Arcobacter butzleri Induces Barrier Dysfunction in Intestinal HT-29/B6 Cells

Roland Bücker,†* Hanno Troeger,†* Josef Kleer,† Michael Fromm,‡ and Jörg-Dieter Schulzke

Departments of †Gastroenterology, Infectious Diseases and Rheumatology, and ‡General Medicine, and ‡Institute of Clinical Physiology, Charité Berlin; and ‡Institute of Food Hygiene, Freie Universität, Berlin, Germany

Background. Arcobacter butzleri causes watery diarrhea and bacteremia. Although, recently, more cases of diarrhea have been caused by Arcobacter species, very little is known about its pathogenesis, the identification of which is the aim of this study.

Methods. Human HT-29/B6 colonic epithelial monolayers were apically inoculated with A. butzleri. Transepithelial resistance and macromolecule fluxes were measured in Ussing chambers. Tight junction protein expression was analyzed by Western blotting, and subcellular distribution was analyzed by confocal laser-scanning microscopy.

Results. Infection of HT-29/B6 caused a decrease in transepithelial resistance to 30% and an increase in paracellular permeability to fluorescein (10.8 ± 3.5 10⁻⁵ cm/s vs. 1.8 ± 0.6 10⁻⁵ cm/s in control; P<.05) and dextran-4 kDa (0.036 ± 0.005 10⁻⁵ cm/s vs. 0.015 ± 0.002 10⁻⁵ cm/s in control; P<.01). This effect was time and dose dependent and was also caused by bacterial lysates showing heat and proteinase-K sensitivity. As structural correlate, expression of the tight junctional proteins claudin-1, -5, and -8 was reduced, and claudin-1 and -8 were redistributed off the tight junctional strands forming intracellular aggregates. Furthermore, A. butzleri induced epithelial apoptosis (3-fold).

Conclusions. A. butzleri induces epithelial barrier dysfunction by changes in tight junction proteins and induction of epithelial apoptosis, which are mechanisms that are consistent with a leak flux type of diarrhea in A. butzleri infection.

Arcobacter are gram-negative, motile and spiral-shaped bacteria with a length of ∼4 μm. Arcobacter were described for the first time in 1977 as aerotolerant Campylobacter-like micro-organisms [1] and were assigned to the Campylobacter group [2, 3]. However, in 1991, the taxonomy was revised, and Arcobacter was introduced as a new genus belonging to the epsilon-proteobacteria [4]. The complete genome sequence of Arcobacter butzleri was recently published [5].

A. butzleri is considered to be an emerging human pathogen that can cause severe diarrhea and bacteremia [6–8]. In humans, A. butzleri causes diarrheal illness associated with abdominal pain and, in some cases, nausea, vomiting, and fever [6, 9]. In addition, watery and persistent diarrheic stools are characteristic of A. butzleri infection [9]. Besides the currently most frequent notifiable Campylobacter infection in Europe [10], Arcobacter are known to be the fourth most common Campylobacter-like organisms isolated from diarrheic stool samples from patients in Belgium and France [9, 11]. The association of A. butzleri with human gastroenteritis was also reported from other countries, and such gastroenteritis is considered to be increasing in incidence [12]. The global prevalence of Arcobacter infection is rather underestimated, because no routine diagnostic of these bacteria has been performed [11]. The exact routes of transmission are unknown. Arcobacter have been considered as food- and water-borne pathogens [13, 14]. The organisms were detected in feces and meat of livestock animals, drinking water, seawater plankton, and pets [15–18]. An epidemic of Arcobacter infection was assumed to spread through contaminated meat (mainly poultry) [19, 20]. Although evidence was found for the association of
Arcobacter with human and animal illness, there is almost no information on the pathophysiology of this disease. In general, intestinal pathogens can induce diarrhea by induction of active ion secretion, via malabsorption, or by impairment of the epithelial barrier function of the intestine. Barrier impairment is often caused by structural changes of the epithelial tight junction (TJ) or by induction of epithelial gross lesions, including induction of apoptosis and necrosis, erosions, or ulcer-type lesions [21], all of which lead to a passive efflux of water and solutes from the circulation into the lumen of the intestine (leak flux diarrhea). The dysfunction of the TJ is characterized either by expression changes or by redistribution or disruption of TJ proteins that seal the paracellular space between neighboring epithelial cells [22, 23].

