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**The influence of environmental conditions on
the infectivity of non-growing *Campylobacter
jejuni*.**

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This investigation has been performed by order and for the account of the Directory Board of RIVM, within the framework of project 251825, Quantitative safety aspects of exposure to *Campylobacter jejuni*.

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Abstract

Campylobacter (C.) jejuni is a causative agent of gastro-enteritis. Growth of this Gram negative bacterium is restricted to a limited number of environmental conditions. Under conditions where growth of *C. jejuni* is not possible, e.g. at temperatures below 30°C, *C. jejuni* can remain viable for a certain period of time, but its culturability decreases. In this study we examined the effect of this loss of culturability on the infectivity in cell-lines. We hypothesised that if non-culturable cells affect the process of infection then the infectivity per culturable cell used in the infection assay is not constant, whereas if only culturable cells are infectious then the infectivity per culturable cell is constant. *C. jejuni* was stored for 40 days in nutrient poor and nutrient rich medium at three temperatures. During this period we monitored its culturability, viability and infectivity. A decrease in culturability in time was observed. At low temperature (4°C) and in nutrient poor medium cells remained culturable for longer periods than at higher temperatures (12°C, 25°C) or in nutrient rich medium. Non-culturable cells remained viable, as determined by tetrazolium chloride staining. The absolute level of adhesion and invasion showed a decrease in time. At low temperatures and in nutrient poor medium cells retained their capability to adhere and invade longer than at higher temperatures or in nutrient rich medium. While the absolute level of adhesion and invasion decreased, the infectivity per culturable cell added seemed to increase during the first 5 days of storage at 4°C. This result confirmed our hypothesis that non-culturable *C. jejuni* can affect the process of infection. However, the result can also be explained by the presence of a limited number of binding sites for *C. jejuni* in our test model. This suggested existence of binding sites has great importance for the research into the influence of non-culturable cells on the infectivity. If non-culturable cells are not infectious but can block binding sites, then they can play a protective role by competing with the culturable cells for the limited number of binding sites.

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Summary

Under non-growing conditions the culturability of *Campylobacter (C.) jejuni* decreases, but cultures seem to retain their viability. The importance of viable non-culturable cells in the contamination cycle is questioned. This study was conducted to examine whether or not these non-culturable cells of *C. jejuni* are infective, and if the infectivity depends upon the environmental conditions under which the non-culturable cells were formed.

During 40 days the culturability, viability and infectivity were determined of *C. jejuni* stored in two media at three temperatures. At low temperature (4°C) and in low nutrient medium (Phosphate Buffered Saline; PPBS) cells remained culturable for longer periods than at higher temperatures (12°C, 25°C) or in high nutrient medium (Brain Heart Infusion; BHI). Temperature did have a more profound effect than medium on survival. Non-culturable cells remained viable, as determined by tetrazolium chloride staining.

Infectivity was determined by the adhesion/invasion and Interleukine-8 (Il-8) assay. The Il-8 secretion was below the level of detection at all time points. Adhesion and invasion were only measurable when the number of cells in the suspension was over 10^5 per ml. The absolute level of adhesion and invasion showed a decrease in time. At low temperatures and in PPBS cells retained their capability to adhere to and to invade in Caco-2 cells for longer periods than at higher temperatures or in BHI.

The infectivity per culturable cell was calculated. Of an overnight culture 0.16 % adhered to and invaded in Caco-2 cells. This percentage increased during the first 5 days at 4°C and decreased thereafter. This result confirms our hypothesis that non-culturable *C. jejuni* can be infectious. However, the result can also be explained by the presence of a limited number of binding sites for *C. jejuni* in our testmodel. As long as the initial number of culturable *C. jejuni* exceeds the number of binding sites, an apparent increase in infectivity can be observed during storage as the number of culturable *C. jejuni* decreases.

The suggested existence of bindingsites has great importance for the research into the influence of non-culturable cells on the infectivity. If non-culturable cells are not infective but can block the bindingsites, culturable cells have to compete with non-culturable cells for the bindingsites. Then a totally different effect of non-culturable cells would be shown, as non-culturable cells than would play a protecting rather than an infectious role.

In future research the infectivity assays have to be optimised. With the optimised assays the existence of bindingplaces will be studied. If the existence of bindingplaces can be proven, the influence of the presence of non-culturable cells on the infectivity will be studied further.

Samenvatting

Campylobacter jejuni is de meest frequente bacteriële veroorzaker van voedselinfecties. Onder omstandigheden waarbij *C. jejuni* niet kan groeien, bijvoorbeeld bij temperaturen lager dan 30°C, verliest de bacterie zijn kweekbaarheid, maar lijkt de bacterie niet dood te gaan. In deze studie is onderzocht of een niet-kweekbare *C. jejuni* een infectie kan veroorzaken, en of de omstandigheden waaronder de kweekbaarheid verloren is gegaan van invloed zijn op een eventuele infectie. Gedurende 40 dagen zijn kweekbaarheid, levensvatbaarheid en vermogen om een infectie te veroorzaken bepaald van *C. jejuni* die was opgeslagen in twee verschillende media bij drie verschillende temperaturen. Wanneer opgeslagen in een nutriënt-arm medium bij lage temperatuur (4°C) bleek *C. jejuni* langer zijn kweekbaarheid te behouden dan bij hogere temperaturen (12°C, 25°C) of in een rijk medium. Bacteriën bleven onder alle condities levensvatbaar. De mate van adhesie en invasie van de opgeslagen cultures nam af in de tijd; bij lage temperatuur in buffer behielden de bacteriën het langst hun vermogen om aan darmepitheelcellen te hechten of om erin binnen te dringen. Terwijl de infectiviteit van de totale cultuur afnam, nam de infectiviteit per kweekbare bacterie in die cultuur toe gedurende de eerste 5 dagen om daarna weer af te nemen. Dit resultaat suggereert dat een niet-kweekbare *C. jejuni* infectieus kan zijn. Echter, er kan ook een alternatieve verklaring zijn, namelijk dat er slechts een beperkt aantal specifieke bindingsplaatsen voor de hechting van *C. jejuni* aanwezig zijn. De eventuele aanwezigheid van specifieke bindingsplaatsen voor *C. jejuni* heeft mogelijk grote gevolgen voor onderzoek aan de invloed van niet-kweekbare cellen. Als niet-kweekbare cellen niet meer infectief zijn, maar wel kunnen hechten aan een bindingsplaats, dan kunnen zij met kweekbare cellen concurreren om een bindingsplaats. Dit zou een geheel nieuwe rol betekenen voor niet-kweekbare cellen: niet-kweekbare cellen zouden dan de kans op een infectie verlagen!

