

Effect of *Lactobacillus* spp. on adhesion, invasion, and translocation of *Campylobacter jejuni* in chicken and pig small-intestinal epithelial cell lines

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Abstract

Background: *Campylobacter* spp. are a major cause of bacterial food-borne diarrhoeal disease. This mainly arises through contamination of meat products during processing. For infection, *Campylobacter* spp. must adhere to epithelial cells of the mucus layer, survive conditions of the gastrointestinal tract, and colonise the intestine of the host. Addition of probiotic bacteria might promote competitive adhesion to epithelial cells, consequently reducing *Campylobacter jejuni* colonisation. Effect of *Lactobacillus* spp. (PCS20, PCS22, PCS25, LGG, PCK9) on *C. jejuni* adhesion, invasion and translocation in pig (PSI cl.1) and chicken (B10XI) small-intestine cell lines, as well as pig enterocytes (CLAB) was investigated. Results Overall, in competitive adhesion assays with PSI cl.1 and CLAB cell monolayers, the addition of *Lactobacillus* spp. reduced *C. jejuni* adherence to the cell surface, and negatively affected the *C. jejuni* invasion. In addition, *Lactobacillus* spp. significantly impaired *C. jejuni* adhesion in three-dimensional functional PSI cl.1 and B10XI cell models. Also, *C. jejuni* did not translocate across PSI cl.1 and B10XI cell monolayers when co-incubated with probiotics. Among selected probiotics, *L. rhamnosus* LGG was the strain that reduced adhesion efficacy of *C. jejuni* most significantly under co-culture conditions. Conclusion The addition of *Lactobacillus* spp. to feed additives in livestock nutrition might be an effective novel strategy that targets *Campylobacter* adhesion to epithelial cells, and thus prevents colonisation, reduces the transmission, and finally lowers the incidence of human campylobacteriosis.

Background

Campylobacter jejuni is the most reported food-borne pathogen in human gastrointestinal infections worldwide [1]. *C. jejuni* are frequently found in the gastrointestinal tract of healthy animals that are destined for human consumption, especially chickens, which naturally harbour *Campylobacter* spp. in their gastrointestinal tracts [1-3].

The intestinal epithelium of the host represents the first barrier against such food-borne pathogens and is supported by the response of the mucosal immune system that is tightly connected with the gastrointestinal barrier. However, to establish an infection, *Campylobacter* spp. must first adhere to and persist in the mucus layer that covers the intestinal epithelium, and they need to survive the adverse conditions of the gastrointestinal tract, to finally colonise the jejunum and ileum of the host [4,5]. Adhesion to epithelial cells of the animal gastrointestinal tract is thus the first important step for successful colonisation. This can then promote transmission of *C. jejuni* to humans, which occurs mainly through handling and consumption of contaminated poultry and pork meat products during slaughter and carcass processing [2,6,7]. It is therefore imperative to reduce the prevalence and colonisation of *Campylobacter* at the farm level, where good hygiene and biosecurity practices are not sufficient [8]. In particular, control of *Campylobacter* spp. in poultry is the most important concern for consumers [1,9]. However, this needs to be achieved without increased use of antibiotics and for that reason, alternative strategies for the reduction of colonisation of *Campylobacter* spp. are urgently needed. In this context, an effective approach that targets *Campylobacter* adhesion to the intestinal mucus will prevent colonisation

and thus reduce the bacterial load of *Campylobacter* spp. in live animals. The consequent reduced transmission from animal carcasses to humans will thus lower the risk to consumers.

Several strategies with limited efficacy have been applied to reduce the burden of *Campylobacter* spp. in the intestine of pig and poultry including vaccination, passive immunisation, bacteriophage therapy, bacteriocin application, organic acids, and medium chain fatty acids [1,9-11]. On the other hand, probiotic bacteria can have high affinity for adherence to the mucosal wall, where they promote the integrity of the healthy functioning of the gastrointestinal barrier by decreasing paracellular permeability through strengthening the tight junctions [12-14]. In that way, selected probiotic strains could offer an alternative method to reduce *Campylobacter* spp. load in animal farming.