**MATERIAL AND METHODS**

**Growth conditions of A. butzleri.** Clinical isolates of *A. butzleri* ATCC 49616 (from human diarrheal stool) and CCUG 10373 (from human blood) were cultured in Mueller-Hinton media with selective supplement (cefoperazone, amphotericin B, teicoplanin, selective supplement; Oxoid) for 72 h at 30°C under microaerobic conditions (CampyGen; Oxoid). For quantification of *A. butzleri*, the liquid-cultured bacteria were microscopically counted.

**Lysates.** *A. butzleri* were harvested from log-phase cultures (200 mL), washed with phosphate-buffered saline, and resuspended in 5 mL of ice-cold bathing solution (RPMI 1640 cell culture medium; PAA Laboratories). The intracellular content of the bacteria was released into the bathing solution after cell wall disruption by means of a hydraulic press (FRENCH Press; Thermo Spectronic). After cell lysis, cell debris and cell membrane remnants were removed by centrifugation (20,000 g for 30 min at 4°C).

**Epithelial cell culture.** HT-29/B6 cells were cultured as described elsewhere [24] and were seeded on polycarbonate filters with a pore size of 3 μm (effective membrane area, 0.6 cm²; Millicell-PCF; Millipore). Monolayers of HT-29/B6 cells form an epithelial barrier between the apical compartment with the insert and the basolateral compartment outside. Confluent monolayers were used for the experiments 7 or 8 days after seeding, when transepithelial electrical resistance was 500–800 Ohm cm². Cells were shifted to antibiotic-free RPMI 1640 before infection. In parallel to the infection with *A. butzleri* strains, 4 following types of controls were performed: (1) without adding bacteria and/or bacterial lysates, (2) with addition of heat-inactivated *A. butzleri* and/or *A. butzleri* lysates (60 min at 56°C), (3) with *Escherichia coli* K12, and (4) with *Campylobacter jejuni* ATCC 33560.

**Electrophysiological studies.** After bacterial infection with *A. butzleri* (MOI of 10) for 40 h, HT-29/B6 cell monolayers were mounted into modified Ussing-type chambers, as described elsewhere [25]. Short circuit current and transepithelial electrical resistance (R') were determined by using a computerized automatic clamp device (Fiebig Hard & Software).

**Epithelial permeability.** Unidirectional tracer flux studies from mucosal to serosal were performed under short-circuit conditions in Ussing chambers with fluorescein and fluorescein isothiocyanate (FITC)–labeled dextran-4000 (Sigma), as described elsewhere [26]. The medium in the basolateral chamber was initially free of dyes. Medium was withdrawn from the basolateral chamber at specific intervals, and fluorescence was measured in a spectrophotofluorimeter (SpectraMaxGemini; Molecular Devices). Permeability was calculated from flux over concentration difference.

**Cytotoxicity.** Lactate dehydrogenase (LDH) release from HT-29/B6 cells was measured according to the method of Madara and Stafford [27]. The MTT assay (MTT Cell Proliferation Kit; Roche) was performed on subconfluent HT-29/B6 cells in 96-well plates in accordance with manufacturer’s instructions and was measured by a spectrophotometer (SpectraMax340PC; Molecular Devices). TUNEL staining (In situ Cell Death Detection Kit; Roche) was performed in accordance with the manufacturer’s instructions.

**Western blot analyses.** Immunoblots were performed and analyzed as described elsewhere [23]. Detergent-soluble protein fractions were prepared from monolayers incubated with or without *A. butzleri*. HT-29/B6 cells were lysed in ice-cold lysis buffer (10 mmol/L Tris; pH, 7.5; 150 mmol/L sodium chloride, 0.5% Triton X-100, 0.1% SDS, and complete protease inhibitor mixture [Roche]) and were incubated for 30 min on ice. The lysate was centrifuged (15,000 g for 15 min at 4°C), and the supernatant was used as whole cell extract. The following antibodies were used: anti-occludin (1:2000; Zymed), anti–claudin-1–5 and -8 (1:1000; Zymed), anti–β-actin (1:5000; Sigma), and anti–caspase-3 (1:1000; Cell Signaling Technology).

