Proteomic Profiles of Campylobacter jejuni and Enterococci during Co-incubation and under Bile Acid Stress

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submitted by:

Annika Dreyer

Born in Gehrden, Germany

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Thesis advisory Committee
Apl. Prof. Dr. Andreas Zautner, Institut für Medizinische Mikrobiologie und Krankenhaushygiene, Medizinische Fakultät, Otto-von-Guericke-Universität Magdeburg

Prof. Dr. Rolf Daniel, Abtl. Genomische und Angewandte Mikrobiologie & Göttingen Genomik Labor, Institut für Mikrobiologie und Genetik, Georg-August Universität Göttingen

PD Dr. Michael Hoppert, Abtl. für Allgemeine Mikrobiologie, Institut für Mikrobiologie und Genetik, Georg-August Universität Göttingen

Members of the Examination Board
Referee: Apl. Prof. Dr. Andreas Zautner, Institut für Medizinische Mikrobiologie und Krankenhaushygiene, Medizinische Fakultät, Otto-von-Guericke-Universität Magdeburg

2nd Referee: Prof. Dr. Rolf Daniel, Abtl. Genomische und Angewandte Mikrobiologie & Göttingen Genomik Labor, Institut für Mikrobiologie und Genetik, Georg-August Universität Göttingen

Further members of the Examination Board
PD Dr. Michael Hoppert, Abtl. für Allgemeine Mikrobiologie, Institut für Mikrobiologie und Genetik, Georg-August Universität Göttingen

Prof. Dr. Kai Heimel, Abtl. Mikrobielle Zellbiologie, Institut für Mikrobiologie und Genetik, Georg-August Universität Göttingen

Prof. Dr. Uwe Groß, Abtl. Medizinische Mikrobiologie, Institut für medizinische Mikrobiologie und Virologie, Universitätsmedizin Göttingen

Prof. Dr. Jan de Vries, Abtl. Angewandte Bioinformatik, Institut für Mikrobiologie und Genetik, Georg-August Universität Göttingen

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Summary

The human gut harbors a complex ecosystem of microbial communities, consisting of commensal inhabitants, which play crucial roles in maintaining gut homeostasis, regulating host metabolism, and modulating immune responses. When a pathogenic bacterium colonizes the gut, it is exposed to various environmental stressors, including the presence of commensal bacteria but also exposure to toxic substances such as bile acids. Investigation of the responses of gut pathogens to the natural inhabitants is essential for the understanding of the mechanisms of microbial colonization and the host’s health. Furthermore, the comprehension of the reaction of these microorganisms to the challenging environment of the gut is relevant to examine microbial adaptions to harsh conditions, such as bile acid stress.

This thesis investigates the proteomic adaptions of *Campylobacter jejuni* and two *Enterococci* species with a particular focus on their coexistence and their response to bile acid stress. The thesis covers four different research topics:

Project I: Bacterial communication is a relevant mechanism for interplay among various microbial species, especially when a pathogen enters the human gastrointestinal tract. To examine the proteomic response of *C. jejuni* to the presence of *Enterococcus faecalis*, *Enterococcus faecium* and *Staphylococcus aureus*, co-incubation experiments of *C. jejuni* with these bacteria were performed. The impact of *S. aureus* on the proteome of *C. jejuni* was most significant, resulting in the up-expression of 215 proteins and the down-expression of 230 proteins. These counts nevertheless remained notably lower compared to the 526 up-expressed and 516 down-expressed proteins observed during exposure to deoxycholic acid (DCA). Within the co-incubation, in all three microbial species, a subgroup of 54 distinct proteins exhibited significant differential expression, indicating a shared co-incubation response by *C. jejuni*. Although this shared proteomic response partially overlapped with the DCA response, distinct proteins were exclusive in the co-incubation response. Co-incubation unveiled three membrane-interactive proteins among the top 20 up-expressed proteins, suggesting that the presence of other bacteria might enhance environmental virulence. Furthermore, the exposure to both stressors, co-incubation and DCA revealed a reciprocal influence, resulting in a unique synergistic proteomic reaction that differed from the individual responses induced by each stimulus.

Project II: The proteomic response towards high concentrations of cholic acid (CA), chenodeoxycholic acid (CDCA) and DCA of the gut inhabitants *E. faecalis* and *E. faecium*
after long-term incubation was analyzed and compared, to simulate bile acid concentrations these bacteria are exposed to during biliary tract infections. Both species show similarities in the proteomic response, however, species-specific differences were also found. In *E. faecalis*, DCA and CDCA strongly down-expressed proteins involved in translation, transcription, and replication, whereas the effect was less significant in *E. faecium*. *

*E. faecium* seems to be slightly more resistant towards CDCA and DCA, nevertheless, a general bile acid response in both species consisting of the up-expression of V-type ATPase subunits, different ABC-transporters, multi-drug transporters and proteins related to cell wall biogenesis were detected in *E. faecalis* as well as in *E. faecium*.

Additionally, adaptations of *E. faecalis* in aerobic as well as microaerophilic environments were analyzed. Interestingly, bile acid adaption in *E. faecalis* seems to be independent from the oxygen level.

**Project III:** Genes encoding for proteins that are known to play a role in bile acid resistance in *C. jejuni* were knocked out and a proteomic analyses of these knockout mutants was performed in comparison to the parental *C. jejuni* strain in the presence and absence of bile acids. The targets chosen for deletion were CmeB, which is a subunit of the *Campylobacter* multidrug efflux CmeABC, CmeR which regulates the CmeABC transporter, and CbrR a *Campylobacter* bile acid resistance regulator. The results indicate that the lack of CmeB results in a notable shift in the proteome, while the impact of CmeR and CbrR lead to less proteomic alterations. Besides, deletion of the respective genes unveils potential alternative involvements in metabolic pathways.

**Project IV:** Co-incubation of *C. jejuni* in the presence of DCA with various Gram positive bacteria such as *E. faecalis*, *E. faecium* and *S. aureus* generates an environment that leads to increased bile acid resistance of the Gram positive bacteria. Therefore, a proteomic analysis was conducted to identify *C. jejuni* proteins that are specifically induced under these conditions. This study provides potential target proteins that might be involved in inter-bacterial communication processes leading to the observed increased bile acid resistance of these Gram positive bacteria.

Overall, this work contributes to the understanding of microbial adaptions to the challenging gut environment consisting of stressors such as varying concentrations of bile acids and the presence of commensal or pathogenic bacteria.
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List of Abbreviations

ANOVA - Analysis of variance
CA - Cholic acid
CDCA - Chenodeoxycholic acid
CDT - Cytolethal distending toxin
CbrR - Campylobacter bile resistance regulator
CG Quant - Cell Growth Quantifier
CmeABC - Campylobacter multidrug efflux ABC
CmeR - Campylobacter multidrug efflux regulator
COG - Clusters of orthologous groups
DCA - Deoxycholic acid
DDA - Data-dependent acquisition
DIA - Data-independent acquisition
DIA-MS - Data-independent acquisition mass spectrometry
DNA - Deoxyribonucleic acid
ECDC - European Centre for Disease Prevention and Control
EFSA - European Food Safety Authority
FDR - False-discovery-rate
g - Gram
h - Hours
HF - High fidelity
IC{sub 50} - Half maximal inhibitory concentration
IPEC - intestinal pathogenic Escherichia coli
kV - Kilovolt
L - Litre
LB - Luria-Bertani
µF - Microfarad
µg - Microgram
mg - Milligram
MH - Mueller-Hinton

min - Minutes

µL - Microlitre

mL - Millilitre

µm - Micrometer

mm - Millimeter

mM - Millimolar

MS - Mass spectrometry

NEB - New England Biolabs

nm - Nanometre

OD - Optical density

PBS - Phosphate-buffered saline

PMF - Proton motif force

ProBAS - Protection from bile acid stress

PVP - Polyvinylpyrrolidone

rpm - Revolutions per minute

SDS-PAGE - Sodium Dodecyl Sulfate – PolyAcrylamid Gel Electrophoresis

T3SS - type-III secretion system

T4SS - type-IV secretion system

T6SS - TIIISS - type-VI secretion system

TPF - 1,3,5-triphenylformazan

TTC - Triphenyl tetrazolium chloride

UV - Ultraviolet

WHO - World Health Organization
1 Introduction

1.1 Overview - infectious diseases

Emerging and re-emerging infectious diseases are a major challenge on global health, causing considerable morbidity and mortality in the population [1]. Usually, infectious diseases are caused by pathogenic microorganisms, such as bacteria, viruses, fungi and parasites (Figure 1) [2, 3]. Transmission of these pathogens can occur via multiple routes, including direct contact, respiratory droplets, contaminated food or water. Among the burdens of infectious diseases are factors as the population density, healthcare infrastructure, socio-economic conditions and environmental factors [4]. Prevention plays a crucial role in controlling the spread of infectious diseases. The promotion of hygiene practices is vital for preventing the transmission of diseases. Furthermore, the rational use of antibiotics, antivirals, and antifungal drugs can help prevent the development of resistance [5, 6]. Moreover, public health surveillance systems worldwide play an important role in early detection, monitoring, and response to infectious diseases, also with respect to sharing data to detect trends, patterns, and potential outbreaks [7, 8]. Clinical and basic research is the fundament for comprehension of pathogen biology, exploration of potential therapeutic approaches and developing novel interventions, including vaccines, diagnostics, and antiviral drugs.

1.2 Gastrointestinal pathogens

Among the most important emerging infectious diseases are different foodborne gastrointestinal infections, representing a significant public health concern [9]. These infections are caused by diverse microbial pathogens or chemicals on contaminated food, having a wide range of gastrointestinal diseases with varying degrees of severity. [10]. It is estimated that approximately 60 % of all infections between 1940 and 2004 were of zoonotic origin (see Figure 1) [11]. As stated in the World Health Organisation's (WHO) report, around 525.000 fatal diarrheal disease cases of children under the age of five are registered each year, a significant number of cases could be prevented through clean drinking water and appropriate hygiene conditions [12].
Figure 1: Examples for origin and spread of zoonotic diseases from livestock and wildlife. Rate of zoonotic diseases in the European Union: *Campylobacter* and *Salmonella* infections are the most prevalent in the population. Statistics adapted from the European Food Safety Authority (EFSA) zoonoses report 2021 [3].
In consequence, the molecular mechanisms underlying bacterial pathogenicity and the evolution of drug resistance are crucial areas of research in combating infectious diseases. Advancements in genomic sequencing, proteomics, and bioinformatics have recently enhanced the understanding of bacterial virulence factors, adaptive responses, and the dynamics of transmission [13, 14].

The human gastrointestinal tract is frequently exposed to a diverse array of pathogenic bacteria, viruses, and parasites. Among the most prevalent bacterial pathogens associated with gastrointestinal infections are Salmonella spp., intestinal pathogenic Escherichia coli (IPEC), Shigella spp., Clostridioites difficile and Campylobacter spp. [15, 16].

1.3 Campylobacter - a human and animal pathogen

According to the European Food Safety Authority and European Centre for Disease Prevention and Control (EFSA and ECDC), Campylobacter is one of the four most common bacteria that cause human gastroenteritis [17]. The reported incidence of C. jejuni and C. coli cases is between one and 160 per 100,000 inhabitants in developed countries, which is above the number of described cases of Salmonella sp. and IPEC [18–26]. Moreover, the number of reported Campylobacteriosis cases has increased over the last years. However, detailed epidemiological data remain incomplete worldwide.

The most common sources for human infection are contaminated water, unpasteurized milk and primarily undercooked or raw chicken meat and also meat from other agriculture-related animals [27–29].

A taxonomic classification of Campylobacter involves its placement in the family Campylobacteraceae within the order Campylobacterales, belonging to the class Epsilonproteobacteria and the phylum Proteobacteria [30]. The genus Campylobacter comprises a group of Gram negative, motile, microaerophilic, spiral or rod shaped bacteria, that are widely distributed in nature. In 1886, Theodor Escherich discovered spiral bacteria in children’s intestines who suffered from a diarrhoeal disease and these observations were published in the Münchener Medizinische Wochenschrift [31]. Campylobacter was first cultivated and described by Smith in 1919 as Vibrio fetus [32]. In 1963, Sebald and Veron renamed Vibrio fetus to Campylobacter fetus and introduced this strain as the type strain of the
genus *Campylobacter* [33]. Members of the genus are usually catalase negative and oxidase positive [34]. Typically, *Campylobacter* species are characterized as non-spore forming and motile bacteria that range in size from approximately 0.2 to 0.8 µm in width and 0.5 to 5 µm in length. The motility in *Campylobacter* is an important virulence and survival factor [35]. *Campylobacter* employs flagella for its motility, which can be either unipolar or bipolar [18]. Not only are flagella playing a crucial role for motility, but also in biofilm formation and autoagglutination [36–38]. Furthermore, *Campylobacter* flagella are involved in the intestinal colonization [39, 40]. In addition to the flagella, the morphological features of *Campylobacter* also contribute to its ability to move through the mucus layer, specifically, the spiral shape of the bacteria [41].

Besides its flagella, *C. jejuni* uses several chemoreceptors to facilitate chemotaxis. Chemotaxis is a relevant mechanism for *C. jejuni* to sense and respond to chemical gradients in the environment, allowing the bacteria to navigate towards favorable conditions or avoid harmful substances [42].

Furthermore, adhesion and invasion of the host cells are important factors of *C. jejuni*’s pathogenicity. The adhesion of *C. jejuni* to intestinal epithelial cells is promoted by multiple factors, such as the motility of the bacteria, the composition of the bacterial surface and the presence of adhesins [43]. Adhesins play an important role for enabling interactions between a pathogen and a host and thus for colonization and persistence of the host. By targeting molecules on the host’s cell surface or surface receptors, adhesion can be facilitated. Using the two proteins FlpA and CadF, *C. jejuni* is able to bind to fibronectin, a host-cell molecule, which consequently enables adherence and invasion [44–46].

Invasion of *C. jejuni* into the host cell underlies two different mechanisms, namely the "zipper" and the "trigger" mechanism [47]. The "zipper" mechanism is launched by different bacterial surface proteins such as adhesins or invasins. These proteins can bind to a host cell receptor, enabling close contact between the bacteria and the host’s cell and internalization via engulfment [48]. The "trigger" mechanism is driven by the above mentioned type-III secretion system (T3SS) and type-IV secretion systems (T4SS). These secretion systems are utilized to secrete bacterial proteins into the host cell which
eventually lead to rearrangement of the cytoskeleton [47]. Consequently, membrane ruffles are formed that finally lead to an engulfment of the bacterial cell [47].

However, the method of invasion depends on the host’s cytoskeleton composition [49]. Once *Campylobacter* enters the host cell, the bacteria are able to manipulate various cell processes and interfere with immune responses, to promote its survival and replication. *Campylobacter* can produce a variety of toxins, including the cytolethal distending toxin (CDT), which causes cell cycle arrest and DNA damage [50, 51]. This can result in apoptosis and cause the inflammatory response in the intestinal mucosa, leading to the disruption of the epithelial barrier, which results in diarrhea.

The preferred growth conditions of *Campylobacter* are temperatures between 37 and 42 °C and concentrations of oxygen around 5 %, CO₂ at 10 %, nitrogen at 85 %, however, the ability to tolerate varying oxygen levels can exhibit inter-specific variation [52, 53].

### 1.3.1 *C. jejuni* infection and epidemiology

Among the *Campylobacter* species, *Campylobacter jejuni* is the most commonly identified cause of bacterial gastroenteritis worldwide, alongside the significantly less frequent *C. coli* [17, 54]. A campylobacteriosis is distinguished by colonization of the small intestine, and the clinical presentation of the infection can range from asymptomatic carriage to a severe enteritis with painful abdominal cramps, fever, nausea and most importantly, severe watery or bloody diarrhea [55]. An acute diarrhea is typically the primary symptom observed within the initial days of a *C. jejuni* infection [56, 57]. Usually, it is a self-limiting a infection, where the clinical manifestations gradually abate over a span of about one or two weeks, without requiring therapeutic intervention [55]. Nevertheless, severe infections where hospitalization is needed can arise and especially in children or immunocompromised individuals, a rare case of fatality might occur (according to WHO 2018). Typically, therapeutic intervention involves the use of ciprofloxacin and erythromycin or azithromycin [58–60]. However, 75-90 % of all *C. jejuni* strains have developed a fluoroquinolone resistance [61, 62]. Moreover, a concerning rise in resistance rates against macrolides was reported as a consequence of the primary therapeutic use of macrolides [63, 60, 64, 65]. Alternative treatment strategies for a *C. jejuni* infection are prebiotics or probiotics. While the effects of prebiotic
treatments remain uncertain [66, 67], the use of probiotics seems to be more promising [68, 69]. Specifically species belonging to *Bacillus* and *Lactobacillus* have been shown to reduce *C. jejuni* colonization in broilers and mice [69–71]. A vaccine for *C. jejuni* is currently not available, all attempts to develop vaccines were not successful in human clinical trials [72, 73]. However, attempts have been made to administer vaccines in broiler chickens [74].

In some cases, infections with *C. jejuni* may lead to a secondary disease such as the Guillain-Barré Syndrome. The Guillain-Barré Syndrome is a serious neural disease characterized by muscle weakness, numbness and paralyses [75, 76]. The Guillain-Barré Syndrome can develop rapidly and lead to potentially life-threatening complications, such as respiratory paralyses [77]. A rare variant of the Guillain-Barré Syndrome is the Miller Fisher syndrome, where the paralysis is affecting the head and is characterized by a loss of reflexes and coordination, as well as paralysis of the eye muscles [78, 79].

Considering these various factors, *C. jejuni* is one of the most prominent and influential bacterial pathogens on a global scale and thus of high medical relevance.

### 1.4 *Enterococci* - opportunistic pathogens

In 1899, Thiercelin, MaCallum and Hastings first isolated and described the genus *Enterococcus*, isolated from a fatal instance of endocarditis, [80, 81]. First, bacteria belonging to the genus *Enterococcus* were assigned to the *Streptococcus*, but renamed *Enterococcus* in 1984 [82, 83]. *Enterococcaceae* belong to the order of *Lactobacillae* in the class *Bacilli* and are part of the phylum *Firmicutes*. *Enterococci* are a Gram-positive, facultative anaerobic, coccal shaped large group of lactic acid bacteria which do not form spores and are oxidase- and catalase-negative [80, 84–87]. Due to their high adaptability, *Enterococci* are able to grow and survive in several different environmental conditions [88]. Diverse *Enterococci* species were isolated from a wide range of environmental habitats, including soil, environmental water, but most importantly, gastrointestinal tracts of various animals [89, 90]. Certain strains of *Enterococci* are employed as probiotic bacteria and can be found in a variety of dairy products, including milk and cheese [91–93]. Currently, *Enterococci* have gained attention as bacteria primarily responsible
for a variety of nosocomial infections [94, 95]. One of the major concerns regarding *Enterococci* is their high potential for antibiotic resistances [96–99].

### 1.4.1 *E. faecalis* and *E. faecium* as commensals

The human microbiome is highly diverse with approximately 5000 different known species of microorganism [100, 101]. However, a wide range of microbes and their specific function is unknown. *E. faecalis* and *E. faecium* are microbial commensals in the human or animal intestinal tract [87]. As members of the microbial gut community, they can be found in the intestines of healthy individuals. *E. faecalis* and *E. faecium* can play a role in various physiological processes, including glycerol-, citrate-, pyruvate- and carbohydrate-metabolism, especially in the fermentation of sugars [102–105]. Moreover, *Enterococci* have the ability to regulate pH levels. Furthermore, they possess the capability to synthesize essential vitamins or other metabolites that play crucial roles in maintaining regular physiological functions [106, 100]. These functions might also prevent the attachment and growth of other potential pathogenic bacteria and are thus considered as probiotic characteristics [107]. Beneficial effects on the host’s health and immunity were previously reported [108, 109]. However, concerns regarding the utilization of these bacteria as probiotics emerge due to their classification as important opportunistic pathogens.

### 1.4.2 *E. faecalis* and *E. faecium* pathogenicity

As natural inhabitants of the human gut, *E. faecalis* and *E. faecium* usually do not harm the host. However, a high number of severe and fatal cases of infections with these bacteria occur each year, especially cases of infective endocarditis as well as infections of the urinary tract [84, 110]. Particularly *E. faecium*, due to its acquired antibiotic resistance towards vancomycin and linezolid, is involved in causing lethal infections [96]. A significant number of infections with *Enterococci* are nosocomial, as they are able to survive and persist in a hospital environment. The enhanced capacity to endure nutrient-depleted environments is due to their inherent resilience to disinfectants, as well as their unusual ability to withstand UV radiation [111–113].
Typically, the duration of an infection spans approximately five to six weeks [114]. Transmission primarily occurs during surgery, through direct contact with healthcare workers or environmental surfaces [115]. The main risk factor for these nosocomial infections is treatment with antibiotics during the patient’s hospitalization [114]. Prevention strategies are hygiene, isolation of patients and resistance screening [116]. To successfully colonize the human body and manifest a disease, Enterococci must overcome multiple barriers. First, different host defense mechanisms such as other commensal microbes or bile acids must be overcome and replication must be possible. If the colonization is successful, Enterococci can damage cells by secreting different harmful substances and thus cause inflammation. E. faecalis for example can secrete hydrogen peroxide as well as superoxide, which can damage the DNA of epithelial cells in the colon [117]. Furthermore, an increased abundance of Enterococci has been correlated with Crohn’s disease, a form of inflammatory bowel disease characterized by chronic and persistent inflammation of the gastrointestinal tract [118, 119].

1.5 Bile acids - antimicrobial action

Bile is a complex mixture of water, electrolytes, organic compounds, and bile acids. Bile acids are bioactive molecules that play a crucial role in various physiological processes, including lipid digestion and absorption, cholesterol homeostasis, and the regulation of energy metabolism. They are synthesized in the liver from cholesterol, stored in the gallbladder and secreted into the bile, facilitating the emulsification and absorption of fats in the small intestine [120]. Apart from their role in lipid metabolism, bile acids also act as signaling molecules through activation of nuclear receptors, which regulate various cellular processes, including glucose and lipid metabolism [121, 122]. Moreover, bile acids have significant antimicrobial properties, influencing the composition and function of the gut microbiome but also pathogenic bacteria [123].

The composition of bile depends on several different factors including diet, general health and stress-level of the individual. However, the liver of an average man synthesizes and secretes approximately 0.5 g per day [123]. Roughly 95 % of the bile acids are recycled via resorption into the hepatocytes, while 5 % are excreted with feces [123].
Three major types of bile acids are part of the human bile acid pool: the primary bile acids cholic acid (CA) and chenodeoxycholic acid (CDCA), as well as the secondary bile acid deoxycholic acid (DCA). The composition of the human bile acid pool is estimated to consist of approximately 40% CA, 40% CDCA and 20% DCA [123]. While primary bile acids (CA, CDCA) are synthesized in the liver, secondary bile acids are products of different intestinal bacteria (Figure 2). One example of a bacterium that is capable of the necessary \( 7 \alpha \)-dehydroxylation is \( C. \) scindens, an inhabitant of the human gut, which 7-dehydroxylates CA to DCA [124]. Conjugation of primary or secondary bile acids with glycine and taurine takes place within the liver, where the bile acid molecules are modified through the covalent linkage of amino acids [125]. The presence of a broad spectrum of bile acids provides protection from diverse pathogenic bacteria such as \( C. \) difficile [126]. Therefore, bile acids are considered important endogenous antimicrobials.

**Figure 2:** Primary bile acids (cholic acid and chenodeoxycholic acid) are synthesized in the liver through the enzymatic conversion of cholesterol. Secondary bile acids (deoxycholic acid and lithocholic acid) are derived from primary bile acids through bacterial-mediated dehydroxylation reactions in the intestine. Additionally, conjugated bile acids are formed in the liver through the process of conjugation with glycine and taurine, where amino acids or other molecules are attached to the bile acid structure.
Most gastrointestinal bacteria exhibit bile acid resistance due to their permanent exposure to different concentrations of bile. Additionally, some pathogenic bacteria have acquired resistance mechanisms against bile acids, similar to antibiotic resistance mechanisms. For instance, \textit{C. jejuni} is resistant against high concentrations of most bile acids, including DCA. The major bile acid resistance factor of \textit{C. jejuni} is the resistance nodulation division (RND) type multidrug efflux CmeABC, which consists of three proteins; the membrane fusion protein CmeA, the inner membrane transporter CmeB and the outer membrane protein CmeC [127, 128]. Knockout mutants of CmeABC show strongly decreased resistance towards bile acids [127, 128], while overexpression of CmeABC by knockout of the CmeABC regulator CmeR results in increased resistance [129]. The development of these resistance mechanism provides a strong advantage in the gastrointestinal environment and contributes to the virulence of \textit{C. jejuni}.

