameed et al., 2020). Of these genes, we were able to detect the following proteins in both tested isolates of **Chapter 3** (*C. jejuni* NCTC11168 and *C. jejuni* 286): Cj1136, Cj1138, NeuA1 and Cst-III, while the proteins WlaN and Cj1137c were only detected in *C. jejuni* NCTC11168. Thus, not only the proteins Wlan was linked to the higher invasion counts that were observed for isolate *C. jejuni* NCTC11168, but also the protein Cj1137c.

Several proteins with glycosidase activity were found significantly differentially expressed when cells were cultured in the presence of L-fucose, although most were putative proteins (highlighted in the schematic overview in Figure 2). The most notable protein was the glycosyltransferase Cj1136, highlighting changes in the outer core of the LOS when fuc+ *C. jejuni* was grown in the presence of L-fucose. The putative protein functions require further research to investigate how these proteins affect LOS synthesis in *Campylobacter*. Other proteins with putative function in the LOS synthesis were Cj0200, Cj1626, Cj0649 and Cj0633, and will be described here briefly. The protein Cj0200 possesses characteristics of a glycosyltransferase and is

part of the DccSR regulon and was significantly downregulated in the presence of L-fucose and D-arabinose in isolate *C. jejuni* NCTC11168 and *C. jejuni* 286 (Wösten et al., 2010). Next to the protein Cj0200, also the protein Cj1626 was linked to the DccR-regulon (Wösten et al., 2010). Furthermore, the protein Cj0649, encodes a beta-barrel LptD-like protein and functions in the assembly of LPS in *Pseudomonas aeruginosa* (Hoang et al., 2012), suggesting similar functions in *C. jejuni*. Lastly, the putative protein Cj0633 is involved in the development of the outer membrane via its N-linked glycoprotein biosynthesis.

The role of N-glycosylation in *C. jejuni*

Furthermore, apart from the genes in the Cj1132c – Cj1153 gene cluster, several other detected proteins were linked to changes in the membrane and periplasmic space. In **Chapter 3,** the galactosyltransferase PglC (Cj1124c) was only detected in isolate *C. jejuni* NCTC11168, while being absent in *C. jejuni* 286. Notably, this protein was significantly upregulated in the presence of L-fucose. This protein is part of the N-glycosylation system (*pgl*), which produces the heptasaccharide GalNAc-α1,4-GalNAc-α1,4-(Glc-β1,3-)GalNAc-α1,4-GalNAc-α1,4-GalNAc-α1,3-diNAcBac-β1,N-Asn (diNAcBac is 2,4-diacetamido-2,4,6-trideoxyglucopyranose) (Glover et al., 2005; Glover et al., 2006). In *C. jejuni* this heptasaccharide is conserved and found on more than 80 periplasmic and membrane-bound proteins (Scott et al., 2011; Cain et al., 2019). Mutagenesis conducted on the *pgl* genes revealed that the glycosylation system has a significant impact on several cellular functions, such as the ability to colonize chickens and mice, the ability to adhere to and invade epithelial cells, the proper functioning of the multidrug efflux complex CmeABC, the stability of the type IV secretion system and interactions with the immune system (Nothaft & Szymanski, 2013; Dubb et al., 2020; Duma et al., 2020). Interestingly, the multridrug efflux system CmeABC has high similarities to the MacABC efflux system (Guérin et al., 2020), of which MacC (Cj0608) was significantly upregulated in the presence L-fucose and D-arabinose in *C. jejuni* NCTC11168 (Chapter 3). MacC is part of the MacABC macrolide-specific pump and is linked to the DccSR regulon, which is involved in adaption to new environments, like as early colonization of chicken intestine (Flint et al., 2010; Wösten et al., 2010). The MacABC system is understudied in *Campylobacter*, however, it is a system that is well conserved and is linked to many different processes in different bacteria, including

infections, flagella stability and biofilm formation (Nishino et al., 2006; Bogomolnaya et al., 2013; Shirshikova et al., 2021; Gibson et al., 2022; Robin et al., 2022). With both the PglC and MacC protein being significantly upregulated in *C. jejuni* NCTC11168, it is likely that PglC acts on MacC, and that the *pgl* system enables glycosylation of the MacABC system efflux system, which is needed for optimal functioning (Dubb et al., 2020).