**Immunofluorescence.** To test for the integrity of the TJ meshwork, the proteins occludin, claudins, and *Zonula occludens* protein-1 were visualized using confocal laser-scanning microscopy (Zeiss LSM 510) and were analyzed with Carl Zeiss LSM Image Examiner software according to prior descriptions [23]. The length of the TJ meshwork per exposed tissue area was measured microscopically using ImageJ software with the NeuronJ plug-in by tracking the TJ. For detection, antibodies raised against TJ proteins (1:50; Zymed) were used.

**Quantitative polymerase chain reaction (PCR).** Total RNA was obtained from HT-29/B6 cells with use of RNAzol reagent (WAK Chemie). Complementary DNA (cDNA) was synthesized by reverse-transcription PCR with use of the High-Capacity cDNA Archive Kit (Applied Biosystems) with oligo(dT) primer. Real-time PCR was performed according to the manufacturer’s instructions with an ABI 7900HT PCR device using the TaqMan Gene Expression Assay (no. Hs00533949_s1; clau-
Figure 1. Effects of *Arcobacter butzleri* on transepithelial resistance. 
A, Time-dependent decrease in transepithelial resistance of HT-29/B6 monolayers. Confluent HT-29/B6 cells grown on permeable supports were apically infected with *A. butzleri* at a multiplicity of infection (MOI) of 10 or with *A. butzleri* lysate by FRENCH Press with proteins >50 kDa (residue after ultrafiltration with 50,000 MWCO Millipore filter unit). 
B, Dose-dependent decrease in transepithelial resistance of HT-29/B6 monolayers. Confluent HT-29/B6 cells were apically infected with *A. butzleri* at initial MOIs of 1, 10, and 100. Data (mean error values ± standard error of the mean; for each group) were analyzed using Student’s *t* test, and asterisks indicate statistically significant differences between controls and infected cell samples. *P < .05, **P < .01, and ***P < .001.

Functional analyses of epithelial permeability. To further characterize epithelial barrier function, the permeability to fluorescein (332 Da) and FITC-dextran-4000 (4 kDa) was measured with FAM dye-labeled primers. Glyceraldehyde 3-phosphate dehydrogenase cDNA was quantified using VIC reporter dyes as endogenous control (all Applied Biosystems). Differential expression was calculated according to the $2^{-\Delta\DeltaCT}$ method [28].

Statistical analysis. Data were analyzed using GraphPad software. Data are expressed as mean values ± standard error of the mean. Statistical analysis was performed using Student’s *t* test, adjusted by Holm-Bonferroni correction for multiple comparisons. *P < .05 was considered to be statistically significant.

RESULTS

Infection with *A. butzleri* reduces R'. To investigate the effect of *A. butzleri* on epithelial integrity, R' of polarized HT-29/B6 monolayers was measured after *A. butzleri* infection. Highly motile bacteria were subcultured in RPMI liquid medium and were added to the apical compartment of confluent HT-29/B6 cell monolayers to yield an initial concentration of $1 \times 10^7$ colony-forming units per mL, equaling a multiplicity of infection of 10. As shown in figure 1, there was an incremental decrease in R' that began 24 h after bacterial inoculation (at 24 h, *P < .05; at 40 h, *P < .001; n = 4 each) with both strains used. All control groups remained at stable R' values. *A. butzleri* 49616 showed the strongest effects on R' and was used in subsequent studies. Lysates from *A. butzleri* caused a similar effect on R' as viable, motile bacteria (figure 1A). HT-29/B6 monolayers, which were apically infected with *A. butzleri* at multiplicities of infection of 1, 10, and 100, showed a dose-dependent decrease in R' after 48 h (figure 1B).

Physicochemical properties of *Arcobacter* lysate. *A. butzleri* lysate derived after FRENCH Press treatment led to a dose-dependent decrease in R'. After 48 h of incubation with diluted *A. butzleri* lysate, R' was reduced to 58% ± 2% (1:5) and 79% ± 3% (1:10) of initial resistance (*P < .01; n = 4). By ultrafiltration, the major active compound of the bacterial lysates was found to have a molecule mass >50 kDa. The activity of this bacterial compound was tested for heat sensitivity. *A. butzleri* lysate treated at 56°C for 60 min did not further affect R' after 48 h of incubation with HT-29/B6 cells. Likewise, proteinase-K treatment abolished the effect of *A. butzleri* lysate on R'. *A. butzleri* supernatants had no effect on R' (figure 2).