1. Introduction

Campylobacter jejuni has been identified as the major cause of bacterial gastro-enteritis in the Netherlands¹. *C. jejuni* is a Gram-negative, motile microorganism, of which the morphology varies from spiral-shaped to coccoid. *C. jejuni* is primarily micro-aerophilic and grows well in environments containing 3-5% CO₂ and 3-15% O₂. *C. jejuni* grows within a short temperature range, being unable to multiply at temperatures either above 45°C or below 30°C. Under non-growing conditions the culturability of *C. jejuni* decreases, often accompanied by a transformation from the spiral form into a coccoid form^{2,3,4}. Temperatures below 15°C^{2,3} and nutrient limitation^{2,4} give lower transformation rates from culturable to non-culturable cells.

All required growth conditions are present in the gastrointestinal tract of warm-blooded animals. Poultry is identified as one of the major sources of *C. jejuni*. Pigs and cattle are a source of *C. coli*, but have not been identified as important reservoirs for *C. jejuni*⁵. However, strains found in poultry have not been found in the human population, and only few of the human isolates have also been detected in poultry⁶. For this apparent contradiction, various reasons can be thought of:

- non-culturable cells might play a role as a source of contamination in the contamination cycle⁷⁻¹¹.
- other sources and/or reservoirs exist, but have not been identified yet.
- both chickens and humans harbour a large population of genotypically different campylobacters, but the dominating, and thus most likely isolated genotype from poultry differs from the dominating genotype in human patients¹²⁻¹⁵.
- the genotype of *Campylobacter* is not a constant, but changes^{12, 16-18}.

This study was conducted to examine the infectivity of non-culturable cells of *C. jejuni*.

For risk assessment it is very important to know if non-culturable cells are infective. In practice the exposure is often measured as the number of culturable *C. jejuni* in a product, but when non-culturable cells are infective the risk is largely underestimated. In literature many contradictory articles on the infectivity of non-culturable cells of *C. jejuni* can be found. Some authors showed that non-culturable cells were not infective in chicks, mice and human volunteers¹⁹⁻²². Other authors demonstrated that non-culturable cells were infectious in chicks and mice⁷⁻⁹. The inconsistency in literature about the infectivity of non-culturable cells might be the result of:

- different methods by which non-culturability was determined. In some articles selective enrichment instead of non-selective enrichment was used⁸⁻¹⁰. The selective agents will negatively influence the culturability, as sub-lethally injured cells are sensitive to these agents^{23,24}.
- different conditions under which the non-culturable cells were formed. In all studies where non-culturable cells were infective, the non-culturable cells were formed at 4°C⁷⁻⁹.

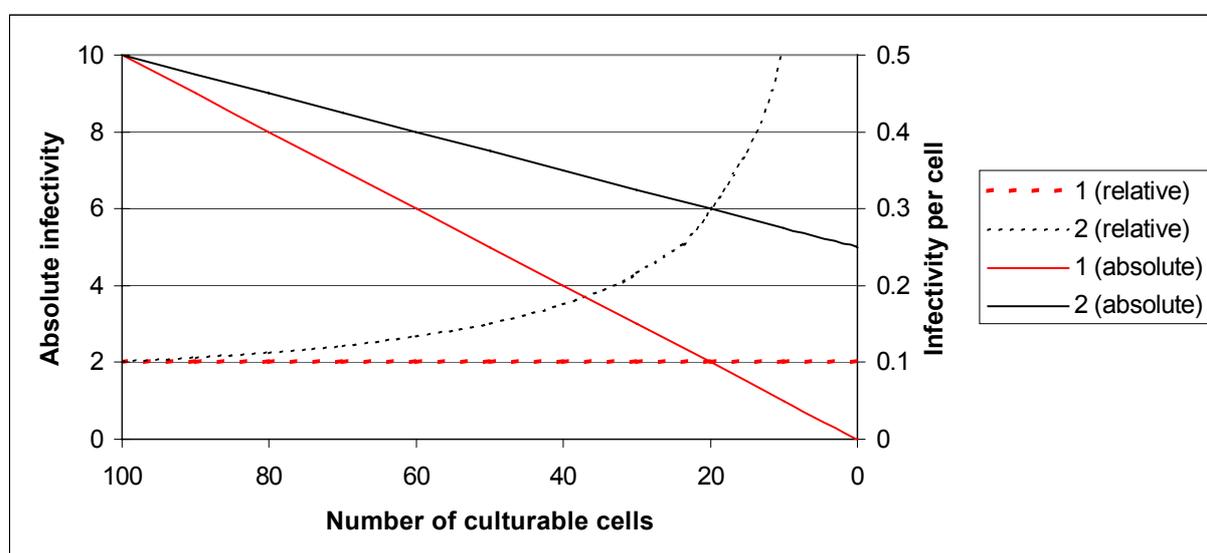
In experiments using higher temperatures no infectivity was found^{19,21}. These results suggest that non-culturable cells formed at low temperatures are distinct from non-culturable cells formed at higher temperatures. Interestingly, Hazeleger *et al.*² showed diversities between non-culturable cells formed at various temperatures. Non-culturable cells formed at 4°C showed characteristics, including intracellular / extracellular ATP-ratio and membrane fatty acid composition, comparable to culturable cells, whereas those of non-culturable cells formed at 25°C were clearly different.