The protective role of probiotic bacteria against pathogens mostly lies in their competition for adhesion sites and nutrients, and their production of antibacterial substances [15]. With the emergence of serious antibiotic resistance in livestock breeding, farmers are considering the use of probiotics as feed additives in livestock nutrition, as this might confer health benefits to the host animal [16]. However, host–pathogen–probiotic interactions must be defined before the application of any alternative strategy for pathogen reduction at the farm level, as defined by the regulatory agencies in many countries, including the European Union [7]. To avoid unnecessary usage of animals in the research projects along with ethical considerations, many highly differentiated cell lines were developed with the aim to establish comparable functionality to their *in vivo* counterparts. In contrast to the more expensive animal trials, cell-line models are cost effective and allow massive screening. In addition, cell-line models are in line with the three R paradigm (Reduce, Refine, Replace) [17]. *Campylobacter jejuni* is able to adhere to the gut epithelium, induce cell death, and disrupt mucosal barrier function. Although *Lactobacillus* spp. can modulate epithelial cell invasion by *C. jejuni*, how this is achieved is still not well understood [15,18,19]. A few studies have reported reduced *C. jejuni* adhesion when such probiotics have colonised the mucus, which has been studied in models with mucin [20] and with chicken intestinal mucus [21,22]. However, to the best of our knowledge, there have not been any *in-vitro* studies that have evaluated the efficacy of *Lactobacillus* spp. for prevention of *C. jejuni* adhesion and invasion of pig and chicken intestinal epithelium.

We have thus here used *Lactobacillus* spp. to modulate *Campylobacter* host-cell interactions using pig and chicken small-intestine cell-line models *in vitro*. The aim was to prevent/reduce the *C. jejuni* K49/4 adhesion, invasion and translocation through competitive adhesion and immunomodulatory activities of *L. plantarum* (PCS20, PCS22, PCS25), *L. rhamnosus* LGG and *Lactobacillus plantarum* (PCK9), using pig (PSI cl.1) and chicken (B10XI) epithelial small intestine cells, and pig enterocytes (CLAB cells).

Results

Cytotoxicity

The co-incubation of the probiotic bacteria *L. plantarum* (PCS20, PCS22, PCS25), *L. rhamnosus* LGG and *Lactobacillus plantarum* PCK9 (1×10^8 CFU/mL) to the pig PSI cl.1, chicken B10XI, and pig CLAB epithelial cell monolayers did not show any cytotoxic effects after 24 h. The viability of PSI cl.1, B10XI and CLAB cells ranged from 90% to 100% when the probiotic bacteria were added, compared to non-treated monolayers (Fig 1). As expected, co-incubation of pathogenic *C. jejuni* K49/4 with the PSI cl.1, B10XI and CLAB cells resulted in cytotoxic effects, with disruption of the monolayers, when compared to untreated cells. This was most significant for the PSI cl.1 cells, which showed only 40% viability after 24 h (Fig 1), thus indicating that the PSI cl.1 cells were the most sensitive cells to *C. jejuni* infection. Addition of the probiotic strains in combination with *C. jejuni* showed protected effects for the PSI cl.1, B10XI and CLAB cells viability. The viability of B10XI and CLAB cells remained > 90% after the co-incubations of the combinations of the PCS22, PCS25, LGG and PCK9 bacteria with *C. jejuni* for 24 h (Fig 1), and more than 80% after 48 h (data not shown). However, these combinations of the probiotic strains and *C. jejuni* showed greater detrimental effects with the PSI cl.1 cells, resulting in only 50% to 70% cell viability after 24 h of co-incubation (Fig 1), which was even more reduced after 48 h of co-incubation, with only 20% cell viability.