1.6 Bacterial communication - a driving force in pathogenicity

One of the key factors contributing to the remarkable success of bacteria lies in their ability for inter-cellular communication. Therefore, bacteria utilize different mechanisms that enable inter- and intraspecific communication and allow for coordination of their activities, enhancing their adaptability and survival in various environments [130, 131]. Over the past few decades, research about bacterial communication, revealed its fundamental role in shaping microbial communities, host-microbe interactions, and ecological processes. The understanding of bacterial communication has unveiled a high diversity of signaling systems and regulatory networks across bacterial species. One of these communication mechanisms is quorum sensing, where bacteria release and detect small signaling molecules into their environment to coordinate gene expression and collective behavior based on population density [132, 133]. Another method for bacteria to communicate with each other is horizontal gene transfer, which is the exchange of genetic material, such as plasmids or transposons, to transfer features and acquire new capabilities [134, 135]. Furthermore, contact-dependent signaling is a strategy where bacteria directly interact with neighboring cells via physical contact, transferring signals and molecules through specialized structures like pili [136]. Moreover, specific secretion systems such as the Type III secretion system are specialized structures for the injection
of effector molecules directly into a host cell, leading to a modulation of host responses [137, 138]. Intercellular communication is also possible using extracellular vesicles, where bacteria release membrane-bound vesicles containing various signaling molecules, which can be taken up by neighboring cells to influence their behavior [139–141]. Another strategy for bacteria to communicate with each other is quinolone signaling, where certain species produce and respond to quinolone molecules, which can regulate gene expression [142].

These diverse communication strategies can enable advantages for pathogenic bacteria against their host and help to establish an infection[143].

Bacterial communication represents a broad area of research that continues to expand the knowledge of microbial behavior and interactions. To better understand these interactions, co-incubation experiments can be utilized.

1.7 Aims of the project

As one of the major causes for gastrointestinal infections worldwide, C. jejuni is an important pathogen with clinical relevance. When C. jejuni enters the human gastrointestinal tract, it is constantly exposed to numerous constituents, including bile acids, but also the presence of other bacteria. The resistance mechanisms of C. jejuni towards bile acid were previously described [128], including a study on proteomic responses to DCA [144]. However, the current data do not provide comprehensive proteomic studies on co-incubation scenarios in C. jejuni.

Furthermore, the genomic and transcriptomic reactions of Enterococci towards bile acids were reported previously, while their proteomic changes have not been investigated so far [145, 146]. In order to characterize the proteomic alterations of C. jejuni, E. faecalis, and E. faecium with regard to bile acid resistance, different experimental approaches were designed and the thesis was separated into four distinct projects. Overall, this thesis contributes to the understanding of the effects of co-incubation and potential interaction of bacterial species specifically concerning long- and short-term bile acid exposure on a proteomic level.
Specifically, the four major projects were:

I. Co-incubation proteome: This study was designed to investigate the alterations occurring in *C. jejuni* as a result of co-incubation with *E. faecalis*, *E. faecium* and *S. aureus*. Through comparative statistical analyses, the differential proteomic response induced by co-incubation and compared to the response triggered by DCA was analyzed.

II. *Enterococci* under bile acid stress: The aim was to investigate the proteomic profiles of *Enterococci* (specifically *E. faecalis* and *E. faecium*) after long-term exposure to different bile acids, mimicking the bile acid concentrations in the gallbladder. This approach aimed to replicate the environmental conditions experienced by *Enterococci* during gallbladder infections and their proteomic response to it. CA, CDCA and DCA were used and compared the susceptibility towards each bile acid as well as the differences and similarities in the stress response between the bile acids but also between the closely related bacterial species.

An additional aim of this study was the investigation of differences in the *E. faecalis* proteome after incubation in aerobic and microaerophilic conditions. Furthermore, the proteomic response in both conditions with DCA was examined and compared, aiming to find a potential influence of oxygen on the bile acid resistance.

III. Proteomic changes in knockout-mutants of bile acid protection related genes: Lastly, the objective was to explore the proteomic alterations following the inactivation of specific genes associated with bile acid protection in *C. jejuni*. To achieve this, knockout mutants targeting the genes *cmeB*, *cbrR*, and *cmeR* were generated, and assessed their susceptibility towards CA and DCA. Comparative proteomic analyses between the knockout mutants and the wildtype strain were employed to show differences in protein expression in the mutants lacking the respective genes. Additionally, the proteomic response after bile acid exposure was analyzed.

IV. Through previous observations, a potential mediation of bile acid resistance from *C. jejuni* towards other bacterial species was noted. Regarding the hypothesis that a
protein present in the supernatant may play a role in facilitating the induction of bile acid resistance, proteomic analyses of co-incubation approaches was conducted. The putative factor responsible for this phenomenon has been designated as the ProBAS (Protection from bile acid stress) factor. The aim of this project was to identify proteins that potentially mediate bile acid resistance to other bacteria to confirm the effect in future experiments.
2 Manuscript I: Characterization of *Campylobacter jejuni* Proteome Profiles in Co-Incubation Scenarios

The manuscript is currently under review in the journal Frontiers in Microbiology, Food Microbiology (August 25, 2023).

Author contribution:

Annika Dreyer planned, optimized and performed cultivation of the bacteria, co-incubation experiments, growth experiments and protein isolation and processing, except for the DIA-MS measurements. Additionally, she did the data analysis using Python3, Perseus and Excel. Furthermore, Annika Dreyer prepared all figures and tables and the supplementary material. Moreover, she wrote the draft of the manuscript, excluding the chapter DIA-MS in the Material and Methods section, which was provided by Dr. Christof Lenz. The format of the manuscript equals the format of the version submitted to Frontiers in Microbiology, Food Microbiology and may differ from the version that will finally be published (Date of submission: June 25, 2023).

Supplementary files for this manuscript version are available on a data drive and will be accessible online after publication.
Characterization of *Campylobacter jejuni* Proteome Profiles in Co-Incubation Scenarios

Authors: Annika Dreyer¹, Christof Lenz²,³, Uwe Groß¹, Wolfgang Bohne¹,‡, Andreas Erich Zautner¹,⁴,⁵,‡

¹: Institute for Medical Microbiology and Virology, University Medical Center Göttingen, Göttingen, Germany.

annika.dreyer@med.uni-goettingen.de

²: Bioanalytical Mass Spectrometry Group, Max Planck Institute for Multidisciplinary Sciences, Göttingen, Germany.

⁴: Department of Clinical Chemistry, University Medical Center Göttingen, Göttingen, Germany.

⁵: Institute of Medical Microbiology and Hospital Hygiene, Medical Faculty, Otto-von-Guericke University Magdeburg, Magdeburg, Germany.

†: These authors contributed equally to this work

* Correspondence:
Andreas Zautner
azautne@gwdg.de

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Abstract

In dynamic microbial ecosystems, bacterial communication is a relevant mechanism for interactions between different microbial species. When *C. jejuni* resides in the intestine of either avian or human hosts, it is exposed to diverse bacteria from the microbiome. This study aimed to reveal the influence of co-incubation with *Enterococcus faecalis*, *Enterococcus faecium*, or *Staphylococcus aureus* on the proteome of *C. jejuni* 81-176 using data-independent-acquisition mass spectrometry (DIA-MS). We compared the proteome profiles during co-incubation with the proteome profile in response to the bile acid deoxycholate (DCA) and investigated the impact of DCA on proteomic changes during co-incubation, as *C. jejuni* is exposed to both factors during colonization. We identified 1375 proteins by DIA-MS, which is notably high, approaching the theoretical maximum of 1645 proteins. *S. aureus* had the highest impact on the proteome of *C. jejuni* with 215 up-regulated and 230 down-regulated proteins. However, these numbers are still markedly lower than the 526 up-regulated and 516 down-regulated proteins in the DCA condition.
regulated proteins during DCA exposure. We identified a subset of 54 significantly differentially expressed proteins that are shared after co-incubation with all three microbial species. These proteins were indicative of a common co-incubation response of C. jejuni. This common proteomic response partly overlapped with the DCA response; however, several proteins were specific to the co-incubation response. In the co-incubation experiment, we identified three membrane-interactive proteins among the top 20 up-regulated proteins. This finding suggests that the presence of other bacteria may contribute to increased virulence in the environment. Furthermore, a conjugative transfer regulon protein was typically expressed during co-incubation. Exposure to both co-incubation and DCA triggers showed that the two stressors had an impact on each other, leading to a distinct synergistic proteomic response that differed from the response to each stimulus individually.

Contribution to the field

To date, there have been no proteomic studies on co-incubation of Campylobacter jejuni with other bacteria. The primary aim of this study was to investigate the proteomic profiles of C. jejuni in co-incubation with the Gram-positive bacteria Enterococcus faecalis, Enterococcus faecium, and Staphylococcus aureus that are part of the avian and human intestinal host microbiome, and furthermore the proteomic changes with regard to additional bile acid exposure. Using data-independent-acquisition mass spectrometry (DIA-MS), we identified 1375 proteins of C. jejuni representing 83.5% of the theoretical proteome and demonstrated a unique yet distinct interaction profile between C. jejuni and the other bacteria via membrane-interactive proteins. This suggests that other bacteria contribute to increased virulence in the environment, and conjugative transfer (via pili/plasma bridges) may play a role during co-incubation. We discovered a significant similarity between C. jejuni’s protein-level reaction when co-incubated with Gram-positive bacteria and bile acids, and its response when grown alone with bile acid. However, we identified a unique response when both triggers were present in parallel, highlighting the complexity of cellular interactions and their potential role in C. jejuni proteomic response pathways under specific conditions. This finding enables future research in the field of proteomic analyses under different influences.

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Ethics statements

Studies involving animal subjects

Generated Statement: No animal studies are presented in this manuscript.

Studies involving human subjects

Generated Statement: No human studies are presented in this manuscript.

Inclusion of identifiable human data

Generated Statement: No potentially identifiable human images or data is presented in this study.
Data availability statement

Generated Statement: The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/supplementary material.
Introduction

*Campylobacter jejuni* belongs to the most frequently diagnosed bacterial gastrointestinal pathogens in humans worldwide (Acheson and Allos, 2001). In the developed world, foodborne infections most commonly occur after consumption of cross-contaminated food, prepared in parallel with poultry meat, whereas *Campylobacter* spp. belong to the natural commensal microbiome in poultry (Skirrow, n.d.). Additional sources for infections are water, raw milk or other livestock animals (Blaser et al., 1983, 1980; Szewzyk et al., 2000). Symptoms of campylobacteriosis include severe bloody diarrhea, fever, abdominal cramps and nausea. Furthermore, *Campylobacter* infections are associated with severe follow-up diseases, for example the Guillain-Barré syndrome, a neural disease that can lead to paralyses and damage of the nervous system (Rees and Hughes, 1995; Sejvar et al., 2011).

The ideal growth temperature for the Gram-negative, helical-shaped and microaerophilic bacterium lies between 37 °C and 42 °C. Due to its broad spectrum of virulence factors that enable the survival in varying environmental conditions, *C. jejuni* can successfully colonize the gut. One of these virulence factors is the ability to survive high concentrations of bile acid in the human or animal gut. Among the diverse functions of bile is the solubilization and emulsification of fat, which makes it an important biological detergent (Begley et al., 2005; Chiang, 2017). Under the exposition of bile acids, the composition of fatty acids and phospholipids of the bacterial cell membranes are altered, which leads to instabilities in the cell’s surface and consequently to the disruption of the cell (Taranto et al., 2003). Furthermore, DNA damages might be induced by the presence of bile acid in different bacteria, such as *E. coli* (Begley et al., 2005; Kandell and Bernstein, 1991). To overcome this stress, bacterial gut inhabitants have developed several mechanisms to cope with bile acid and are able to tolerate varying concentrations of bile.

Co-incubation can have several important positive or negative effects on the growth of different bacteria, however, proteomic studies on co-incubation remain rare. A proteomic study by García-Pérez et al. (2017) has shown that co-incubation can reduce the number of extracellular proteins in microbial communities in wounds (García-Pérez et al., 2018). In addition, co-incubation of different bacteria with yeasts, such as *C. albicans*, has shown positive effects on the growth of both species, probably due to the release of nutrients into the medium or beneficial changes in pH (Ellepola et al., 2019). During co-incubation with other bacteria, *C. jejuni* has been shown to interact with a variety of other bacteria, for instance *Bifidobacterium longum* which prevents the adherence of *C. jejuni* to intestinal tract cells (Quinn et al., 2020b, 2020a). A combination of different bacteria that include *E. faecium* can lead to a decrease of *C. jejuni* in the gastro-intestinal tract of poultry (Neveling and Dicks, 2021). Anis et al. (2022) showed that studying the co-incubation of *C. jejuni* with other bacteria might be an interesting topic, as the bacterial interaction might enhance *C. jejuni* survival when exposed to external stresses, such as the presence of oxygen (Anis et al., 2022).

In this study, we aimed to observe the impact of co-incubation on the *C. jejuni* proteome and the possible effects of co-incubation on the bile acid response of the bacterium. Therefore, we analyzed the proteome of *C. jejuni* in co-incubation and under deoxycholate (DCA) stress. DCA is a secondary bile acid, which is a product of dehydroxylation by gut microbiota and has been shown to have inhibiting effects on the growth of *C. jejuni* and other bacteria at a certain concentration (Lertpiriyapong et al., 2012; Vidal et al., 2021) and furthermore substantial effects on the proteome (Masanta et al., 2019). The bacteria chosen for co-incubation were less resistant towards DCA than *C. jejuni*.
One of the bacteria chosen for the co-incubation study was *E. faecalis*, a Gram-positive, facultative anaerobic coccal opportunistic pathogen that belongs to the human commensal microbiome, but can also be found in environmental samples (Fiore et al., 2019), (Lebreton et al., n.d.), (Van Tyne and Gilmore, 2014). Furthermore, we tested a close relative of *E. faecalis*, *E. faecium*, which is also an opportunistic pathogen of global importance due to its high antibiotic resistance potential (Lopes et al., 2006), (Gorrie et al., 2019). The third bacterium used in this study was *Staphylococcus aureus*, another Gram-positive opportunistic pathogen of high clinical relevance due to the high number of severe infections caused by multidrug resistant *S. aureus* (Cheung et al., 2021; Klevens et al., n.d.; Rasigade et al., 2014).

This work aims to provide a deeper look at the co-incubation proteome of the pathogen *C. jejuni* with other bacteria that are usually present in the human body and the respective proteomic changes in presence of DCA. We used data-independent acquisition mass spectrometry (DIA-MS) to systematically compare the proteomic changes in co-incubation of the different bacteria with *C. jejuni* as well as the proteomic response to bile acid (DCA). This technique enables the quantitative analysis of every detectable compound in a sample of proteins and thus provides high reliability in the quantitative results (Huang et al., 2015). To our knowledge, this is the first proteomic co-incubation study on *C. jejuni*.

**Material and methods**

**Bacterial strains and growth conditions**

*Campylobacter jejuni* wildtype strain 81-176 was used for all described experiments. *C. jejuni* was grown overnight on CAM-agar plates from Biomérieux (Marcy-l’Étoile, France) at 42 °C. Mueller-Hinton (MH) broth served as liquid medium at 37 °C. To generate a microaerophilic environment, the Gas Pak™ EZ Campy Container System by BD (Franklin Lakes, NJ, USA) and an anaerobic jar for incubation were used.

*Enterococcus faecalis* strain 700802 (V583), *Enterococcus faecium* TX0016 (ATCC BAA-472) and *Staphylococcus aureus* strain NCTC 8325 (PS 47) were used for co-incubation experiments and grown overnight on Columbia agar plates from Biomérieux (Marcy-l’Étoile, France).

**Co-incubation**

For co-incubation experiments, the optical density at 600 nm (OD<sub>600</sub>) of *C. jejuni* was set to 0.5 and the OD<sub>600</sub> of the respective other bacterium was set to 0.1. Incubation was performed in phosphate buffered saline (PBS) to avoid effects of the medium on the bile acid resistance. DCA was added to the medium at a concentration of 0.1 % for *E. faecalis* and *E. faecium* and 0.075 for *S. aureus*. These concentrations usually lead to death of the Gram-positive bacteria. Incubation was carried out for 3 h at 37 °C and shaking at 150 rpm. After three hours, a spot assay on Müller-Hinton plates was done to show the survival of the bacteria after 3 h in a dilution series. Subsequently, protein extraction was done.
The Gram-positive bacteria without presence of *C. jejuni* served as positive control while the approaches of Gram-positive bacteria with the respective amount of DCA served as negative control. All samples were prepared in biological triplicates.

Protein extraction from pellet

Cultures were centrifuged at 4,000 rpm for 10 minutes at 4 °C. For protein-extraction from the pellet, the supernatant was discarded. For samples containing *C. jejuni*, pellets were resuspended in 2 mL 0.9 % saline and kept on ice over the procedure. Subsequently, the Gram-negative cells were disrupted via sonification using a Branson sonifier 250 from Branson ultrasonics (Brookfield, Connecticut, USA) with the following settings: output control = 3, duty cycler = 30 %. The sonification process was performed five times for 30 seconds followed by 30 seconds of cooling to avoid overheating of the proteins. Afterwards, the Gram-positive cells were disrupted using 0.75 g of 4 mm glass beads that were added to the samples and were subsequently treated in a “Fast prep 96 Homogenizer” (MP Biomedicals Germany GmbH, Schwerne, Germany) for 2 x 20 seconds, followed by centrifugation at 5500 g for one minute. The supernatant was then removed and samples were centrifuged at 13,500 xg for 10 minutes at 4 °C in a tabletop centrifuge. Finally, the supernatant was used for a Pierce assay, that was performed to determine the protein concentration of all samples. After this, the concentrations were adjusted to 1 µg/µL for DIA-MS analysis. For all samples, biological triplicates were prepared.

DIA-MS

Protein samples were loaded onto a 4-12 % NuPAGE Novex Bis-Tris Minigels (Invitrogen) and run into the gel for 1.5 cm. Following Coomassie staining, the protein areas were cut out, diced, and subjected to reduction with dithiothreitol, alkylation with iodoacetamide and finally overnight digestion with trypsin was performed. Tryptic peptides were extracted from the gel, the solution dried in a Speedvac and kept at -20°C for further analysis.

Protein digests were analyzed on a nanoflow chromatography system (nanoElute) hyphenated to a hybrid timed ion mobilityquadrupole-time of flight mass spectrometer (timsTOF Pro, all Bruker). In brief, 250 ng equivalents of peptides were dissolved in loading buffer (2 % acetonitrile, 0.1 % trifluoroacetic acid in water), enriched on a reversed-phase C18 trapping column (0.3 cm × 300 µm, Thermo Fisher Scientific) and separated on a reversed phase C18 column with an integrated CaptiveSpray Emitter (Aurora 25 cm × 75 µm, IonOpticks) using a 50 min linear gradient of 5-35 % acetonitrile / 0.1 % formic acid (v:v) at 250 nl min⁻¹, and a column temperature of 50°C. For identification, representative samples were analysed in PASEF acquisition mode using default manufacturer’s settings [n=12; (Meier et al., 2018)]. For identification and quantification samples were analysed in diaPASEF mode using a customized 16x2 window acquisition scheme (Meier et al., 2020, Skowronek et al., 2022). For each biological replicate, three technical replicates were performed in diaPASEF mode for quantitation.

The data processing was performed using the Spectronaut v16.0.220606.53000 software package (Biognosys AG, Schlieren, Switzerland). Identification of proteins as well as hybrid spectral library generation from 12x2 DDA acquisitions and 12x2 DIA acquisitions experiments were done using the Pulsar search engine against UniProtKB *C. jejuni* 81-176, *E. faecalis* 700802, *E. faecium* TX0016 and *S. aureus* NCTC 8325 proteomes using the default parameters. The False Discovery Rate (FDR) was set to 1% on the spectral, peptide and protein group levels for all samples. DIA quantification was
performed with up to 6 fragments per peptide and up to 10 peptides per protein. A dynamic retention
time alignment was done, as well as dynamic mass recalibration and quartile normalization, for the 1%
FDR. Imputation of global data was executed for the final results table.

Data processing

Perseus v1.6.2.2 was used for the statistical analysis and for generation of volcano plots to compare
the different samples (Storey and Tibshirani, 2003; Tyanova et al., 2016). As significant regulation
level, two-fold up- or down-expression was chosen. Proteins present in 80% of the samples were
considered for further analysis. For volcano-plot generation in Perseus, a t-test was chosen with a
number of randomizations = 250 and a FDR of 0.05 (Storey and Tibshirani, 2003). All proteins that
are described in the following as up- or down-expressed were significantly regulated, if not otherwise
stated.

COG-categories were assigned to the proteins using the online-tool eggNOGmapper v 2.18
(Cantalapiedra et al., n.d.; Huerta-Cepas et al., 2019, 2017). To identify commonly expressed proteins,
Venn diagrams were generated utilizing InteractiVenn (Heberle et al., 2015). All Plots were generated
using matplotlib in python3 (Van Rossum and Drake, 1995).

Results + Discussion

Identification of C. jejuni proteins that are commonly regulated during co-incubation with
different Gram-positive bacteria

The interbacterial communication between Campylobacter jejuni and other bacterial species remains
poorly explored to date, lacking comprehensive investigation. Our research is aimed to investigate
mechanisms of this cross-talk and its potential implications in various ecological and pathogenic
contexts.

We hypothesized that co-incubation of C. jejuni with other bacterial species triggers a proteomic
response in C. jejuni. Three different Gram-positive species were chosen for co-incubation with
C. jejuni, namely E. faecalis, E. faecium and S. aureus, which are all putative inhabitants of the human
gut microbiome. The bacteria were incubated for three hours at 37 °C in PBS, without nutrient supply
since we were not interested in responses due to different degrees of nutrient competition (see scheme
of the workflow (Figure 1). Instead, we aimed to target responses resulting from direct bacterial contact
or from interactions with secreted molecules. Using volcano-plots generated from DIA-MS data, we
compared the proteome of C. jejuni in monoculture with each of the three bacteria with C. jejuni in co-
incubations.

Co-incubation resulted in all cases in an altered proteomic profile, whose dimension depends on the
species used for co-incubation. With S. aureus, the changes in the proteomic profile exhibited the
highest intensity with 445 differentially regulated proteins.
It is well known that *S. aureus* on the one hand produces several toxins and hemolysins that might act against other bacteria (Otto, 2014; Shinefield, 1963). On the other hand, *S. aureus* can also secrete beneficial substances for other microorganisms and co-exist in polymicrobial communities, which can be advantageous for infections (García-Pérez et al., 2018; Karki et al., 2021; Nguyen and Oglesby-Sherrouse, 2016). These characteristics of *S. aureus* might contribute to the increased number of differentially expressed proteins in the co-incubation with *C. jejuni*.

In the co-incubation assay with *E. faecium*, 405 proteins were differentially expressed and in the assay with *E. faecalis*, 241 proteins were differentially regulated. The ratio of up-expressed and down-expressed proteins also varied specifically.

Among the differentially expressed proteins, 54 were commonly up-expressed in all three samples and 100 proteins were commonly down-expressed (Figure 2). The distribution of COG-categories differs between up-expressed and down-expressed proteins (Figure 3). Down-expressed proteins are characterized by a higher proportion of the categories C (Energy production and conversion), E (Amino Acid metabolism and transport), F (Nucleotide metabolism and transport), I (Lipid metabolism) and G (Carbohydrate metabolism and transport). In contrast, up-expressed proteins are characterized by a higher proportion of the categories J (Translation), L (Replication and repair), M (Cell wall/membrane/envelope biogenesis) and T (Signal Transduction).

The differentially expressed proteins in all approaches were sorted according to their difference expression level. We compared the top 20 up- and down-expressed proteins of each co-incubation proteome (See supplemental Excel file), in order to identify commonly regulated proteins with a high degree of regulation. Four commonly up-expressed proteins were found in the top 20 up-expressed proteins: Hemolysin A (A0A0H3PEK7_CAMJJ), a DNA/RNA non-specific endonuclease (A0A0H3PJE6_CAMJJ), a putative lipoprotein (A0A0H3PA71_CAMJJ), and a putative membrane protein (A0A0H3PDB2_CAMJJ).

Among the top20 up-expressed proteins in co-incubation were three membrane-interactive proteins, which might indicate an enhanced virulence in the environment due to contact with other bacteria.

Moreover, four commonly down-expressed proteins were found in the top 20 down-expressed proteins, namely a Translation initiation factor IF-3 (IF3_CAMJJ), a DNA-directed RNA polymerase subunit omega (RPOZ_CAMJJ), an ATP synthase subunit beta (ATPB_CAMJJ) and a 6,7-dimethyl-8-ribityllumazine synthase (RISB_CAMJJ).