- different strains used. Some authors did not find any infectivity of non-culturable cells formed at 4°C²⁰⁻²². This might be explained by strain differences, as Jones *et al.*⁷ reported that the capability of non-culturable cells to infect mice differed between strains.
- presence of very few culturable cells in suspensions supposed to contain only non-culturables.

Our reasoning is as follows:

1. If non-culturable cells are not infective, a linear relationship exists between the number of culturable cells and the infectivity starting with a maximum infectivity at the maximum number of culturable cells down to no infectivity at zero culturable cells. The infectivity per culturable cell is a constant (Figure 1, case 1).

Figure 1. The relation between the infectivity and the number of culturable cells.



1. Non-culturable cells are not infective. 2. Non-culturable cells contribute to the infectivity. Relative means the infectivity per cell, absolute means the total number of infecting bacteria.

2. If non-culturable cells contribute to the infectivity, a linear relation can exist between the number of culturable cells and the infectivity, but at zero culturable cells

infectivity still exists. The infectivity per number of culturable cells is not a constant, it increases, when the number of culturable cells decreases and the number of non-culturable cells increases (Figure 1, case 2).

We measured the decrease in culturability and infectivity of *C. jejuni* in time stored at various temperatures (4°C, 12°C and 25°C, respectively) and in two different media [brain heart infusion (BHI) and phosphate buffered saline (PPBS), respectively]. These environmental conditions were based on the study of Hazeleger *et al.*² who reported dissimilarities between non-culturable cells formed at various temperatures and in two different media. For our experiments *C. jejuni* C356 was chosen, because this strain showed the highest level of adhesion to and invasion in Caco-2 cells in a pilot study (unpublished result).

To be infectious cells at least have to be viable. To assess if there exist non-culturable cells that are still viable, the cellular respiration activity of the cells as measure for the viability was determined. The infectivity was determined by measuring the number of *C. jejuni* cells adhering to and/or invading in human epithelial Caco-2 cells. The culturability was measured by spread plating on Columbia agar plates with 5% lysed horse blood (CAB). A drawback of the use of the adhesion and/or invasion assay is that infectivity is measured by determining the number of adhered and/or invaded culturable cells. If non-culturable cells can adhere or invade but remain non-culturable, no infectivity is measured by the adhesion/invasion assay. Therefore, in addition the IL-8 assay was performed to measure the infectivity by determining the secretion of IL-8, a proinflammatory cytokine²⁹, by Caco-2 cells³⁰. The IL-8 assay is not dependent on whether or not cells are culturable.

2. Materials and Methods

2.1 Experimental set up

2.1.1 Culturing *C. jejuni* and *Salmonella (S.) enteritidis*

Bacterial strains were stored at -70°C in Brain Heart Infusion broth (BHI, Beckton Dickinson) plus 30% (v/v) glycerol in vials. For culturing *C. jejuni* C356, the content of one vial was thawed and put in an Erlenmeyer flask with 50 ml BHI. The Erlenmeyer flask was incubated while shaking in a custom made incubator (NuAire, Plymouth, Minnesota, USA) with a micro-aerobic atmosphere (10% O_2 , 5% CO_2 , 85% N_2) at 37°C for 24 hours. After 24 hours, the strain was subcultured in 100 ml BHI and incubated under the same conditions for 24 hours.

For culturing *S. Enteritidis* 97-198, the content of one vial was thawed and put in a tube with 10 ml BHI. The culture was used after overnight incubation at 37°C without shaking. *S. Enteritidis* was chosen as positive control based on its good adherence and invasion properties and IL-8 induction in Caco-2 cells (unpublished data).

2.1.2 Preparing Erlenmeyer flasks for storage experiments

The 24 hours old (stationary phase) cells were harvested by centrifugation at $3,000 \times g$ and resuspended in either BHI (nutrient rich medium) or 50 mM potassium phosphate buffer containing 8.5 g/l NaCl (PPBS, nutrient poor medium). The cell suspensions were stored in Erlenmeyer flasks with a large opening in the dark, at various temperatures (4°C , 12°C and 25°C). Samples were taken after 5, 11, 17, 25, 32 and 40 days. For each sampling point a new set of Erlenmeyer flasks was used. All the experiments were done in duplicate.

2.2 Measuring culturability, morphology and viability

2.2.1 Culturability

Plate counts were performed by spread plating 0.1 ml of appropriate decimal dilutions of cell suspensions on Columbia Agar Base with 5% (v/v) lysed defibrinated horse blood (CAB). The plates were incubated micro-aerobically in a jar using BBL® Campypak (Becton Dickinson) at 37°C for 72 hours.

2.2.2 Morphology

For morphological analysis, the cells were stained with acridine orange (Molecular Probes Europe BV, Leiden, the Netherlands) according to a modified technique of Hobbie *et al.*²⁵. Of a 1% acridine orange in distilled water, 100 µl was added to 1.0 ml of a bacterial suspension. After incubation for 5 minutes in the dark, the morphology of the stained cells was monitored under a Zeiss epifluorescence microscope.

2.2.3 Viability and total cell number

The *C. jejuni* cells were stained with 5-cyano-2,3-ditolyl tetrazolium chloride (CTC, Polysciences, Warrington, USA) and 4',6-diamino-2-phenylindole (DAPI, Sigma Chemical Co., St Louis, USA) as described by Cappelier *et al.*²⁶. CTC is an indicator of cellular respiratory activity. During respiration CTC is reduced into an insoluble red fluorescent CTC formazan salt, which accumulates intracellularly. DAPI stains DNA of intact cells and was used to determine total cell number.