***Campylobacter jejuni* adhesion and invasion in cell monolayers**

The efficiency of the *Lactobacillus* strains PCS20, PCS22, PCS25, LGG and PCK9 were determined in terms of impairment of competitive *C. jejuni* K49/4 adhesion and invasion to pig PSI cl.1 and CLAB enterocytes using the non-polarised cell models (Fig 2). *L. rhamnosus* LGG generally showed the greatest reduction of *C. jejuni* adherence and invasion to the PSI cl.1 and CLAB cell monolayers. Here, the control adhesion of the starting *C. jejuni* inoculum was 2%, which was reduced approximately to 0.02% by *L. rhamnosus* LGG in both, PSI cl.1 and CLAB cell models (Fig 2 A). Additionally, *L. plantarum* PCS25 reduced this control *C. jejuni* adhesion on PSI cl.1 cells to 0.04% and PCS22 to only 0.01% (Fig 2 A). Despite slightly higher *C. jejuni* adhesion to the CLAB cells (Fig 2 A), the *C. jejuni* invasion into these cells was lower when compared to the PSI cl.1 cells (Fig 2 B). As shown in Figure 2B, < 0.1% of the *C. jejuni* cells invaded the PSI cl.1 cells, and approximately 0.001% invaded the CLAB cells. The results further demonstrate the efficiency of the *Lactobacillus* strains (PCS20, PCS22, PCS25, LGG, PCK9) to reduce *C. jejuni* K49/4 invasion into intestinal epithelial PSI cl.1 and CLAB cells as the monolayer model. The *C. jejuni* invasion rate into these cell monolayers was reduced by 90% when the probiotic bacteria were used (Fig 2 B). Thus, the competitive adhesion assays using PSI cl.1 and CLAB cell monolayers showed that addition of probiotic bacteria efficiently reduces the *C. jejuni* adherence to the surface of intestinal epithelial cells, and their invasion of these cells.

***Campylobacter jejuni* adhesion and invasion using the polarised cell model**

The efficiency of *Lactobacillus* strains PCS20, PCS22, PCS25, LGG and PCK9 to impair *C. jejuni* K49/4 adhesion and invasion were also tested using the three-dimensional functional model of the pig PSI cl.1 and chicken B10XI polarised intestine epithelial cells. An additional file shows this in more detail (see Additional file 1). The post-infection growth kinetics (i.e. 3, 17, 24 h) were determined for *C. jejuni*

adhesion (Fig 3) and invasion (Fig 4). *C. jejuni* adhesion to PSI cl.1 cells was more pronounced, with more adhered *C. jejuni* observed 17 h post-infection, which then dropped off (Fig 3 A). On the other hand, there was an increased number of *C. jejuni* on the chicken B10XI cells from the beginning of the infection, with the highest levels reached at 24 h post-infection (Fig 3 B). Addition of the probiotic bacteria PCS20, PCS22, PC25 and PCK9 significantly decreased *C. jejuni* adherence to PSI cl.1 and B10XI cells early in the observation period (i.e., after 3 h), compared to non-treated cells (Fig 3). Addition of PCS20, PCS22, PCS25 and PCK9 cells also significantly decreased the number of *C. jejuni* adhered to PSI cl.1 cells after 17 h and 24 h (Fig 3 A). However, for the numbers of *C. jejuni* adhered to B10XI cells, these remained at the control levels 24 h post-infection when PCS20 and PCS25 were used, and only the PCS22, LGG and PCK9 significantly reduced these numbers (Fig 3 B). The control *C. jejuni* invasion into PSI cl.1 cells remained constant to 24 h, but was comparable to B10XI cells only at 3 h post-infection (Fig 4). Addition of PCS20, PCS22, PCS25 and PCK9 reduced the invasiveness of *C. jejuni* into PSI cl.1 cells to below the level of detection regardless of the time post-infection (Fig 4 A). *C. jejuni* invasion into B10XI cells was only seen at 3 h post-infection, and addition of PCS20, PCS22 and PCK9 significantly reduced this *C. jejuni* invasiveness (Fig 4 B). Strains PCS25 and LGG were the two most efficient strains in preventing the invasion of *C. jejuni* into B10XI cells when compared to other strains. When these two strains were coincubated with *C. jejuni*, no invasion into B10XI was observed 3 h post-infection (Fig 4 B).

Intestinal epithelium integrity and *C. jejuni* translocation

The efficiency of *Lactobacillus* strains PCS20, PCS22, PCS25, LGG and PCK9 to impair the effects to *C. jejuni* K49/4 on epithelial intercellular integrity were investigated. These were determined by measurements of TEER immediately after infection of polarised PSI cl.1 and B10XI cells, and after 3, 17 and 24 h post-infection.

