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

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ABSTRACT

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

1. Introduction

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

Even though campylobacters can be present in relatively high cell

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concentrations (2–3 $\log_{10}$ cfu/g) on raw poultry meat (Guyard-Nicodeme et al., 2015), the detection of low amounts is crucial since *Campylobacter* cells can be present in low numbers on products that are consumed raw and campylobacters have shown to survive for a long time under adverse environmental conditions (Lee et al., 1998; Park, 2002; Sampers et al., 2010; Sopwith et al., 2008; Wilson et al., 2008) and conceivably still have a high potential to cause disease (Black et al., 1988). Hence, a sensitive yet selective method is necessary to detect *Campylobacter* in food and thereby verifying food safety control measures. In the European Union, the standard ISO 10272-1:2017 is widely applied for the detection of *Campylobacter* spp. from food products. Dependent on the expected amount of campylobacters and their history and the probable presence of competing background microbiota, different procedures are to be followed. If campylobacters are expected to be sub-lethally injured, enrichment is done in Bolton broth (BB; procedure A), while Preston broth is advised if the amount of competing background microbiota is expected to be high (procedure B) (International Organization for Standardization, 2017). In reality, tested poultry products are often stored cold or frozen, which can induce sub-lethal damage to *Campylobacter* cells. It has been shown that the viability of *Campylobacter* spp. decreased by more than one $\log_{10}$ cfu/g when subjected to freezing (Bhaduri and Cottrell, 2004; Georgsson et al., 2006; Haddad et al., 2009; Klančnik et al., 2008; Maziero and De Oliveira, 2010; Sampers et al., 2010; Lanzl et al., 2020). On top of the viability-loss, recovery duration during enrichment also increased significantly after freeze stress compared to cells that had not been stressed before enrichment. Next to that, even though cell concentrations seem not to decline significantly after refrigerated storage, it has been shown that recovery duration during enrichment still increased significantly after refrigerated storage (Lanzl et al., 2020). At the same time contamination of food products with Extended-spectrum beta-lactamase (ESBL)-producing background microbiota cannot be excluded (Jasson et al., 2009). Enrichment in BB (procedure A) appears to be a logical choice for the recovery and growth of sub-lethally injured *Campylobacter* spp. from food products.

BB is assumed to be a nutrient-rich medium composed of enzymatic digest of animal tissues, lactalbumin hydrolysate, and yeast extract and is further supplemented with several organic compounds such as sodium pyruvate, alpha-ketoglutarate, and haemin to enhance the growth of *Campylobacter* spp. (International Organization for Standardization, 2017). However, the exact composition of the medium is not specified. Research has shown that most campylobacters are unable to metabolize carbohydrates due to interrupted Embden-Meyerhof and Pentose Phosphate pathways (Gripp et al., 2011; Kelly, 2001; Line et al., 2010; Stahl et al., 2011). Campylobacters do have a functioning set of enzymes for the citrate (TCA) cycle (Hofreuter, 2014; Stahl et al., 2012) and depend heavily on the TCA intermediates including alpha-ketoglutarate, succinate, fumarate, and malate for their energy needs. Likewise, it has been demonstrated, that *C. jejuni* can utilize the organic acids acetate, lactate, and pyruvate (Guccione et al., 2008; Thomas et al., 2011; Wright et al., 2009). *C. jejuni* can utilize free amino acids as well, with serine being the preferred amino acid followed by aspartate, asparagine, and glutamate (Hofreuter et al., 2008). However, also other amino acids such as proline and threonine can be metabolized (Guccione et al., 2008; Hofreuter et al., 2008; Wright et al., 2009) and it has been suggested that peptides play an important role in amino acid catabolism of *C. jejuni* as well (Gao et al., 2017; Gundogdu et al., 2016; Hofreuter et al., 2006).

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

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

2. Materials and methods

2.1. Bacterial strains and preparation of working cultures

Two *C. jejuni* (WDCM 00005 and 81-176) and one *C. coli* isolate (Ca 2800) were selected for this study (details of all bacterial strains used in this study can be found in table S1 of the supplementary materials). For the preparation of working cultures, *C. jejuni* and *C. coli* were plated from the −80 °C vials onto Columbia agar base (CAB, Oxoid, supplemented with 5% (v/v) lysed horse blood (BioTrading Benelux B.V. Mijdrecht, Netherlands) and 0.5% agar (Bacteriological agar No.1, Oxoid)) and grown microaerobically for 24 h at 41.5 °C. Subsequently, a single colony was resuspended in Heart Infusion broth (Bacto HI, Becton, Dickinson and Company) and cultured for 24 h at 41.5 °C to obtain stationary phase cultures. Afterward, working cultures were prepared by making a 1:500 dilution in unselective BB (Oxoid, supplemented with 5% (v/v) sterile lysed horse blood (BioTrading Benelux B.V., Mijdrecht, the Netherlands)) without the addition of selective supplements and cultured for 24 h at 41.5 °C to reach the stationary phase.