To 0.5 ml of a bacterial suspension, 0.5 ml of BHI and 100 µl of a 0.04 g l⁻¹ pyruvic acid solution (Sigma Chemical Co.) were added to stimulate cellular respiration. CTC was added to a final concentration of 5 mM. The mixture was incubated for 4 hours at 37°C in a microaerobic atmosphere. Cells were subsequently harvested by filtration through an isopore polycarbonate black membrane filter (0.2 µm pore size, 25 mm in diameter; Millipore, Ireland) and covered with a 5 µg ml⁻¹ DAPI solution for 5 minutes. Finally, stains were removed by filtration. After washing the filter two times with 1 ml distilled water, the filter was air-dried and mounted in non-fluorescent immersion oil (Olympus, Japan) where after a coverslip was added. The number of respiring cells and the total number of cells were counted under a Zeiss epifluorescence microscope with a 405 nm excitation filter and a 455 nm dichromic mirror allowing simultaneous visualisation of both dyes.

2.3 Measuring infectivity

2.3.1 Caco-2 cell line, growth media and conditions

Caco-2 cells, human colon adenocarcinoma cells isolated from a primary colonic tumour in a 72-year-old Caucasian male were obtained from the American Type Culture Collection (Caco-2, ATCC HTB-37). In the experiments passage 30 to 45 were used.

We used Dulbecco's Modified Eagle Medium 25 mM HEPES, containing 4500 mg l⁻¹ D-glucose but no sodiumpyruvate (DMEM, GibcoBRL, Life Technologies Ltd, Paisley, Scotland) supplemented with 10% heat inactivated (30 minutes at 60°C) fetal calf serum (FCS, Integro b.v., Zaandam, the Netherlands), 0.1% MEM non-essential amino-acids (Gibco), 6 mM L-glutamine (Gibco) and 50 µg ml⁻¹ gentamycin (Gibco) [DMEM10%].

Cells were stored at -135°C in DMEM 10%, plus 10% (v/v) DMSO (Sigma Chemical Co., St Louis, USA). After quickly thawing in a waterbath at 37°C , the content of one vial (10^6 cells) was put in a culture-flask with 10 ml DMEM 10%. The cells were grown in a CO_2 5% (v/v) incubator at 37°C . For experimental assays, confluent cultures were harvested by mild trypsinisation and seeded into 12-wells tissue culture plates (Costar, Corning Costar Europe, Badhoevedorp, the Netherlands) at 160 000 cells/well and incubated in a CO_2 5% (v/v) incubator at 37°C . Medium was changed 3 times a week and plates were used at 11-13 days post-confluence.

2.3.2 Adhesion/Invasion assay

The adhesion / invasion assay as developed in a former study was used²⁷. Briefly, 12 to 13 day old Caco-2 cells in the 12-well plates were inoculated with 40 μl bacterial suspension. The bacteria were allowed to adhere to and invade in the cells for 3 hours in a CO_2 5% (v/v) incubator at 37°C .

To study adhesion and invasion, after incubation, the monolayers were rinsed 3 times with TCM and the cells were lysed with 1 ml 1% (v/v) Triton-x100 (Merck, Amsterdam, the Netherlands) in distilled water.

To study invasion, after incubation, the bacterial suspension was replaced by TCM containing 300 $\mu\text{g ml}^{-1}$ gentamycin. After incubation for 1 hour, the monolayers were rinsed 3 times with TCM and the cells were lysed with 1 ml 1% (v/v) Triton-x100 in distilled water.

Adhesion/invasion and invasion alone were determined by plating serial dilutions of lysed Caco-2 cell suspensions on CAB and counting the resulting colony forming units (cfu), after 72 hours incubation at 37°C under micro-aerobic conditions.

S. enteritidis 97-198 was used as positive control. The assays were performed in triplicate.

2.3.3 Interleukin-8 assay

The IL-8 assay as developed in a former study was used²⁷. Briefly, 12 to 13 day old Caco-2 cells in 12-well plates were inoculated with 40 μl bacterial suspension. The bacteria were allowed to adhere to and invade in the cells for 3 hours in a CO_2 5% (v/v) incubator at 37°C . After incubation, the monolayers were rinsed 3 times with TCM. Finally, TCM with 50 $\mu\text{g/ml}$ gentamycin was added to the cells, followed by incubation for 24 hours in a CO_2 5% (v/v) incubator at 37°C . After incubation, supernatants were collected and stored at -70°C to be analysed later. IL-8 concentrations were determined using IL-8 ELISA essentially according to Garssen *et al.*²⁸. Briefly, 96-well plates (NUNC-Immuno Plate, Roskilde, Denmark) were coated with 1 $\mu\text{g/ml}$ antihuman IL-8 (Biosource, Nivelles, Belgium) in coating buffer (0.04 M carbonate buffer, pH 9.6). After 24 hours incubation at 4°C , the plates were incubated in blocking buffer [1% Bovine Serum Albumin (BSA, Sigma, Axel, the

Netherlands) plus 0.05% Tween-20 (Merck) in PBS] 24 hours at 4°C and washed in PBS plus 0.05% Tween-20. Recombinant human IL-8 (Medgenix diagnostics SA, Fleurus, Belgium) was used as a standard. Standard as well as serial dilutions of culture supernatant and 0.2 µg ml⁻¹ biotinylated antihuman IL-8 (Medgenix diagnostics SA) was added to the plates. After incubation for 2 hours at 21°C, plates were washed and 0.1 µg ml⁻¹ poly horseradish peroxidase-labeled streptavidin (Central Laboratory of the Blood Transfusion Service, Amsterdam, the Netherlands) was added. After incubation for 30 minutes at 21°C, plates were washed again and 50 µl 0.1 mg ml⁻¹ tetra methyl benzidine (Sigma Chemical Co, St. Louis, MO) plus 0.006% H₂O₂ in 0.1M Na-acetate, pH 5.5 was added. The colour-reaction was stopped after 5 minutes by adding 50 µl 2N H₂SO₄ (Merck). Plates were read at 450 nm using a Titertek Multiskan MCC/340 ELISA reader.