In addition to cellular conjugation, some *Campylobacter* strains possess the capability to employ a type 6 secretion system which can be used for communication with their surrounding environment but also other bacteria (Chen et al., 2015; Gallique et al., 2017). However, *C. jejuni* strain 81-176 does not harbor a type 6 secretion system (Liaw et al., 2019), which implies the utilization of alternative mechanisms for bacterial communication – probably using conjugation. However, other *C. jejuni* strains, for example strain 488, 43431 or RC039 utilize a type 6 secretion system (Liaw et al., 2019), indicating that cross-talk via type-6 secretion system-dependent protein secretion would be possible in some *C. jejuni* strains.
The co-incubation response and the bile acid stress response partly overlap

In order to identify proteins that are specifically regulated during co-incubation, we compared the changes in the proteomic profile after co-incubation with the stress response during incubation with bile acids, which was previously shown to trigger a strong proteomic stress response in C. jejuni (Masanta et al., 2019). After 3 h incubation with 0.1 % DCA, a substantial proportion of C. jejuni proteins were differentially expressed (Figure 6). A total of 526 proteins were identified among the up-expressed proteins, which is ~10-fold more than the 54 up-expressed proteins during co-incubation with Gram-positive bacteria. Likewise, 516 proteins were down-expressed after DCA incubation, which is ~5-fold more than the number during co-incubation with Gram-positive bacteria.

This leads to the assumption that the exposure to DCA provokes a significantly more pronounced proteomic response compared to the co-incubation scenarios.

Venn diagrams show the overlapping proteins between both approaches (Figure 4 and Suppl. Figures 2 & 3). Out of the 54 commonly up-expressed proteins during co-incubation, 36 proteins were also found in C. jejuni monoculture with DCA. This indicates that only the 18 remaining proteins are specific for co-incubation (see suppl. Table 1). Moreover, from the 516 down-expressed proteins in C. jejuni in presence of DCA, 78 were shared with the 100 down-expressed proteins in the co-incubation approach (Figure 4), indicating that the 22 remaining proteins are specifically down-expressed in co-incubation (see suppl. Table 6).

The pattern of the COG categories of differentially proteins in the monoculture approach with DCA differs from commonly expressed proteins in co-incubation (Figure 5, 7 & 8). The percentage of up-expressed proteins assigned to the categories J (Translation), L (Replication and repair) and T (Signal transduction) is higher in the co-incubation proteome, while categories C (Energy production and conversion), G (Carbohydrate metabolism and transport), M (Cell wall/ membrane / envelope / biogenesis) and V (Defense mechanisms) are more resent in the monoculture of C. jejuni and DCA. Categories C, E, F and J are more down-expressed in the co-incubation approach.

In C. jejuni the most relevant mechanism to survive bile acid stress is the CmeABC multidrug efflux, resistance nodulation-division (RND) type multidrug efflux (Lin et al., 2003). CmeABC consists of a three-gene operon encoding for a membrane fusion protein - CmeA, the efflux pump membrane transporter - CmeB and CmeC, which is the outer membrane lipoprotein (Lin et al., 2002). Knockout mutants of these genes led to significant loss of bile acid resistance (Lin et al., 2003). In a proteomic study, Masanta et al. showed that the proteins belonging to the CmeABC multidrug efflux pump were up-expressed under bile acid stress exposure (Masanta et al., 2019). Thus, the presence of CmeA, B or C in all our samples with DCA served as indicator that the proteome under bile acid stress is depicted. In the co-incubation approach without DCA, none of the the CmeABC proteins was detected (suppl. Table 2).

Among the 22 specifically down-expressed proteins during co-incubation are mostly general metabolic proteins. In the 18 commonly up-expressed proteins during co-incubation, we found proteins that might play a role in the interaction between C. jejuni with other bacteria. For example, a Conjugative transfer regulon protein (Q9KIR9_CAMJJ) was detected among the up-expressed proteins in all three samples. The presence of this protein indicates that horizontal gene transfer may be occurring between these bacteria, whereby genetic material is exchanged between different species (Llosa et al., 2002). This mechanism of genetic exchange could allow for the acquisition of novel genetic traits, such as antibiotic
resistance or other beneficial genes and indicates a potential for cross-communication between bacteria.

Additionally, a chaperone protein DnaJ was found among these proteins (DNAJ_CAMJ), indicating an active response towards stress. DnaJ and related Hsp proteins are highly conserved among species and play a role in diverse processes such as folding and unfolding of proteins, translation and ATPase activity of specific chaperones (Qiu et al., 2006). This indicates that the bacteria might be stressed by either the presence of other bacteria or the absence of nutrients.

**Co-incubation of C. jejuni with Gram-positive bacteria in the presence of bile acids triggers a unique proteomic response different from the single stimuli**

We also studied the proteomic response in the presence of both triggers, DCA plus co-incubation with Gram-positive bacteria. This should reveal the relative influence of the individual triggers on the common response. Among the 18 up-expressed proteins that were specific to co-incubation, only two were up-expressed in the approach of co-incubation with DCA (Figure 4). These proteins were a Histidine kinase (A0A0H3PE96_CAMJ) and a tRNA modification GTPase MnmE (MNME_CAMJ) (Suppl. Table 1). Furthermore, from 22 down-expressed proteins that were specific for co-incubation, only four proteins remained down-expressed when DCA was added. The limited number of commonly regulated proteins in co-incubation with and without DCA indicates that DCA seems to suppress the specific co-incubation response to a large extent.

Comparing the co-incubation plus DCA approach to the monoculture of C. jejuni with DCA, 185 proteins occurred commonly among the up-expressed candidates, which corresponded to ~37.8% of the 490 proteins that were up-expressed in the monoculture with DCA, excluding the 36 proteins, that also occurred in co-incubation without DCA (Figure 4). This lead to the assumption that the additional trigger of co-incubation might also inhibit the expression of a certain amount of the DCA response specific proteins in C. jejuni. Moreover, 77 proteins were uniquely down-expressed in the approach of co-incubation plus DCA (Figure 4), while 196 of the 277 down-expressed proteins in this approach were shared with the C. jejuni monoculture with DCA.

The proteomes in co-incubation with and without DCA exhibit significant dissimilarities. In total, 152 proteins were found to be specifically up-expressed when both triggers, co-incubation plus DCA, are present. Due to the fact that these 152 proteins occurred only in the approach co-incubation plus DCA, and were not a combination of both triggers, it can be assumed, that the proteomic response in presence of both, DCA and another bacterium possesses a unique character.

Moreover, the respective COG-categories were assigned to these 152 proteins (Figure 7). Compared to the monoculture proteome with DCA, the categories M (Cell wall / membrane envelop / biogenesis), P (inorganic ion transport and metabolism) and U (Intracellular trafficking) were increased in co-incubation with DCA. A detailed analysis of these 152 proteins revealed a high number of ABC-transporter associated proteins, proteins related to antibiotic resistance, efflux and transport proteins and general membrane proteins (suppl. Table 5).

Furthermore, the COG categories of the 77 proteins commonly exclusively down-expressed in the approach of co-incubation with DCA were determined. When compared to the 516 down-expressed proteins in C. jejuni with DCA and the 277 commonly down-expressed proteins in co-incubation with
DCA, the pattern of the 77 proteins shows similarities but also differences (Figure 8). An increase of 385 proteins belonging to the category E (Amino acid metabolism and transport) was observed and a decrease of proteins belonging to the category J (translation) was observed when compared to the other samples.

**Conclusion**

In summary, our investigation highlights the proteomic response of *C. jejuni* to co-incubation as well as bile acid stress. We cover a high percentage of the total proteome of *C. jejuni* in our DIA-MS analysis, which demonstrates a small but distinct interaction potential between *C. jejuni* and the other bacteria via membrane-interactive proteins, indicating that the other bacteria contribute to an increased virulence in the environment. Also, conjugative transfer seems to play a role during the co-incubation. We also report a remarkable overlap between the proteomic response of *C. jejuni* in co-incubation in presence of DCA and the approach of *C. jejuni* monoculture with bile acid.

However, we were able to identify a unique response when both triggers (co-incubation and DCA) are present. This distinct response highlights the complexity of cellular interactions and shows the potential role of *C. jejuni* in proteomic response pathways under these specific conditions and enables future research in the field of proteomic analyses under different influences.

**Limitations of the study**

A limitation of this study is the difficulty in undertaking additional research in this experimental setup due to the labor-intensive nature of DIA-MS analysis. Additionally, the use of a single strain (81-176) limits the generalizability of the findings to other *Campylobacter* strains. Since our focus in this study was primarily on the *C. jejuni* proteome, there is a lack of comprehensive analysis regarding the proteomic responses of the other bacteria involved in the co-incubation. Future research should aim to explore this aspect to provide a more holistic understanding of the interactions and proteomic dynamics within the complex co-incubation system.
Figure 1: Scheme of the workflow: 8 different approaches of mono- or co-incubation were prepared and incubated for 3 h at 150 rpm and 37°C. Subsequently, the approaches were centrifuged, and proteins were extracted, followed by acetone precipitation. DIA-MS was performed and the data analysis including statistical analysis was done afterwards.

Figure 2: Venn diagrams of the commonly up- and down-expressed proteins of in *C. jejuni* co-incubation in the pellet with *E. faecalis*, *E. faecium* and *S. aureus*. 54 proteins are commonly up-expressed in all three co-incubation approaches while 100 proteins are commonly down-expressed.

Figure 3: COG-categories of the 54 commonly up-expressed and 100 commonly down-expressed proteins in all three co-incubation approaches. Samples were normalized. The different colors in the stacked bar plot represent the percentual distribution of the COG-categories.

Figure 4: Venn diagram that show the comparison of the commonly up-expressed proteins (left) of *C. jejuni* in co-incubation with *E. faecalis*, *E. faecium* and *S. aureus* with and without DCA and the up-expressed proteins of *C. jejuni* with DCA in monoculture. We detected 152 proteins that occur specifically in co-incubation with DCA and not in the other approaches. The down expressed proteins are shown at the right. The red boxes highlight proteins that are specifically and unique expressed in the approach of co-incubation and DCA.

Figure 5: Stacked bar plots of the up- and down-expressed proteins of *C. jejuni* in co-incubation with DCA. In total, 343 up-expressed and 277 down-expressed proteins of the pellet were assigned to their respective COG categories. The different colors in the stacked bar plot represent the percentual distribution of the COG-categories.

Figure 6: Venn diagrams of the commonly up- and down-expressed proteins of *C. jejuni* in co-incubation in the pellet with *E. faecalis*, *E. faecium* and *S. aureus* after the addition of DCA. 343 proteins are commonly up-expressed in all three co-incubation approaches while 277 proteins are commonly down-expressed.

Figure 7: COG categories of the up-expressed proteins in the *C. jejuni* mono-culture approach with DCA, the commonly expressed proteins in co-incubation with DCA and the unique up-expressed proteins of the co-cultivation approach. For comparison, the approach of the 54 up-expressed proteins in co-incubation without DCA is depicted on the right.

Figure 8: COG categories of the down-expressed proteins in the *C. jejuni* mono-culture approach with DCA, the commonly expressed proteins in co-incubation with DCA and the unique up-expressed proteins of the co-cultivation approach. For comparison, the approach of the 100 down-expressed proteins in co-incubation without DCA is depicted on the right.
Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Author Contributions


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References


Data Availability Statement
The datasets for this study can be found in the PRIDE databank. Upload is still pending and will be handed in as soon as possible.

Supplementary Material
Supplementary Material including Suppl. Table 1 - 7 & Suppl. Figure 1 - 4 Supplementary excel file including proteomic raw data
**Figure 1**

- **C. jejuni**
- **C. jejuni + DCA**
- **C. jejuni & E. faecalis**
- **C. jejuni & E. faecalis + DCA**
- **C. jejuni & E. faecium**
- **C. jejuni & E. faecium + DCA**
- **C. jejuni & S. aureus**
- **C. jejuni & S. aureus + DCA**

**3 h incubation**

37°C in PBS

- shaking (150 rpm)
- microaerophilic

- centrifugation
- protein extraction
- acetone precipitation
- DIA-MS
- statistical analyses
Figure 2

Up-expressed

- C. jejuni & E. faecalis (79)
- C. jejuni & E. faecium (235)
- C. jejuni & S. aureus (215)

- 54
- 12
- 3
- 59

Down-expressed

- C. jejuni & E. faecalis (162)
- C. jejuni & E. faecium (176)
- C. jejuni & S. aureus (236)

- 100
- 29
- 23
- 59
Figure 3
Figure 4

Up-expressed

- Co-incubation (54)
- C. jejuni + DCA (525)
- Co-inubation + DCA (343)

16
32
4
2
185
305

152

Down-expressed

- Co-incubation (100)
- C. jejuni + DCA (516)
- Co-inubation + DCA (277)

16
32
4
2
185
164

77
Figure 6

Up-expressed

C. jejuni & E. faecalis + DCA (506)

C. jejuni & E. faecium + DCA (406)

36

7

8

343

120

48

C. jejuni & S. aureus + DCA (652)

Down-expressed

C. jejuni & E. faecalis + DCA (302)

C. jejuni & E. faecium + DCA (554)

5

14

158

277

6

40

155

C. jejuni & S. aureus + DCA (486)
Figure 7
3 Manuscript II: Comparative analysis of proteomic adaptations in *Enterococcus faecalis* and *Enterococcus faecium* after long term bile acid exposure

The manuscript is currently under review in the journal BMC Microbiology (August 25, 2023).

Author contribution:

Annika Dreyer planned, optimized and performed cultivation of the bacteria, growth experiments, growth curve acquisition as well as protein isolation and processing, except for the DIA-MS measurements. In addition, she performed the data analysis using Python3, Perseus and Excel. Furthermore, Annika Dreyer prepared all figures and tables and the supplementary material. Additionally, she wrote the draft of the manuscript, except for the chapter DIA-MS in the Material and Methods section, which was friendly provided by Dr. Christof Lenz. The format of the manuscript equals the format of the version submitted to BMC Microbiology and may differ from the version that will finally be published (Date of submission: May 30, 2023).

Supplementary files for this manuscript version are available on a data drive and will be accessible online after publication.
Comparative analysis of proteomic adaptations in *Enterococcus faecalis* and *Enterococcus faecium* after long term bile acid exposure

Authors: Annika Dreyer¹, Christof Lenz²,³, Uwe Groß¹, Wolfgang Bohne¹,†, Andreas Erich Zautner¹,4,5,†,*

¹ Institute for Medical Microbiology and Virology, University Medical Center Göttingen, Göttingen, Germany.
² Bioanalytical Mass Spectrometry Group, Max Planck Institute for Multidisciplinary Sciences, Göttingen, Germany.
³ Department of Clinical Chemistry, University Medical Center Göttingen, Göttingen, Germany.
⁴ Institute of Medical Microbiology and Hospital Hygiene, Medical Faculty, Otto-von-Guericke University Magdeburg, Magdeburg, Germany.
⁵ Center for Health and Medical Prevention (CHaMP), Otto-von-Guericke University Magdeburg, Magdeburg, Germany.
† These authors contributed equally to this work
* Correspondence: Andreas E. Zautner, email: azautne@gwdg.de

Abstract

Background

All gastrointestinal pathogens, including *Enterococci*, undergo adaptation processes during colonization and infection. In this study, we investigated two crucial proteomic adaptations.

Firstly, we examined the adjustments to cope with high bile acid concentrations that the pathogens encounter during a potential gallbladder infection. Therefore, we chose the primary bile acids cholic acid (CA) and chenodeoxycholic acid (CDCA) as well as the secondary bile acid deoxycholic acid (DCA), as these are the most prominent bile acids. Secondly, we investigated the
adaptations from an aerobic to an anaerobic/microaerophilic environment, as encountered after oral-fecal infection, in the absence and presence of deoxycholic acid (DCA).

Results

Our findings showed similarities, but also species-specific variations in the response to the different bile acids. Both species showed a similar IC$_{50}$ for DCA and CDCA in growth experiments and both were highly resistant towards CA. DCA and CDCA had a strong effect on down-expression of proteins involved in translation, transcription and replication in *E. faecalis*, but to a lesser extent in *E. faecium*. Proteins commonly significantly altered in their expression in all bile acid treated samples were identified for both species and represent a “general bile acid response”. Among these, various subunits of a V-type ATPase, different ABC-transporters, multi-drug transporters and proteins related to cell wall biogenesis were up-expressed in both species and thus seem to play an essential role in bile acid resistance.

Most of the differentially expressed proteins were also identified when *E. faecalis* was incubated with low levels of DCA at microaerophilic conditions in comparison to aerobic conditions, indicating that adaptations to bile acids and to a microaerophilic atmosphere can occur simultaneously.

Conclusions

Overall, these findings provide an extensive insight into the proteomic stress response of two *Enterococcus* species and help to understand the resistance potential and the stress-coping mechanisms of these important gastrointestinal bacteria.
Introduction

The genus *Enterococcus* is a large group of Gram-positive, facultative anaerobic, non-spore-forming, coccal bacteria that were first described in 1899 by MacCallum and Hastings. Usually, various *Enterococci* species are present in the human gastrointestinal tract, but they are also found in animals and in environmental samples. Some *Enterococci* are used as probiotic bacteria or in a variety of dairy products such as cheese or milk. Particularly *Enterococcus faecalis* and *Enterococcus faecium* belong to the natural commensal bacteria of the human intestinal tract. As opportunistic pathogens, they have become a relevant cause for community-acquired and nosocomial infections worldwide. Especially *E. faecium* has become one of the most frequently reported sources for life-threatening hospital-acquired infections due to its potential antibiotic resistance to vancomycin and linezolid. As intestinal inhabitants, *E. faecalis* and *E. faecium* are permanently exposed to bile acids. Human bile roughly consists of ~40% cholic acid (CA) and ~40% chenodeoxycholic acid (CDCA), the primary bile acids, as well as ~20% of the secondary bile acids deoxycholic acid (DCA) and, to a minor proportion, lithocholic acid. These bile acids are conjugated with glycine or taurine in the liver cells so that a total of eight possible conjugated bile acids are present. Among the diverse functions of bile is the solubilization and emulsification of fat, which makes bile an important biological detergent. It is assumed that the exposure to bile acids can lead to alternations in the fatty acid- and phospholipid-composition of bacterial cell membranes and causes thus instabilities in the cell surface. Moreover, DNA damage may be induced by bile acids. As a consequence, many bacteria that inhabit the gastrointestinal tract have evolved mechanisms to cope with bile acid stress. Genome and transcriptome studies have shown in Gram-positive bacteria, that the expression of genes encoding for transporters that
Excrete bile salts is regulated by bile salts. Other genes that are regulated by the presence of bile are involved in general stress response or carbohydrate metabolism.

*Enterococci* are typical pathogens in cholecystitis and are particularly associated with common bile duct (CBD) stones. They also play a significant role in iatrogenically induced infections such as cholangiopancreatography (ERCP) induced cholangitis, acute pancreatitis, postoperative pancreatic fistulae, and other post-surgery biliary tract infections. In particular, disease progression of primary sclerosing cholangitis (PSC) has been associated with the presence of enterococci.

In case of an acute cholecystitis, the bacteria entering the biliary tract must adapt to the high and varying bile acid concentrations between approximately 15 and 272 mmol/L.

Genomic and transcriptomic data have shown interesting results about the bile acid response in *E. faecalis* and *E. faecium*. Transcriptional analyses in *E. faecium* to bile salts stress have identified major changes in the transcriptomic response when analyzed after five and fifteen minutes, where genes involved in nucleotide transport and metabolism were downregulated. Genes responsible for carbohydrate metabolism and posttranslational modifications, protein turnover and chaperones were found to be upregulated. Moreover, a study by Solheim et al. in 2007 analyzed the transcriptomic response between 10- to 60 minutes after bile acid exposure. A high number of genes that are responsible for cell envelope or fatty acid and phospholipid metabolism were repressed, while genes that encode for multidrug-resistance transporters or V-type ATPases were found to be induced. In contrast, only few data on proteomic changes after bile acid exposure exist for *Enterococcus* species. In 2010, Bøhle et al. analyzed the *E. faecalis* proteome with exposure to 1% bovine bile over 20, 60 or 120 minutes. In mass spectrometric analyses, they...
found mainly proteins involved in fatty acid and phospholipid biosynthesis pathways to be down-expressed. All of these studies were focused on the effects of bile over a short time period, while studies on the long-term effects are lacking.

Furthermore, data-independent acquisition mass spectrometry (DIA-MS) has not been applied to analyze the *Enterococcus* bile acid response so far, although this technique enables quantitative analysis of every detectable compound in a sample of proteins and thus provides a high reliability in the quantitative results. In this study we used DIA-MS to systematically compare the long-term proteomic changes (18 h) of *E. faecalis* and *E. faecium* after incubation with high concentrations of chenodeoxycholic acid (CDCA) and cholic acid (CA) as primary bile acids, as well as deoxycholic acid (DCA) as a secondary bile acid, assuming a similar stress response in both microbial species.

When considering colonization or infection of the biliary tract by a new fecal-orally transmitted enterococcal strain, the transition from aerobic conditions in the duodenum to microerophilic and finally to anaerobic conditions in the gallbladder must be considered in addition to the bile acid load. Therefore, we conducted a second independent experiment, in which we examined and compared the impact of aerobic and microaerophilic conditions on bile acid stress in *E. faecalis*, both with and without exposure to a low concentration of DCA. This investigation aimed to reveal the potential adaptations of the bacteria to these conditions, highlighting their relevance in scenarios such as fecal-oral uptake of these bacteria, which can occur especially in infants.
Material and Methods

Enterococcus strains and growth conditions

*E. faecalis* ATCC 700802 (V583) and *Enterococcus faecium* TX0016 (ATCC BAA-472) were grown in M17 broth (Thermo Fisher Scientific, Waltham, Massachusetts, USA), as previous experiments had shown that both organisms exhibit optimal growth in M17. Sublethal concentrations of 0.05 % CA, CDCA or DCA were added to the medium before incubation. The control sample was grown without bile salts. Stock solutions of 1 % sodium-CA, sodium-CDCA and sodium-DCA (Merck, Darmstadt, Germany) were prepared in dH₂O. Cultures were grown for 18 h, respectively.

Growth curves were generated by measuring the optical density at 600 nm (OD₆₀₀) every 30 min for the first five hours after inoculation and finally after 24 hours. In the growth experiments, biological triplicates of 0 %, 0.01 %, 0.025 %, 0.038 % and 0.05 % of either DCA, CA or CDCA were analyzed. The IC₅₀ was determined with with GraphPad Prism version 6 (GraphPad Software, La Jolla, California, USA) using nonlinear regression with the model $Y = \text{Bottom} + \frac{\text{Top} - \text{Bottom}}{1 + 10^{((\log IC_{50} \cdot X) \cdot \text{HillSlope})}}$.

To analyze the adaptation to microaerophilic conditions, bacteria were first grown in normal atmosphere and then diluted to an OD₆₀₀ of 0.05 and incubated in parallel for 18 h either in normal atmosphere or under microaerophilic conditions with and without 0.01 % DCA, respectively. The OD₆₀₀ was measured every hour for six hours and after 24 hours. The microaerophilic environment was created using anaerobe containers with BD gaspaks (Becton Dickinson, Franklin Lakes, New Jersey, USA).
Protein purification and quantification

After 18 hours of growth, the cultures were transferred to ice immediately and protein purification was started. Cultures were centrifuged at 3500 x g for 10 minutes at 4 °C. Afterwards, the cells were resuspended in 1 mL 0.9 % NaCl aqueous solution. In the next step, 0.75 g 4 mm glass beads were added and samples were treated in a “Fast prep 96 Homogenizer” (MP Biomedicals Germany GmbH, Eschwege, Germany) for 2 x 20 seconds, followed by centrifugation at 5500 x g for one minute. The supernatant was removed and the samples were centrifuged at 13,500 x g for 10 minutes at 4 °C. The supernatant was taken and used for further procedures.

A Pierce assay (Thermo Fisher Scientific, Waltham, Massachusetts, USA) was used to determine the protein concentration in each sample. For DIA-MS analysis, concentrations were adjusted to 1 µg/µL of protein. All samples were prepared in triplicate.

DIA-MS

Samples were purified by short-run SDS-PAGE with Coomassie stain (in-gel tryptic digestion). For the library, a pre-fractionation of a pooled reference sample was divided into 12 fractions by basic pH-reversed phase chromatography. Spiking was performed with a Biognosys iRT peptide standard.

For mass spectrometric analysis, identification was done by data-dependent acquisition (DDA) on a TripleTOF 5600+ (Sciex, Darmstadt, Germany). Therefore, 1000 ng equivalent were loaded, followed by a 90 min gradient, and the Top25 method. Two technical replicates were made per RP fraction. Quantification and ID by DIA-MS were performed using Thermo Q Exactive. Three
technical replicates per sample were prepared. Data processing was done with the Spectronaut v16.0.220606.53000 software package (Biognosys AG, Schlieren, Switzerland).

Protein identification and hybrid spectral library generation from 12x2 DDA acquisitions and 12x2 DIA acquisitions experiments were performed using Pulsar search engine against UniProtKB *E. faecalis* 700802 and *E. faecium* TX0016 proteomes with default parameters. A False Discovery Rate (FDR) of 1% on the spectral, peptide and protein group levels was set for all samples. DIA quantification was done using up to 6 fragments per peptide and up to 10 peptides per proteins. Dynamic retention time alignment was done, as well as dynamic mass recalibration and quartile normalization, for 1% FDR. Global data imputation was done for the final results table.