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

2.2. Application of stress treatments

For mono-culture enrichments, 1 ml of the working culture of each

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

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

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

Infusion bottles were filled with 42 ml of BB, closed with a rubber stopper and aluminium cap, and sterilized. Subsequently, bottles were supplemented with 5% sterile horse blood and 450 µl of the selective supplement (Oxoid SR0208E). Bottles were filled with reference or freeze-stressed cultures using an inoculation level ($\log_{10}$ N_0) of 6–7 $\log_{10}$ cfu/ml to be able to observe outgrowth and possible growth suppression of *Campylobacter* spp. in co-culture while still being able to quantify changes in extracellular metabolite concentrations. In all other mono- and co-culture experiments (see Sections 2.4 and 2.5) $\log_{10}$ N_0 was approx. 2 $\log_{10}$ cfu/ml. In all experiments, the addition of fluids to sterilized infusion bottles was achieved using syringes to puncture the rubber stopper of the bottles. The headspace of infusion bottles was flushed for 2 min with a gas mixture of 5% O_2, 10% CO_2, and 85% N_2 by a homemade gas flushing device using needles to puncture the rubber stopper. Inoculated infusion bottles were incubated in water baths at 37 °C for the first 5 h and subsequently transferred to 41.5 °C for the remaining 43 h following ISO 10272-1:2017. After inoculation (t_0), and after 2, 4, 6, 8, 24, and 48 h samples were taken from the bottles using a syringe and after 0, 4, 8, and 24 h bottles were flushed with the appropriate gas mixture.

2.3.1. Determination of growth kinetics

For mono-culture enrichments, samples were immediately decimally diluted in PPS (Tritium Microbiologie), plated onto CAB for *Campylobacter* spp. and incubated microaerobically for 48 h at 41.5 °C. For ESBL-*E. coli* monoculture experiments, samples were plated onto BHI-agar and incubated aerobically for 24 h at 37 °C. For co-culture enrichments, the plating was done onto RAPID *Campylobacter* agar (RCA; Biorad) and Brilliance ESBL agar (BEA; Oxoid) for campylobacters and ESBL-*E. coli*, respectively. All *Campylobacter* cells were cultured under microaerobic conditions (5% O_2, 10% CO_2, 85% N_2) in flushed jars (Anoxomat WS9000, Mart Microbiology, Drachten, Netherlands) unless stated otherwise. After incubation, cfu were counted and cell concentrations were calculated as $\log_{10}$ cfu/ml for each time point and sample. Three biologically independent reproductions per strain and stress treatment were performed on different days. In parallel to $\log_{10}$ counts determination, samples were taken for High-Performance Liquid Chromatography (HPLC) and Ultra-high Performance Liquid Chromatography (UPLC) analysis and transferred to two sterile Eppendorf tubes (2 × 0.5 ml), snap-frozen in liquid nitrogen, and stored at −20 °C until quantification of extracellular metabolites by HPLC and UPLC.

2.3.2. Sample preparation for HPLC and UPLC analysis

For the quantification of acetate, formate, lactate, propionate, pyruvate, citrate, fumarate, a-ketoglutarate, malate, succinate, ethanol, glycerol, cellobiose, and glucose, 500 µl of the defrosted sample was deproteinized by addition of 250 µl cold Carrez A (0.1 M potassium ferrocyanide trihydrate). After mixing, 250 µl cold Carrez B (0.2 M zinc sulfate heptahydrate) was added, followed by mixing and centrifugation at 17,000 ×*g* for 10 min. 200 µl of the deproteinated sample was injected on an UltiMate 3000 HPLC (Dionex, Germany) equipped with an Aminex HPX-87H column (300 × 7.8 mm) with guard-column (Biorad). As mobile phase, 5 mM H_2SO_4 was used at a flow rate of 0.6 ml/min. The column temperature was kept at 40 °C. Compounds were detected by a refractive index detector (RefractoMax 520). Amino acids (alanine, asparagine, aspartate, cysteine, glutamate, glutamine, arginine, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, valine) were quantified by UPLC. 40 µl of the defrosted sample was deproteinated by addition of 50 µl 0.1 M HCl, containing 250 µM norvaline as internal standard and 10 µl 30% sulfosalicylic acid (SSA). Subsequently, the solution was mixed and centrifuged at 17,000 ×*g* for 10 min at 4 °C. Amino acids were derivatized using the AccQ•Tag Ultra derivatization kit (Waters, USA). 20 µl of the deproteinated supernatant or standard amino acids mixture was mixed with 60 µl AccQ•Tag Ultra borate buffer in glass vials. For deproteinated samples, 75 µl of 4 M NaOH was added to 5 ml borate buffer to neutralize the addition of SSA. Subsequently, 20 µl of AccQ•Tag reagent dissolved in 2 ml AccQ•Tag reagent diluent was added and immediately vortexed for 10 s. Then, the sample solution was heated at 55 °C in a heat block for 10 min. Amino acids were quantified by UPLC by injection of 1 µl sample on an UltiMate 3000 (Dionex, Germany) equipped with an AccQ•Tag Ultra BEH C18 column (150 mm × 2.1 mm × 1.7 µm) (Waters, USA) with BEH C18 guard column (5 mm × 2.1 mm × 1.7 µm) (Waters, USA). The column temperature was set at 55 °C and the mobile phase flow rate was maintained at 0.7 ml/min. Eluent A was 5% AccQ•Tag Ultra concentrate solvent A and Eluent B was 100% AccQ•Tag Ultra solvent B. The separation gradient was 0–0.04 min 99.9% A, 5.24 min 90.9% A, 7.24 min 78.8% A, 8.54 min 57.8% A, 8.55–10.14 min 10% A, 10.23–17 min 99.9% A. Compounds were detected by UV measurement at 260 nm. Glutamine and arginine could not be separated in the UPLC analysis due to overlapping peak areas.