Initially, the effects of the probiotic bacteria on epithelial intercellular integrity of the PSI cl.1 and B10XI cells were determined. At 1×10^7 CFU/mL, the probiotic bacteria generally increased the TEER of the PSI cl.1 (data not shown) and B10XI (Fig 5 A) polarised monolayers over the first 3 h of exposure (except for PCS22), as compared to the control ($p < 0.01$). By 24 h post-infection, the TEER in the presence of the probiotic bacteria PCS20, PCS22 and PCS25 decreased to below the control value. The exceptions of LGG and PCK9 maintained significantly increased TEER to 24 h post-infection with the B10XI polarised monolayer, as compared to control ($p < 0.01$) (Fig 5 A).

The *C. jejuni* adherence to PSI cl.1 and B10XI (Fig 5 B) polarised cells decreased the TEER to 24 h post-infection. For the B10XI polarised cells, the addition of all of the probiotics to *C. jejuni* significantly decreased TEER in the first 3 h, a situation generally maintained to 24 h post-infection, for PCS20, PCS22 and PCS25. In contrast, addition of LGG and PCK9 resulted in increased TEER of B10XI polarised cells beyond these first 3 h post-infection (Fig 5 B). Incubation of PSI cl.1 polarised cells with *C. jejuni* caused additional decline in TEER values when compared to the probiotic bacteria only. However, no significant differences were observed for PSI cl.1 polarised cells between the probiotic strains ($p > 0.01$). The TEER

of PSI cl.1 monolayers with no bacteria added was relatively constant over the 24 h of monitoring ($\approx 1850 \Omega/\text{cm}^2$).

Campylobacter jejuni K49/4 translocated to the basolateral compartment after apical infection of PSI cl.1 and B10XI cells, as seen at 3 h post-infection (Fig 6). Differences were seen when comparing *C. jejuni* translocation through these PSI cl.1 and B10XI polarised monolayers. By 24 h, 1×10^2 *C. jejuni* were present in the basolateral compartment for PSI cl.1 cells. No translocated bacteria were detected after 17 h post-infection for B10XI cells (Fig 6). At 3 h post-infection, addition of PCS20, PCS22, PCS25, LGG and PCK9 significantly impaired *C. jejuni* translocation to the basolateral chamber of PSI cl.1 and B10XI cells (Fig 6). Of note, although *C. jejuni* adhered in high numbers to PSI cl.1 cells to 24 h (Fig 3), the probiotic bacteria prevented *C. jejuni* invasion into these cells (Fig 4), and consequently the *C. jejuni* translocation to basolateral chamber. These monolayers were not disrupted over the first 17 h post-infection, as was observed under the microscope. In addition, the TEER stayed relatively constant over this period, which indicated that the *C. jejuni* translocation was mostly transcellular.

Discussion

Adhesion represents a virulence factor in human/animal infections that is important for *Campylobacter* survival out of the host and during host–pathogen interactions. *Campylobacter* adhesion to epithelial cells involves contact between the bacterial and the eukaryotic cell surfaces. It is also crucial for further *Campylobacter* invasion of the intracellular space and traversing of the host barriers, and thus for the *Campylobacter* pathogenicity [3,34]. *Campylobacter* spp. of different origins can adhere to and invade human, porcine and avian cell lines [32,35-37]. The data in the present study on *C. jejuni* adhesion to and invasion of PSI cl.1, B10XI and CLAB cell lines are in agreement with this. Furthermore, when non-polarised PSI cl.1 and CLAB cells were used, greater *C. jejuni* adhesion was seen for CLAB cells, which reflects the differences in the structural and functional characteristics of the cell surface. However, when intestinal cell lines are cultivated on microporous membranes such as in transwells, they can spontaneously differentiate and polarise. In this way they serve as a much better model of the *in-vivo* intestinal environment. Another advantage of transwells is that they can be used for studies of transport through the polarised epithelial monolayer.