Caco-2 cells without bacteria and bacteria without Caco-2 cells were used as negative controls, Caco-2 cells with *S. Enteritidis* 97-198 were used as positive control. The assays were performed in triplicate.

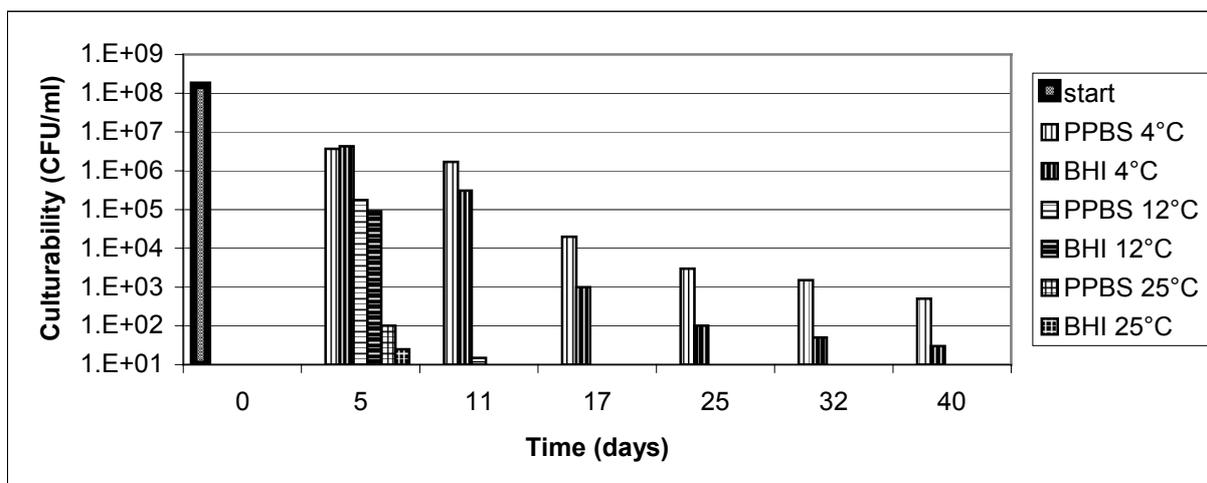
3. Results

3.1 Culturability, morphology and viability

C. jejuni C356 resuspended in PPBS and BHI and stored at different temperatures showed a decrease in culturability in time (Figure 2). The suspensions incubated at 4°C remained culturable for over 40 days, while the suspensions incubated at 12°C and 25°C were below the detection limit of culturability in respectively 17 and 11 days. Besides the temperature effect a medium effect was seen. At all time points the suspensions incubated in PPBS showed a higher number of culturable cells than the cells stored in BHI.

The decrease in culturability did not have an effect on the morphology or viability. The shape of *C. jejuni* C356 cells was from the beginning rod-shaped and did not change in time. The viability, measured as number of CTC-stained cells, was at all time-points $\pm 95\%$. The number of cells stained by DAPI diminished slightly in time (not shown).

Figure 2. Culturability of *C. jejuni* C356 (CFU ml⁻¹) kept in two media (PPBS or BHI) at various temperatures (4°C, 12°C or 25°C)



Both PPBS and BHI were inoculated from the same start culture.

3.2 Infectivity

Invasion plus adhesion and invasion of Caco-2 cells by *C. jejuni* C356 stored in PPBS and BHI and incubated at different temperatures were measured (Figure 3 and 4). The results showed a decrease in adhesion/invasion at longer storage times. Cells incubated at 4°C retained their ability to adhere and invade Caco-2 cells for 11 days, while cells incubated at 12°C and 25°C were below the detection limit of adhesion/invasion in 11 and 5 days, respectively. No clear medium effect was seen.

Figure 3. Invasion plus adhesion of Caco-2 cells by *C. jejuni* C356 kept in two media (PPBS or BHI) at various temperatures (4 °C, 12 °C or 25 °C)

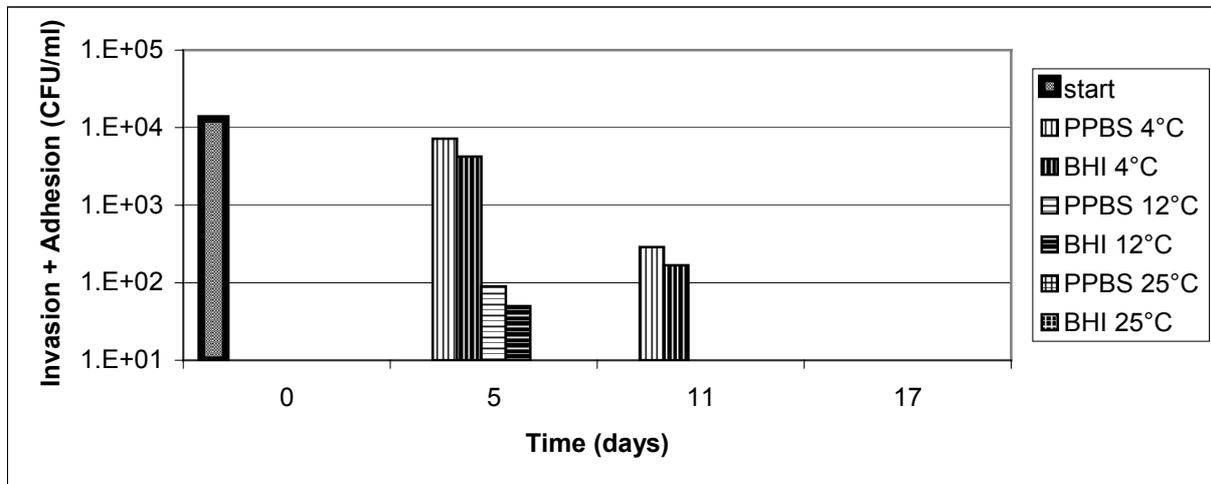
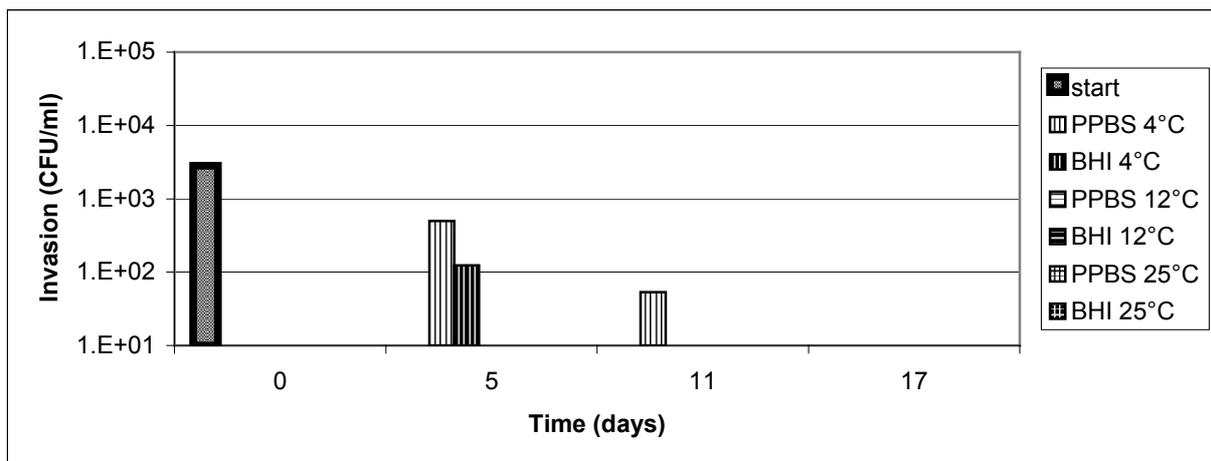