2.4. Preparation of spent BB and enrichment in spent medium

After enrichments were performed as described in 2.3, 45 ml of spent media of ESBL-*E. coli* strain RIVM 2 in monoculture and co-culture with *C. jejuni* strain 81-176 were collected by transferring the (co-)culture to 50 ml Greiner tubes, and subsequent centrifugation at 17,000 ×*g* for 5 min and filter-sterilization (0.2 µm filter, Sartorius Minisart™ Plus Syringe Filters, Thermo Fisher Scientific). The spent BB was then transferred into sterile 45 ml infusion bottles and used for enrichments in spent BB. For mono-culture enrichments of *C. jejuni* strain 81-176 and *C. coli* strain Ca 2800 in spent medium, working cultures were serially diluted in PPS until approx. 10^3 cfu/ml and inoculated in spent medium to reach an inoculation concentration ($\log_{10}$ N_0) of approx. 2 $\log_{10}$ cfu/ml, after which infusion bottles were flushed and incubated, and samples were taken after 0, 24, and 48 h.

2.5. Oxygen availability during enrichment

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

start of enrichment and after 24 h of incubation.

2.6. Data and statistical analysis

For all experiments conducted to assess the composition of BB as well as the utilization of compounds in BB during enrichment, three biological reproductions were taken and means and standard errors were determined for each time point, strain, compound, and history. For the determination of cell concentrations (log_{10} cfu/ml), the mean and standard error of three biological reproductions were calculated. To determine whether differences in compound availability after 48 h (compared to t_0) were significant, Student's *t*-tests were performed and significance was determined with *p*-values with Bonferroni-correction ($p = 0.05$ / (amount of t-tests performed) $= 0.05/132 = 0.00038$). To determine whether the increase in cell concentrations of *C. jejuni* and *C. coli* after 24 and 48 h of enrichment C-spent BB and C&E-spent BB (Section 2.4) was significantly different compared to growth in fresh BB, Student's *t*-tests were performed using a significance value of $p = 0.05$. Similarly, to determine whether differences in cell concentration of *C. jejuni* and *C. coli* after 24 h of enrichment were significant in different atmospheric settings (Section 2.5), Student's t-tests were performed using a significance value of $p = 0.05$.

3. Results

3.1. Compound availability in selective BB

At the start of each monoculture enrichment, the available amounts of the 20 essential amino acids, organic acids (acetate, formate, lactate, propionate, and pyruvate), TCA-cycle intermediates (citrate, fumarate, a-ketoglutarate, malate, and succinate), alcohols (ethanol and glycerol) and carbohydrates (glucose and cellobiose) were measured and an overview is given in Fig. 1A. Interestingly, BB contained all essential amino acids, of which the amounts were in most cases lower than 1 mM except for glycine (1.19 ± 0.15 mM). Acetate, pyruvate, a-ketoglutarate, malate, ethanol, and the disaccharide cellobiose were present in relatively high amounts (between 1.11 ± 0.21 mM for pyruvate and 2.47 ± 0.67 mM acetate) and some other organic compounds were present in even higher amounts, as BB contains approximately 5 mM of lactate (5.09 ± 0.3 mM) and more than 3 mM of succinate (3.1 ± 0.35 mM). The concentration of each compound in BB can also be found in a table format in table S2 of the supplementary materials.