Effect of glucose on the LOS of the glucose-negative isolate *C. jejuni* NCTC11168

In **Chapter 4,** we performed proteomics analyses with *C. jejuni* isolates grown in DMEM-G compared to normal DMEM. Next to the water isolate *C. jejuni* 18-440, we also tested the human isolate *C. jejuni* NCTC11168 (data not shown), which is lacking the D-glucose utilization cluster. Surprisingly, the glycosyltransferase Cj1137c was 125 fold-change upregulated in the presence of D-glucose in strain NCTC11168 (Suppl. Table 1), highlighting significant changes in the outer core of the LOS. It would be interesting to further analyze the LOS production in the presence of D-glucose, even with isolates that are not carrying the D-glucose utilization cluster, and assess whether the upregulation of this LOS protein affects the colonization and virulence of *C. jejuni* NCTC11168.

Iron/metal acquisition of *C. jejuni*

Although only phenotypic virulence data was obtained for the human isolates *C. jejuni* NCTC11168 and *C. jejuni* 286, our proteomic results of the water isolate *C. jejuni* 18-440 provided new insights in the virulence of *C. jejuni*. A study from 1985 highlighted that water isolates were less invasive and less cytoxic to HeLa cells than clinical isolates (Newell et al., 1985). In water isolates, nutrient insufficiency was a powerful stress factor which significantly affected the virulence of *Campylobacter*, however, cells were able to recover their virulence within 24-48 hours (Mihaljevic et al., 2007). We screened 193 virulence proteins in the proteomics dataset of the three isolates that were tested in **Chapters 3 and 4**. Of the 193 tested virulence proteins, 169 were detected in the human *C. jejuni* NCTC11168 isolate, 166 were detected in the human *C. jejuni* 286 isolate and 140 were detected in the water *C. jejuni* 18-440 isolate (Suppl. Table. 2). In the water isolate, the cytolethal distending toxin (CDT) complex, which is a major player in *Campylobacter's* virulence as it causes DNA double-strand breaks

that result in cell cycle arrests (He et al., 2021), was completely absent, suggesting less cytotoxicity in this isolate. These findings are in line with the observation of previous studies in which water isolates were less invasive and cytotoxic (Newell et al., 1985). Interestingly, the water isolate produced 10 virulence proteins, including five different flagellar proteins, which the human isolate *C. jejuni* NCTC11168 did not produce, suggesting changes in the motility of this isolate. **Chapter 4** highlighted that this water isolate significantly upregulated numerous of metal acquisition proteins. A paper by Liu et al. (2018) described many of our observed proteins. The authors highlighted the proteins CfbpA, CeuB, ChuC and the cluster CJJ81176_1649–1655 (Cj1658-Cj1664 in *C. jejuni* NCTC11168, which includes the protein P19). Based on knockout mutant studies of the cluster Cj1658-Cj1664, the authors observed an iron-sensitive phenotype, with reduced growth in acidic medium, increased sensitivity to streptomycin and higher resistance to H_2O_2 stress (Chan et al., 2010; Liu et al., 2018). Furthermore, in iron-restricted medium, the Cj1658-Cj1664 genes were required for optimal growth when using human fecal extracts as an iron source. Notably, in the presence of human fecal extracts, these genes were more highly expressed compared to chicken cecal extracts, highlighting the importance of these genes in iron scavenging and stress survival in the human intestine environment (Liu et al., 2018). In **Chapter 4**, in the presence of L-fucose, we observed significant upregulation of the proteins Cj1658, Cj1659/p19, Cj1661, Cj1663, ChuD, ChuC, CeuD, CeuE, Cj0173c, HugZ, Cj0414, Cj0415, MoaD and CfbpC. Notably, most of these proteins were part of multicomponent transport systems; Cj1658, Cj1659/p19, Cj1661 and Cj1663 are part of the rhodotorulic acid uptake system (Cj1658-Cj1663), ChuD and ChuC are part of a heme uptake system (ChuA,B,C,D,Z), CeuD and CeuE are part of the Enterochelin uptake system (CeuBCDE), and Cj0173c is part of the ferritransferrins uptake system (Cj0173c-Cj0178) (Miller et al., 2009). Furthermore, the proteins HugZ, Cj0414, Cj0415, MoaD and CfbpC are annotated as heme oxygenase, oxidoreductase, oxidoreductase, putative molybdopterin converting factor and putative iron-uptake ABC transport system, respectively. These findings suggest that the presence of L-fucose give fuc+ *C. jejuni* isolates an adaptation advantage towards the human gut environment by upregulation a high quantity of metal acquisition/transport proteins needed for the survival and colonization of the gut.