**Data processing**

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE 31–33 partner repository with the dataset identifier PXD040819. For statistical analysis, Perseus v1.6.2.2 was used to generate volcano plots for comparison between different samples 34. Two-fold expression changes were defined as significant. Only proteins that were regulated in five out of six samples were considered. For generation of volcano-plots in Perseus, a t-test was chosen with a number of randomizations = 250 and a FDR = 0.05 35. If not otherwise stated, all proteins that are subsequently described as up- or down-expressed were significantly regulated.

The respective COG-categories were assigned to the proteins using eggNOGmapper v 2.18 36–38. Venn diagrams were generated using InteractiVenn 39 to identify proteins that were consistently up- or down regulated in all bile salt treated samples. For comparison, the whole theoretical
proteome from UniProtKB was used for both organisms. Growth-curves, donut-plots and heatmaps were generated using matplotlib in python3.

Results

Growth rate comparison between *E. faecalis* and *E. faecium* in the presence of DCA, CDCA and CA

We compared the growth rates of *E. faecalis* and *E. faecium* in the presence of 0%, 0.01%, 0.025%, 0.038% and 0.05% of DCA, CDCA and CA, respectively. Growth gradually decreased with increasing DCA and CDCA concentrations. At 0.05% DCA and CDCA, only a weak increase of the OD$_{600}$ was detectable after 24 h (Figure 1), indicating a strong inhibitory effect. The IC$_{50}$ for DCA and CDCA was similar for both species and in the range of 0.01 – 0.023% when determined at three different time points at 3 h, 5 h and 24 h (Table 1). In contrast, growth rates were almost unaffected by CA in both species, even at the highest concentration of 0.05% (Figure 1), suggesting a high resistance of both Enterococcus species towards this primary bile acid.

Proteomic stress response towards DCA, CDCA and CA in *E. faecalis* and *E. faecium*

The similar sensitivity pattern of *E. faecalis* and *E. faecium* towards the three tested bile acids leads to the assumption that their adaptation processes are likely to be similar, as well. To investigate the involved stress response more thoroughly, we decided to analyze the proteome profile changes of *E. faecalis* and *E. faecium* after individual exposure with the three bile acids (0.05% for 24 h) in comparison to an untreated control. The high concentration of bile salts was
deliberately chosen to simulate a proteome under significant stress, similar to the concentrations encountered in the gallbladder environment during colonization of this organ. For *E. faecalis* samples, a total of 1410 proteins were identified in DIA-MS which represented 43.5 % of the whole theoretical proteome. 1400 proteins were identified for *E. faecium* samples, which represented 45.8 % of the whole theoretical proteome (Table 2).

The number of proteins with significantly altered expression level was similar in all bile acid treated samples. DCA resulted in 631 differentially expressed proteins in *E. faecalis* and 622 in *E. faecium*. CDCA treatment resulted in 608 differentially expressed proteins in *E. faecalis* and 565 in *E. faecium*. Interestingly, after CA exposure the number of differentially expressed *E. faecalis* proteins (644) and *E. faecium* proteins (633) was in the same range as with DCA and CDCA, although the latter bile acids mediated a markedly stronger growth inhibition (Table 2, Figure 1).

When differentially expressed proteins were separated into up-expressed and down-expressed proteins, the number of down-expressed proteins exceeded the number of up-expressed proteins (Table 2). Specifically, the fraction of down-expressed proteins on the overall differentially expressed proteins was 67 % for DCA, 62 % for CDCA and 58 % for CA.

**Clusters of Orthologous Groups of proteins (COG) categories**

Differentially expressed proteins were assigned to their respective COG categories and significantly up- or down-expressed proteins were depicted in doughnut plots (suppl. Figure 2).

The relative proportion of the individual COG-categories shows a species-specific pattern. We furthermore determined the proportion of up- and down-expressed proteins for each bile acid within the individual COG-categories (Figure 2).
In both species, the response to DCA shows higher similarity to the CDCA response than to the response towards the more hydrophilic primary bile acid CA. DCA and CDCA result in a massive down-expression of proteins in the COG categories “translation” (J), “transcription” (K) and “replication” (L) in *E. faecalis*. These three categories are also down-expressed in *E. faecium*, but to a lesser extent, suggesting that *E. faecium* is more tolerant towards DCA/CDCA stress than *E. faecalis*. In contrast, CA shows a less pronounced effect on the COG categories “translation (J), “transcription” (K) and “replication” (L). This is in accordance with its higher growth rates in the presence of CA when compared to DCA or CDCA. The proteins of the categories “cell wall/membrane/envelope biogenesis” (M) and “post-translational modification, protein turnover, and chaperones” (O) are relatively up-expressed under bile acid stress conditions. The number of commonly up-expressed proteins by all three bile acids was similar for *E. faecalis* (71) and *E. faecium* (74). Likewise, the number of commonly down-expressed proteins is 212 for *E. faecalis* and 162 for *E. faecium* (Figures 3 & 4). The distribution of these proteins in COG categories is different, suggesting that the general bile stress response varies between the two microbial species (Suppl. Figure 2).

**E. faecalis in microaerophilic vs aerobic conditions, with and without DCA exposure**

As an intestinal inhabitant, *E. faecalis* is adapted to microaerophilic and anaerobic habitats. However, in case of an oral uptake of *E. faecalis*, potentially originating from fecal sources, the bacteria must undergo adaptations to transition from aerobic to microaerophilic and anaerobic conditions. Moreover, the bacteria are exposed to bile acid in presence and absence of oxygen in the different environments of the gastrointestinal tract. We thus compared the *E. faecalis* growth
rate and its alterations of the proteome in aerobic versus microaerophilic conditions in an independent experiment. *E. faecalis* displayed a similar growth behavior under both conditions up to 6 h. However, at 24 h a markedly higher final OD$_{600}$ was observed under microaerophilic conditions than with normal oxygen concentration (Figure 5).

Proteomic analysis revealed 59 differentially expressed proteins in response to a microaerophilic atmosphere, with 27 up-expressed and 32 down-expressed proteins under microaerophilic conditions compared to aerobic conditions (Tables 3 & 4, suppl. Figure 1). In samples grown under microaerophilic conditions, several ribosomal proteins were up-expressed compared to normal oxygen concentration. On the other hand, various proteins involved in glycolysis and carbohydrate catabolism were down-expressed under microaerophilic conditions, for example glyceraldehyde-3-phosphate dehydrogenase, components of the pyruvate dehydrogenase complex, an aldose epimerase and a glycosyl hydrolase family protein.

Treatment with 0.01 % DCA resulted in a moderate growth inhibition compared to untreated controls, in both, microaerophilic and aerobic conditions (Figures 1 & 5). As observed for DCA-untreated samples, DCA-treated samples also displayed increased growth under microaerophilic conditions compared to aerobic conditions after 24 h (Figure 5). Proteome analysis revealed that under aerobic conditions, 419 proteins were up-expressed and 245 down-expressed at 0.01 % DCA, compared to DCA-untreated controls grown at aerobic conditions. Similarly, in microaerophilic conditions, 396 proteins were up-expressed, and 251 proteins were down-expressed in 0.01 % DCA treated samples compared to DCA-untreated controls grown at microaerophilic conditions (Table 5).
Interestingly, the 0.01 % DCA-treated samples under microaerophilic conditions show 46 of the 259 differentially expressed proteins that were identified in the DCA untreated sample under microaerophilic conditions (Suppl. Figure 4). This indicates that DCA stress does not prevent the up- and down-expression of the majority of proteins that occur as an adaptation to microaerophilic conditions.

Identification of a general bile stress response based on *E. faecalis* and *E. faecium* proteins commonly significantly altered in their expression

As described above, treatment with 0.05 % DCA, CDCA and CA identified 71 commonly up-expressed proteins. Proteomic data for the approaches with 0.05 % and with 0.01 % DCA were obtained from independent experiments performed at different time points and can thus not be directly compared. Nevertheless, of the 71 commonly up-expressed proteins identified from the 0.05 % bile acid samples, 37 proteins were also up-expressed in the two samples using 0.01 % DCA with either aerobic or microaerophilic atmosphere (Table 6 & 7, suppl.-Figure 3). This suggests a strong conservation of the general stress response towards DCA, independent from atmospheric conditions.

From the 37 up-expressed proteins, four proteins were subunits of a V-type ATP synthase (Table 8), namely alpha chain, beta chain, subunit E and subunit I, suggesting an important role of this protein complex in bile acid stress adaptation (Table 6). In total, nine *E. faecalis* V-type ATPase related proteins were identified in samples with 0.05 % of DCA, CDCA or CA. In *E. faecium*, eight V-type ATPase associated proteins were detected in total. However, these proteins were not as frequently up-expressed during bile acid stress as in *E. faecalis*, and only one (V-type ATPase
subunit F) was up-expressed in all three bile acids (Table 8, suppl excel file 1). Functional analysis of V-type ATPases in bile acid stress adaptation would greatly benefit from the availability of specific inhibitors for this protein class. In contrast to eukaryotes, specific V-type ATPase inhibitors were not described for prokaryotes yet. In eukaryotic cells, bafilomycin A and archazolid A were shown to act as V-type ATPases inhibitors. We tested these compounds in growth assays up to a concentration of 10 µM on *E. faecalis*, but could not find any inhibitory effect (data not shown). Furthermore, a combination of 10 µM bafilomycin or archazolid with 0.01% DCA did not lead to stronger growth inhibition as the 0.01% DCA control, indicating that these compounds do not inhibit the bile acid adaptation in *E. faecalis*.

A unique pattern seen in both species was the up-expression of membrane transporters. In *E. faecalis*, three ABC-transporters and one multidrug-resistance transporter were commonly up-expressed in all bile acid treated samples (Table 6). In *E. faecium*, five ABC-transporter and one multidrug-resistance systems were collectively up-expressed (suppl excel-file1).

Furthermore, four proteins involved in peptidoglycan metabolism and murein synthesis were up-expressed in *E. faecalis*. These are a UDP-N-acetylglucosamine 1-carboxyvinyltransferase 1, a UDP-N-acetylglucosamine 1-carboxyvinyltransferase 1, a UDP-N-acetylmuramoyl-tripeptide--D-alanyl-D-alanine ligase and a penicillin-binding protein (Table 6). These proteins were also up-expressed in *E. faecium* after exposure with DCA, CDCA or CA (suppl Excel-file 1).

Among the 24 down-expressed proteins were central elements of the pyruvate and citrate metabolism, including two components of the pyruvate dehydrogenase complex (dihydrolipoyl dehydrogenase; dihydrolipoamide acetyltransferase) and a [citrate [pro-3S]-lyase] ligase, which is involved in the cleavage of citrate into acetate and oxaloacetate. Furthermore, down-
expression of a key enzyme of the shikimate pathway (AROA_ENTFA) indicates decreased biosynthesis of folates and amino acids. This is in line with reduced expression levels of dihydrofolate reductase, also involved in folate metabolism and of 4-hydroxy-tetrahydrodipicolinate synthase, which is a key enzyme for lysine biosynthesis (Table 7). These proteins were not found among the down-expressed proteins in *E. faecium* (Suppl. Excel-file 1), which supports the assumption that the bile acid stress response is unique in both organisms.

**Discussion**

Tolerance against bile acid stress and microaerophilic conditions are key factors for pathogens and commensals to colonize the intestinal or the biliary tract.

The most abundant bile acid, CA, which is the precursor for other secondary bile acids, is synthesized by the liver from cholesterol. CA has a steroid structure with three hydroxyl groups and a carboxyl group and it has a hydrocarbon side chain. The other primary bile acid CDCA differs from CA in its structure, it lacks one hydroxyl group. DCA, which is synthesized from CA has only one hydroxyl group.

We systematically investigated in this study adaptation processes that occur in *E. faecalis* and *E. faecium* after exposure to the three major bile acids in the human intestinal tract with a quantitative proteomic approach and correlated the obtained data with the inhibitory potential of these bile acids on the bacterial growth rate.
Similarities and differences in the bile acid adaptation processes between *E. faecalis* and *E. faecium*

Both microbial species displayed comparable susceptibility in their replication rate towards DCA and CDCA with an IC$_{50}$ in the range of 0.01 % - 0.023 %. Although the effect of the three bile acids on the growth rate is similar in both species, DIA-MS revealed differences in the proteomic response between the two *Enterococcus* species. Most strikingly, DCA and CDCA at 0.05% had a very strong effect on down-expression of proteins assigned to the COG categories translation (J), transcription (K), and replication (L) in *E. faecalis*. Such an extreme down-expression of these fundamental functions indicates a particular high stress level, which brings the cells to their adaptation limits. Due to the reduced growth with 0.05 % of DCA and CDCA, the down-expression of these categories is not surprising. A linear relationship between growth rate and abundance of ribosomal proteins has been studied in *E. coli* and in other bacteria before. This effect might also be present in *Enterococci*. Furthermore, the extreme reduction of growth might mask the stress response towards DCA and CDCA. However, this was not the case with CA.

Apparently, with CA, the stress level in both organisms was not as high as in DCA and CDCA, as the COG-categories J, K, L were not as much down-expressed. In the approach of *E. faecalis* with CA, the COG-category J (translation) is even highly up-expressed. It is worth to mention at this place that we determined the long-term effects of bile acids after 24 h exposure, while in many other transcriptomic or proteomic studies changes at much shorter time periods were analyzed. The number of down-expressed proteins associated with translation, transcription, and replication was increased only moderately in *E. faecium*, suggesting a higher robustness to long term DCA and CDCA exposure.
The COG categories cell wall biogenesis (M) and chaperone production (O) were significantly up-expressed in both organisms, when exposed to DCA and CDCA (Figure 2), indicating that the maintenance and regeneration of the cell wall, the membrane and the protection of proteins via chaperones are of high importance under bile acid stress. Previous studies showed that bile acids disrupt the bacterial cell membrane \cite{10-12,45}, thus, the proteomic response of the Enterococci fits to these findings. With exposure to CA, these COG-categories were not as much regulated as in DCA and CDCA, suggesting that CA does not have the same impact on the cells.

We found that both Enterococcus species are particularly well adapted to the primary bile acid CA, which is the bile acid with the highest concentration in the human gall-bladder but also in the gut \cite{10,26}. In contrast to DCA and CDCA, growth rates were almost unaffected by 0.05 % CA.

Proteins commonly altered in their expression – a general (but species-specific) bile acid stress response

Comparative analysis of the samples exposed to DCA, CDCA and CA identified a subset of 283 commonly regulated proteins in \textit{E. faecalis} and of 236 commonly regulated proteins in \textit{E. faecium}.

These proteins define the general stress response towards bile acids and thus are particularly useful for the identification of shared strategies by both species, but also allow the identification of species-specific mechanisms. A subset of 71 up-expressed proteins is shared at a concentration of 0.05 % DCA, CDCA and CA in \textit{E. faecalis}. Of these, 37 proteins are also up-expressed at a lower concentration of 0.01 % DCA.
a) **V-type ATP-synthases**

Among these shared up-expressed proteins are four subunits of a V-type ATPase. Namely, these are ATP synthase alpha and beta chain, which form the catalytic hexamer\(^ {46-49}\), the subunit C, which is responsible for control of the assembly of the V-type ATPase\(^ {50}\), the subunit E and G, which are playing a role in the assembly of the ATPase and function as stalk\(^ {46}\), and of subunit D and I, whose exact function remains still unknown. In total, nine V-type ATPase subunits are present in the genome of *E. faecalis*, we were able to identify all of them by DIA-MS. In *E. faecium*, eight V-type ATPase subunits are currently known. We found all eight by mass spectrometry.

V-type ATPases are membrane-bound proteins that are actively pumping ions, usually H\(^+\), out of the cell using ATP\(^ {48,49}\). These proton gradients are highly conserved in nature and have been shown to be crucial for survival in bile acid mediated stress before\(^ {27,51}\). This function has also been shown in *Lactobacillus plantarum* and *Bifidobacterium* sp.\(^ {16,52,53}\). The maintenance of a proton motive force in presence of bile also plays a role in other organisms. In *E. coli*, it has been shown that a bile acid secretion system might be driven by a proton motive force\(^ {54}\). In *Enterococcus hirae*, V-type ATPases are known for proton or sodium transport either inside or outside of the cell\(^ {55}\). It is unclear whether the *Enterococcus* V-type ATPase transports H\(^+\) or Na\(^+\) across the plasma membrane. A previous study showed that V-type ATPases in *E. hirae* are responsible for Na\(^+\) transport\(^ {55}\). This might also be the case in *E. faecalis* and *E. faecium*. However, it is reasonable to assume that the V-type ATPase also contributes to an ion motive force that in turn can energize other plasma membrane transporters, which might be important to transport bile acids out of the cell.
In both organisms, the up-expression of V-type ATPase subunits was observed, however, the up-expression is seen only at a moderate level in *E. faecium*. From the eight detected V-type ATPase subunits in *E. faecium*, only one was up-expressed in all bile acids. This indicates that the contribution of V-type ATPase to the bile acid induced stress response might be slightly different for *E. faecium* and *E. faecalis*.

**b) ABC transporters**

Several ABC transporter-related proteins as well as multidrug efflux proteins were found in the group of commonly up-expressed proteins in both, *E. faecalis* and *E. faecium*. These proteins might be relevant to actively transport bile acids out of the cell. This seems to be a similarity between both species but also fits to the observations in other species, such as *E. coli*, *Bifidobacterium longum* or *Campylobacter jejuni*, where bile acids are exported from the cell. The up-expression of different transporters in both species as a response to bile acid exposition indicates that the process of transporting bile acids out of the cell is a conserved mechanism between bacteria.

The connection between antimicrobial resistance mechanisms and bile acid resistance mechanisms has been observed before, which explains the up-expression of the multidrug efflux pump proteins. In 2017, Wulkersdorfer et al. showed that the effectivity of antibiotics decreases in the presence of bile acids in *E. faecalis* and *E. coli*. Thus, it is likely, that the ABC transporters and multidrug resistance transporters we found to be up-expressed in *E. faecalis* and *E. faecium* are not only playing a role in antimicrobial resistance but also in bile resistance.
Proteins involved in peptidoglycan metabolism and murein synthesis were commonly up-expressed in all *E. faecalis* and *E. faecium* samples with bile acids. As bile acids disrupt the bacterial cell wall and membrane \(^{11,12,26,65}\), the synthesis of peptidoglycan and murein is thus a compensatory response to bile acid stress. This indicates that the integrity and maintenance of the bacterial cell wall plays an important role in adaption to bile acids in both species. In contrast, down-expression of proteins involved in pyruvate-, citrate- and folate metabolism was only observed in *E. faecalis*, but not in *E. faecium*.

Together, our analysis of the proteomic response indicates similarities, but also significant differences in the adaptation towards bile acid stress in *E. faecalis* and *E. faecium*, even though these species are closely related \(^{59,60}\). Whether these differences are adaptations to different microenvironments in the intestinal tract is currently unclear.

**Adaptation to the microaerophilic environment**

*E. faecalis* usually inhabits the human gut, where the oxygen concentration is 1-2 %. However, faeco-oral transmission is a common route for enterococcal infections, especially in infants. Due to its facultative anaerobic nature, *E. faecalis* is able to survive in normal oxygen conditions as well as in microaerophilic or anaerobic environments.

In fact, our growth comparison revealed a higher OD\(_{600}\) in microaerophilic environment than under aerobic conditions for *E. faecalis*. This suggests that *E. faecalis* is well adapted to a low...
oxygen atmosphere, which was also found in previous studies \textsuperscript{61–63}. We found several ribosomal proteins among the up-expressed proteins under microaerophilic conditions, which suggests increased protein synthesis under these conditions. In samples with aerobic conditions, proteins involved in glycolysis and carbohydrate catabolism were upregulated when compared to microaerophilic samples. Among these proteins were a glyceraldehyde-3-phosphate dehydrogenase and components of the pyruvate dehydrogenase complex. This supports the observations of Portela et al. in 2014, who described an enhanced glycolysis metabolism of \textit{E. faecalis} in an aerobic environment \textsuperscript{64}. Most of the microaerophilic adaptations were also observed in the presence of DCA. This indicates that DCA has a strong influence on the bacteria in an aerobic as well as in microaerophilic atmosphere but does not prevent the microaerophilic proteomic response.

\textbf{Declarations}

\textbf{Ethics approval and consent to participate}

Not applicable.

\textbf{Consent for publication}

Not applicable.

\textbf{Availability of data and materials}

Data are available via ProteomeXchange with identifier PXD040819. Submission details:
Project Name: Comparative analysis of proteomic adaptations in Enterococcus faecalis and Enterococcus faecium after long term bile acid exposure. Project accession: PXD040819

Project DOI: Not applicable.

Reviewer account details: Username: reviewer_pxd040819@ebi.ac.uk Password: tSZJmLHN

Competing interests

The authors declare that they have no competing interests.

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Authors’ Contributions

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References

1. W. G. MacCallum and T.W. Hastings, “A Case of Acute Endocarditis Caused by Micrococcus zymogenes (nov. spec.), With a Description of the Microorganism.,” A preliminary communi-
cation appeared in the Bulletin of the Johns Hopkins Hospital, p. 46.,(1899).


Table 1: IC\textsubscript{50} of E. faecalis and E. faecium after 3, 5.5 and 24 hours of growth in the three different bile acids. IC\textsubscript{50} was determined via graph pad prism after measurement of the OD\textsubscript{600}.

<table>
<thead>
<tr>
<th></th>
<th>IC\textsubscript{50} E. faecalis</th>
<th>IC\textsubscript{50} E. faecium</th>
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</thead>
<tbody>
<tr>
<td>after 3 h of growth</td>
<td>DCA 0.01 %</td>
<td>0.015 %</td>
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<tr>
<td></td>
<td>CDCA 0.011 %</td>
<td>0.013 %</td>
</tr>
<tr>
<td>after 5.5 h of growth</td>
<td>DCA 0.012 %</td>
<td>0.011 %</td>
</tr>
<tr>
<td></td>
<td>CDCA 0.013 %</td>
<td>0.014 %</td>
</tr>
<tr>
<td>after 24 h of growth</td>
<td>DCA 0.011 %</td>
<td>0.013 %</td>
</tr>
<tr>
<td></td>
<td>CDCA 0.014 %</td>
<td>0.023 %</td>
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Table 2: Number of up- or down-expressed proteins of E. faecalis and E. faecium in three different bile acids and the respective percentage amount of the total identified proteins in DIA-MS.

<table>
<thead>
<tr>
<th></th>
<th>total</th>
<th>DCA</th>
<th>CDCA</th>
<th>CA</th>
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<tr>
<td></td>
<td>up</td>
<td>down</td>
<td>up</td>
<td>down</td>
</tr>
<tr>
<td>E. faecalis</td>
<td>1410</td>
<td>207 (15 %)</td>
<td>424 (17 %)</td>
<td>232 (16 %)</td>
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<tr>
<td></td>
<td>631 (45 %)</td>
<td></td>
<td>608 (43 %)</td>
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<tr>
<td>E. faecium</td>
<td>1400</td>
<td>260 (19 %)</td>
<td>362 (26 %)</td>
<td>174 (12 %)</td>
</tr>
<tr>
<td></td>
<td>622 (44 %)</td>
<td></td>
<td>565 (40 %)</td>
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Table 3: Up-expressed proteins in *E. faecalis* under microaerophilic conditions when compared to aerobe conditions. Yellow-marked proteins are constituents of ribosomes.

<table>
<thead>
<tr>
<th>Uniprot ID</th>
<th>Protein function</th>
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<tbody>
<tr>
<td>H7C6Z5_ENTFA</td>
<td>2-dehydropantoate 2-reductase</td>
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<tr>
<td>Q82Z23_ENTFA</td>
<td>pheromone cAD1 lipoprotein</td>
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<td>Q82Z24_ENTFA</td>
<td>FAD:protein FMN transferase</td>
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<td>Q82Z45_ENTFA</td>
<td>Dps family protein</td>
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<td>Q830A9_ENTFA</td>
<td>transcriptional regulator, MarR family</td>
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<td>Q830E0_ENTFA</td>
<td>uncharacterized protein</td>
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<td>Q830L9_ENTFA</td>
<td>PSP1 C-terminal domain-containing protein</td>
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<td>5'-methylthioadenosine/S-adenosylhomocysteine nucleosidase</td>
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<td>fumarate reductase flavoprotein subunit, putative</td>
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<td>uncharacterized protein</td>
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<td>RL29_ENTFA</td>
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Table 4: Down-expressed proteins of *E. faecalis* in microaerophilic conditions when compared to aerobe conditions. Green-marked proteins represent proteins involved in glycolysis and pyruvate metabolism.