In the present study, the most sensitive cell line to addition of *C. jejuni* was the PSI cl.1 cells, while the B10XI cells tolerated exposure to prolonged probiotic–pathogen interactions. At 3 h post-infection, *C. jejuni* adhesion to polarised PSI cl.1 cells was greater in comparison to that with polarised B10XI cells, which is in agreement with earlier studies [38,39]. The numbers of *C. jejuni* that adhered to the polarised PSI cl.1 cells decreased towards the end of observation period (i.e., 24 h post-infection). In contrast, the numbers of adhered *C. jejuni* increased from the beginning of the infection on B10XI cells, which demonstrates the host-specific tropism of this pathogen. This is in agreement with other studies that have demonstrated that within an avian host, *Campylobacter* behave as commensals and do not proliferate intracellularly [40,41]. However, differences in *C. jejuni* adhesion indicate strain-specific

responses, and also different mechanisms of adhesion for these different cell lines, which is in agreement with other studies [21,40,42].

Furthermore, we aimed to prevent/reduce this adhesion to the non-polarised PSI cl.1 and CLAB cells and the polarised PSI cl.1 and B10XI intestinal epithelial cells by addition of probiotics, and thus to potentially control *Campylobacter* contamination and/or transmission. With the main emphasis on human health, this can be particularly effective in the first segment of the food chain – in animal breeding for meat production. This has become more necessary because of the high *Campylobacter* prevalence, increased antibiotics usage and consequent bacterial resistance on livestock farms, and especially in broiler production [43]. Thus, one of the options is to reduce the prevalence of broiler flocks colonised with *Campylobacter*, and to reduce the intestinal *Campylobacter* load of the broiler chickens prior to slaughter [1,8].

To avoid selection pressure for the emergence of resistant bacteria and to avoid deleterious effects that antibiotics can have on the protective microbiota, research is ongoing into alternative strategies, including phage therapy and vaccination; however, none of these approaches have proven effective in practice to date [7,44]. Here, we have explored the use of probiotics as an alternative strategy. Probiotics are known to promote the integrity of the healthy gastrointestinal barrier, and also to enhance the gut microbiota. This might allow the prevention, and potentially the treatment, of a variety of human diseases, through delaying *C. jejuni* colonisation and the signal transduction that arises during *C. jejuni* infection [45,46].

In the present study we investigated the use of *Lactobacillus spp.* (PCS20, PCS22, PCS25, LGG, PCK9) to modulate *Campylobacter*–host cell interactions using *in-vitro* pig and chicken small-intestine cell models. First, we demonstrated no cytotoxic activities of these probiotic bacteria towards pig PSI cl.1, chicken B10XI epithelial small intestine cells, and pig CLAB enterocytes. We then aimed to prevent or reduce *C. jejuni* adhesion, invasion and translocation through the competitive adhesion and immunomodulatory activities of the combination of these probiotic bacteria with the pathogen, *C. jejuni*. As observed, these *Lactobacillus spp.* reduced *C. jejuni* adhesion to pig PSI cl.1 epithelial small intestine cells and CLAB enterocytes using a non-polarised cell model, with the greatest effects seen for *L. rhamnosus* LGG. We confirmed that the presence of these probiotic bacteria hindered the adherence of *C. jejuni* to the cell surface. We thus support the conclusion of Mohan (2015), who reported that particular probiotic strains can out-compete *C. jejuni* through different mechanisms of actions. Mohan (2015) particularly noted that the probiotics reached the adhesion sites more rapidly, with competitive exclusion against *C. jejuni* through occupation of the adhesion sites [47]. The present data (e.g. Fig 2,3) show that *L. rhamnosus* LGG was the most effective of these probiotics for impaired adherence of *C. jejuni* to both polarised PSI cl.1 and B10XI cells. This would appear to be mediated via the adherence of *L. rhamnosus* LGG to these intestine cells and its persistence there for prolonged periods after administration, as was also indicated by the study of Alander et al. (1999) [48]. Once adhered to the host cell, *C. jejuni* can then invade the cell, and hence survive for long periods inside both phagocytes and epithelial cells [33,49]. This is a further important factor in disease pathogenesis, and it is mainly due to cell death and/or disruption of the mucosal barrier function, and correlates with both pathogen virulence and disease severity [19].

When the probiotic bacteria were added in the present study, *C. jejuni* invaded the non-polarised PSI cl.1 cells in greater numbers than for the CLAB cells, which suggests that these probiotic strains were more efficient in preventing the invasion of the CLAB cells. According to other studies and knowing that *Campylobacter* spp. are commensals in poultry, we hypothesised that *C. jejuni* would not invade the chicken B10XI epithelial intestinal cells *in vitro* [38,39]. The data here show that *C. jejuni* invaded polarised PSI cl.1 cells in higher numbers than for the B10XI cells, and also persisted in the PSI cl.1 cells through the whole observation period; instead, there were no *C. jejuni* in the B10XI cells 17 h post-infection.