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

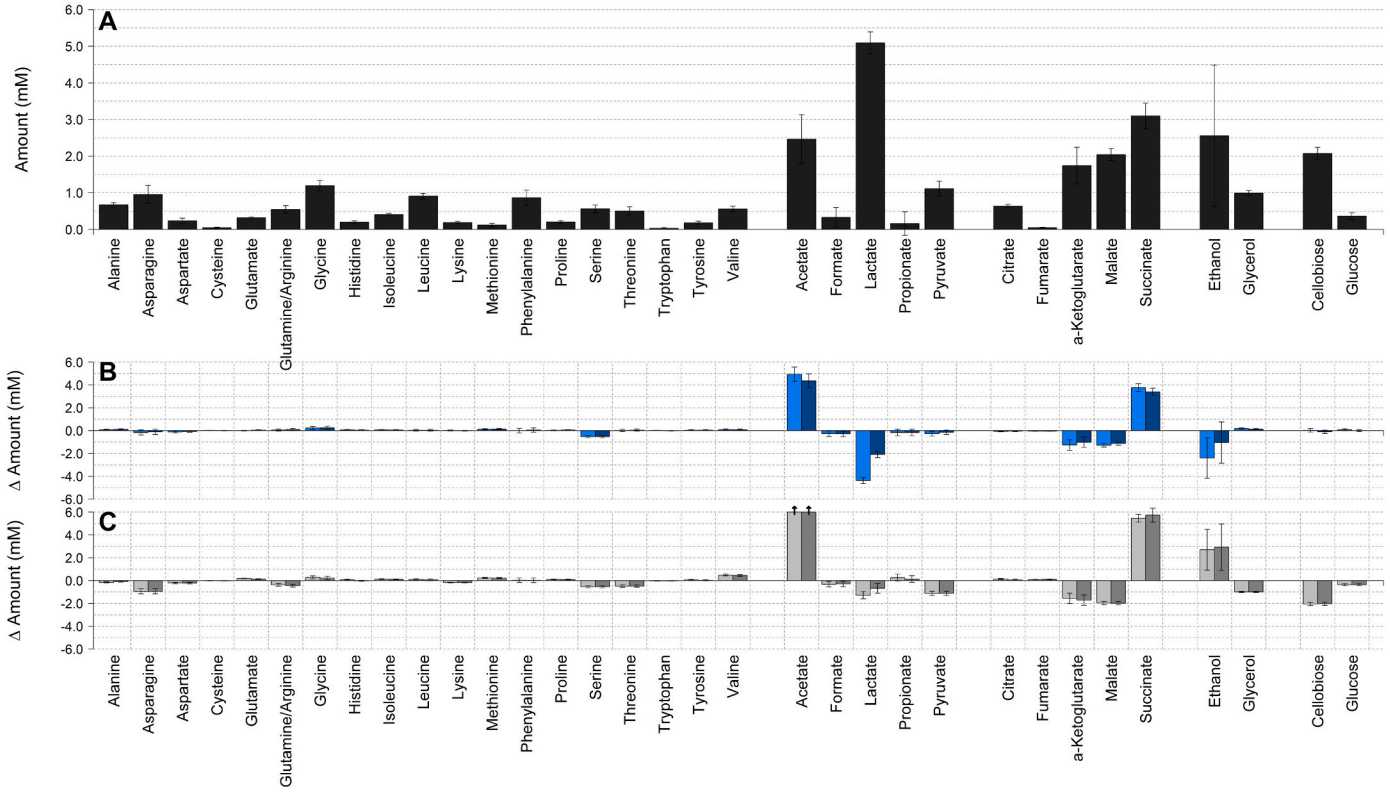

Fig. 1. Composition of BB (A) and change of compound concentration after 48 h of enrichment of reference and freeze-stressed *Campylobacter* spp. (B) and ESBL-*E. coli* (C) cells. The composition of selective BB before enrichment is depicted in graph **A**. The amount of each compound (in mM) is indicated in black-colored bars. The error bars depict the standard deviation ($n = 36$). Graph **B** shows the difference in compound amount after 48 h of mono-culture enrichment of reference (light blue) and freeze-stressed cells (dark blue) relative to the start of the enrichment of *Campylobacter* spp. (bars depict the average of three reproductions of two *C. jejuni* and one *C. coli* species). The error bars depict the standard deviation ($n = 3$). Graph **C** shows the difference in compound amount after 48 h of mono-culture enrichment of reference (light grey) and freeze-stressed cells (dark grey) relative to the start of the enrichment of ESBL-*E. coli* (bars depict the average of three reproductions of 3 ESBL-*E. coli* strains). The error bars depict the standard deviation ($n = 9$). In graph C, the arrows (for the compound acetate) indicate that the increase in amount was above 6 mM (17.2 ± 0.6 mM and 15.3 ± 1.7 mM for reference and freeze-stressed cells, respectively). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

changes in compound utilizations are represented as the overall mean of the genus *Campylobacter*.