Metabolic changes in *C. jejuni*

Apart from the L-fucose utilization cluster proteins, many different metabolic proteins were affected in the presence of L-fucose, including the proteins Acetyl-CoA and Mq0, which possibly link to fatty acid and cell envelope biosynthesis. Furthermore, the significantly differentially expressed proteins LeuD (L-leucine metabolism), Cj0920c (involved in aspartate and glutamate metabolism), Peb1C (involved in aspartate and glutamate metabolism), Cj0546 (involved in vitamin K2 synthesis), AscA (involved in acetate metabolism) and PutA (involved in proline metabolism), highlight a metabolic rewiring of *C. jejuni* upon exposure to L-fucose. In addition, the three downregulated D-glucose metabolism proteins Glk, Pgi2 and Pgl, further suggest a metabolic rewiring that affects the LOS production of *C. jejuni*.

Flagella, chemotaxis, toxin production and other virulence related proteins of *C. jejuni*

As mentioned before, the flagella of *C. jejuni* have multiple purposes: they have a role in migration towards less hostile environments, reaching the gut, and penetrating the viscous mucosa lining of the human epithelial cells. Therefore, the flagella not only promote motility, but also chemotaxis, adhesion and invasion of the human gut (McSweegan & Walker, 1986; Black et al., 1988; Grant et al., 1993; Szymanski et al., 1995; Konkel et al., 2004). Multiple flagellar proteins were significantly differentially expressed when *Campylobacter* was grown in the presence of L-fucose, however, expression was strain dependent, suggesting that the changes in motility differ amongst isolates. Furthermore, several proteins of the CDT complex were found significantly upregulated in the presence of L-fucose. The upregulation of the CDT complex highlights possible epithelial disruption, as displayed in Figure 3. However, as many isolates do not carry the CDT complex, such as the isolate *C. jejuni* 18-440, the toxin production response upon exposure to L-fucose might be strain dependent. Lastly, many virulence proteins were affected by the presence of L-fucose. However, due to the difference in observed invasion phenotypes and the difference in upregulated virulence proteins between the isolates, some virulence responses to L-fucose might be strain dependent, highlighting the need for virulence testing on a larger number of *Campylobacter* isolates.

Future perspectives and concluding remarks

The presented results in this thesis showed that metabolism of L-fucose by fuc+ *C. jejuni* isolates affects physiology and performance in multiple ways including shifts in the composition of cytoplasmic and putative membrane and periplasmic proteins, in line with metabolic rewiring and conceivable modulation of lipooligosaccharide production, metal acquisition, flagella synthesis and chemotaxis, and toxin production. In addition, insight was obtained on the impact of L-fucose and D-glucose metabolism in a fuc+ and gluc+ *C. jejuni* isolate. Based on the role of these parameters in *Campylobacter* growth performance and survival, colonization, proliferation and invasion of the human gut, additional directions for studies on the role of L-fucose and D-glucose utilization in fuc+ and gluc+ *C. jejuni* and *C. coli* are listed below:

1. In the tested isolates, different LOS production proteins were significantly differentially expressed in the presence of L-fucose. Interestingly, only parts of the outer core (which is the most variable part of the LOS) were affected in the presence of L-fucose or D-glucose. In depth biochemical analyses are needed to unravel the impact of L-fucose on the LOS and CPS of *C. jejuni* and *C. coli.*
2. Although the D-glucose utilization cluster is very rare in *C. jejuni*, we provided evidence that even in non gluc+ *C. jejuni* isolates LOS biosynthesis proteins were differentially expressed. How D-glucose affects the LOS biosynthesis of *C. jejuni* also requires further characterization of the LOS of *C. jejuni*. Combination with scanning and transmission electron microscopy will add details about cell surface characteristics.
3. It is clear that fuc+ *C. jejuni* and *C. coli* isolates undergo metabolic rewiring, possibly linked to the survival and colonization of the human gut. Interestingly, this metabolic rewiring was strain specific in the tested conditions, possibly related to the origin of the isolate. More isolates should be studied to get a full understanding of the metabolic rewiring of *C. jejuni* and *C. coli* upon exposure to L-fucose.
4. In the different tested fuc+ *C. jejuni* isolates, many putative membrane and periplasmic space proteins were significantly differentially expressed in the presence of L-fucose. To obtain a better understanding of the effect that L-

fucose has on the membrane, knockout studies have to be performed to characterize these hypothetical putative encoded membrane and periplasmic related proteins, as it is likely that they are involved in the survival and virulence of *C. jejuni*. A special emphasis on the role of MacABC is required to assess its role in *Campylobacter*.

5. L-fucose metabolism affected expression of multiple virulence proteins, such as the toxin producing CDT complex. To get better insights in the effect of L-fucose utilization on the virulence of fuc+ *C. jejuni* isolates, extended virulence testing has to be done, including chemotaxis, motility assays, toxin production, and, *in vitro* adhesion, invasion and translocation assays using epithelial cells.

In conclusion, this thesis provided in depth insights in the L-fucose and D-glucose utilization clusters in *C. jejuni* and *C. coli*, and highlighted the role of these compounds in growth, survival, metabolism and virulence. This included the identification of multiple metabolism-associated, and virulence and membrane-related proteins that are likely to be involved in the colonization of the human gut and invasion of epithelial cells. These phenotypic observations (**Chapter 3 and 4**), together with the significant correlation between fuc+ isolates and human isolates (**Chapter 5**), highlight the importance of the L-fucose utilization cluster in a broad range of processes that ultimately help *Campylobacter* to colonize and proliferate inside the human gut, conceivably leading to increased pathogenesis in the presence of L-fucose.

Supplements

Suppl. Table 1 Differentially expressed proteins in *C. jejuni* NCTC11168 when grown in DMEM-G medium, compared to DMEM medium.

Function	Fold-change
Putative glycosyltransferase	124.99
Chaperone	47.16
Hypothetical protein	2.60
Adenylosuccinate lyase	2.60
NADH dehydrogenase I chain M	2.38
Putative integral membrane protein	1.88
50S ribosomal protein L3	1.84
NifU protein homolog	1.71
Purine nucleoside phosphorylase	-1.61
Serine transporter	-1.62
Molybdopterin molybdenumtransferase	-1.62
Peptidyl-prolyl cis-trans isomerase	-1.63
Cytidine diphosphoramidate kinase	-1.66
Geranyltranstransferase	-1.83
3-deoxy-manno-octulosonate cytidylyltransferase	-1.91
Putative periplasmic protein	-1.92
DNA helicase	-2.02
Putative integral membrane protein	-2.08
Putative acetyltransferase	-2.17
Uncharacterized protein	-2.66
Putative methyl-accepting chemotaxis signal transduction protein	-3.94

Suppl. Table 2 Presence and absence protein list of all tested virulence proteins in *C. jejuni*.