Figure 4. Invasion of Caco-2 cells by *C. jejuni* C356 kept in two media (PPBS or BHI) at various temperatures (4 °C, 12 °C or 25 °C)



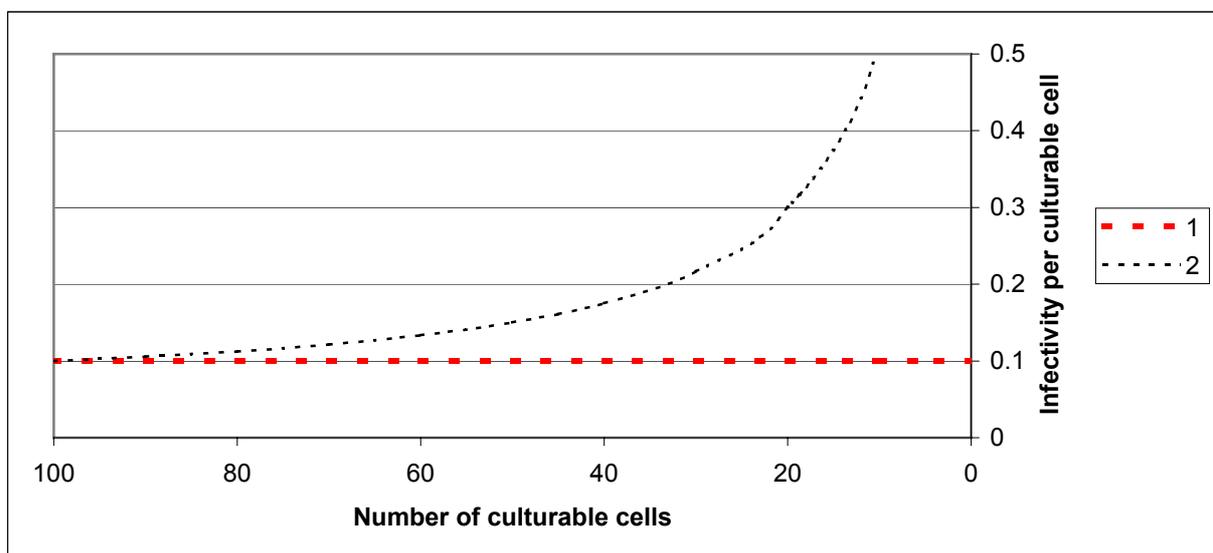
No Il-8 response was measured after exposure of the Caco-2 cells to *C. jejuni* C356, the secreted Il-8 was around the detection limit of 10 pg ml⁻¹. In response to exposure to *S. enteritidis* 97-198, the positive control, the Caco-2 cells secreted 70 pg Il-8 ml⁻¹ (not shown).

4. Discussion

This study was undertaken to find out whether or not non-culturable cells can be infective, and if so, does the infectivity depend on the environmental conditions under which the non-culturable cells were formed.

To determine the relation between the infectivity and the number of culturable cells, the culturability and infectivity, during storage under different environmental conditions, were measured. A decrease in culturability in time was detected, which mostly depended on the temperature and slightly on the medium used. At low temperatures cells remained culturable for longer periods than at higher temperatures. A transition from a spiral to a coccoid shape, often accompanying the decrease in culturability^{2-4, 21}, was not observed. In our experiments most non-culturable *C. jejuni* cells ($\pm 95\%$) showed cellular respiration, as described before^{4, 26}, and thus were viable. Using CTC-staining as a measure for viability, this result confirmed the existence of non-culturable cells that were still viable and might be infectious. We hypothesized that if the number of culturable cells decreases during storage, the infectivity per culturable cell: 1.) will remain constant if the non-culturable cells are not infective; or 2.) will increase if the non-culturable cells contribute to the infectivity. This is shown in figure 5.

Figure 5. The hypothesized relations between the infectivity and the number of culturable cells.



1. Non-culturable cells are not infective. 2. Non-culturable cells contribute to the infectivity

During measuring the infectivity by the adhesion/invasion assay and the Il-8 assay, we encountered two problems:

- only at a limited number of time points the infectivity could be measured by the adhesion/invasion assay. When the culturability of the suspension was below

10^5 CFU/ml, giving an infection dose below 10^{-2} culturable *C. jejuni* cells / Caco-2 cell, no adhesion/invasion could be detected.