Trends in compound utilization were comparable after 48 h of enrichment of reference and freeze-stressed campylobacters but observed changes (both positive and negative) were higher, yet insignificantly for reference cells compared to freeze-stressed cells during enrichment for all but one compound. The only exception was lactate, which was consumed significantly ($p < 0.001$) more by reference cells than freeze-stressed cells. Likewise, a delay in compound utilization could be observed consistently for freeze-stressed cells throughout enrichment, which correlates with an increased lag duration of freeze-stressed campylobacters during enrichment (details on the kinetics of compound utilization in monoculture enrichments can be found in Figs. S1 to S4 of the supplementary materials and details on the growth kinetics of cells during enrichment can be found in Fig. S9 of the supplementary materials). The same observations could be made for the trends in compound utilization of ESBL-*E. coli* as well, although differences were less obvious.

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

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

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

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

ESBL-*E. coli* was able to utilize a wider range of amino acids present in BB (namely asparagine, glutamine/arginine, lysine, threonine, and tryptophan). Next to that, both, the target pathogens *C. jejuni* and *C. coli* and the competitors ESBL-*E. coli* were able to utilize aspartate, serine, formate, a-ketoglutarate, and malate present in BB during enrichment. Results showed that most compounds that were utilized by *Campylobacter* spp. were too, and more rapidly utilized, by ESBL-*E. coli*. Furthermore, the latter was able to utilize pyruvate, glycerol, and the two carbohydrates cellobiose and glucose during enrichment, while on the other hand, lactate, citrate, and fumarate decreased from the medium by *Campylobacter* spp., exclusively.

Utilization of all compounds was also investigated during co-culture enrichments, whereby no differentiation could be made between utilization by campylobacters or ESBL-*E. coli* (an overview can be found in Figs. S5–8 of the supplementary materials). Comparison of compound utilization patterns between mono- and co-culture enrichments revealed that trends in utilization of compounds during co-culture were more similar to those of ESBL-*E. coli* than campylobacters.

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

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

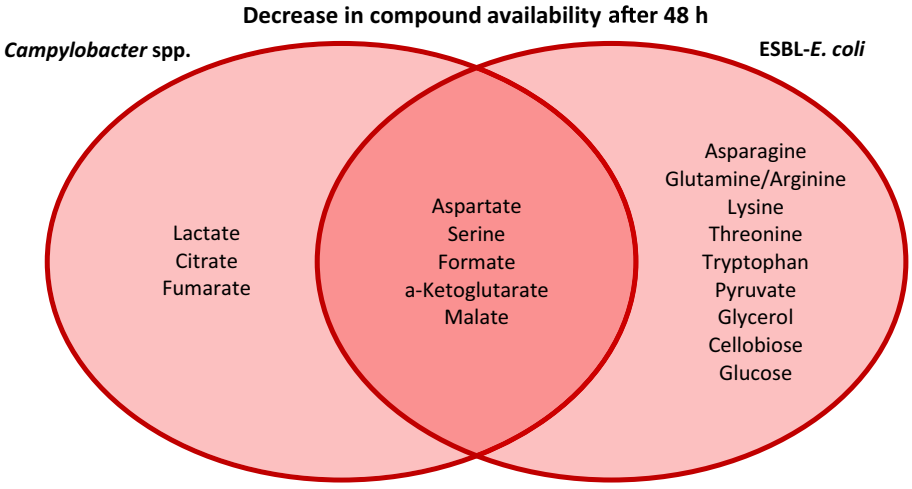

Fig. 2. Significant decrease in compound availability after 48 h of enrichment in BB of *Campylobacter* spp. or ESBL-*E. coli*. The decrease of measured compounds is depicted in the red VENN diagram. Compounds depicted in the left and right circles are significantly taken up by *Campylobacter* spp. and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