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<td>Q834E5_ENTFA</td>
<td>transcriptional regulator, LysR family</td>
</tr>
<tr>
<td>Q834IS_ENTFA</td>
<td>short chain dehydrogenase family protein</td>
</tr>
<tr>
<td>Q834J1_ENTFA</td>
<td>branched-chain alpha-keto acid dehydrogenase, E1 component, alpha subunit</td>
</tr>
<tr>
<td>Q834V5_ENTFA</td>
<td>glyceraldehyde-3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>Q835M3_ENTFA</td>
<td>pyruvate dehydrogenase complex, E1 component, beta subunit</td>
</tr>
<tr>
<td>Q835M4_ENTFA</td>
<td>pyruvate dehydrogenase E1 component subunit alpha</td>
</tr>
<tr>
<td>Q835Q8_ENTFA</td>
<td>N-acetylglucosamine-6-phosphate deacetylase</td>
</tr>
<tr>
<td>Q836P1_ENTFA</td>
<td>aldose 1-epimerase</td>
</tr>
<tr>
<td>Q836T6_ENTFA</td>
<td>N-acetyltransferase domain-containing protein</td>
</tr>
<tr>
<td>Q836T7_ENTFA</td>
<td>glycosyl hydrolase, family 1</td>
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<tr>
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<td>oxido-reductase, Gfo/Idh/MocA family</td>
</tr>
<tr>
<td>Q836V7_ENTFA</td>
<td>penicillin-binding protein C</td>
</tr>
<tr>
<td>Q837B9_ENTFA</td>
<td>uncharacterized protein</td>
</tr>
<tr>
<td>Q838A6_ENTFA</td>
<td>glyoxalase family protein</td>
</tr>
</tbody>
</table>
Table 5: The number of up- or down-expressed proteins in aerobe and microaerophilic *E. faecalis* approaches with 0.01 % DCA and the respective percentage amount of the total identified proteins in DIA-MS.

<table>
<thead>
<tr>
<th></th>
<th>total</th>
<th>aerobic + DCA</th>
<th>microaerophilic + DCA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>up</td>
<td>down</td>
<td>up</td>
</tr>
<tr>
<td><em>E. faecalis</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1051</td>
<td>419 (40 %)</td>
<td>245 (23 %)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>245 (23 %)</td>
<td>150 (38 %)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>251 (24 %)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>251 (24 %)</td>
</tr>
<tr>
<td></td>
<td>664</td>
<td>647 (63 %)</td>
<td>647 (62 %)</td>
</tr>
</tbody>
</table>

Table 6: 37 proteins which are commonly up-expressed in all five *E. faecalis* approaches in the presence of 0.05 % DCA, CDCA or CA and 0.01 % DCA under aerobic as well as microaerophilic conditions. Proteins involved in murein or peptidoglycan synthesis are marked in green, transporter proteins are marked in blue and V-type ATPase subunits are marked in yellow.

<table>
<thead>
<tr>
<th>Uniprot ID</th>
<th>Protein function</th>
</tr>
</thead>
<tbody>
<tr>
<td>H7C6V7_ENTFA</td>
<td>penicillin-binding protein 4</td>
</tr>
<tr>
<td>H7C713_ENTFA</td>
<td>cell division protein DivIVA</td>
</tr>
<tr>
<td>Q82Y9_ENTFA</td>
<td>peptidase, U32 family, putative</td>
</tr>
<tr>
<td>Q82ZA8_ENTFA</td>
<td>hydrolase, haloacid dehalogenase-like family</td>
</tr>
<tr>
<td>Q82ZH5_ENTFA</td>
<td>iron compound ABC transporter, substrate-binding protein</td>
</tr>
<tr>
<td>Y2866_ENTFA</td>
<td>probable transcriptional regulatory protein EF_2866</td>
</tr>
<tr>
<td>Q830N7_ENTFA</td>
<td>lipoate--protein ligase</td>
</tr>
<tr>
<td>Q8304_ENTFA</td>
<td>diacylglycerol kinase catalytic domain protein</td>
</tr>
<tr>
<td>Q831B8_ENTFA</td>
<td>ABC transporter, ATP-binding/permease protein</td>
</tr>
<tr>
<td>Q831B9_ENTFA</td>
<td>ABC transporter, ATP-binding/permease protein</td>
</tr>
<tr>
<td>RF1_ENTFA</td>
<td>peptide chain release factor 1</td>
</tr>
<tr>
<td>Q831R2_ENTFA</td>
<td>PTS system, IIA component</td>
</tr>
<tr>
<td>EFTS_ENTFA</td>
<td>elongation factor Ts</td>
</tr>
<tr>
<td>Q832A0_ENTFA</td>
<td>uncharacterized protein</td>
</tr>
<tr>
<td>Q832N1_ENTFA</td>
<td>dTDP-glucose 4,6-dehydratase</td>
</tr>
<tr>
<td>Q833B2_ENTFA</td>
<td>oxidoreductase, pyridine nucleotide-disulfide family</td>
</tr>
<tr>
<td>MURC_ENTFA</td>
<td>UDP-N-acetylmuramate--L-alanine ligase</td>
</tr>
<tr>
<td>Q8343_ENTFA</td>
<td>DUF4097 domain-containing protein</td>
</tr>
<tr>
<td>Q834G9_ENTFA</td>
<td>DegV family protein, putative</td>
</tr>
<tr>
<td>Q834T0_ENTFA</td>
<td>TPR domain protein</td>
</tr>
<tr>
<td>VATB_ENTFA</td>
<td>V-type ATP synthase beta chain</td>
</tr>
<tr>
<td>VATA_ENTFA</td>
<td>V-type ATP synthase alpha chain</td>
</tr>
<tr>
<td>Q834Y2_ENTFA</td>
<td>V-type ATPase, subunit E</td>
</tr>
<tr>
<td>Q834Y4_ENTFA</td>
<td>V-type ATP synthase subunit I</td>
</tr>
<tr>
<td>DNAK_ENTFA</td>
<td>chaperone protein DnaK</td>
</tr>
<tr>
<td>GRPE_ENTFA</td>
<td>protein GrpE</td>
</tr>
<tr>
<td>Q835V8_ENTFA</td>
<td>sulfatase domain protein</td>
</tr>
<tr>
<td>MURA1_ENTFA</td>
<td>UDP-N-acetylglycosamine 1-carboxyvinyltransferase 1</td>
</tr>
<tr>
<td>QUEA_ENTFA</td>
<td>S-adenosylmethionine:trNA ribosyltransferase-isomerase</td>
</tr>
<tr>
<td>Q8373_ENTFA</td>
<td>UDP-N-acetylmuramoyl-tripeptide--D-alanyl-D-alanine ligase</td>
</tr>
<tr>
<td>TIG_ENTFA</td>
<td>trigger factor</td>
</tr>
</tbody>
</table>
Table 7: 24 proteins which are commonly down-expressed in all five *E. faecalis* approaches in the presence of 0.05 % DCA, CDCA or CA and 0.01 % DCA under aerobic as well as microaerophilic conditions. Proteins associated with pyruvate and citrate metabolism are marked in orange, proteins involved in biosynthesis of folic acid and amino acids are marked in purple.

<table>
<thead>
<tr>
<th>Uniprot ID</th>
<th>Protein function</th>
</tr>
</thead>
<tbody>
<tr>
<td>H7C718_ENTFA</td>
<td>single-stranded DNA-binding protein</td>
</tr>
<tr>
<td>AROA_ENTFA</td>
<td>3-phosphoshikimate 1-carboxyvinyltransferase</td>
</tr>
<tr>
<td>Q82YW0_ENTFA</td>
<td>citrate [pro-3S] -lyase ligase</td>
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<tr>
<td>Q82Z79_ENTFA</td>
<td>isochorismatase family protein</td>
</tr>
<tr>
<td>Q82ZD3_ENTFA</td>
<td>uncharacterized protein</td>
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<td>Q82ZF0_ENTFA</td>
<td>peptide ABC transporter, ATP-binding protein</td>
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<td>Q82ZF1_ENTFA</td>
<td>peptide ABC transporter, ATP-binding protein</td>
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<tr>
<td>Q82ZF2_ENTFA</td>
<td>peptide ABC transporter, permease protein</td>
</tr>
<tr>
<td>Q82ZK6_ENTFA</td>
<td>phosphosugar-binding transcriptional regulator, RpiR family, putative</td>
</tr>
<tr>
<td>Q830J7_ENTFA</td>
<td>NAD_binding_9 domain-containing protein</td>
</tr>
<tr>
<td>Q831L7_ENTFA</td>
<td>UDP-galactopyranose mutase</td>
</tr>
<tr>
<td>Q833L4_ENTFA</td>
<td>uncharacterized protein</td>
</tr>
<tr>
<td>Q834I9_ENTFA</td>
<td>branched-chain phosphotransacetylase</td>
</tr>
<tr>
<td>Q834J0_ENTFA</td>
<td>dihydrolipoamide acetyltransferase component of pyruvate dehydrogenase complex</td>
</tr>
<tr>
<td>Q834J2_ENTFA</td>
<td>dihydrolipoamide acetyltransferase component of pyruvate dehydrogenase complex</td>
</tr>
<tr>
<td>Q834R2_ENTFA</td>
<td>dihydrofolate reductase</td>
</tr>
<tr>
<td>Q834W2_ENTFA</td>
<td>PTS system, IIABC components</td>
</tr>
<tr>
<td>Q835H7_ENTFA</td>
<td>cadmium-translocating P-type ATPase</td>
</tr>
<tr>
<td>DAPA_ENTFA</td>
<td>4-hydroxy-tetrahydrodipicolinate synthase</td>
</tr>
<tr>
<td>Q836S2_ENTFA</td>
<td>nucleoside diphosphate kinase</td>
</tr>
<tr>
<td>Q836T6_ENTFA</td>
<td>N-acetyltransferase domain-containing protein</td>
</tr>
<tr>
<td>Q837A3_ENTFA</td>
<td>uncharacterized protein</td>
</tr>
<tr>
<td>Q837H3_ENTFA</td>
<td>glyoxalase family protein</td>
</tr>
</tbody>
</table>
Table 8: V-type ATPases identified in *E. faecalis* and *E. faecium* samples with 0.05 % bile acids and *E. faecalis* samples with 0.01 % DCA in aerobic and microaerophilic conditions. Up-expressed proteins are labelled in grey. Proteins that were not regulated are labeled in white. n.i. = not identified in DIA-MS. \(^1\) = absent in genome.

<table>
<thead>
<tr>
<th>Identified protein</th>
<th><em>E. faecalis</em></th>
<th><em>E. faecium</em></th>
<th><em>E. faecalis</em></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.05% DCA</td>
<td>0.05% CDCA</td>
<td>0.05% CA</td>
</tr>
<tr>
<td>V-type ATP synthase alpha chain</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>V-type ATP synthase beta chain</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>V-type ATPase subunit C</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>V-type ATPase subunit D</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>V-type ATPase subunit E</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>V-type ATPase subunit F</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>V-type ATPase subunit G</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>V-type ATP synthase subunit I</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>V-type ATPase subunit K</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 1: Growth curves of *E. faecalis* (blue) and *E. faecium* (green) with DCA, CDCA and CA at 0 %, 0.01 %, 0.025 %, 0.038 % and 0.05 % bile acid concentration. The OD₆₀₀ was measured every half hour for 5.5 hours and after 24 hours for *E. faecalis* and every hour for 12 h and after 24 h for *E. faecium*.
Figure 2: The total number of detected proteins as well as the regulated proteins were assigned to their respective COG-category. The percentage of regulated proteins in relation to the total number was calculated for each COG-category and visualized in a heatmap. A: Up-expressed proteins B: down-expressed proteins. Yellow = 40-50 %, green = 25-40 %, bright blue = 10-25 %, darkblue = 0-10 % higher than in the whole proteome.
Figure 3: Venn diagrams of proteins that are commonly up-expressed in all approaches with 0.05 % bile salts in *E. faecalis* (A) and *E. faecium* (B). In *E. faecalis*, 71 proteins are commonly up-expressed, while in *E. faecium*, 74 proteins are commonly up-expressed.
Figure 4: Venn diagrams of proteins that are commonly down-expressed in all approaches with 0.05 % bile salts in *E. faecalis* (A) and *E. faecium* (B). In *E. faecalis*, 212 proteins are commonly down-expressed, while in *E. faecium*, 162 proteins are commonly down-expressed.
Figure 5: Growth curves of *E. faecalis* with (blue) and without (red) oxygen and with (bright colors) and without 0.01 % DCA (dark colors). The OD$_{600}$ was measured every hour for six hours and after 24 hours. After 24 hours, growth was higher in microaerophilic conditions than with normal oxygen concentration.
4 Additional results - Comparative proteomic analysis of *Campylobacter jejuni* insertional gene inactivation mutants and their bile acid induced stress proteome

4.1 Background

In *C. jejuni*, different genes are associated with bile acid resistance. One of the most prominent bile acid resistance mechanisms is the resistance nodulation-division (RND) type multidrug efflux pump CmeABC, which consists of a three-gene operon that encodes for the proteins CmeA, CmeB and CmeC [128]. In this efflux pump, CmeA plays the role of the membrane fusion protein, CmeB is the efflux pump membrane transporter and CmeC is the outer membrane lipoprotein [127, 147]. In 2003, Lin *et al.* generated knockout mutants of the genes *cmeB* and *cmeC* to show the effect on bile acid resistance in *C. jejuni* [128]. These knockout mutants were extremely susceptible to bile acids and several antibiotics compared to the parental strain and showed significantly reduced growth. CmeABC seems to enable the transport of bile acids and other antimicrobials out of the cell [148, 127]. In a proteomic study, Masanta *et al.* showed that the proteins belonging to the CmeABC multidrug efflux pump were up-expressed under bile acid stress exposure [144]. CmeR acts as a transcriptional repressor for CmeABC, it has been demonstrated that knockout mutants of *cmeR* show overexpression of CmeABC and an increased resistance towards antibiotics [129]. The *cmeR* gene is located upstream of *cmeA* and has a similar structure as members of the TetR family of transcriptional repressors. In 2005, Raphael *et al.* showed that knockout mutants of the protein named *Campylobacter* bile resistance regulator (CbrR) were highly susceptible to bile salts using the paternal *C. jejuni* strain F38011 [149]. However, it remains unknown in which other processes CbrR is involved as a response regulator. It was stated, that CbrR play a role in the regulation of flagellar motility in *C. jejuni* as knockout mutants seemed to be highly motile [150].

In this part of the thesis, *C. jejuni* 81-176 insertional gene inactivation mutants for the bile acid resistance associated genes ∆*cmeB*, ∆*cmeR* and ∆*cbrR* were generated and analyzed for proteomic changes by DIA-MS.
The proteome profiles were compared to the parental strain to identify potential other alternative functions or activities linked to the respective genes. Furthermore, the proteomic changes of these mutants after long-term incubation with sublethal concentrations of cholic acid (CA) were analyzed. Moreover, the motility of these mutants and the ability to form biofilms as well as their autoagglutination potential were compared, as these mechanisms play a critical role in survival of Campylobacter [36, 151–153].
4.2 Material and Methods

4.2.1 Bacteria and growth conditions

Campylobacter jejuni strain 81-176 was used for all experiments. Bacteria were usually grown on CAM-agar plates from Biomérieux (Marcy-l’Étoile, France) at 42 °C. Mueller-Hinton (MH) broth served as liquid medium at 37 °C. For ΔcmeB, specific blood-agar plates with MH, 5 % sheep blood, 15 % agar and 50 µg/mL kanamycin as single antibiotic were used. For creation of a microaerophilic environment, Gas Pak™ EZ Campy Container System by BD (Franklin Lakes, NJ USA) and an anaerobic jar for incubation were used. 

NEB-5-α E. coli cells were grown on LB (Luria-Bertani) agar plates supplemented with 50 µg/mL ampicillin for selection or in liquid LB. Growth curves were obtained by measuring the backscatter of the optical density at 600 nm (OD$_{600}$) using a CG Quant (Aquila biolabs/scientific bioprocessing, Pittsburgh, Pennsylvania, USA). DCA or CA were added to the medium at a concentration of the respective half IC$_{50}$ of the mutants (see Table 2) before incubation, the control was grown without bile salts. The IC$_{50}$ was determined using GraphPad Prism version 6 (GraphPad Software, La Jolla, California, USA) using nonlinear regression with the model:

$$ Y = \frac{\text{Bottom} + (\text{Top} – \text{Bottom})}{1 + 10^{(\text{Log} (\text{IC}_{50}) – X) \cdot \text{HillSlope}}} $$

4.2.2 Generation of insertional gene inactivation mutants of CmeB, CmeR and CbrR

Mutants were generated by double homologous recombination resulting in the insertion of a kanamycin resistance cassette into the target gene, using a pBlueScript II SK (psk II) vector plasmid (Stratagene, San Diego, California, USA) with an ampicillin resistance cassette. The vector was linearized using the high-fidelity restriction enzymes BamHI – HF and EcoRI – HF by New England Biolabs (NEB - New England Biolabs, Ipswich, Massachusetts, USA). The plasmid was constructed according to the description of previous publications [154].

Genomic DNA extraction of C. jejuni 81-176 was performed by automated isolation using a MagNA Pure instrument (Roche, Basel, Switzerland). Hybrid-primers containing a
5’ and 3’ fragment of the respective target genes as well as a fragment of the multiple cloning site in the pBlueScript vector, were generated using Geneious Prime version 2021.1.1 (Geneious, Auckland, New Zealand). Primers were ordered from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). For each fragment, two primers forward (5’→3’) and reverse (3’→5’) were ordered, see table 1 for details. In addition, primers for the amplification of a kanamycin cassette were used.

Table 1: Primers used for the amplification of the fragments for insertion in the vector. ∆cmeB, ∆cmeR and ∆cbrR refer to the insertional gene inactivation mutants of the genes cmeB, cbrR and cmeR, respectively. F and R refers to the direction of the amplification of the primer, forward direction from 5’ to 3’ (F) and reverse direction from 3’ to 5’ (R). The primers kan F/R primers were used for amplification of the kanamycin cassette.

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence (5’ → 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5’ΔcmeB F</td>
<td>AGGTCGACGGTATCGATAAGCTTGATATCGCACTCCAAGCTATGGAATAATCATCCCCTA</td>
</tr>
<tr>
<td>5’ΔcmeB R</td>
<td>TCTCGTTTTCATACCTCGGTATAATCTTACCAAAACAAAAGCGGAAAAAGCTATGATGAA</td>
</tr>
<tr>
<td>3’ΔcmeB F</td>
<td>TACTGGATGAATTGTTTTAGTACCTAGATTGAAATTAATGACATCATAATCA</td>
</tr>
<tr>
<td>3’ΔcmeB R</td>
<td>GCGGTGGCGGCCGCTCTAGAACTAGTGGATCAGATGCAGTTAAAAAACTTGGAGTAACAG</td>
</tr>
<tr>
<td>5’ΔcmeR F</td>
<td>AAGGTAGACGTTAGCTAGATCGACTTGAGGTTTTAATAATGACATCATAATCA</td>
</tr>
<tr>
<td>5’ΔcmeR R</td>
<td>TCTCGTTTTCATACCTCGGTATAATCTTACCAAAACAAAAGCGGAAAAAGCTATGATGAA</td>
</tr>
<tr>
<td>3’ΔcmeR F</td>
<td>TACCTGGATGAATTGTTTTAGTACCTAGATTGAAATTAATGACATCATAATCA</td>
</tr>
<tr>
<td>3’ΔcmeR R</td>
<td>GCGGTGGCGGCCGCTCTAGAACTAGTGGATGTAAGTGAATTATATAATATTTCAAATGTT</td>
</tr>
<tr>
<td>5’ΔcbrR F</td>
<td>AGGTCGACGGTATCGATAAGCTTGATATCGGGATTTTTTATTTTACTATGTTAGAATATA</td>
</tr>
<tr>
<td>5’ΔcbrR R</td>
<td>TCTCGTTTTCATACCTCGGTATAATCTTACCAAAACAAAAGCGGAAAAAGCTATGATGAA</td>
</tr>
<tr>
<td>3’ΔcbrR F</td>
<td>TACCTGGATGAATTGTTTTAGTACCTAGATTGAAATTAATGACATCATAATCA</td>
</tr>
<tr>
<td>3’ΔcbrR R</td>
<td>GCGGTGGCGGCCGCTCTAGAACTAGTGGATGTAAGTGAATTATATAATATTTCAAATGTT</td>
</tr>
<tr>
<td>kan F</td>
<td>GTAAGATTTAACCAGGAAAGAAACG</td>
</tr>
<tr>
<td>kan R</td>
<td>AATCTAGGACTAAACACATATCCA</td>
</tr>
</tbody>
</table>

Assembly of the vector was done with the four different fragments using the NEBuilder HiFi DNA Cloning kit (NEB) according to the manufacturer’s instructions. The assembled vector then contained the 5’ and 3’ overlapping Campylobacter DNA regions of the respective target gene and the kanamycin resistance cassette which was placed between both fragments. After the assembly, the plasmid was transferred to chemo competent NEB-5-α E. coli cells. Successful transformation was ensured via selective ampicillin LB agar plates (50 µg/mL ampicillin). Final confirmation of the vector was done via sanger sequencing by Microsynth SeqLab GmbH (Göttingen, Germany).

The generation of the knockout-mutants ∆cmeB and ∆cmeR was accomplished by Maja Andiel under the instruction and supervision of Annika Dreyer as part of Maja Andiel’s Bachelor’s Thesis [155].

Complementation of ∆cmeB was tried according to Karlyshew and Wren [156], but was not successful. To generate electrocompetent C. jejuni cells, bacteria were harvested and
washed three times via centrifugation at 13,000 rpm in a tabletop centrifuge using an ice-
cold washing buffer which contained 15 % Glycerol and 272 mM Sucrose. The cells were
immediately used for electroporation. Electroporation of the competent \textit{C. jejuni} cells was
done using the Electro Cell Manipulator 600 with an Electroporation Safety Stand 630A
by BTX Electroporation System (Holliston, Massachusetts, USA). Prior to use, cuvettes
were stored at -20 °C and transferred to ice before the electroporation. Electroporation
was performed with the following settings: Resistance = 2.5 kV, resistance timing =
186 Ω, capacitance timing = 25 μF and pulse = 2.50. After electroporation, the cells were
transferred to a CAM agar plate without kanamycin and incubated overnight at 42 °C.
The next day, the bacteria were transferred to selective plates containing kanamycin
(50 μg/mL) for the knockout mutants or chloramphenicol for the complementary mutants
and incubated at 42 °C for two days. Single colonies were picked and used for further
experiments.

4.2.3 Autoagglutination assay

To compare the autoagglutination potential of the different knockout mutants, an assay
as previously described by Misawa and Blaser [157] was done with slight modifications.
The parental strain \textit{C. jejuni} and the mutants were grown on their respective agar plates
overnight at 42 °C under microaerophilic conditions. Bacteria were harvested from the
plate and resuspended in 1 ml 1x PBS. The OD$_{600}$ was adjusted to 1 for the inoculation.
2 ml of the bacterial suspensions were transferred into glass tubes and incubated for
24 h at 37 °C without shaking under microaerophilic conditions. After 24 h, 1 ml of the
supernatant was carefully drawn out and the OD$_{600}$ was measured and compared to the
start OD$_{600}$. Experiments were carried out for each strain in biological triplicates and
technical duplicates per biological experiment.

4.2.4 Biofilm assay

The biofilm formation potential of the knockout mutants was observed in a biofilm
assay. Bacteria were harvested from the plates and resuspended in liquid MH broth
and the OD$_{600}$ was adjusted to 0.05. Wells in a 96-well plate (flat-bottom, Greiner
Bio-one, Frickenhausen, Germany) were filled with 100 μl of the bacterial suspension
and incubated for 48 h at 37 °C under microaerophilic conditions without shaking. After 48 h, the bacterial suspensions were carefully drawn out of the wells and the plate was dried for 30 min at 60 °C. For staining of the cells, 100 µL of 0.1 % crystal violet was used and the cells were incubated for 15 min at room temperature. Unbound crystal violet solutions were discarded, and the wells were rinsed with 100 µl dH2O. After this, the plates were dried for 15 min at 60 °C. For quantification of the biofilm formation, 100 µl of a 20 % acetone and 80 % ethanol dissolving solution was added to the wells and the wells were incubated for 15 min at room temperature. For measurement of the absorbance, 80 µl of the dissolved crystal violet were transferred to a new 96-well plate. The plate was read at an absorbance of 570 nm using a microplate spectrophotometer (epoch 2, BioTek, Santa Clara, CA, USA). Experiments were carried out for each strain in biological triplicates and technical quadruplicates per biological experiment.

4.2.5 Motility

The motility of the mutants was checked using a soft-agar swarming-assay as described by Kearns in 2010 [158]. Therefore, 0.3 % soft agar with 5 % sheep-blood was prepared. A spot of 3 µL C. jejuni 81-176 parental strain and the respective mutants was placed in the middle of a plate. After two days of growth, swarming was examined. Swarming is a collective form of motion in which cells migrate rapidly over surfaces, where they are able to form dynamic patterns.