Uniprot	Protein	*C. jejuni* NCTC11168	*C. jejuni* 286	*C. jejuni* 18-440
Q0P8G7	**Cj1448c**	nd	nd	nd
Q0P8Q8	**pldA/NCTC11168**	nd	nd	nd
Q0P8T4	**Cj1323**	nd	nd	nd
Q0P8T6	**Cj1321**	nd	nd	nd
Q0P8T6	**Cj1321/pseudogene**	nd	nd	nd
Q0P977	**fliR/Cj1179c**	nd	nd	nd
Q0PAD4	**cfrA/Cj0755**	nd	nd	nd
Q0PAI2	**flgG2/Cj0697**	nd	nd	nd
Q0PBW1	**Cj0178**	nd	nd	nd
Q0PC83	**flgE**	nd	nd	nd
Q0P7V2	**cj1677/capB**	nd	nd	nd
Q0P7V4	**fliQ/Cj1675**	nd	nd	nd
Q0P814	**chuA/Cj1614**	nd	nd	present
Q0P8C6	**CjE1664/Cj1491c**	nd	nd	present
Q0P8G8	**tagH/CCC13826/1187**	nd	nd	present
Q0P9C0	**C8J/1074/htrB**	nd	nd	present
Q0PA11	**flaD/Cj0887c**	nd	nd	present
Q0PAW7	**fliS/Cj0549**	nd	nd	present
Q0PC85	**fliK/Cj0041**	nd	nd	present
Q0PC85	**Cj0041**	nd	nd	present
Q9PPM0	**flgH/Cj0687c**	nd	nd	present
Q0PAJ4	**CipA/Cj0685c**	nd	present	present
Q0PAY8	**flgB/Cj0528c**	present	nd	nd
Q0P8S5	**pseD/Cj1333**	present	nd	nd
Q0P9B5	**wlaN/Cj1139c**	present	nd	nd
Q0P9B7	**Cj1137c**	present	nd	nd
Q9PHP0	**cj0627/capA**	present	nd	nd
Q0P9D0	**pglC/Cj1124c**	present	nd	present
Q0P8H7	**Cj1437c**	present	present	nd
Q0PC58	**cdtC/Cj0077c**	present	present	nd
Q0P870	**Cj1556**	present	present	nd
Q0P8H3	**Cj1441c**	present	present	nd
Q0P8H4	**Cj1440c**	present	present	nd
Q0P8H8	**Cj1436c**	present	present	nd
Q0P8H9	**Cj1435c**	present	present	nd
Q0P8I0	**Cj1434c**	present	present	nd
Q0P8I1	**Cj1433c**	present	present	nd
Q0P8I3	**Cj1431c**	present	present	nd
Q0P8I4	**Cj1430c**	present	present	nd

Q0P8I6	**Cj1428c**	present	present	nd
Q0P8I7	**Cj1427c**	present	present	nd
Q0P8I8	**Cj1426c**	present	present	nd
Q0P8J1	**Cj1423c**	present	present	nd
Q0P8J3	**Cj1421c**	present	present	nd
Q0P8N8	**Cj1371**	present	present	nd
Q0P8R9	**maf6/Cj1340c**	present	present	nd
Q0P8S2	**pseE/Cj1337**	present	present	nd
Q0P8T2	**Cj1325**	present	present	nd
Q0P8U4	**pseH/Cj1313**	present	present	nd
Q0P914	**Cj1242/CiaC**	present	present	nd
Q0P9A9	**C8J/1091/waaV**	present	present	nd
Q0P9C8	**pglB/Cj1126c**	present	present	nd
Q0P9D1	**pglD/Cj1123c**	present	present	nd
Q0P9I5	**Cj1069**	present	present	nd
Q0P9L0	**Cj1042c**	present	present	nd
Q0PAI1	**flgG/Cj0698**	present	present	nd
Q0PAK4	**rpoN/Cj0670**	present	present	nd
Q0PAY9	**flgC/Cj0527c**	present	present	nd
Q0PC56	**cdtA/Cj0079c**	present	present	nd
Q0PC57	**cdtB/Cj0078c**	present	present	nd
Q0P7V5	**mqnD/Cj1674**	present	present	nd
Q0P7Y2	**iamB/Cj1646**	present	present	nd

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A

Appendix

Summary

Campylobacter is a zoonotic bacterium and is the main cause of bacterial foodborne gastroenteritis worldwide, with most human campylobacteriosis cases being the result of infection with *Campylobacter jejuni* or *Campylobacter coli*. Transmission via handling and consumption of (poultry) meat only contributes to half of all campylobacteriosis cases, highlighting other transmission routes such as contact with live animals, contact with pets, and via environmental waters.