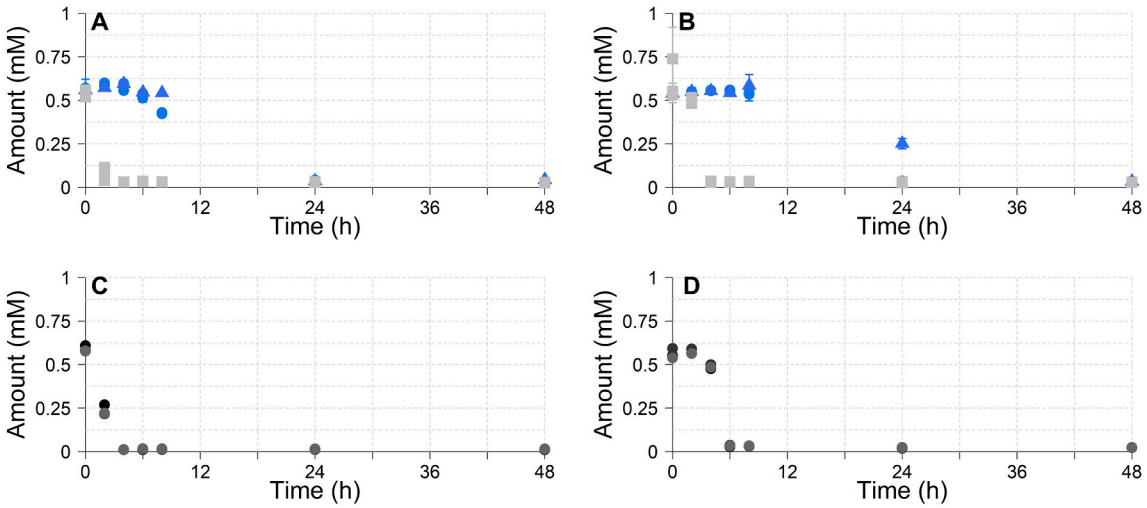

Fig. 3. Utilization of serine by *C. jejuni*, *C. coli* and ESBL-*E. coli* during mono-culture (panels A and B) and co-culture (panels C and D)-enrichment in BB. Serine utilization during mono-culture enrichments is depicted in graphs A for reference cells and B for freeze-stressed cells. The amount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

constituted approximately 6–7% of serine initially available in BB. Notably, during the mono-culture growth of all three ESBL-*E. coli* strains, serine was taken up from the medium almost until exhaustion (~95%) already after 2 and 4 h of enrichment in BB of reference and freeze-stressed cells, respectively, and co-culture experiments showed similar serine utilization trends as ESBL-*E. coli* in mono-culture.

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

With serine being a critical growth substrate for campylobacters, we hypothesized that a competition-induced lack of serine could be the reason for the faster transition of *Campylobacter* into stationary phase during co-culture enrichments resulting in a lower cell concentration (growth kinetics are depicted in Fig. S9 of the supplementary materials). As a next step, BB was supplemented with 1 mM of L-serine and a mono-culture enrichment was performed with one *C. jejuni* and one *C. coli* strain. Observed differences in cell concentrations in BB and BB supplemented with 1 mM L-serine were less than 0.5 $\log_{10}$ cfu/ml after 24 and 48 h (data not shown). Since results showed that L-serine was rapidly utilized by ESBL-*E. coli* in mono-culture and that utilization trends during co-culture looked rather similar, a follow-up experiment was conducted to test whether supplementation of BB with 5 and 25 mM of L-serine could result in higher cell concentration of reference cells of *C. jejuni* 81-176 and *C. coli* Ca 2800 during co-culture enrichment with