Figure 2. Transepithelial electrical resistance of HT-29/B6 monolayers. Confluent HT-29/B6 cells were apically treated with *Arcobacter butzleri* lysate by FRENCH Press with proteins >50 kDa or with *A. butzleri* supernatants. Heat treatment (60 min at 56°C) or proteinase-K treatment on *A. butzleri* lysate abolished the effect on transepithelial resistance (n = 4 for each group). **P < .01.
Epithelial Dysfunction by *A. butzleri* Infection

**Figure 3.** Effects of *Arcobacter butzleri* on paracellular permeability. Paracellular permeability after 40 h of *A. butzleri* infection when trans-epithelial resistance (R') and short circuit current (Isc) were decreased. Fluxes of tracer macromolecules (fluorescein, fluorescein isothiocyanate (FITC)-dextran-4000) through *A. butzleri* infected HT-29/B6 monolayers were measured in Ussing chambers. *P < .05, **P < .01, and ***P < .001.

Figure 4. A, Effects on the expression of tight junction proteins in HT-29/B6 cells 40 h after *Arcobacter butzleri* infection. Western blots revealed an obvious increase of occludin, whereas claudin-1, -3, -5, and -8 were decreased. B, Densitometry on occludin and claudins showed a decrement in claudin-1, claudin-5, and claudin-8 and an increase of occludin expression after normalization with β-actin. *P < .05 (n = 4; Student’s *t* test).

as tracer flux from the apical to the basolateral compartment in Ussing chambers. After 40 h of mucosal exposure to *A. butzleri*, R' of the HT-29/B6 monolayers had decreased to 30%, whereas the short circuit current was not induced but rather was slightly diminished (0.14 ± 0.03 µmol h⁻¹ cm⁻² after *A. butzleri* infection vs. 0.33 ± 0.01 µmol h⁻¹ cm⁻² in control; *P < .001; n = 6*). Concomitantly, permeability to fluorescein and FITC-dextran-4000 was increased after *A. butzleri* infection (for fluorescein, from 1.8 ± 0.6 10⁻⁶ cm/s in control to 10.8 ± 3.5 10⁻⁶ cm/s [*P < .05; n = 6*]; for FITC-dextran, from 0.015 ± 0.002 10⁻⁶ cm/s in control to 0.036 ± 0.005 10⁻⁶ cm/s [*P < .01; n = 4*]) (figure 3).

**Structural alteration of TJ organization after infection with *A. butzleri.*** In Western blot analysis, expression of claudin-1, -5, and -8 was decreased 40 h after infection (figure 4). To investigate the cellular distribution of these TJ proteins, HT-29/B6 monolayers were stained at the same time, when the R' had decreased to 30% of the initial value. The localization of endogenous claudins was determined by immunofluorescence with use of confocal laser-scanning microscopy (figure 5). First, visualization of the TJ proteins *Z. occludens* protein-1 and occludin showed an intact tight junctional meshwork without any gaps or other types of gross lesions (data not shown). Then,
Figure 5. Confocal laser-scanning microscopy (z-axis stack) of HT-29/B6 monolayers 40 h after infection with Arcobacter butzleri immunostaining with anti–Zonula occludens protein-1 (green) or anti-claudin (red) antibodies (merge is shown in yellow) illustrates characteristic loss of claudin-1 (A) and claudin-8 (C) off the tight junction, as well as accumulation of claudin-1 and -8 in the cytosol and a strong reduction of claudin-5 (B).