Moreover, the motility of the mutants was compared to the parental strain at different growth stadiums under a microscope. Therefore, growth was tracked using the CG Quant system. At the peak phase of growth, the bacteria were carefully transferred to PBS with 5 % polyvinylpyrrolidone (PVP) (K 90 polyvinylpyrrolidone ordered from Carl Roth, Karlsruhe, Germany). PVP was used due to the observation, that in presence of high molecular weight molecules, the motility of other gastrointestinal bacteria such as C. difficile is enhanced [159]. Furthermore, C. jejuni seems to show enhanced motility with increased viscosity [41].

Additionally, motility was investigated using TTC (triphenyl tetrazolium chloride) assays, where a medium containing 2.8% brucella broth (Sigma Aldrich, St. Louis, Missouri, USA) with 2.5 % agarose and 5 mg/mL TTC was filled into 15 mL falcon tubes. Due to
the presence of different active dehydrogenases in living cells, TTC is reduced to TPF (1,3,5-triphenylformazan), a red colored substance, which serves as stain. 50 µL bacterial suspension at an OD600 of 1 were added on top of the cooled agar in the falcon tubes. The bacteria were incubated for 24 h at 42 °C. Motility was checked via the staining depth which correlates with the capability of motility.

4.2.6 Protein purification and quantification

*C. jejuni* 81-176 parental strain and the knockout mutants ΔcmeB, ΔcbrR and ΔcmeR were incubated overnight in 10 mL liquid MH broth with or without the respective amount of DCA or CA. After incubation, the cultures were centrifuged at 4,000 rpm for 10 min at 4 °C. The supernatant was discarded, and the pellets were resuspended in 2 mL 0.9 % saline and kept on ice. Subsequently, the Gram-negative cells were disrupted via sonification using a Branson sonifier 250 from Branson ultrasonics (Brookfield, Connecticut, USA) with the following settings: output control = 3, duty cycler = 30 %. Sonification was performed five times for 30 seconds followed by 30 seconds cooling. The disrupted cells were pre-centrifuged for 10 min at 4,000 rpm at 4 °C. The supernatant was centrifuged at 12,000 rpm for 15 min at 4°C in a tabletop centrifuge. A Pierce assay was performed to determine the protein concentration of the samples. Afterwards, the concentrations were adjusted to 1 µg/µL. For all samples, biological triplicates were prepared.

4.2.7 DIA-MS

DIA-MS was performed as described in Manuscript I (chapter 2) and II (chapter 3). The respective protein samples were purified by short-run SDS-PAGE with Coomassie stain. Digestion of samples was done via in-gel tryptic digestion. For the library, a pre-fractionation of a pooled reference sample was divided into 12 different fractions via basic pH-reversed phase chromatography. In the next step, the spiking was performed with a Biognosys iRT peptide standard.

For the following mass spectrometric analysis, the identification of proteins was done by data-dependent acquisition (DDA) on a TripleTOF 5600+ (Sciex, Darmstadt, Germany). Therefore, 1000 ng equivalent were loaded. Afterwards, a 90 min gradient was performed,
and the Top25 method followed. Per RP fraction, two technical replicates were prepared. Quantification and ID determination by DIA-MS were done utilizing the Thermo Q Exactive. Three technical replicates were prepared of each biological replicate. Data processing was done using the Spectronaut v16.0.220606.53000 software package (Biognosys AG, Schlieren, Switzerland).

Identification of proteins as well as hybrid spectral library generation from 12x2 DDA acquisitions and 12x2 DIA acquisitions experiments were done using Pulsar search engine against UniProtKB the \textit{C. jejuni} 81-176 proteome default parameters. For every sample, a False Discovery Rate (FDR) of 1 \% on the spectral, peptide and protein group levels was chosen. DIA quantification was performed utilizing up to 6 fragments per peptide and up to 10 peptides per protein. A dynamic retention time alignment was done, as well as dynamic mass recalibration and quartile normalization, for 1 \% FDR. For the final results tables, global data imputation was done. The data produced in this project can be viewed on request from the PRIDE database [160].

\subsection*{4.2.8 Data processing}

For the statistical analysis that followed DIA-MS, Perseus v1.6.2.2 was employed for generation of volcano plots to compare the different samples [161, 162]. As significant regulation level, two-fold up- or down-expression was chosen. Only those proteins that were present in five out of six samples were considered for further analysis. Using Perseus, log2(x) transformed intensity values were used for analyses. For volcano-plot generation, a t-test was chosen with a number of randomizations = 250 and a FDR of 0.05. All proteins that are described in the following as up- or down-expressed were significantly regulated, if not otherwise stated.

Further analysis was done using MS Excel (Microsoft) and python3. An R script was used to perform an ANOVA (Analysis of Variance) analysis [163]. Generation of Venn diagrams was done using InteractiVenn to identify proteins that were commonly up- or down regulated different samples [164]. Plots were generated using matplotlib as well as plotly in python3 [165].
4.3 Results

4.3.1 \( \Delta cmeB \) shows significant proteomic and phenotypic changes

The phenotypic analysis of the insertional gene inactivation mutant \( \Delta cmeB \) revealed a reduced fitness when compared to the parental strain. \( \Delta cmeB \) growth was significantly slower than the parental strain. Moreover, \( \Delta cmeB \) was unable to grow on CAM-agar plates due to the enhanced susceptibility to antibiotics. Therefore, blood-agar plates containing kanamycin as only antibiotic were manufactured. \( \Delta cmeB \) was highly susceptible to antibiotics and bile salts. Therefore, it was difficult to determine an IC\(_{50}\) for this knockout mutant. \( \Delta cmeB \) was incubated with different concentrations of DCA and CA and growth was only visible at concentrations lower than 0.006 % of CA and 0.001 % of DCA (Table 2).

Table 2: Half IC\(_{50}\) of the parental strain and each mutant. Percentage of CA or DCA present in the medium.

<table>
<thead>
<tr>
<th>Half IC(_{50})</th>
<th>wt</th>
<th>( \Delta cmeB )</th>
<th>( \Delta cbrR )</th>
<th>( \Delta cmeR )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholic acid (CA)</td>
<td>0.25 %</td>
<td>0.003 %</td>
<td>0.125 %</td>
<td>0.275 %</td>
</tr>
<tr>
<td>Deoxycholic acid (DCA)</td>
<td>0.15 %</td>
<td>0.0005 %</td>
<td>0.185 %</td>
<td>0.27 %</td>
</tr>
</tbody>
</table>

Interestingly, microscopy revealed an enhanced general motility of \( \Delta cmeB \) in liquid MH with 5 % PVP. \( \Delta cmeB \) seems to be highly motile compared to the slightly motile parental strain and the other insertional gene inactivation mutants. This effect was demonstrated in different independent knockout mutants of the CmeB protein in this study. Motility was also tested in swarming assays (Figure 3), where bacteria migrate over a surface. Furthermore, motility was analyzed in TTC assays (Figure 4), where the staining depth of the agar showed the ability of the bacteria to move. However, the motility of \( \Delta cmeB \) in these assays was similar to the motility of the parental strain.

Furthermore, the biofilm formation of the mutant was tested and revealed high similarities to the parental strain (Figure 5). The autoagglutination potential of \( \Delta cmeB \) was slightly enhanced, but not significant (Figure 6).

Despite multiple attempts using the method described by Karlyshev and Wren [156], a complementary mutant of \( \Delta cmeB \) could not be generated.
Proteomic changes

When compared to the proteome of the parental strain, 83 proteins were significantly up-expressed and 62 proteins were down-expressed (See Table 3). Differentially expressed proteins in $\Delta cmeB$ were assigned to their respective COG categories using eggnogmapper v 2.18. Significantly up- or downexpressed proteins were depicted in a bar plot that shows the percentual amount of these proteins compared to the whole proteome identified in DIA-MS. The relative proportion of the individual COG-categories shows a percentual

![Figure 3: Motility assays of C. jejuni parental strain (wt) and knockout mutants. Bacterial swarming is a social behavior where bacteria migrate over the agar surface. It is a type of multicellular surface movement powered by rotating helical flagella [157]. The swarming potential of $\Delta cmeB$ is enhanced compared to the parental strain while $\Delta cbrR$ and $\Delta cmeR$ show no swarming motility at all. Yellow bars represent the radii of the swarming distance after 48 h. The ANOVA test showed no significance in the swarming distance of $\Delta cmeB$ compared to the parental strain.](image-url)
Figure 4: Motility-assay, TTC-based: The Staining depth of the TTC assay of *C. jejuni* wt and knockout mutants is depicted. The staining depth correlates with the capability of motility. Δ*cmeB* shows enhanced motility when compared to the parental strain and the other two mutants. Δ*cbrR* shows decreased motility and Δ*cmeR* shows significantly reduced motility. The ANOVA test showed the following p-values: * = significant, p ≤ 0.05, ** = significant, p ≤ 0.01. P-value wt-ΔcmeR = 0.035, p-value ΔcmeB-ΔcbrR = 0.005, p-value ΔcmeR-ΔcmeB = 0.002.

decrease of proteins assigned to the categories P (inorganic ion transport and metabolism), T (signal transduction) and V (defense mechanisms). On the other side, the categories C (energy production), D (cell cycle control and mitosis), G (carbohydrate metabolism and transport), H (co-enzyme metabolism), M (cell wall/membrane/envelope biogenesis), N (Motility), O (Post-translational modification, protein turnover, chaperone functions) and Q (Secondary Structure) show a percentual increase.

The highest up-expressed proteins were an ABC transporter, periplasmic substrate-binding protein (A0A0H3PAR5_CAMJJ), a putative Phosphate ABC transporter, periplasmic phosphate-binding protein (A0A0H3PEG8_CAMJJ), and several flagellar proteins (Figure 7). Moreover, CmeA, the membrane fusion protein of the multidrug efflux system was up-expressed. Among the 62 down-expressed proteins, CmeB, the inner membrane transporter and CmeC, the outer membrane lipoprotein were found. This confirmed
Figure 5: Biofilm formation potential of \textit{C. jejuni} wt and knockout mutants. The biofilm formation potential of \textit{\Delta}cmeB is similar to the parental strain while \textit{\Delta}cbrR and \textit{\Delta}cmeR show slightly increased biofilm formation potential. Bars represent the means of biological triplicates, which consist of technical duplicates, respectively. Error bars represent the standard deviation of the biological triplicates. The ANOVA analysis showed no significance between any of the mutants or the parental strain.

the successful knockout of CmeB and also the lack of expression of CmeC, which is located downstream of CmeB in the genome of \textit{C. jejuni}. Without these two proteins, the multidrug efflux system is non-functional as demonstrated by high bile acid sensitivity of \textit{\Delta}cmeB. Interestingly, the enhanced motility of \textit{\Delta}cmeB is also visible in the proteome. Several flagellar proteins are up-expressed when compared to the slightly motile parental strain. Especially five proteins CJJ81176_1338 (flagellin), flgE (Flagellar hook protein), flgD (basal-body rod modification protein), pseA (flagellin modification protein) and flaA (flagellin) were strongly upexpressed in \textit{\Delta}cmeB, indicating, that these proteins might be influenced by cmeB or cmeABC.
Figure 6: Relative autoagglutination potential of *C. jejuni* wt and knockout mutants. The autoagglutination potential of Δ*cmeB* is strongly increased when compared to the parental strain. Moreover, Δ*cbrR* and Δ*cmeR* show a slightly increased autoagglutination potential. The bars represent the means of biological triplicates, which consist of technical duplicates, respectively. Error bars represent the standard deviation of the biological triplicates. The ANOVA analysis showed no significance between any of the mutants or the parental strain.
Table 3: The 83 up- and 62 down-expressed proteins in ΔcmeB compared to the parental strain, sorted by difference, from high to low values. Green-marked proteins represent flagellum-associated proteins and pink-marks represent members of the CmeABC efflux. Blue marked proteins are ABC transporters.

<table>
<thead>
<tr>
<th>Up-expressed proteins</th>
<th>Protein description</th>
<th>Protein names</th>
<th>Down-expressed proteins</th>
<th>Protein description</th>
<th>Protein names</th>
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<tr>
<td>A0A0H3PAS5_CAMJJ</td>
<td>Peroxisome proliferator-activated receptor (PPAR) translocator, PPAR alpha</td>
<td>NAPA_CAMJJ</td>
<td>A0A0H3PDA9_CAMJJ</td>
<td>Peroxisome proliferator-activated receptor (PPAR) translocator, PPAR alpha</td>
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| A0A0H3PAS5_CAMJJ       | Peroxisome proliferator-activated receptor (PPAR) translocator, PPAR alpha | NAPA_CAMJJ | A0A0H3PDA9_CAMJJ         | Perox...
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<td>Delta-aminolevulinic acid dehydratase</td>
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<td>2-acylglcyerocephothanolamine acyltransferase / acyl-acetyl carrier protein synthetase</td>
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<tr>
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<td>Oxidoreductase, short chain dehydrogenase/reductase family</td>
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</table>
Figure 7: Volcano plot of the knockout mutant ΔcmeB compared to the parental strain (wt). The Y-axis shows the -Log of the p-value and the X-axis shows the scale of difference between both proteomes. Pink marked squares represent proteins belonging to the CmeABC multidrug efflux. Green squares represent flagellar proteins. Blue squares represent ABC-efflux proteins.
4.3.2 ΔcbrR phenotypic and proteomic changes

Phenotypic changes

The knockout mutant ΔcbrR shows similar growth as the parental strain and is not as reduced in the general fitness as ΔcmeB. When incubated with the respective half IC$_{50}$ of DCA or CA, ΔcbrR shows a similar susceptibility towards DCA as the parental strain but is more susceptible towards CA.

Motility experiments showed that ΔcbrR is not motile. While the parental strain and ΔcmeB showed a strong swarming ability, ΔcbrR did not show swarming-motility (Figure 3). In addition, TTC-assays showed that ΔcbrR did not move through the agar (Figure 4). Microscopy confirmed the inability of ΔcbrR to move. In ΔcbrR, the autoagglutination assays revealed a similar autoagglutination-potential as the parental strain (Figure 6).

Proteomic changes

In contrast to ΔcmeB, less proteins were significantly differentially expressed in ΔcbrR when compared to the parental strain proteome (Table 4, Figure 8). Three proteins were up-expressed, namely A0A0H3PB35_CAMJJ, a putative sugar transferase, A0A0H3PDV4_CAMJJ, a putative methyltransferase and A0A0H3PES6_CAMJJ, an uncharacterized protein. Among the seven down-expressed proteins were A0A0H3PJ41_CAMJJ, the response regulator CbrR, which confirmed the successful knockout. Furthermore, two flagellar proteins, A0A0H3PDN2_CAMJJ, a putative methyltransferase, A0A0H3PAC8_CAMJJ, a 3-deoxy-D-manno-octulosonate 8-phosphate phosphatase KdsC, A0A0H3PHN8_CAMJJ, an amino acid-binding protein and A0A0H3PAA9_CAMJJ, a putative sugar transferase were found in the down-expressed proteins.
Table 4: The three up- and seven down-expressed proteins in ΔcbrR when compared to the wildtype C. jejuni, sorted by difference, from high values to low values. Green-marked proteins represent flagellum-associated proteins. A0A0H3PJ41_CAMJJ, marked in dark blue, is one of the top down-expressed proteins and a synonym for CbrR.

<table>
<thead>
<tr>
<th>Up-expressed</th>
<th>Protein names</th>
<th>Protein descriptions</th>
<th>Down-expressed</th>
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<td>Putative sugar transferase</td>
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<td>Q2M5R2_CAMJJ</td>
<td>Flagellin</td>
<td></td>
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</tbody>
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Figure 8: Volcano plot of the knockout mutant ΔcbrR compared to the parental strain (wt). The Y-axis shows the -Log of the p-value and the X-axis shows the scale of difference between both proteomes. Darkblue marked square shows CbrR. Green squares represent flagellar proteins.
4.3.3 ΔcmeR phenotypic and proteomic changes

**Phenotypic changes**

The knockout mutant ΔcmeR shows similar growth as the parental strain and is not reduced in the general fitness as ΔcmeB. According to current knowledge, autoagglutination has not been studied yet in CmeR knockout mutants. No significant changes were observed in the autoagglutination potential of ΔcmeR, as the measured optical density after 24 h incubation was similar to the parental strain (Figure 6). Furthermore, in this study, biofilm assays revealed a slightly enhanced but not significant ability of ΔcmeR to form biofilms compared to the parental strain (Figure 5). Motility experiments showed that ΔcmeR is not motile, as ΔcbrR. No swarming-motility was observed (Figure 3) and in TTC-assays, motility was significantly reduced compared to the parental strain (Figure 4). Microscopy of the mutant confirmed the immotility. ΔcmeR is more resistant against CA and DCA and has thus a higher IC\textsubscript{50} than the parental strain.

**Proteomic changes**

In the proteome of ΔcmeR, 12 proteins were up-expressed and 22 proteins were down-expressed in comparison to the parental strain (Table 5, Figure 9). The CmeR protein as well as flagellin (flaA) were among the downexpressed proteins. CmeA, CmeB and CmeC on the other hand, were significantly upexpressed. Among the up-expressed proteins in ΔcmeR an uncharacterized protein designated as A0A0H3PA13_CAMJJ and also known as Cj0561c was found to be highly up-expressed (Figure 9), this protein was also up-expressed in the parental strain, ΔcmeB and ΔcbrR in presence of CA. A study from 2008 showed that Cj0561c seems to be strongly induced by bile salts, and is probably regulated by CmeR [166]. Other slightly up-expressed proteins found in the sample were A0A0H3PA35_CAMJJ, a thiol-disulfide interchange protein DsbA, an AccP protein (Q2M5Q4_CAMJJ), an UPF0033 domain-containing protein (A0A0H3P9W6_CAMJJ), flagellin subunit protein FlaC (A0A0H3PDD9_CAMJJ), a putative methyltransferase (A0A0H3PDV4_CAMJJ), a malate dehydrogenase (A0A0H3PBR0_CAMJJ) and two uncharacterized proteins (A0A0H3PCX6_CAMJJ and A0A0H3PCI2_CAMJJ).
Table 5: The 12 up- and 22 down-expressed proteins in ∆cmeR when compared to the wildtype C. jejuni, sorted by difference, from high values to low values. Green-marked proteins represent flagellum-associated proteins, pink-marked candidates represent members of the CmeABC efflux and the orange-marked protein is A0A0H3PAI3_CAMJJ, a synonym for cj0561c, which was remarkably high up-expressed in this sample. A0A0H3PED0_CAMJJ, marked in bright blue, is the top down-expressed protein and a synonym for CmeR.

<table>
<thead>
<tr>
<th>Up-expressed</th>
<th>Protein names</th>
<th>Protein description</th>
<th>Down-expressed</th>
<th>Protein names</th>
<th>Protein description</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A0A0H3PAI3_CAMJJ</td>
<td>Uncharacterized protein (Cj0561c)</td>
<td></td>
<td>A0A0H3PED0_CAMJJ</td>
<td>CmeR (Transcriptional regulator, TetR family)</td>
</tr>
<tr>
<td></td>
<td>A0A0H3PB79_CAMJJ</td>
<td>Efflux pump membrane transporter CmeB</td>
<td></td>
<td>A0A0H3PDN2_CAMJJ</td>
<td>Putative methyltransferase</td>
</tr>
<tr>
<td></td>
<td>A0A0H3PIS5_CAMJJ</td>
<td>RND efflux system, membrane fusion protein CmeA</td>
<td></td>
<td>A0A0H3JB7_CAMJJ</td>
<td>Succinate dehydrogenase, iron-sulfur protein subunit</td>
</tr>
<tr>
<td></td>
<td>A0A0H3PAE4_CAMJJ</td>
<td>RND efflux system, outer membrane lipoprotein CmeC</td>
<td></td>
<td>Q2M5R2_CAMJJ</td>
<td>Flagellin</td>
</tr>
<tr>
<td></td>
<td>A0A0H3PA35_CAMJJ</td>
<td>Thiol-disulfide interchange protein DsbA</td>
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<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Q2M5Q4_CAMJJ</td>
<td>A0A0H3PED0_CAMJJ</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>A0A0H3PD9_CAMJJ</td>
<td>Flagellin subunit protein Flac</td>
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</tr>
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<td>A0A0H3P9W6_CAMJJ</td>
<td>Uncharacterized protein</td>
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<td></td>
<td></td>
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<td>A0A0H3PC2_CAMJJ</td>
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<td></td>
<td>A0A0H3PV4_CAMJJ</td>
<td>Putative methyltransferase</td>
<td></td>
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<td></td>
<td>A0A0H3PC6_CAMJJ</td>
<td>Uncharacterized protein</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>A0A0H3PR0_CAMJJ</td>
<td>Malate dehydrogenase</td>
<td></td>
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<td></td>
</tr>
<tr>
<td></td>
<td>Q29VV3_CAMJJ</td>
<td>A0A0H3PAJ3_CAMJJ</td>
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<td></td>
</tr>
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<td></td>
<td>A0A0H3PA50_CAMJJ</td>
<td>Lipoprotein, putative frameshift</td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td>A0A0H3PL7_CAMJJ</td>
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<td></td>
<td>A0A0H3PCN0_CAMJJ</td>
<td>Uncharacterized protein</td>
<td></td>
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<td></td>
<td>A0A0H3PHS_CAMJJ</td>
<td>D-3-phosphoglycerate dehydrogenase</td>
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<td>A0A0H3PHS_CAMJJ</td>
<td>Methyl-accepting chemotaxis protein</td>
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<td>Q29VV3_CAMJJ</td>
<td>Putative glycosyl transferase</td>
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<td>A0A0H3PC10_CAMJJ</td>
<td>Disulphide bond formation protein, DsbB family</td>
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<tr>
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<td>A0A0H3PC6_CAMJJ</td>
<td>NADP-dependent malic enzyme, truncation</td>
<td></td>
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<td></td>
<td>A0A0H3PD6_CAMJJ</td>
<td>HDOD domain-containing protein</td>
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</tr>
<tr>
<td></td>
<td>A0A0H3NI6_CAMJJ</td>
<td>Amino acid-binding protein</td>
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<td></td>
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<tr>
<td></td>
<td>A0A0H3PA44_CAMJJ</td>
<td>Lipoprotein, putative frameshift</td>
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<tr>
<td></td>
<td>A0A0H3PI0_CAMJJ</td>
<td>Quinone-reactive Ni/Fe hydrogenase, cytochrome b subunit</td>
<td></td>
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<td></td>
</tr>
<tr>
<td></td>
<td>DER_CAMJJ</td>
<td>GTPase Der</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>A0A0H3PA52_CAMJJ</td>
<td>Periplasmic serine endoprotease</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>A0A0H3PI8_CAMJJ</td>
<td>CjaC protein</td>
<td></td>
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<td></td>
</tr>
<tr>
<td></td>
<td>PSEB_CAMJJ</td>
<td>UDP-N-acetylgucosamine 4,6-dehydratase (inverting)</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>A0A0H3PI91_CAMJJ</td>
<td>Major outer membrane protein</td>
<td></td>
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</tbody>
</table>
Figure 9: Volcano plot of the knockout mutant ∆cmeR compared to the parental strain (wt). The Y-axis shows the -Log of the p-value and the X-axis shows the scale of difference between both proteomes. Pink marked squares represent proteins belonging to the CmeABC multidrug efflux. Brightblue square represents CmeR. Green squares represent flagellar proteins. Orange marked square represents A0A0H3PAI3_CAMJJ (also known as: CJJ811176_0586 or Cj0561c), which showed pronounced increase in its expression levels, indicating that CmeR might regulate expression of this protein.
4.3.4 Proteomic changes after long-term bile acid exposure

The parental *C. jejuni* strain and all mutants were incubated in their respective half IC$_{50}$ of CA over 24 h. DIA-MS showed proteomic adaptions towards CA. CA did not have an influence on the motility of the bacteria. In general, not many proteins are needed to adapt towards CA. The presence of the CmeABC efflux in the parental strain, ∆cbrR and ∆cmeR was necessary and sufficient for overcoming CA stress.

Proteomic response to cholic acid of the parental strain

After incubation with the respective half IC$_{50}$ of CA over 24 h, the wild-type *C. jejuni* 81-176 showed a bile-acid specific proteomic response. The number of regulated proteins was rather low, indicating that only these few up-expressed proteins are relevant to cope with bile acid stress. In total, 7 proteins were up-expressed and five proteins were down-expressed (Table 6, Figure 10). CmeABC subunits were significantly up-expressed and also Cj0561c (A0A0H3PAI3_CAMJJ) was among the up-expressed proteins. Other up-expressed proteins were an arylsulfate sulfotransferase (A0A0H3P9J4_CAMJJ), a cystathionine beta-lyase (A0A0H3PAV5_CAMJJ) and a putative dihydroorotase (A0A0H3P9D3_CAMJJ). Among the five down-expressed proteins were two uncharacterized proteins (A0A0H3PCN0_CAMJJ and A0A0H3P9L7_CAMJJ), a 5-hydroxyisourate hydrolase (A0A0H3PHJ0_CAMJJ), a cytochrome c family protein (A0A0H3P9N5_CAMJJ) and a periplasmic serine endoprotease, DegP-like (A0A0H3PA52_CAMJJ).