C. jejuni and *C. coli* were previously considered asaccharolytic, but are now known to carry specific saccharides metabolization pathways, the so-called L-fucose and D-glucose utilization clusters. Approximately 52% and 75% of the investigated *C. jejuni* and *C. coli* isolates carry the L-fucose utilization cluster (*Cj0480c – Cj0489*), while 0.25% and 6.3% carry the D-glucose utilization cluster (*glcP*, *pgi2 glk*, p*gl*, *zwf, edd* and *eda*), respectively. This thesis describes research on *Campylobacter* to investigate whether these clusters are beneficial for *Campylobacter* niche adaptation and help establish human infection.

In **Chapter 2** the L-fucose utilization cluster in *C. jejuni* and *C. coli* isolates is characterized including roles in growth, survival and metabolism. After incubation for seven days, comparative analysis of performance in L-fucose-enriched MEMα medium compared to MEMα, showed an enhanced survival and prolonged spiral cell morphology for all tested isolates, with the *C. coli* isolate having the most robust phenotype. Furthermore, HPLC analysis indicated that L-fucose utilization was linked to acetate, lactate, pyruvate and succinate production. Genetic analysis of the L-fucose utilization cluster revealed a frameshift in the *Cj0489* gene, resulting in two open reading frames encoding for putative 77 amino acid N-terminal (Cj0489-S) and 394 amino acid C-terminal (Cj0489-L) proteins, in the reference isolate *C. jejuni* NCTC11168, which did not result in a different phenotype of growth, survival and metabolism compared to the other tested *Campylobacter* isolates.

Chapter 3 describes an extended phenotypic testing of the L-fucose utilization cluster in two closely related human *C. jejuni* isolates, *C. jejuni* NCTC11168 and *C. jejuni* 286, and next to L-fucose metabolism, also D-arabinose metabolism was studied. D-arabinose is a sugar that is similar to L-fucose and was found to be metabolized via

the L-fucose utilization cluster of *C. jejuni*. L-fucose and D-arabinose metabolism, including metabolite production, and its impact on Caco-2 cell interaction and binding to fibronectin were investigated. Increased survival and changes in metabolism were observed in both tested isolates when cultured with L-fucose and D-arabinose, and resulted in production of acetate, pyruvate and succinate, and the respective signature metabolites lactate and glycolic acid, which was in line with the upregulation of L-fucose cluster proteins. *In vitro* Caco-2 cell studies and fibronectin-binding experiments showed significantly higher invasion and fibronectin binding efficacy of *C. jejuni* NCTC11168 cells grown with L-fucose or D-arabinose, compared to cells grown without these substrates, while no significant differences were found with *C. jejuni* 286 between all tested media. The fibronectin binding proteins CadF and FlpA were both detected in the two isolates, however, not significantly differentially expressed in cells grown with L-fucose or D-arabinose. The protein Cj0608, which was more than 135-fold upregulated in *C. jejuni* NCTC11168, was linked to this observed phenotype. Cj0608, a putative TolC-like component MacC, is part of the tripartite secretion system MacABC, previously characterized in a range of Gram-negative pathogens, and functions in diverse cellular processes, including antibiotic resistance, cell division and lipoprotein trafficking. Additionally, *C. jejuni* NCTC11168 uniquely produced five virulence proteins, namely, FlgB, PseD, PglC, WlaN and Cj1137c. As the proteins PgLC, WlaN and Cj1137c are involved in cell wall lipooligosaccharide (LOS) synthesis, it is conceivable that L-fucose and D-arabinose utilization affected cell surface structure, resulting in enhanced fibronectin-mediated invasion of this isolate. Further studies on the MacABC system are required to elucidate its possible role in LOS synthesis and virulence of *C. jejuni*.