The length of the TJ network per exposed tissue area was measured and was not altered in infected monolayers (11.1 ± 0.5 μm/cell after A. butzleri infection vs. 10.5 ± 0.2 μm/cell in control; n = 3; the difference was not statistically significant). This is important, because an altered cell size with a change in the exposed TJ length would have caused a change in tightness of the monolayer, even if TJ protein measurements per mg protein in Western blots were unaltered. Thereafter, TJ proteins were quantified by densitometry of Western blots. As the main result, claudin-1, -5, and -8 expression levels were decreased, and furthermore, cellular distribution of these claudins was altered. Z-axis scans (xz plane) of control monolayers revealed that these tight junctional proteins were present in colocalization with Z. occludens protein-1 within the TJ strands of HT-29/B6 cells, whereas immunostaining of infected monolayers revealed a reduction of these claudins in the TJ, most pronounced for claudin-1 and -5 (figure 5A and 5B) and a redistribution of claudin-1 and claudin-8 off the TJ, with a concomitant appearance of intracellular claudin aggregates (figure 5A and 5C). Claudin-2, -3, and -4 showed no obvious alterations (data not shown). Twenty-four hours after infection, the claudin-5 messenger RNA level measured by real-time reverse-transcriptase PCR was
reduced to 57% ± 10% (P < .05; n = 4), indicating expression regulation from the gene.

**Cytotoxicity.** The barrier dysfunction after infection could result, on the one hand, from changes or disruption of TJ strands but, on the other hand, also from a loss of epithelial cells by necrosis or apoptosis induction. Therefore, LDH release was determined as a marker for cytotoxicity. We found a slight increase in LDH release, which suggests moderate cytotoxic effects caused by *A. butzleri*. Two days after infection, LDH release was elevated to 1.4% ± 0.2%, compared with 0.6% ± 0.2% in control (P < .001; n = 6 (figure 6A)). In subconfluent HT-29/B6 cells, viability was decreased to 60% ± 2% of control, as measured by MTT assay after 1 day of exposure to *A. butzleri* lysate (P < .001; n = 12) (figure 6B). Furthermore, apoptosis was examined histologically in TUNEL staining (figure 7A and 7B), indicating an increased apoptotic rate in *A. butzleri*-infected HT-29/B6 monolayers. Twenty-four hours after infection, the apoptotic rate was still similar to the control level (0.9% ± 0.2%); however, after 48 h, infected monolayers showed a 3-fold increase in epithelial apotoses to 3.4% ± 0.9% (P < .05; n = 5) (figure 7B). In parallel, pro-caspase-3 band intensity decreased to 68% ± 8% of the control level (P < .05; n = 3) (figure 7D). The caspase inhibitor N-benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl-ketone (Z-VAD-FMK; 50 μmol/L) attenuated the *A. butzleri* lysate-induced effect on R1 at 48 h (*A. butzleri* reduced R1 to 39% ± 1%, and the decrease in R1 was partially blocked by Z-VAD-FMK, resulting in a R1 of 53% ± 3%; P < .01; n = 4 each) (figure 7C). In parallel, apoptosis induction was blocked by Z-VAD-FMK to the control level (figure 7B).

**DISCUSSION**

In taxonomy, *A. butzleri* is a relative of *C. jejuni*, one of the most common causes of bacterial diarrhea in humans. From

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**Figure 7.** Effects of *Arcobacter butzleri* on epithelial apoptosis. A, Apoptotic effects of *A. butzleri* on confluent HT-29/B6 cells visualized with TUNEL staining and fluorescence microscopy. Approximately 2 days after *A. butzleri* infection, the number of cell rosette formations was increased, condensed or fragmentized nuclei were visible, and a more generalized loss of cells was observed. B, After 48 h of incubation with *A. butzleri*, TUNEL staining of confluent HT-29/B6 monolayers revealed an increase in the number of apoptotic cells (P < .05; n = 5). Caspase inhibitor (N-benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl-ketone [Z-VAD]) completely inhibits apoptotic cell death, and transepithelial resistance was partially although not completely reconstituted by Z-VAD (50 μmol/L; C). D, Western analysis. Increased apoptosis was indicated by caspase-3 activation 48 h after infection. Band intensity of pro-caspase-3 (35 kDa) was significantly diminished (P < .05; n = 3).