The extent of inhibition of this *C. jejuni* invasion was dependent on the probiotic strain. Each probiotic strain prevented the invasion of PSI cl.1 cells; however, only *L. plantarum* PCS25 and *L. rhamnosus* LGG completely inhibited *C. jejuni* invasion of B10XI cells. Other *in-vitro* studies have shown that the extent of reduction in adhesion and/or invasion rates due to probiotic use is highly strain specific [19,21,40,42]. Campana et al. (2012) observed inhibitory activity of *L. acidophilus* ATCC4356 on *C. jejuni* adhesion to and invasion of Caco-2 cells [50]. More recently, Wang et al. (2014) isolated four adhesive *Lactobacillus* strains that inhibited *C. jejuni* adhesion to and invasion of human HT29 cells [51]. The present data show that the invasion of intestinal cells *in vitro* can be completely prevented by the use of particular probiotic bacteria. Moreover, in agreement with other studies, inhibition of *C. jejuni* invasion of intestinal epithelial cells might be attributed to competitive exclusion by the probiotic bacteria [52]. However, other mechanisms of action of probiotic bacteria in addition to competitive exclusion should be taken into account. This is particularly evident in the present study because better *C. jejuni* adhesion did not necessarily result in better invasion rates, and *vice versa*.

In addition to different adherence properties of the probiotic bacteria to the intestinal epithelial cells *in vitro*, the production of various metabolites (e.g., bacteriocins) or alterations in the signalling cascades involved in the invasion should also be taken into account when interpreting these data, as these aspects are also likely to be modified by the probiotic bacteria. We would speculate that the *C. jejuni* adhesion and invasion rate *in vivo* might indeed be even lower due to other factors, such as innate and adaptive immunity, the presence of other microorganisms in the gut, the presence of the protective mucus layer, and peristalsis; these are not accounted for in experimental *in-vitro* studies with cell lines. For example, increased mucins might interfere with *C. jejuni* invasion of the epithelial cells. Simplified models with mucin or chicken intestinal mucus have demonstrated that *C. jejuni* adhesion can be reduced when probiotics colonise mucus before addition of *C. jejuni* [18,20].

Finally, the probiotic strains were tested for their efficacies for the prevention of *C. jejuni* translocation using the three-dimensional functional model of the pig (PSI cl.1) and chicken (B10XI) polarised intestine epithelial cells. It would be reasonable to assume that prevention of *C. jejuni* adhesion to eukaryotic cells would prevent the translocation of this pathogen. Alemka et al. (2010) showed that probiotics attenuated *C. jejuni* adhesion to, invasion of, and translocation across polarised HT29-MTXE12 cells, which is a subclone of the human colon carcinoma HT29 cell line that secretes mucus [40]. Contrary to expectation, at 3 h post-infection, *C. jejuni* efficiently translocated across the polarised chicken B10XI intestinal cells;

however, the translocation level was much lower when compared to PSI cl.1 cells. Similar observations were reported by Konkel et al. (1992), who initially showed the highest rate of *C. jejuni* translocation at 4 h post-infection for polarised Caco2 cells [49]. When the probiotics in the present study were incubated with *C. jejuni*, the levels of translocated *C. jejuni* were similar in both the PSI cl.1 and B10XI cell lines (Fig 6). Later, despite high levels of adhered *C. jejuni* (Fig 3 B), no *Campylobacter* spp. were detected in the basolateral chamber of the B10XI cells (Fig 6 B) and no monolayer disruption was observed under the microscope which suggested that no translocation occurred and that *C. jejuni* did not proliferate in the basolateral chamber. The route of *C. jejuni* translocation to the basolateral chamber of transwells has been the subject of a lot of research, and some studies have described *C. jejuni* transcellular transcytosis [53], while others have implicated a paracellular route of translocation [40,54]. Changes in TEER of the infected PSI cl.1 cells during the first 24 h post-infection, and persistence of *C. jejuni* in the basolateral chamber of the transwells, indicated here that *C. jejuni* used the transcellular rather than a paracellular route of translocation. There were also no correlations between the variations in the TEER of the cell lines and the translocation of *C. jejuni* across the polarised intestinal epithelial cell monolayers. Furthermore, an increase in TEER was observed for the polarised intestinal epithelial cell monolayers when the probiotic bacteria were co-incubated with *C. jejuni* indicating that latter contributed to the integrity of the intestinal epithelium.