Table 6: The seven up-expressed and five down-expressed proteins of the wildtype *C. jejuni* after longterm incubation with CA. Pink-marked candidates represent members of the CmeABC efflux and the orange-marked protein is A0A0H3PAI3_CAMJJ, a synonym for cj0561c.

<table>
<thead>
<tr>
<th>Up-expressed Protein names</th>
<th>Protein descriptions</th>
<th>Down-expressed Protein names</th>
<th>Protein descriptions</th>
</tr>
</thead>
<tbody>
<tr>
<td>A0A0H3PB79_CAMJJ</td>
<td>Efflux pump membrane transporter CmeB</td>
<td>A0A0H3PCN0_CAMJJ</td>
<td>Uncharacterized protein</td>
</tr>
<tr>
<td>A0A0H3PSSS_CAMJJ</td>
<td>RND efflux system, membrane fusion protein CmeA</td>
<td>A0A0H3PHJ0_CAMJJ</td>
<td>5-hydroxyisourate hydrolase</td>
</tr>
<tr>
<td>A0A0H3PAE4_CAMJJ</td>
<td>RND efflux system, outer membrane lipoprotein CmeC</td>
<td>A0A0H3P9N5_CAMJJ</td>
<td>Cytochrome c family protein</td>
</tr>
<tr>
<td>A0A0H3P9J4_CAMJJ</td>
<td>Arylsulfate sulfotransferase, degenerate</td>
<td>A0A0H3PL7_CAMJJ</td>
<td>Uncharacterized protein</td>
</tr>
<tr>
<td>A0A0H3PAI3_CAMJJ</td>
<td>Uncharacterized protein (Cj0561c)</td>
<td>A0A0H3PA52_CAMJJ</td>
<td>Periplasmic serine endoprotease DegP-like</td>
</tr>
<tr>
<td>A0A0H3PAV5_CAMJJ</td>
<td>Cystathionine beta-lyase</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A0A0H3P9D3_CAMJJ</td>
<td>Dihydroorotase, putative</td>
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</table>
Figure 10: Volcano plot of the parental *C. jejuni* (wt) compared to the parental strain with CA. The Y-axis shows the -Log of the p-value and the X-axis shows the scale of difference between both proteomes. Pink marked squares represent proteins belonging to the CmeABC multidrug efflux. The orange marked square represents A0A0H3PAI3_CAMJJ (also known as: CJJ81176_0586 or Cj0561c).
ΔcmeB proteomic response to low concentrations of cholic acid

Due to the fact that ΔcmeB is highly susceptible for bile acids, only very low concentrations of CA were used to determine the stress proteome after long-term exposure. ΔcmeB was able to survive 0.003 % CA, and still showed a proteomic stress response, when compared to ΔcmeB without bile acid exposure (Table 7, Figure 11). The usual up-expression of the multidrug efflux CmeABC was not possible for this mutant. However, Cj0561c (A0A0H3PAI3_CAMJJ) was among the five significantly up-expressed proteins, as in the parental strain. The other up-expressed proteins were a putative membrane protein (A0A0H3PAJ1_CAMJJ), a putative lipoprotein (A0A0H3PBE5_CAMJJ), a 3-deoxy-D-manno-octulosonate 8-phosphate phosphatase KdsC (A0A0H3PAC8_CAMJJ) and a DUF4261 domain-containing protein (A0A0H3PE85_CAMJJ). Seven proteins were down-expressed in CA exposure.

Table 7: The five up-expressed and seven down-expressed proteins of ΔcmeB after longterm incubation with CA. The orange-mark represents the protein A0A0H3PAI3_CAMJJ, a synonym for cj0561c.

<table>
<thead>
<tr>
<th>Up-expressed Protein names</th>
<th>Protein descriptions</th>
<th>Down-expressed Protein names</th>
<th>Protein descriptions</th>
</tr>
</thead>
<tbody>
<tr>
<td>A0A0H3PAI3_CAMJJ</td>
<td>Uncharacterized protein (Cj0561c)</td>
<td>A0A0H3PAJ3_CAMJJ</td>
<td>Citrate transporter, authentic frameshift</td>
</tr>
<tr>
<td>A0A0H3PAJ1_CAMJJ</td>
<td>Membrane protein, putative</td>
<td>A0A0H3PCN0_CAMJJ</td>
<td>Uncharacterized protein</td>
</tr>
<tr>
<td>A0A0H3PAC8_CAMJJ</td>
<td>Lipoprotein, putative</td>
<td>A0A0H3PA50_CAMJJ</td>
<td>Lipoprotein, putative</td>
</tr>
<tr>
<td>A0A0H3PE85_CAMJJ</td>
<td>3-deoxy-D-manno-octulosonate 8-phosphate phosphatase KdsC</td>
<td>A0A0H3PAC8_CAMJJ</td>
<td>Uncharacterized protein</td>
</tr>
<tr>
<td></td>
<td>DUF4261 domain-containing protein</td>
<td>A0A0H3P9H5_CAMJJ</td>
<td>D-3-phosphoglycerate dehydrogenase</td>
</tr>
<tr>
<td></td>
<td></td>
<td>A0A0H3PHJ0_CAMJJ</td>
<td>5-hydroxisourate hydrolase</td>
</tr>
<tr>
<td></td>
<td></td>
<td>A0A0H3PID6_CAMJJ</td>
<td>NADP-dependent malic enzyme, truncation</td>
</tr>
</tbody>
</table>
Figure 11: Volcano plot of the knockout mutant ΔcmeB compared to ΔcmeB with CA. The Y-axis shows the -Log of the p-value and the X-axis shows the scale of difference between both proteomes. The knockout of CmeABC results in the deregulation of these proteins, thus, CmeABC proteins are not present in the plot. The orange marked square represents A0A0H3PAI3_CAMJJ (also known as: CJJ81176_0586 or Cj0561c).
\(\Delta cbrR\) proteomic response to cholic acid

In the \(\Delta cbrR\) mutant, the up- and down-expressed proteins under CA exposure are similar to the proteins expressed in the parental strain. In total, eight proteins were up-expressed and two proteins were down-expressed (Table 8, Figure 12). The mutant and the parental strain share six commonly expressed proteins. These proteins were the three proteins involved in the CmeABC efflux, Cj0561c (A0A0H3PAI3_CAMJJ) and a degenerate arylsulfate sulfotransferase (A0A0H3P9J4_CAMJJ) among the up-expressed proteins and a periplasmic serine endoprotease, DegP-like (A0A0H3PA52_CAMJJ) was found as commonly down-expressed protein.

Table 8: The eight up-expressed and two down-expressed proteins of \(\Delta cbrR\) after longterm incubation with CA. The orange-mark represents the protein A0A0H3PAI3_CAMJJ, a synonym for cj0561c and the pink-marked proteins represent proteins belonging to the CmeABC efflux.

<table>
<thead>
<tr>
<th>Up-expressed Proteins</th>
<th>Protein descriptions</th>
<th>Down-expressed Proteins</th>
<th>Protein descriptions</th>
</tr>
</thead>
<tbody>
<tr>
<td>A0A0H3PA9J_CAMJJ</td>
<td>Efflux pump membrane transporter CmeB</td>
<td>A0A0H3PAJ3_CAMJJ</td>
<td>Citrate transporter, authentic frameshift</td>
</tr>
<tr>
<td>A0A0H3PIS5_CAMJJ</td>
<td>RND efflux system, membrane fusion protein CmeA</td>
<td>A0A0H3PA52_CAMJJ</td>
<td>Periplasmic serine endoprotease DegP-like</td>
</tr>
<tr>
<td>A0A0H3P9J4_CAMJJ</td>
<td>Arylsulfate sulfotransferase, degenerate</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A0A0H3PAE4_CAMJJ</td>
<td>RND efflux system, outer membrane lipoprotein CmeC</td>
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<tr>
<td>A0A0H3PA35_CAMJJ</td>
<td>Thiol:disulfide interchange protein DsbA</td>
<td></td>
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</tr>
<tr>
<td>A0A0H3PAI3_CAMJJ</td>
<td>Thiol:disulfide interchange protein DsbA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A0A0H3PCI2_CAMJJ</td>
<td>Uncharacterized protein (Cj0561c)</td>
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<td></td>
</tr>
<tr>
<td>Q69B8S_CAMJJ</td>
<td>Uncharacterized protein</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Q69B8B_CAMJJ</td>
<td>Citrate transporter, authentic frameshift</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 12: Volcano plot of the knockout mutant ∆cbrR compared to ∆cbrR with CA. The Y-axis shows the -Log of the p-value and the X-axis shows the scale of difference between both proteomes. Pink marked squares represent proteins belonging to the CmeABC multidrug efflux. The orange marked square represents A0A0H3PAI3_CAMJJ (also known as: CJJ81176_0586 or Cj0561c).
$\Delta cmeR$ proteomic response to cholic acid

Interestingly, the $\Delta cmeR$ knockout-mutant showed no significant up-expression of proteins under bile-acid stress, however, five proteins were down-expressed (Table 9, Figure 13). A possible reason for this is the general high expression of the CmeABC efflux in this mutant, which leads to a higher resistance towards CA. The expression of CmeABC is already high and is thus not enhanced by the presence of CA, as it is the case for the parental strain and $\Delta cbrR$.

Table 9: The five down-expressed proteins of $\Delta cmeR$ after longterm incubation with CA. No significantly up-expressed proteins were found in this approach.

<table>
<thead>
<tr>
<th>Up-expressed Protein names</th>
<th>Protein descriptions</th>
<th>Down-expressed Protein names</th>
<th>Protein descriptions</th>
</tr>
</thead>
<tbody>
<tr>
<td>A0A0H3P9P2_CAMJJ</td>
<td>Acyl carrier protein, putative</td>
<td>A0A0H3PAM5_CAMJJ</td>
<td>4-oxalocrotonate tautomerase family protein</td>
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<tr>
<td>A0A0H3PAM5_CAMJJ</td>
<td>Toluene tolerance protein, putative</td>
<td>A0A0H3PC13_CAMJJ</td>
<td>CheX domain-containing protein</td>
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<td>A0A0H3PHT3_CAMJJ</td>
<td>UPF0234 protein CJJ81176_0398</td>
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</table>
Figure 13: Volcano plot of the knockout mutant $\Delta cmeR$ compared to $\Delta cmeR$ with CA. The Y-axis shows the -Log of the p-value and the X-axis shows the scale of difference between both proteomes. No proteins are significantly up-expressed. Only five proteins are significantly down-expressed.
5 Additional results - *C. jejuni* conferred bile salt resistance

5.1 Background

In previous co-incubation experiments carried out by a doctoral student (Ruben Leonhard Ullrich, unpublished data), the observation that *C. jejuni* seems to be able to increase bile acid stress resistance of *E. faecalis* and other bacteria was made. Therefore, *C. jejuni* 81-176 was incubated in a 5:1 ratio with *E. faecalis* for three hours with or without 0.1 % of DCA. Afterwards, a spot assay in a dilution series from $10^{-1}$ to $10^{-6}$ was performed to check for survival of *E. faecalis* (Figure 14).

When incubated without *C. jejuni*, *E. faecalis* usually does not survive in presence of DCA, and no or only little growth is visible on the spot assay. When co-incubated with *C. jejuni* in presence of DCA, survival rates similar to the incubation without DCA is visible. This effect was called the “ProBAS” effect (Protection from bile acid stress). In order to analyze the mechanism behind this effect, different experiments were conducted.

It was observed that the protective effect can be mediated by the supernatant from a previous ProBAS experiment, as incubation of the bacteria with the supernatant leads to a similar but slightly reduced effect. When the supernatant was treated with proteinase K,

![Figure 14](image)

*Figure 14*: Example of a ProBAS assay: A spot assay in a dilution series after mono- or co-incubation of *E. faecalis* 700802 and *C. jejuni* 81-176 or 11168 with or without 0.1 % DCA. In presence of DCA, *E. faecalis* shows minimal to negligible survival rates. When co-incubated with *C. jejuni* 81-176 or other *C. jejuni* in presence of DCA, survival of *E. faecalis* is enhanced and similar to the control without bile acid.
the effect did not occur. This lead to the assumption that a protein present in the supernatant is responsible for the increased bile acid resistance of *E. faecalis*.

In this work, the ProBAS effect was consequently tested in various different *Enterococci* strains. Furthermore, *S. aureus* and *S. agalactiae* were tested for ProBAS. Due to the findings in the previous work and in this work, it was decided that a proteomic analyses of the supernatant of the ProBAS experiment would be appropriate to find the potential ProBAS factor. Furthermore, ProBAS is also possible with different *C. jejuni* strains such as A17, 81116 and 11168 and is not limited to strain 81-176. Therefore, comparison of co-incubation approaches of *C. jejuni* with either *E. faecalis*, *E. faecium* or *S. aureus* NCTC 8325 with DCA and without DCA were planned (see section Material and Methods below).
5.2 Material and Methods

5.2.1 Bacterial growth conditions and strains

*C. jejuni* 81-176 was cultivated overnight on CAM-agar plates from Biomérieux (Biomérieux, Marcy-l’Étoile, France) at 42 °C. *E. faecalis* 700802, *E. faecium* TX0016 and *S. aureus* NCTC 8325 were growing overnight on COS-agar plates from Biomerieux at 37 °C. One day prior to the ProBAS experiment, *Enterococcus faecalis*, *Enterococcus faecium* and *S. aureus* were transferred to 10 mL liquid BHI medium (Thermo Fisher Scientific, Waltham, Massachusetts, USA) and incubated overnight at 37 °C.

Table 10: Strains used for ProBAS experiments: different *C. jejuni* strains were used to test their ability to induce enhanced bile acid resistance in several *Enterococci* species, a *S. aureus* strain, a *S. agalactiae* strain and a *S. pyogenes* strain. Some of the bacteria were clinical isolates and were thus not assigned to their specific strain.

<table>
<thead>
<tr>
<th>Organism and strain designation</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Campylobacter jejuni</em> 81-176 / ATCC-BAA-2151</td>
</tr>
<tr>
<td><em>Campylobacter jejuni</em> ATCC 81116 / NCTC 11828 / DSM 24189</td>
</tr>
<tr>
<td><em>Campylobacter jejuni</em> ATCC 700819 / NCTC 11168</td>
</tr>
<tr>
<td><em>Campylobacter jejuni</em> A17, clinical isolate</td>
</tr>
<tr>
<td><em>Enterococcus faecalis</em> strain ATCC 700802 / V583</td>
</tr>
<tr>
<td><em>Enterococcus faecium</em> strain ATCC BAA-472 / TX0016</td>
</tr>
<tr>
<td><em>Enterococcus casseliflavus</em> clinical isolate</td>
</tr>
<tr>
<td><em>Enterococcus avium</em> clinical isolate</td>
</tr>
<tr>
<td><em>Enterococcus gillus</em> clinical isolate</td>
</tr>
<tr>
<td><em>Enterococcus durans</em> clinical isolate</td>
</tr>
<tr>
<td><em>Enterococcus hirae</em> clinical isolate</td>
</tr>
<tr>
<td><em>Enterococcus raffinosus</em> clinical isolate</td>
</tr>
<tr>
<td><em>Enterococcus thailandicus</em> clinical isolate</td>
</tr>
<tr>
<td><em>Enterococcus gallinarium</em> clinical isolate</td>
</tr>
<tr>
<td><em>Enterococcus ecorum</em> clinical isolate</td>
</tr>
<tr>
<td><em>Enterococcus pallens</em> clinical isolate</td>
</tr>
<tr>
<td><em>Enterococcus caninitesti</em> clinical isolate</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em> strain ATCC 35556 / NCTC 8325 / DSM 4910</td>
</tr>
<tr>
<td><em>Streptococcus pyogenes</em> clinical isolate</td>
</tr>
<tr>
<td><em>Streptococcus agalactiae</em> strain ATCC 13813 / NCTC 8181 / DSM 2134</td>
</tr>
</tbody>
</table>

5.2.2 ProBAS experimental procedure

*C. jejuni* was rinsed from the plate and resuspended in 1 x PBS. *Enterococci* and *S. aureus* were pelleted via centrifugation in a tabletop centrifuge at 6,000 x g for 1 min. The pellet was resuspended in 1 x PBS. The OD$_{600}$ was determined and adjusted to a final OD$_{600}$ of 0.5 for *C. jejuni* and 0.1 for the respective co-incubated bacterium. Subsequently, the
bacteria were incubated with or without 0.1 % DCA at 37 °C for 3 h shaking at 150 rpm. Biological triplicates were prepared for each experiment.

5.2.3 Protein purification

After incubation, the samples were directly kept on ice and centrifuged at 5,000 xg in a Megafuge 16R centrifuge (Thermo Fisher Scientific, Waltham, Massachusetts, USA) for 10 min at 4 °C. Subsequently, the pellet was discarded, and the supernatant was filtered through a 0.2 µm filter membrane (SARSTEDT AG& Co. KG, Nümbrecht, Germany), to remove remaining bacteria from the supernatant. The supernatant was precipitated overnight at -20 °C with Acetone at a ratio of 1:3. At the next day, the precipitation was centrifuged at 13,000 xg in a tabletop centrifuge (centrifuge 5424, Eppendorf, Hamburg, Germany) and the pellet was discarded. Subsequently the protein concentration was measured using a Pierce BCA protein assay kit (Thermo Fisher Scientific, Waltham, Massachusetts, USA) and the protein concentration was adjusted to 1 µg/µL for DIA-MS.

5.2.4 DIA-MS

DIA-MS was performed as described previously (section 4.2), with the exception that the samples were measured on a QExactive mass spectrometer (Thermo Fisher Scientific, Waltham, Massachusetts, USA) instead of a TripleTOF 5600+ (Sciex, Darmstadt, Germany). Access to the data generated in this project can be provided on request from the PRIDE database [160].

5.2.5 Data evaluation

Data were analysed as described before (section 4.2).
5.3 Results

5.3.1 ProBAS candidates

In order to demonstrate that the ProBAS effect occurs in co-incubation with different Enterococci species, several Enterococci were tested for ProBAS activity with C. jejuni (Table 11). ProBAS was observed in all of the tested species. However, the DCA tolerance of the species was varying and thus, ProBAS occurred mostly at lower DCA concentrations than in E. faecalis and E. faecium, ranging from 0.025 % to 0.1 %.

Table 11: ProBAS activity different Enterococci species and the respective DCA concentration where the effect became visible.

<table>
<thead>
<tr>
<th>Organism</th>
<th>DCA concentration [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enterococcus faecalis</td>
<td>0.1 %</td>
</tr>
<tr>
<td>Enterococcus faecium</td>
<td>0.1 %</td>
</tr>
<tr>
<td>Enterococcus casseliflavus</td>
<td>0.075 %</td>
</tr>
<tr>
<td>Enterococcus avium</td>
<td>0.075 %</td>
</tr>
<tr>
<td>Enterococcus gilnus</td>
<td>0.075 %</td>
</tr>
<tr>
<td>Enterococcus durans</td>
<td>0.075 %</td>
</tr>
<tr>
<td>Enterococcus hirae</td>
<td>0.075 %</td>
</tr>
<tr>
<td>Enterococcus raffinosus</td>
<td>0.075 %</td>
</tr>
<tr>
<td>Enterococcus thailandicus</td>
<td>0.075 %</td>
</tr>
<tr>
<td>Enterococcus gallinarium</td>
<td>0.05 %</td>
</tr>
<tr>
<td>Enterococcus cecorum</td>
<td>0.025 %</td>
</tr>
<tr>
<td>Enterococcus pallens</td>
<td>0.05 %</td>
</tr>
<tr>
<td>Enterococcus caninitesti</td>
<td>0.075 %</td>
</tr>
</tbody>
</table>

As ProBAS occurred in all of the tested Enterococci species, other bacterial species were additionally tested for ProBAS (Table 12). S. aureus and S. agalactiae were ProBAS positive, while S. pyogenes did not show any ProBAS effect.

Table 12: ProBAS activity in species other than Enterococci and the DCA concentration at which the effect becomes apparent. ProBAS was not visible in S. pyogenes. However, S. aureus and S. agalactiae were ProBAS positive.

<table>
<thead>
<tr>
<th>Organism</th>
<th>DCA concentration [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Staphylococcus aureus NCTC 8325</td>
<td>0.075 %</td>
</tr>
<tr>
<td>Streptococcus agalactiae ATCC 13813</td>
<td>0.075 %</td>
</tr>
<tr>
<td>Streptococcus pyogenes clinical isolate</td>
<td>-</td>
</tr>
</tbody>
</table>

5.3.2 Identification of potential ProBAS candidates

In order to find proteins that might be responsible for the transfer of bile acid resistance from C. jejuni to other bacteria in the supernatant of co-incubation approaches, up- and
down-expressed proteins were analyzed. In the co-incubation approaches of \textit{C. jejuni} with \textit{E. faecalis}, \textit{E. faecium} or \textit{S. aureus}, no commonly expressed proteins were found, neither up- nor down-expressed. However, 22 proteins were differentially expressed in co-incubation with \textit{E. faecalis}, 16 with \textit{E. faecium} and 137 with \textit{S. aureus}.

In the approaches of co-incubation with DCA, 167 commonly up-expressed and 74 down-expressed proteins were identified. In the mono-cultivation approach of \textit{C. jejuni} with DCA, 330 proteins were up-expressed and 162 down-expressed.

This study aimed to find the protein causally linked to the transfer of bile acid resistance. In order to find the potential ProBAS factor, proteins that are only present in co-incubation with DCA were analyzed. Therefore, differentially expressed proteins present in \textit{C. jejuni} monoculture with DCA were excluded. In total, 16 up-expressed and 14 down-expressed proteins were detected that were exclusively present in co-incubation with DCA (Figure 15).

Among the 16 up-expressed proteins were mostly metabolism-related proteins, but also proteins with different potential functions, as well as one uncharacterized protein (Table 13). One protein is a surface exposed protein, namely the surface-exposed lipoprotein.

![Figure 15: Venn diagrams that display the comparison of the commonly up-expressed proteins of \textit{C. jejuni} in co-incubation with \textit{E. faecalis}, \textit{E. faecium} and \textit{S. aureus} in presence and absence of DCA and the up-expressed proteins of \textit{C. jejuni} with DCA in monoculture. 30 proteins that occur specifically in co-incubation with DCA and not in the other approaches were detected. The up-expressed proteins are shown on the left and the down-expressed proteins are shown at the right. Red boxes highlight the specifically expressed proteins in co-incubation with DCA.](image)
(A0A0H3P9U7_CAMJJ), making it a potential candidate for the ProBAS effect. This could be investigated in future knockout-studies.

Among the 14 down-expressed candidates, metabolism, but also DNA and transcription-related proteins were detected, as well as one uncharacterized protein (Table 14).

**Table 13:** Exclusively up-expressed proteins in the approach co-incubation with DCA assigned to their specific function.

<table>
<thead>
<tr>
<th>Protein name</th>
<th>Potential function</th>
</tr>
</thead>
<tbody>
<tr>
<td>A0A0H3P9H5_CAMJJ</td>
<td>D-3-phosphoglycerate dehydrogenase</td>
</tr>
<tr>
<td>A0A0H3P9K9_CAMJJ</td>
<td>Oxidoreductase, short chain dehydrogenase/reductase family</td>
</tr>
<tr>
<td>A0A0H3P9P2_CAMJJ</td>
<td>Acyl carrier protein, putative</td>
</tr>
<tr>
<td>A0A0H3P9U7_CAMJJ</td>
<td>Surface-exposed lipoprotein</td>
</tr>
<tr>
<td>A0A0H3PA08_CAMJJ</td>
<td>Pyridoxal phosphate homeostasis protein</td>
</tr>
<tr>
<td>A0A0H3PA4J_CAMJJ</td>
<td>Histidine biosynthesis bifunctional protein HisIE</td>
</tr>
<tr>
<td>A0A0H3PAK2_CAMJJ</td>
<td>Nitrogen fixation protein NifU</td>
</tr>
<tr>
<td>A0A0H3PAL4_CAMJJ</td>
<td>Flagellar motor switch protein FlIG</td>
</tr>
<tr>
<td>A0A0H3PAJX_CAMJJ</td>
<td>Thiol peroxidase</td>
</tr>
<tr>
<td>A0A0H3PDH6_CAMJJ</td>
<td>3-deoxy-D-manno-octulosonate cytidylyltransferase</td>
</tr>
<tr>
<td>A0A0H3PDJ1_CAMJJ</td>
<td>Oxidoreductase, zinc-binding dehydrogenase family</td>
</tr>
<tr>
<td>A0A0H3PEL5_CAMJJ</td>
<td>Uncharacterized protein</td>
</tr>
<tr>
<td>A0A0H3PJH9_CAMJJ</td>
<td>Carboxyl-terminal protease</td>
</tr>
<tr>
<td>A0A0H3P1M2_CAMJJ</td>
<td>Saccharopine dehydrogenase</td>
</tr>
<tr>
<td>TAL_CAMJJ</td>
<td>Transaldolase</td>
</tr>
<tr>
<td>RL29_CAMJJ</td>
<td>50S ribosomal protein L29</td>
</tr>
</tbody>
</table>

**Table 14:** Exclusively down-expressed proteins in the approach co-incubation with DCA assigned to their specific function.