**Figure 6.** Effects of *Arcobacter butzleri* on epithelial necrosis. Cell death (epithelial necrosis) was induced in *A. butzleri*-exposed HT-29/B6 cells. A, *A. butzleri* increased lactate dehydrogenase (LDH) release as a marker for necrosis after 48 h of coincubation (n = 6). B, *A. butzleri* lysate (1:10) decreased cell viability on subconfluent HT-29/B6 cells (measured with MTT cell viability assay; n = 12). ***P < .001.
a microbiological perspective, both species show some homology, share similar hosts, and are regarded as pathogens in humans but as commensals in animals. In particular, poultry is thought to be the main reservoir of both pathogens [15, 29]. Despite the close relationship between the pathogens, they are unique organisms with independent factors of pathogenicity, although C. jejuni has also been shown to down-regulate the function of the epithelial TJs [30]. It is well known that several enteropathogenic bacteria can change sealing properties of the epithelial TJ, leading to diarrhea, as caused by, for example, Clostridium difficile [31] or the attenuated Vibrio cholerae strain CVD101. The latter, depleted of the chloride secretion-inducing cholera toxin gene, still induced diarrhea in human volunteers, and this is caused by a second toxin (Z. occludens toxin), which affects TJ structure; consequently, ions and water are passively transported from the circulation into the intestinal lumen [32]. Also, epithelial barrier dysfunction in human immunodeficiency virus enteropathy and in Giardia lamblia infection contributes to diarrhea by a leak flux mechanism [33, 34].

As the first important result of our present study, A. butzleri was shown to be capable of inducing epithelial barrier impairment, which has been shown here for the first time to our knowledge. In our cell culture model, increased macromolecular permeability via the paracellular pathway and a decrease in epithelial resistance was observed. Of importance, infection with A. butzleri was not accompanied by an increase in short circuit current, which is a direct measure of active anion secretion. On the one hand, this excludes an opening of apical chloride channels as source of the R’ change, and on the other hand, it suggests that diarrheal mechanisms other than secretion are important (e.g., leak flux diarrhea induction).

As a structural correlate of the barrier impairment, a change in TJ protein composition and distribution was identified, which is the second important finding of the present study. With respect to barrier function, the claudin protein family is most important, even if not all claudins have sealing properties [35]. Some claudins are even pore forming, and the individual role of a single claudin will also depend on the background of the other TJ proteins in a TJ strand. Claudin-1, for example, has important sealing properties [22], which is supported by functional analysis of claudin-1 knockout mice showing severe transepidermal water loss, which points to a crucial role for barrier function [36]. We found claudin-1 expression to be reduced by A. butzleri, and furthermore, its cellular distribution changed significantly. Claudin-1 was redistributed off the TJ and internalized. A similar effect on TJs was obtained by Muza-Moons et al [37] for claudin-1 disruption in enteropathogenic E. coli–infected human intestinal T84 monolayers. The intracellular claudin-1 protein aggregation in response to A. butzleri may be the result of changes in TJ assembly and/or turnover. Ubiquitinylation as a mechanism of claudin assembly and disassembly has recently been discussed to play a central role in posttranslational claudin regulation [38].

In contrast to claudin-1, claudin-2 induces the formation of cation-selective channels and is thereby able to increase epithelial permeability [39]. This has been discussed to represent a protective mechanism when expressed in the inflamed intestinal mucosa, by which noxious agents are rinsed off from the mucosal surface, but clearly contributes to leakiness of the epithelium. Claudin-3 and claudin-4 have sealing function and have receptor function for Clostridium perfringens enterotoxin [40, 41]. C. perfringens enterotoxin is a well-characterized modulator of barrier integrity. It binds to claudins and induces disruption of the TJ strands. However, claudins-2, -3, and -4 were not altered by A. butzleri in our HT-29/B6 cell model. As shown in MDCK cells, C. perfringens enterotoxin can remove claudin-4 from the TJ [41].

A. butzleri specifically caused removal of claudin-5 from the TJ. Claudin-5 is also a sealing TJ protein. Overexpression of claudin-5 in Caco-2 cells with low R’ background led to sealing of the barrier [23]. In A. butzleri–treated HT-29/B6 cells, claudin-5 expression was reduced in Western blots, and this seemed to also be the case in immunofluorescence microscopy, even if this is not a quantitative method and can yield only indirect evidence. Investigation of the regulatory influences revealed that the claudin-5 mRNA level in real-time reverse-transcriptase PCR analysis was reduced, which paralleled the diminished claudin-5 protein expression and gives evidence for A. butzleri to affect claudin-5 via transcriptional regulation from the gene.