Conclusions

Contrary to the acute enterocolitis caused by *C. jejuni* in humans, poultry exposed to *Campylobacter* show prolonged colonisation without pathological changes in the intestine or clinical signs of infection. Furthermore, despite several control measures, animals (and especially chickens) remain the most common source of *Campylobacter* spp. Addition of *Lactobacillus* spp. to feed during animal nutrition might represent an effective novel strategy to reduce the prevalence in broiler chickens of *Campylobacter* spp. and the colonisation of intestinal *C. jejuni* in animals prior to slaughter.

To the best of our knowledge, the present study is the first that uses functional pig and chicken cell line models of non-tumorous origins to study the effects of probiotics on *Campylobacter* adhesion, invasion and translocation and on the mechanism(s) used by *Lactobacillus* spp. against *C. jejuni*. *Lactobacillus* spp. impaired *C. jejuni* adhesion to PSI cl.1 pig epithelial small intestine cells and CLAB pig enterocytes as cell monolayers, and modulated *C. jejuni* invasion into these cells. Additionally, the probiotics impaired *C. jejuni* adhesion, invasion and translocation across three-dimensional functional PSI cl.1 and B10XI polarised intestine epithelial cell models. We thus propose that the addition of *Lactobacillus* spp. to animal feed would represent an effective novel strategy to target *Campylobacter* adhesion to epithelial cells, prevent *Campylobacter* colonisation, reduce *Campylobacter* transmission, and finally, lower incidence of human campylobacteriosis. Further research is necessary to clarify the mechanism(s) of these probiotic–*Campylobacter* interactions and to investigate specific/ effective probiotic strains for attenuation of the virulence properties and to combat *C. jejuni* infections.

Methods

Bacterial strains and growth conditions

Lactobacillus strains used in this study were *Lactobacillus plantarum* PCS20, PCS22, PCS25 (cheese isolates, from the collection of the Department of Biochemistry and Nutrition, Faculty of Medicine, University of Maribor, Slovenia), *Lactobacillus rhamnosus* LGG (from American Type Culture Collection; ATCC53103) and *Lactobacillus plantarum* PCK9 (from a cheese isolate, obtained in the European research project PathogenCombat; FP6-007081) [23]. The strains applied on the intestinal epithelial cell lines in our study were selected on the basis of previous *in vitro* and *in vivo* research. All *Lactobacillus* strains were previously tested for their probiotic characteristics and showed good adhesive properties to intestinal epithelial cells [24-27]. The tested strains were grown in De Man Rogosa, Sharpe (MRS) broth (Merck, Darmstadt, Germany) for 24 h at 37 °C, and under anaerobic conditions. The final bacterial suspensions for the competition assays contained approximately 1×10^8 CFU/mL.

C. jejuni K49/4, a poultry meat isolate was grown at 42 °C microaerophilically (5% O₂, 10% CO₂, 85% N₂) on Columbia agar (Oxoid, Hampshire, UK) supplemented with 5% defibrinated horse blood (Oxoid, Hampshire, UK). *C. jejuni* were transferred to Preston broth (Oxoid) at 42 °C, and grown microaerophilically for 9 h. These *C. jejuni* cultures in exponential phase were diluted in cell culture media containing no antibiotics to approximately 2×10^8 CFU/mL and were used for cell culture assays.