- the adhesion/invasion assay only detects culturable cells. If non-culturable cells could adhere or invade but remain non-culturable, they will not be detected by this assay. The Il-8 assay, which does not depend on culturability, did not show sign of infectivity. No Il-8 was produced by Caco-2 cells after infection with *C. jejuni* C356.

The limited number of time points at which adhesion and invasion could be measured, showed a decrease in absolute adhesion and invasion in time. The decrease in infectivity depended mostly on the temperature and less on the medium used. At low temperatures cells retained their capability to adhere to and to invade in Caco-2 cells for longer periods than at higher temperatures.

The infectivity per culturable cell was calculated for the few time points at which we measured infectivity by the adhesion/invasion assay (Figure 6 and 7).

Of an overnight culture 0.2 % adhered and invaded. This percentage increased to 4.8 % and 2.4 % after 5 days storage at 4°C in PPBS and BHI, respectively. Finally, the percentages decreased to 1.4 and 0.4% after 11 days and no adhesion and invasion was measurable after 17 days. The same trend was seen for invasion only (Figure 7).

Figure 6. The percentage of invading plus adhering bacteria relative to the number of culturable bacteria during storage at 4°C in PPBS or BHI

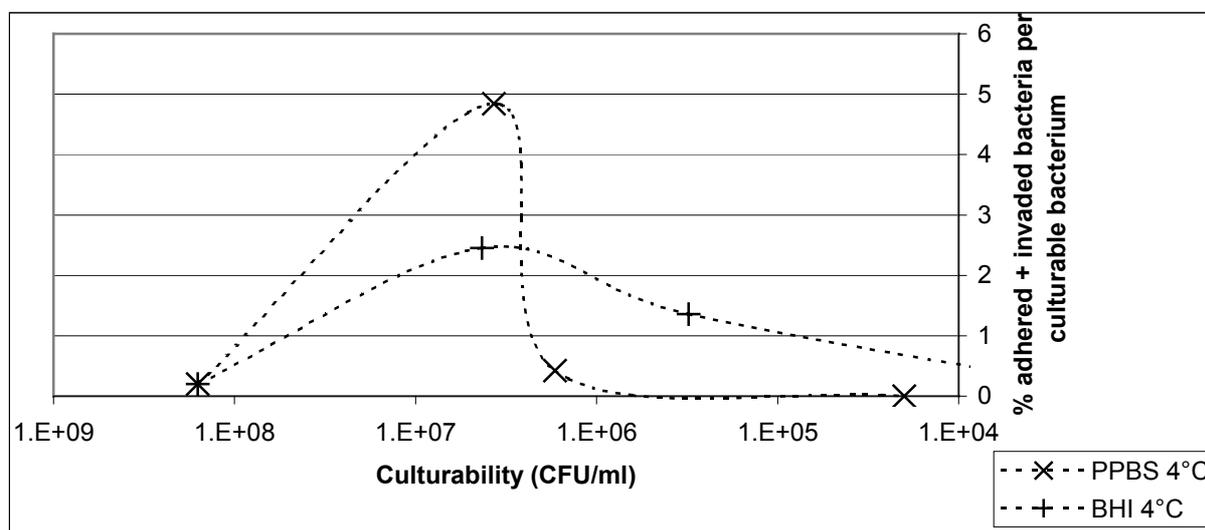
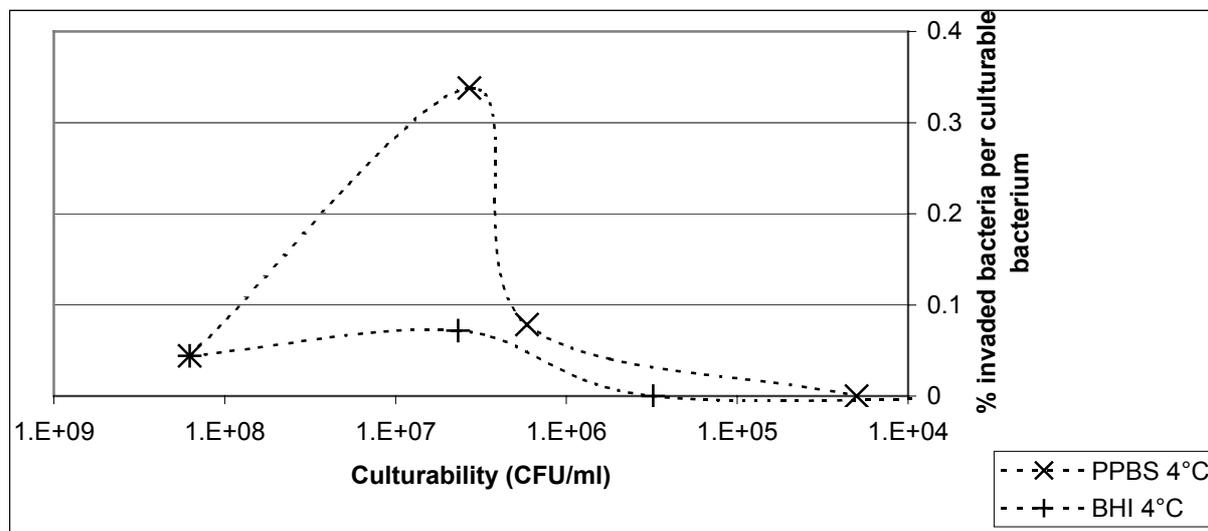


Figure 7. The percentage of invading bacteria relative to the number of culturable bacteria during storage at 4 °C in PPBS or BHI



The decrease in culturability and concomitant rise in infectivity per bacterium indicated that non-culturable *C. jejuni* were infective. An increase in the efficiency at lower doses was shown by Hu and Kopecko³¹. They found the highest invasion efficiency of INT-cells by *C. jejuni* 81-176 (3.5%) at 0.02 bacteria per epithelial cell, and a decrease in the efficiency at higher doses. Their results suggest the existence of a maximum level of adhesion and invasion, or a limited number of bindingsites per Caco-2 cell for *C. jejuni* to adhere and invade.