Finally, we investigated claudin-8, which also has sealing properties within the TJ and which represents a paracellular barrier protein for cations [42]. Similar to expression of claudin-1, claudin-8 expression was found to be diminished in A. butzleri–exposed HT-29/B6 cells, and its distribution in immunostainings was changed from the TJ strands toward intracellular aggregates.

Although expression of the TJ protein occludin was up-regulated, R’ could not be maintained at its original level in infected monolayers. However, this was not surprising, because the function of occludin is not sealing of the TJ, even if it is present within TJ strands. This is directly supported by experimental evidence showing that epithelial barrier function was not significantly affected in occludin-deficient mice [43, 44].

Confocal laser-scanning microscopy showed that the length of the TJ meshwork per exposed serosal area in our measuring chamber was not affected by A. butzleri. Therefore, we could rule out that an increase in exposed TJ strand length as the result of a decrease in cell size has contributed to the reduction in R’. Taken together, the reduction of claudin-1, -5, and -8 levels and their redistribution off the TJ are responsible for the functional changes after A. butzleri infection and may contribute to diarrhea by a leak flux mechanism. This is also in line
with the clinical experience of watery diarrhea being the main manifestation of Arcobacter infection [9, 45, 46].

In addition to TJ alteration, effects on epithelial apoptosis were observed 48 h after infection, which is an additional relevant observation of this study. In general, apoptosis can be induced via death receptors, which activate caspase-8 as the first step. Then, activation of the caspase network occurs through caspase-3 activation. In addition, caspase-independent apoptosis can be induced by bacterial pathogens through activation of p38 or by interference with NF-κB signaling. In our study, A. butzleri was shown to induce apoptosis in a caspase-3-dependent manner. However, the functional relevance of epithelial apoptosis for the barrier function of an epithelium has often been a matter of debate. For the colon, our group has yielded direct evidence in the past that an increased apoptotic rate of this extent is important for epithelial barrier function [47] and that apoptotic foci directly contribute to barrier dysfunction under inflammatory conditions (ulcerative colitis) [21]. This interpretation is also supported by our present finding of a partial reconstitution of A. butzleri–induced barrier dysfunction by inhibition of apoptosis with Z-VAD-FMK, which enabled us to distinguish the barrier impairment caused by TJ disruption from apoptotic influences. Both play a significant role in the late effect of A. butzleri on R, but the effect on TJs appears to be predominant.

A. butzleri also hampered the viability of subconfluent HT-29/B6 cells, as indicated by MTT assay. In other cell culture models, A. butzleri lysate had few toxic effects, and it does not possess a cytolethal-distending toxin, as does C. jejuni [48]. Cytotoxic effects of A. butzleri were first described by Musmanno et al [49] and could be confirmed in this study. LDH release from confluent monolayers increased 48 h after infection, providing evidence for a late necrotic effect.

Lysate of A. butzleri had a similar effect on R, TJs, and the viability of HT-29/B6 cells as intact bacteria. This has been indicated in experiments in which the effect of the lysate was abolished under protein-degrading conditions, as well as by exposure to heat (heat sensitivity), and this implicates that the active compound of A. butzleri lysate is a protein. Bacterial supernatants showed no effect on R, which indicates that a toxin is cell associated, rather than secreted. In general, for the mechanisms of TJ regulation in response to pathogens, different types of signaling have been described thus far (e.g., changes in half-life time, cleavage of claudins, ubiquitinylation, and gene regulation). We were able to show that A. butzleri can affect claudin expression from the gene. Furthermore, A. butzleri compounds can activate the caspase pathway, as outlined above.

Moreover, we can presume that the virulence of A. butzleri has 2 phases. First, an initial effect on TJs was observed, and then, we observed a late effect on cytotoxicity because of necrosis and induction of apoptosis. The inherent pathomechanisms of Arcobacter enteritis are, thus far, still unknown. Our findings provide evidence for A. butzleri to be a host-adapted pathogen, characterized by defined interactions with human epithelial cells and distinct pathomechanisms. In summary, we have demonstrated for the first time to our knowledge that A. butzleri induces impairment of the intestinal barrier function via TJ regulation and apoptosis induction, which are mechanisms that are well known to cause leak flux diarrhea.

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References