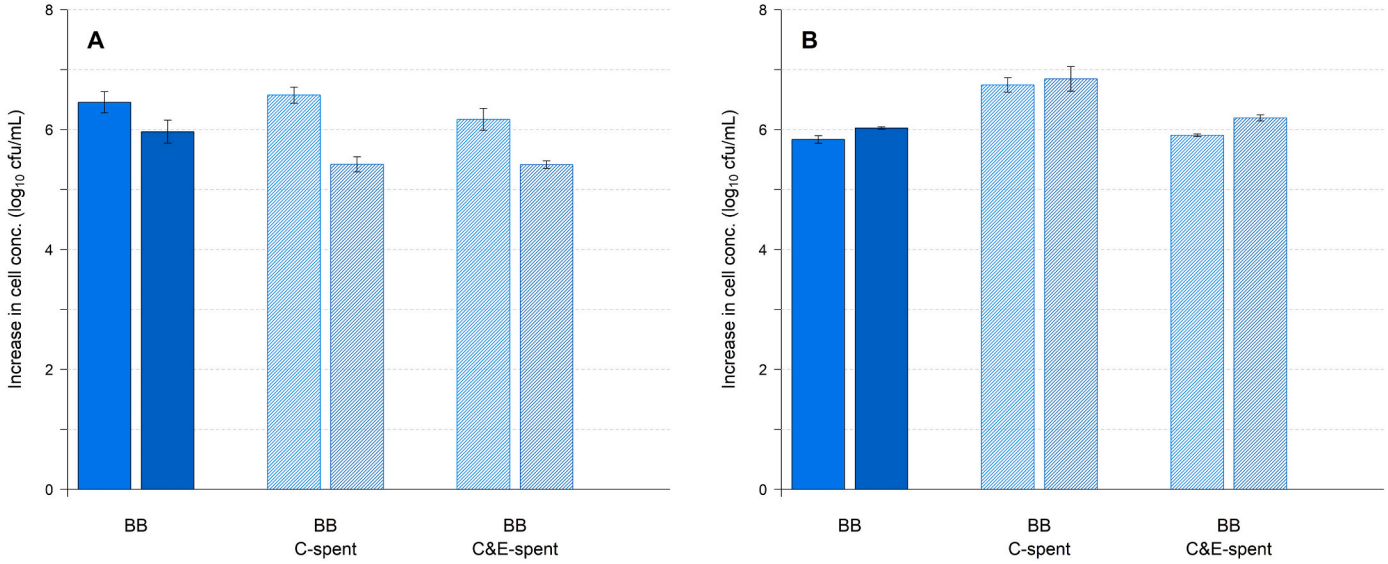

Fig. 4. Increase in cell concentrations (after 24 and 48 h) of *C. jejuni* strain 81-176 (A) and *C. coli* strain Ca 2800 (B) during monoculture enrichment in fresh BB and spent BB. The increase in cell concentrations in BB and different spent media are depicted after 24 and 48 h of mono-culture enrichment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

ESBL-*E. coli* RIVM 2. No obvious beneficial effect of supplementation with 5 or 25 mM of L-serine could be observed since the increase in cell concentration between the start of enrichment and the reach of the stationary phase (Δt_{24}-t_0) was less than 0.5 $\log_{10}$ cfu/ml for both *Campylobacter* strains compared to BB (Fig. S10). To assess whether the observed growth arrest of campylobacters during co-culture growth with ESBL-*E. coli* might be due to growth-induced lack of other medium compounds, mono-culture enrichments of *C. jejuni* and *C. coli* were conducted in spent BB that was obtained from 48 h-monoculture and co-culture incubations of *Campylobacter* and/or ESBL-*E. coli* (Fig. 4). The log-change in growth between stationary phase and initial cell concentration (Δt_{24}-t_0) was determined to compare the growth ability of *C. jejuni* and *C. coli* in different spent BB compared to fresh BB (Fig. 4). After 24 h of enrichment of strain 81-176 in C-spent and C&E-spent BB, an increase in cell numbers of 6.6 ± 0.13 and 6.2 ± 0.18 $\log_{10}$ cfu/ml, respectively, could be observed, which was comparable to the increase in cell concentrations of the same strain in fresh BB (6.5 ± 0.18 $\log_{10}$ cfu/ml). Outcomes of Student's *t*-tests also confirmed that differences in log-increase after 24 h between both spent BB and fresh BB were insignificant ($p = 0.52$ and $p = 0.25$, respectively). The same trend could be observed after 48 h, as differences in log-increase remained similar ($p = 0.08$ and $p = 0.06$, respectively). For strain Ca 2800, after 24 h, a log-change of 6.7 ± 0.12 and 5.9 ± 0.02 $\log_{10}$ cfu/ml could be observed in C-spent and C&E-spent BB, respectively, compared to 5.8 ± 0.06 $\log_{10}$ cfu/ml in fresh BB. Correspondingly, significance testing showed, that growth was comparable in C&E-spent BB ($p = 0.28$), but even better in C-spent BB ($p = 0.01$). Comparably, log-changes after 48 h showed the same trends ($p = 0.05$ and $p = 0.03$, respectively). Since growth of both strains was similar in spent BB compared to fresh BB, this indicates not only that BB contains a surplus of essential substrates for the growth of campylobacters but also that the growth suppression during co-culture enrichment is not due to competition-induced lack of critical growth substrates or production of inhibitory compounds.

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

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

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

4. Discussion

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

Likewise, glutamate, proline, pyruvate, acetate, and succinate have been identified as possible growth substrates for *C. jejuni* (Hofreuter, 2014; Stahl et al., 2012), and our study showed that the concentration of extracellular pyruvate decreased only for the *C. coli* strain tested, and this could be only observed for reference cells. It has been recognized that pyruvate plays an important role in the metabolism of *C. jejuni* (Velayudhan and Kelly, 2002), although no pyruvate transporters have been identified yet. However, it is known that pyruvate can be produced intracellularly through the metabolism of serine and lactate, and for some isolates also L-fucose (Stahl et al., 2012). Previous studies have identified acetate and succinate as growth substrates for *C. jejuni* (Stahl et al., 2012). Wright et al. observed that acetate, which was previously secreted during the exponential phase, was taken up in late stationary phase (after 28 h) and used as a growth substrate by *C. jejuni* when