In **Chapter 4** studies are described with a *C. jejuni* isolate which was identified during the *in silico* horizontal gene transfer analyses described in **Chapter 5**, namely, the whole genome-sequenced water isolate *C. jejuni* 18-440. This isolate carries an atypical L-fucose utilization pathway and a D-glucose utilization pathway. Upon L-fucose or D-glucose consumption by this isolate, increased growth and prolonged spiral shaped morphology and prolonged survival was observed up to 14 days, which was linked to increased concentrations of signature products lactate and pyruvate in the medium. Proteomics analyses revealed that the proteins of the L-fucose utilization cluster were upregulated in L-fucose-grown cells, while only the D-glucose transporter, GlcP, of

the D-glucose utilization cluster was upregulated when cells were cultured in the presence of D-glucose. In L-fucose-grown cells, in total 75 proteins were significantly differentially epressed, with putative functions in metal acquisition (including multicomponent transport systems), LOS production, metabolism, next to a range of membrane/periplasmic located proteins. In D-glucose-grown cells only 22 proteins were significantly differential expressed, with putative functions in metal acquisition and membrane/periplasmic located proteins. Notably, in both L-fucose and D-glucose-grown cells, protein Ppg2 (Peptidoglycan L,D-Carboxypeptidase) was upregulated, which was in line with the observed prolonged helical morphology of the *C. jejuni* 18-440 cells. Based on the findings that L-fucose improves growth and survival of *C. jejuni*, together with the significant different expression of proteins involved in iron/metal acquisition, metabolism, extracellular lipooligosaccharides and morphology, it was speculated that L-fucose causes a rewiring of the metabolism linked to the human gut environment.

In **Chapter 5** an *in silico* approach is used to investigate the distribution of D-glucose utilization and L-fucose utilization clusters among ~9,600 *C. jejuni* and *C. coli* genomes of different isolation sources in the Netherlands, the United Kingdom, the United States of America and Finland. Integration sites of the L-fucose and D-glucose utilization cluster were investigated, which pointed to several isolates that acquired their L-fucose utilization cluster from the other *Campylobacter* species via horizontal gene transfer. L-fucose cluster alignments revealed multiple frameshift mutations supporting the hypothesis that the L-fucose cluster was integrated multiple times across the *C. coli*/*C. jejuni* phylogeny. Next, a significant correlation was observed between human *C. jejuni* isolates and *C. jejuni* isolates carrying the L-fucose utilization cluster using the Dutch dataset and the combined dataset of the four countries. These findings highlight a potential selective advantage for *C. jejuni* isolates carrying the L-fucose utilization cluster in human infection and during survival and proliferation within the human host, possibly after transmission from any reservoir.

Chapter 6 discusses the experimental results obtained in the research chapters. First the phenotypic results of proliferation, survival, metabolism and virulence are discussed. This is followed by a discussion of the proteomic results of L-fucose, D-arabinose or D-glucose-grown *C. jejuni* cells, which gave new insights into the LOS

production, metal acquisition, metabolic rewiring, membrane/periplasmic proteins, flagella's and chemotaxis, and toxin production of *Campylobacter*. These insights were used in recommendations for future research fields.

In conclusion, this thesis provided in depth insights into the importance of the L-fucose utilization cluster to *Campylobacter*. Throughout the chapters it was highlighted that isolates carrying the L-fucose cluster have improved growth, prolonged spiral shape morphology, increased survival, several metabolic changes, increased invasion to Caco-2 cells and increased binding to fibronectin in the presence of L-fucose. Proteomic analyses suggest that ultimately L-fucose utilization is involved in virulence, LOS synthesis and metabolic rewiring, possibly linked to the human gut environment. Furthermore, phenotypic evidence of D-glucose utilization by *C. jejuni* isolates carrying the D-glucose utilization cluster was provided, which was linked to the upregulation of the GlcP transporter. This thesis highlights the capability of *Campylobacter* to utilize L-fucose, D-arabinose and D-glucose, that support niche adaptation and help establish human infection.