<table>
<thead>
<tr>
<th>Protein name</th>
<th>Potential function</th>
</tr>
</thead>
<tbody>
<tr>
<td>A0A0H3P9C0_CAMJJ</td>
<td>ABC transporter, ATP-binding protein</td>
</tr>
<tr>
<td>A0A0H3P9J7_CAMJJ</td>
<td>ATP synthase F0, B' subunit</td>
</tr>
<tr>
<td>A0A0H3P9P3_CAMJJ</td>
<td>UDP-N-acetylglucosamine 2-epimerase</td>
</tr>
<tr>
<td>A0A0H3PAL0_CAMJJ</td>
<td>Fibronectin-binding protein</td>
</tr>
<tr>
<td>A0A0H3PAS0_CAMJJ</td>
<td>UvrABC system protein B</td>
</tr>
<tr>
<td>A0A0H3PBA7_CAMJJ</td>
<td>GDP-L-fucose synthase</td>
</tr>
<tr>
<td>A0A0H3PBF3_CAMJJ</td>
<td>DNA-binding response regulator</td>
</tr>
<tr>
<td>A0A0H3PHG1_CAMJJ</td>
<td>Coenzyme A biosynthesis bifunctional protein CoaBC</td>
</tr>
<tr>
<td>A0A0H3PI91_CAMJJ</td>
<td>Major outer membrane protein</td>
</tr>
<tr>
<td>GREA_CAMJJ</td>
<td>Transcription elongation factor GreA</td>
</tr>
<tr>
<td>RISB_CAMJJ</td>
<td>6,7-dimethyl-8-ribityllumazine synthase</td>
</tr>
<tr>
<td>RL34_CAMJJ</td>
<td>50S ribosomal protein L34</td>
</tr>
<tr>
<td>PANB_CAMJJ</td>
<td>3-methyl-2-oxobutanoate hydroxymethyltransferase</td>
</tr>
<tr>
<td>Q2A945_CAMJJ</td>
<td>Uncharacterized protein</td>
</tr>
</tbody>
</table>
6 Discussion

In this thesis, multiple aspects of bile acid resistance mechanisms in \textit{C. jejuni} and \textit{Enterococci} were investigated. One aim was the understanding of the unique proteomic responses of these bacterial species when exposed to bile acid stress. Furthermore, the proteomic response to different co-incubation scenarios was characterized. Identification of differentially expressed proteins was done by utilization of the advanced proteomic analysis technique DIA-MS. Each manuscript chapter (Chapters 2 and 3) contains a detailed discussion regarding the specific topics. In this final discussion, the manuscripts as well as the additional results (Chapters 4.3 and 5.3) are contextualized in relation to each other.

6.1 Bile acid stress response in \textit{Enterococci} and \textit{C. jejuni}

6.1.1 Different types of transporters are crucial for \textit{Enterococci} and \textit{C. jejuni}

The ability to survive high bile acid concentrations over a longer time period (24 h) is crucial for survival of gut microbes but also for pathogens that colonize the gastrointestinal tract \cite{148, 167}. This also includes organs such as the bile acid-rich biliary tract \cite{125}. Both \textit{E. faecalis} and \textit{E. faecium} were isolated in 21\% of acute cholangitis cases \cite{168}. Furthermore, reports showed that \textit{E. faecium} can cause acute cholecystitis \cite{169}. Therefore, the study of the ability to survive prolonged high bile acid exposure of the opportunistic pathogenic \textit{Enterococci} is of high importance.

The results of this study unveiled differences in the susceptibility but similarities in the proteomic response of \textit{E. faecalis} and \textit{E. faecium}, especially the important role of ABC transporters, membrane repair-associated proteins and V-type ATPases in bile acid protection was confirmed. V-type ATPases are highly conserved proteins responsible for generating proton gradients and have been demonstrated to contribute to bile acid resistance in transcriptomic studies \cite{145, 170}. This effect has also been demonstrated in \textit{Lactobacillus plantarum} and \textit{Bifidobacterium} sp., which highlights the importance of V-type ATPases across different species \cite{171–173}. The hypothesized contribution of V-type ATPases in generating an ion motive force suggests its potential involvement in
energizing plasma membrane transporters, which could play a role in facilitating the efflux of bile acids from the cell (Figure 16). It is possible that one of the up-expressed ABC transporters found in the proteome benefits from this ion motive force.

To confirm the role of V-type ATPases, different attempts to inhibit this protein-complex were performed. As bafilomycin A as well as archazolid A (friendly provided by Prof. Dr. Rolf Müller) inhibit V-type ATPases in eukaryotic cells [174–176], both compounds were tested in growth assays on Enterococci. No effect was visible after incubation with DCA and the respective compound. Consequently, ampicillin, an antibiotic for which E. faecalis remains susceptible, was used to increase the solubility of the bacterial cell membranes to facilitate the intracellular transport of bafilomycin and archazolid. However, these experiments were not showing an effect on bile acid tolerance, indicating that bafilomycin and archazolid might not affect bacterial V-type ATPases.

Figure 16: Structure of the V-type ATPase in Enterococci. V-type ATPases consist of several different subunits and generate an ion motive force via proton transport. This ion motive force can be utilized to energize different (ABC) transporters that carry bile acids out of the cell and thus facilitate resistance.
Figure 17: Structure of the CmeABC multidrug efflux. CmeA is the membrane fusion protein while CmeB acts as efflux pump membrane transporter and CmeC serves as outer membrane lipoprotein. The RND family transporter uses energy from a proton motive force (PMF) to transport antibiotics or bile acids out of the cell.

In contrast to Enterococci, C. jejuni does not show enhanced expression of V-type ATPases to withstand bile acid stress. Despite the differences, a similarity in both organisms is the use of transport mechanisms to cope with bile acid stress. While in Enterococci several different ABC transporters seem to be involved in bile acid resistance, C. jejuni uses one specific transporter to export bile acids. The multidrug efflux pump CmeABC plays the key role in the bile acid resistance of C. jejuni. CmeABC belongs to the Resistance-Nodulation-Division (RND) family of transporters and consists of a three-gene operon that encodes for the membrane fusion protein CmeA, the efflux pump membrane transporter CmeB and the outer membrane lipoprotein CmeC (Figure 17) [128, 127, 147]. The efflux pump works by using energy from a proton motive force (PMF). However, the exact mechanisms by which CmeABC uses the PMF to transport substrates such as bile acids or antibiotics out of the cell is not fully understood yet. Another efflux pump that interacts with the predominant efflux pump CmeABC is CmeDEF, which also confers antimicrobial resistance [177]. CmeABC and CmeDEF seem to interact to protect the
cell from antibiotics [178], however, CmeDEF is not involved in bile acid resistance as previously demonstrated [179] and supported by the results of this thesis.

6.1.2 Insertional inactivation mutants of CmeB, CmeR and CbrR show different degrees of proteomic variations

In this work, CmeB was deleted and the phenotypic and proteomic response were analyzed. As previously described by Lin et al. [127, 128], the mutant showed a decreased general fitness and an extremely enhanced susceptibility towards bile acids and antibiotics. Moreover, an increased susceptibility towards several antibiotics and bile acids of a homolog mutant of this efflux system, the AcrAB-TolC efflux pump in E. coli, Salmonella, and Vibrio cholerae was shown before, indicating that these transporters are widely spread among bacterial species [180–182]. The proteome of ΔcmeB showed significant differences to the parental strain, which indicates that CmeABC is important for various processes in the cell and its lack influences protein expression. Furthermore, an up-expression of various ABC transporters was observed in ΔcmeB, suggesting a possible replacement for the deleted multidrug efflux pump. The mutant ΔcmeB experiences increased stress levels in the absence of CmeABC, in contrast to the parental strain. This triggers a potential compensatory protein expression in the mutant to cope with this stress.

The proteomic alterations in the mutant of the CmeABC repressor CmeR were comparatively less significant than those observed in ΔcmeB, suggesting that the absence of cmeR does not impact the organism to the same extent as absence of cmeB. As previously described, cmeR acts as repressor for the CmeABC operon and the loss of cmeR consequently leads to an over-expression of CmeABC compared to the parental strain [129]. Consequently, the mutant shows higher resistance towards bile acids and different antibiotics [127, 183]. The effect was confirmed in this study, as ΔcmeR was more resistant against DCA and CA and showed strong up-expression of CmeA, CmeB and CmeC in the proteome. Another interesting protein was detected among the up-expressed candidates: the uncharacterized protein A0A0H3PAI3_CAMJJ. A0A0H3PAI3_CAMJJ, also assigned as Cj0561c, was already described in a transcriptomic study by Guo et al. in 2008 to be up-expressed in knockout mutants lacking CmeR [166]. This could be confirmed by the
proteomic analysis in this study. In addition, Guo et al. showed that Cj0561c is strongly induced by bile salts, which is also the case in this study. In the parental strain, as well as in ΔcmeB and in ΔcbrR, Cj0561c was significantly up-expressed under exposure to CA. A knockout mutant of Cj0561c resulted in no significant changes in bile salt resistance in a previous study [166]. Furthermore, Guo et al. stated, that CmeR is a regulator that controls the expression of several genes. In the proteomic analysis of this work, only the proteins belonging to the CmeABC multidrug efflux and Cj0561c were found among the most significantly up-expressed candidates in absence of CmeR. This indicates that, other than described in the transcriptomic study, CmeR is not a global regulator, but specific for CmeABC and Cj0561c. The exact role of Cj0561c remains unknown.

In comparison to ΔcmeB, the regulation of proteins was less pronounced in ΔcbrR when compared to the parental strain proteome. This indicates that the lack of CbrR affects the expression of other proteins only on a low level. CbrR was previously described as an important response regulator that is involved in different processes, such as sodium deoxycholate resistance but also chicken colonization. However, the bile acid resistance of ΔcbrR was not significantly lower than in the parental C. jejuni strain.

A putative methyltransferase was strongly down-expressed in ΔcbrR, indicating that this protein might be induced by CbrR. Cox et al. reported decreased biofilm formation potential and increased autoagglutination in ΔcbrR, while this study showed no significant changes in in either of these features.

In all mutants harboring a functional CmeABC efflux, the up-expression of this efflux was sufficient to overcome CA stress. The high susceptibility of ΔcmeB towards CA and DCA was expected, as previous work has shown the strong effects of bile acids on this mutant [147, 127]. It was previously documented that ΔcbrR showed a high sensitivity to bile acids, hence the gene was named accordingly (Campylobacter bile resistance regulator) [149]. However, this study does not support this observation, as the susceptibility was only slightly decreased compared to the parental strain. Moreover, CbrR was not significantly up- or down-expressed in the parental strain when exposed to CA, indicating that it does not play a role in bile acid protection. Raphael et al. used C. jejuni strain F38011, which might be the reason for the different outcomes of the knockout [149].
Interestingly, the cmeR knockout-mutant showed no significant up-expression of proteins in the presence of bile-acid stress, however, five proteins were down-expressed. A possible reason for this is the general high expression of the CmeABC efflux in this mutant, which leads to a higher resistance towards CA [129]. The expression of CmeABC in the ΔcmeR mutant is already increased and is thus not further enhanced by the presence of CA, as it is the case for the wildtype and the ΔcbrR mutant. This also supports the previous hypothesis that presence of CmeABC is sufficient to protect the cell from bile acids.

6.1.3 Motility changes occur likely due to spontaneous phase variability

The ΔcmeB mutant showed increased motility in TTC (triphenyl tetrazolium chloride) assays and in microscopic analyses. Motility is a major virulence and survival factor in C. jejuni [35]. The wildtype C. jejuni is a motile bacterium harboring one or two flagella. Flagella are playing an important role in motility, but also in several other functions, such as biofilm formation and autoagglutination [36], and are known to be crucial for intestinal colonization [39, 40]. CmeABC proteins have not been associated with increased motility before, thus it is unlikely that the multidrug efflux is playing a crucial role in Campylobacter motility. A possible explanation for this effect could be a spontaneous increase of the synthesis of flagellar proteins. Hendrixon et al. have shown that spontaneous phase variation can affect flagellum formation in C. jejuni [184]. This can lead to an unexpected loss or gain of flagella, meaning that flagellar proteins are prone to frequent and significant changes due to high phase variability. Furthermore, this can explain the loss of motility in the mutants ΔcmeR and ΔcbrR. Additionally, C. jejuni 81-176 is tending to have a high genetic instability and underlies spontaneous variations of the MotA gene which encodes a flagellar motor associated protein [185]. However, MotA was not significantly regulated in this study.

The immotility of the ΔcbrR mutant in this study contradicts the observations of Cox et al. who stated that their CbrR-knockout was highly motile [150]. Cox et al. used a special streptomycin-resistant derivative of 81–176 (DRH212) in their work, which might have different characteristics than the C. jejuni 81-176 parental strain used in this work and this could explain the hypermotility they observed. However, it is more likely that the strain they used is also exposed to a high phase variability.
6.2 Co-incubation proteome of *C. jejuni* reveals specific adaptions

6.2.1 Co-incubation leads to a strong common proteomic response

The proteomic response of *C. jejuni* to co-incubation with *E. faecalis*, *E. faecium* as well as *S. aureus* was analyzed. Co-incubation of different species resulted in a distinct proteomic profile alteration in *C. jejuni*, varying based on the species involved. Variations in the number of up- or down-expressed proteins, depending on the respective species, were observed.

Particularly, the presence of *S. aureus* resulted in the highest intensity of proteomic alterations, involving 445 differentially expressed proteins. The production of toxins and hemolysins by *S. aureus* is widely recognized for its potential antimicrobial activity against other bacterial species [186, 187]. Conversely, *S. aureus* is able to secrete substances with beneficial properties for other microorganisms, that promote beneficial interactions with other microorganisms, enabling the establishment of polymicrobial communities that can be an advantage in the context of infectious processes [188, 189].

Nevertheless, these advantageous interactions could also be provided by *E. faecalis* or *E. faecium*, as three membrane-interactive proteins were commonly found in the top 20 up-expressed proteins in all samples. This indicates communication between the different bacterial species.

Among the up-expressed proteins in all co-incubation scenarios, a conjugative transfer regulon protein was present. Under the conditions of co-incubation, it is plausible that bacteria enhance their intercellular communication potentially as a protective or competitive mechanism. As conjugation is a universally conserved transfer mechanism among bacteria, regardless of their Gram classification, it is possible that *C. jejuni* uses this mechanism. Gram negative bacteria such as *C. jejuni* are usually forming pili for conjugation [190]. On the other hand, Gram positive bacteria normally use Type IV secretion systems [191]. Nevertheless, pili exist in Gram positive bacteria as well [192], and conjugation between Gram negative and Gram positive bacteria has been observed and well-examined, for example in case of plasmid transfer [193]. This supports the hypothesis, that the conjugative transfer across species might be possible in this co-incubation scenario. However, the plasmid formation in Gram positive bacteria differs
from the plasmid formation in Gram negative bacteria. Gram-positive bacteria can express two different types of pili, the Sortase Assembled pili and the type IV pili that are similar to those in Gram-negative bacteria [192, 194, 195]. In contrast, Gram negative bacteria form pili by non-covalent homopolymerization of major pilin subunits [136]. Overall, the mechanisms of pili-based conjugation between Gram positive and Gram negative bacteria differ in the structures involved and the details of the transfer process. These differences raise doubts about the possibility of a conjugative pilus between a Gram-positive and a Gram-negative bacterium. Nevertheless, alternative mechanisms exist for conjugation or protein transfer between bacterial species, such as the use of secretion systems or mating pair formation, which does not necessarily require the use of pili [196–199].

The resulting data revealed a general proteome but also unique proteomic reactions to co-incubation. Furthermore, a distinctive response to the two triggers co-incubation and presence of the bile acid DCA, which differed from the mono-cultivation of C. jejuni with DCA response was unveiled.

In presence of bile acids, C. jejuni in mono-cultivation showed up-expression of the CmeABC multidrug efflux, which served as control for bile acid stress response in this study. Previously, Masanta et al. demonstrated the proteomic up-expression of CmeABC under bile acid stress [144]. C. jejuni showed a unique response in co-incubation and presence of DCA, that differed from the response of C. jejuni mono-culture with bile acid and from the co-incubation proteome without DCA. These results indicate that the co-incubation seems to have an influence on the response towards bile acids.

In summary, a strong common proteomic answer towards co-incubation was detected in this study. Furthermore, a distinct proteomic response towards other bacteria in presence of bile acid stress that differs from the co-incubation proteome without DCA and C. jejuni mono-culture with DCA was identified.

6.2.2 Supernatant proteins might enhance bile acid resistance in bacteria co-incubated with C. jejuni

Other than in the co-incubation experiment, the ProBAS (protection from bile acid stress) experiment was focused on the proteome of the supernatant of co-incubation scenarios in presence of DCA. The decision to prioritize the supernatant was based on the hypothesis
that the protein responsible for the potential mediation of bile acid resistance is likely present in the supernatant. In the absence of \textit{C. jejuni}, \textit{Enterococci} showed limited survival at a concentration of 0.1 \% DCA, resulting in minimal or no detectable growth during spot assays. In contrast, when co-incubated with \textit{C. jejuni} in the presence of DCA, their survival rates resemble those in the absence of DCA (unpublished data). It was noticed that the protective influence could be transmitted through the supernatant derived from a previous ProBAS experiment, resulting in a similar but slightly diminished outcome (see chapter 5). Treatment of the supernatant with proteinase K, led to the disappearance of this effect. This suggests that a protein in the supernatant likely confers the enhanced bile acid resistance observed in \textit{Enterococci} or other co-incubated bacteria, such as \textit{S. aureus} or \textit{S. agalactiae}.

In the ProBAS supernatant, 1052 proteins were identified using DIA-MS, while 1375 proteins were detected in the co-incubation pellet. An analysis of the distribution of COG categories (clusters of orthologous genes) of the different proteomes showed a similar outcome (Appendix-Figure 19). No significant variations between the ProBAS supernatant and the co-incubation pellet were visible, suggesting that there is no typical pellet- or supernatant-proteome. A possible reason for this is the damage of the cells by DCA, which leads to cell disruption and enables release of proteins into the supernatant.

Bile acid protection of \textit{E. faecalis} was previously observed in ProBAS assays (see chapter 5). The same cultivation conditions were applied in the experiment investigating co-incubation proteome analysis described in manuscript I (chapter 2) and the supernatant of the ProBAS experiment 5. The aim of the ProBAS project was the identification of proteins associated with the enhanced bile acid resistance in \textit{E. faecalis}, \textit{E. faecium} and \textit{S. aureus} in co-incubation with \textit{C. jejuni} 81-176 (Figure 18). In total, 16 up-expressed and 14 down-expressed proteins were detected specifically in the co-incubation approaches with DCA, but not in \textit{C. jejuni} mono-cultivation with DCA. Among the up-expressed proteins were several interesting candidates that might be involved in the induction in bile acid resistance in the other bacteria. The surface-exposed lipoprotein A0A0H3P9U7_CAMJJ is a potential ProBAS candidate, as the protein is exposed to the surface which might enable communication with the environment and potentially other bacteria. Moreover, the saccharopine dehydrogenase A0A0H3PJM2_CAMJJ might be involved in the ProBAS effect. Previous knockout of the respective gene has demonstrated involvement
of A0A0H3PJM2_CAMJJ in the alternative spermidine pathway [200]. Hanfrey et al. state that the alternative spermidine pathway is crucial for *Campylobacter* survival, as it is essential for the polyamine synthesis and plays an important role in various cellular processes, including DNA replication, transcription, translation, and cell division. Due to the involvement in diverse cellular processes, it is likely that A0A0H3PJM2_CAMJJ might also be involved in the mediation of bile acid resistance. Another interesting candidate is the uncharacterized protein A0A0H3PEL5_CAMJJ. The function of this protein was not characterized so far, thus, it might be involved in the mediation of bile acid resistance. Additionally, it is possible that one of the down-expressed proteins might be responsible for the ProBAS effect. Negative regulation can also be considered as a possible trigger for the induction of bile acid resistance.

The insertional gene inactivation of the respective identified proteins and the experimental examination of the potential involvement in bile acid resistance induction utilizing a ProBAS assay can lead to deeper insight into the role of these proteins.

![Figure 18: The unknown mechanism that induces bile acid protection in Gram positive bacteria by *C. jejuni* could be triggered by one of the proteins that were found to be up- or down-expressed in the ProBAS-experiments. A generation of insertional inactivation mutants could help to find the responsible factor for the observed effect.](image)

In both studies, co-incubation experiments were conducted. However, the focus of both projects was on the proteome of *C. jejuni* 81-176, which is a limitation of the studies. In order to obtain a comprehensive overview, the proteomes of the other bacterial
cultures \((E. \text{faecalis}, E. \text{faecium} \text{ and } S. \text{aureus})\) in the co-incubation approaches should be considered. It is feasible that the proteome of the other bacteria also changes in co-incubation.

Overall, this work contributes to the understanding of proteomics in the context of bile acid stress and provides valuable insights into individual behavior and adaptation of \(C. \text{jejuni}\) and \(\text{Enterococci}\). Additionally, this study provides new insights into the response of \(C. \text{jejuni}\) to different co-incubation scenarios, also with respect to bile acid resistance and the potential mediation of resistance.

### 6.3 Outlook

In summary, all projects of this thesis investigated the proteomic changes of \(C. \text{jejuni}\) or \(E. \text{faecalis}\) and \(E. \text{faecium}\) with respect to bile acid resistance or co-incubation.

The identified proteomic changes in \(C. \text{jejuni}\) co-incubation experiments suggest mutual interactions between species and specific proteomic responses to co-incubation in presence and absence of bile acids. Future research should extend the proteomic analysis to the other bacterial species involved in co-incubation \((E. \text{faecalis}, E. \text{faecium} \text{ and } S. \text{aureus})\), to investigate how they respond and adapt to the presence of \(C. \text{jejuni}\) and bile acids or both triggers at the same time. The finding of specific proteins or factors responsible for inter-species interactions could advance our knowledge of microbiome dynamics and pathogen-pathogen or pathogen-host interactions.

Furthermore, results from this thesis indicate that V-type ATPases play a crucial role in bile resistance for both \(E. \text{faecalis}\) and \(E. \text{faecium}\). Future research should aim to insertionally inactivate V-type ATPases to confirm their role in bile resistance. Furthermore, specific inactivation of V-type ATPases in bacteria could be a future aim for studies addressing \(\text{Enterococci}\) and bile acid resistance, as bafilomycin A and archazolid A were not sufficient to inhibit the V-type ATPase.

Proteomic changes resulting from the insertional inactivation of genes related to bile acid stress in \(C. \text{jejuni}\) were analyzed. As the lack of CmeB leads to a significant change in the proteome, potential regulatory roles of the CmeABC efflux could be studied in future projects. Furthermore, the role of CbrR is not clear yet and could be the aim of future
studies. According to this work, CbrR is not involved in bile acid stress, as previously hypothesized. Additionally, the exact role of Cj0561c, a protein which is regulated by CmeR but also induced by bile acids remains unclear and could be the target of future studies.

Another project of this thesis focused on the identification of \textit{C. jejuni} proteins that were regulated when the two triggers, co-incubation and bile acid stress were simultaneously present. The identified proteins could be involved in the mediation of increased bile acid resistance to \textit{E. faecalis} observed under these conditions. To further investigate this effect, future research should involve insertional inactivation of the identified genes. Especially the surface-exposed lipoprotein (A0A0H3P9U7\_CAMJJ) seems to be a promising candidate for these experiments. Studying the mechanisms by which the increased bile acid resistance is induced could help obtaining important insights into the bacterial adaptive responses and communication under bile acid stress.
References


[197] M. Getino and F. de la Cruz, “Natural and artificial strategies to control the conjugative transmission of plasmids,” *Microbiology Spectrum*, vol. 6, Jan. 2018.


Appendix

In order to find potential differences in the supernatant and pellet proteomes of the two co-incubation experiments (2 and 5.3), the COG-categories of all identified proteins in both experimental approaches were compared. Furthermore, the COG-categories of the whole theoretical proteome of *C. jejuni* 81-176, consisting of 1645 proteins (according to Uniprot), were compared to the pellet and supernatant proteome. The comparison showed that there is no specific supernatant or pellet proteome, both proteomes are similar to the whole theoretical proteome.

*Figure 19:* Distribution of COG-categories in the whole theoretical proteome of *C. jejuni* 81-176, the identified proteins in DIA-MS in the proteome of the co-incubation project (DIA-MS proteome Pellet) and the ProBAS proteome (DIA-MS proteome Supernatant). The theoretical proteome includes 1645 poteins, 1375 were identified in the co-incubation project and 1052 were identified in the supernatant of the ProBAS assay.
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