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

Although mono-culture enrichments of three ESBL-*E. coli* isolates showed that most of the compounds metabolized by campylobacters could be utilized by ESBL-*E. coli* as well, the current study demonstrates that the observed growth arrest of campylobacters during co-culture enrichments with ESBL-*E. coli* could not be explained by a lack of nutrients in BB, since campylobacters grew well in spent medium. One additional reason for conducting enrichments in C&E-spent BB (Fig. 4) was to investigate possible growth inhibition by inhibitory compounds produced by *E. coli* (e.g. colicins) at high cell concentrations to outcompete *Campylobacter* during co-culture enrichments. This inhibitory behaviour was previously described for *E. coli* in co-competition with other microorganisms than *Campylobacter* (Cascales et al., 2007; Kerr et al., 2002; Lenski and Riley, 2002). The fact that *Campylobacter* growth in C&E-spent BB was comparable to that in fresh BB and that an agar plate diffusion assay did not show inhibition zones (data not shown) led to the conclusion that either no colicins were produced or at least not to an extent that would impede the growth of campylobacters. Note that throughout this study, cell concentrations were determined by applying the plating technique. Although it is a widely used method to determine differences in cell concentrations, this method has its limitations (e.g. plating error) and therefore, unless stated otherwise, all experiments were performed using (at least) two biologically independent reproductions. Next to inhibitory compounds, also an increase in acidification of BB was considered, especially concerning the high amounts of acetate and succinate produced by ESBL-*E. coli* during enrichment. However, pH measurements after 0, 24, and 48 h of enrichment in mono- and co-culture showed that acidification of the medium could be ruled out as well, as the pH of BB remained neutral (between 6.5 and 7.5) for all measurements throughout the study (data not shown), which is optimal for growth of campylobacters (Doyle and Roman, 1981). All results gathered until this point led to the conclusion, that BB sufficiently supports the growth of campylobacters during enrichment and that the observed early transition into stationary phase is not caused by lack of growth substrates or presence of inhibitory compounds in BB.

Based on the results of the duplicate experiments (Fig. 5) where a constant availability of a microaerobic environment lead to higher cell concentrations of *C. jejuni* strain 81-176 during co-culture enrichment, it is conceivable that oxygen availability could influence the final cell concentration of *Campylobacter* in co-culture with *E. coli*. Campylobacters are known to grow only under microaerobic conditions. Although they can get some energy from fermentation, low amounts of oxygen are required for the proper functioning of an enzyme (class I ribonucleotide reductase) responsible for DNA synthesis (Sellars et al., 2002). *E. coli* is widely known to be able to grow under both, aerobic and anaerobic conditions. Although energy yields are higher aerobically compared to anaerobically, at high growth rates, *E. coli* has been shown to exhibit overflow metabolism, where it switches from respiration to fermentation to avoid a high density of respiratory proteins in the cell membrane (Szenk et al., 2017). We hypothesized that in microaerobic conditions, ESBL-*E. coli* consumes oxygen during growth depleting the medium and headspace of the infusion bottles of oxygen. As ESBL-*E. coli* are also able to grow anaerobically, their growth would be unaffected, while the growth of campylobacters, being obligate microaerobic, would be impeded.

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

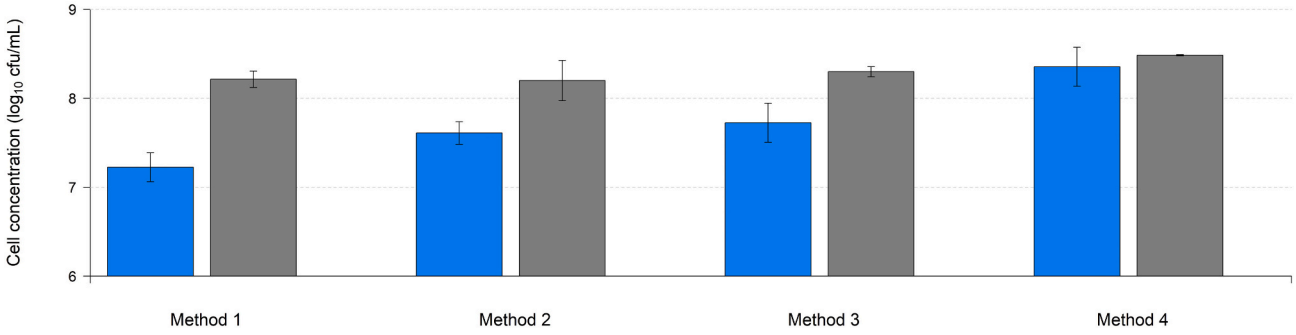

Fig. 5. Increase in cell concentrations ($\Delta_{t24\text{-}t0}$)of *C. jejuni* strain 81-176 (blue bars) and ESBL-*E. coli* strain RIVM 2 (grey bars) after 24 h of co-culture enrichment in four different atmospheric settings. Infusion bottles were flushed at the beginning of enrichment as well as after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

5. Conclusion

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

Declaration of competing interest

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

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijfoodmicro.2021.109518.

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