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About the author

Pjotr S. Middendorf was born on the 27th of March of 1992, in Borger, the Netherlands. After finishing high school at Carmelcollege Emmen, he started a bachelor's degree in Biotechnology at the Van Hall Larenstein in Leeuwarden, the Netherlands. In the second year of his bachelor's degree, he performed an internship at the biochemistry group of the Rijksuniversiteit Groningen, where he worked on protein interactions. In the final year of his bachelor's degree, he undertook an internship at the company Merial, where he worked on virus production in bioreactions. His graduation internship was done at AgResearch in New Zealand, where he investigated bovine in vitro fertilization and cell culture.

After obtaining his bachelor's degree from the Van Hall Larenstein, Pjotr continued his studies by doing a master's degree in Biotechnology, with a specialization in Medical Biotechnology. He completed two thesis studies. His first thesis took place at the department of Virology at Wageningen University, where he characterized long transcripts and miRNAs from the SeMNPV egt gene. His second thesis was performed at the department of Microbiology, where he constructed shuttle vectors containing the novel DISARM anti-phage defense system for transformation of *E. coli*.

In 2017, Pjotr started a PhD at the department of Food Microbiology (FHM) at Wageningen University, under the supervision of Prof. Dr Tjakko Abee, Prof. Dr Heidy M.W. Den Besten and Dr. Wilma F. Jacobs-Reitsma. Here, he worked with the foodborne pathogen *Campylobacter*, and performed a systems biology approach to identify mechanisms contributing to transmission dynamics and virulence of this bacteria.

Currently, Pjotr works at Eurotrol, a company that produces in vitro diagnostics medical devices. Here, he works as product developer and is mainly involved in product development and product changes.

If you would like to connect with Pjotr on LinkedIn, please scan his QR code.

https://www.linkedin.com/in/pjotr-middendorf-326ob362/

Full CV available on LinkedIn.

Overview of completed training activities

Discipline specific activities

KNVM Symposium 2018	KNVM RIVM	Bilthoven (NL)
KNVM Symposium 2018	KNVM TU Delft	Delft (NL)
CHRO 2019	Institute of Food Science & Technology	Belfast (UK)
HPLC Workshop	WUR	Wageningen (NL)
Intestinal microbiome of humans and animals	VLAG	Wageningen (NL)
FEMS 2020	FEMS	Belgrade (Online)
Proteomics workshop	WUR	Wageningen (NL)
KNVM/NVMM Scientific Spring 2021	KNVM/NVMM	Online
Ecophysiology of food-associated micro-organisms: Roles in health and disease 2021	VLAG	Wageningen (NL)
Congress world microbe forum 2021	FEMS & ASM	Online
Webinar A guide to proteomics data analysis using UniProt and InterPro	EMBL & EBI	Online
KNVM Food Microbiology 2022	WUR & KNVM	Online

General courses

VLAG PhD Week	VLAG	Baarlo (NL)
Introduction to R	VLAG	Wageningen (NL)
Applied statistics	VLAG	Wageningen (NL)
Scientific Artwork, Data visualisation and Infographics with Adobe Illustrato	WGS	Wageningen (NL)
Introduction to Python: Absolute Beginner	Edx	Online
Career perspectives	WGS	Wageningen (NL)

Other activities

Preparation of research proposal	WUR, Food Microbiology	Wageningen (NL)
PhD trip to China (+ organisation)	WUR, Food Microbiology	Wageningen (NL)
Department seminars	WUR, Food Microbiology	Wageningen (NL)
Weekly project meetings	WUR, Food Microbiology	Wageningen (NL)
Annual meetings RIVM	WUR, Food Microbiology & RIVM	Wageningen (NL)
Course: Microbial Genomics	Utrecht University	Utrecht (NL)

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