At the start (t=0) of our experiments the number of culturable *C. jejuni* cells per Caco-2 cell was 40, while at t=5 the number of culturable *C. jejuni* cells per Caco-2 cell varied from 2.25×10^{-3} (BHI 12°C) to 1.1 (BHI 4°C). The maximum adhesion/invasion-efficiency (4.8%) was found in PPBS after 5 days at 4°C with an infection dose of ± 0.9 *C. jejuni* C356 per Caco-2 cell, which is higher than reported by Hu and Kopecko³¹. The use of different cell lines might explain this difference.

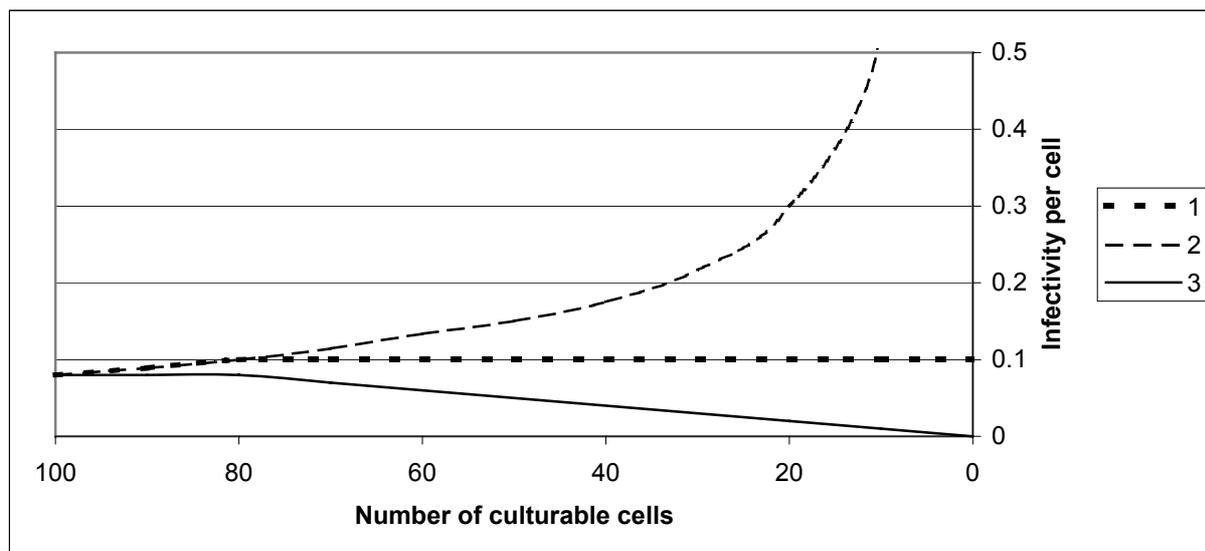
The existence of a limited number of bindingsites has great importance for the research into the influence of non-culturable cells on the infectivity. Until now, we only had two options:

1. the non-culturable cells are not infective.
2. the non-culturable cells can contribute to the infectivity.

Now a third option arises:

3. the non-culturable cells are not infective, but can block a bindingsite. In that case culturable cells have to compete for a bindingsite with the non-culturable cells, and this would result in lower levels of infectivity. This third option is visualised in Figure 8 as line 3.

Figure 8. The relation between the infectivity and the number of culturable cells.



1. Non-culturable cells are not infective. 2. Non-culturable cells contribute to the infectivity. 3. Non-culturable cells are not infective, but can block a part of the limited number of bindingsites.

Figure 8, line 1 shows the relation between the culturability and the infectivity if non-culturable cells are not infective and do not block bindingsites. At first when the number of culturable cells is exceeding the number of bindingplaces the infectivity per cell will rise. Thereafter the infectivity per cell will be constant.

Figure 8, line 2 shows the relation between the culturability and the infectivity if non-culturable cells are infectious. At first when the number of infectious cells (culturable and non-culturable) is exceeding the number of bindingplaces the infectivity per cell will rise. Thereafter, the number of culturable cells diminishes, but as non-culturable cells contribute to an infection, the infectivity per culturable bacterium increases.

Line 3 in Figure 8 shows the relation between culturability and infectivity when non-culturable cells are not infectious, but block a part of the available bindingsites. At $t=0$ when only culturable cells are present, the infectivity is the highest. Thereafter the percentage of non-culturable cells increases. The culturable cells have to compete for binding sites to an increasing number of non-culturable cells, resulting in a decrease in infectivity per cell. The experimental data were too few to determine whether or not non-culturable cells did contribute to infectivity. The data did not allow determination of the maximum level of absolute adhesion and invasion and the subsequent trend after this maximum due to the high dose of *C. jejuni* required for a measurable response.

The results presented in this report however do not exclude a role for non-culturable *C. jejuni* in the infection process. In the non-culturable state cells might remain infectious for a certain period of time. Or, as non-culturable cell, it might lose its capacity to infect a host. If an infection with *C. jejuni* occurs after binding to a specific *C. jejuni* bindingsite and if non-culturable cells retain their capacity to bind to such sites, then the presence of non-culturable *C. jejuni* reduces the probability of infection with *C. jejuni*.

Quantitative exposure data on *C. jejuni* obtained by direct culture techniques therefore will underestimate the real situation in case of non-culturables appear to be infectious. Using molecular techniques, the detection of *C. jejuni* DNA might overestimate the exposure to *C. jejuni* if non-culturables are non-infective. Obviously, the role of non-culturable *C. jejuni* needs to be fully elucidated in order to perform a reliable risk assessment of *C. jejuni*.

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