PSI cl.1, B10XI and CLAB cell monolayers

The normal PSI cl.1 epithelial-derived cell line (partially differentiated cryptic enterocyte-like) was obtained from an adult pig at slaughter, as previously described [17]. These cells represent the closest model to humans in terms of genome, organ development, anatomy, physiology and metabolism of the intestinal tract, and for disease progression, and intestine–microbe interactions [28,29]. The B10XI cells represent normal enterocytes of the chicken small intestine. The B10XI cell line was developed and characterised at the University of Maribor, Slovenia, in the framework of the EU funded 'PathogenCombat' project, to build a three-dimensional functional epithelial cell model of the chicken intestine [23]. The CLAB cells are enterocytes that were obtained from an adult pig at slaughter in Slovenia and represent adult mucin secreting enterocyte-like cell line [30]. Although CLAB cells are epithelial in origin, they do not polarise *in vitro* [23]. These cells are of non-tumorigenic origin, which makes them more suitable as the *in vitro* model to study pathogen–host interactions than tumorigenic cell lines. The phenotypical and functional characterization of the cells was performed with immunocytochemistry and the search for the presence of key epithelial markers. In addition, all cell lines were tested for mycoplasma contamination prior to the experiments.

The PSI cl.1 and B10XI cell lines can form a tightly packed epithelial barrier when grown on microporous inserts, and for this reason, they were chosen for further studies to evaluate the efficacy of the probiotics for prevention of *C. jejuni* K49/4 adhesion, invasion and translocation across polarised cell monolayers. PSI cl.1, B10XI and CLAB cells are of non-tumorigenic origin; instead, they were isolated from dissected

animal tissue using the limiting dilution technique. The functional PSI cl.1 and B10XI intestinal cell models were developed for studies into probiotic/pathogen/gut epithelial interactions in more detail following initial screening for the efficacy of the probiotics for prevention of *C. jejuni* K49/4 adhesion and invasion on PSI cl.1 and CLAB cell monolayers.

Cultivation and propagation of cell lines

PSI cl.1, B10XI and CLAB cell lines were grown in advanced Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich, Grand Island, USA), supplemented with 5% foetal calf serum (Lonza, Basel, Switzerland), 2 mmol/L L-glutamine (Sigma), 100 U/mL penicillin (Sigma) and 1 mg/mL streptomycin (Fluka, Buchs, Switzerland), at 37 °C in a humidified atmosphere of 5% CO₂. Initially, the B10XI cells were cultured at both 37 °C and 42 °C; however, as no differences were seen regarding the growth, appearance or size of the cells and their survival in culture, these were later cultured at 37 °C. To form monolayers, 96-well microplates were seeded with approximately 5.0 × 10⁵ cells/mL PSI cl.1 and CLAB, and incubated for 24 h. The confluent cultures were washed three times with phosphate-buffered saline (PBS) and cultivated in antibiotic free DMEM for the cell adhesion and invasion assays.

PSI cl.1 and B10XI polarised cell model

To obtain polarised monolayers, PSI cl.1 or B10XI cells were seeded on Transwell filter inserts (pore size, 0.4 µm; 12 mm; Corning) that were placed into 12-well plates (22.1 mm; Corning), at a density of 1 × 10⁵ cells/cm². The TEER was measured using an electrical resistance system (Millicell-ERS; Millipore, Bedford, MA, USA). The net TEER was corrected for background resistance by subtraction of the resistance of the microporous membranes with the cell cultures (108 Ω/cm²) from the resistances measured with the system. Functional polarity was established when the TEER between the apical and basolateral surfaces of the monolayers exceeded 1600 Ω/cm² for PSI cl.1 cells and 240 Ω/cm² for B10XI cells. TEER was measured before the cells reached confluence, after the addition of the bacteria to the medium (with or without gentamicin), and 24 h post-infection. The TEER of cell monolayers without bacteria was used as the control for each experiment.

Effects of probiotic bacteria and *C. jejuni* K49/4 on viability of cell cultures

To test the effects of the bacteria on the viability of the PSI cl.1, B10XI and CLAB cell monolayers, the cells were seeded separately in microplates at a density of 6 × 10⁵ cells/well. Later, each strain of viable probiotic bacteria (100 µL; 1 × 10⁸ CFU/mL) and *C. jejuni* K49/4 (100 µL; 2 × 10⁸ CFU/mL) were simultaneously added to pre-washed monolayers of the PSI cl.1, B10XI and CLAB cells, and the cell monolayers were incubated for 24 h at 37 °C in a humidified atmosphere of 5% CO₂. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay was used to determine cell viability, as described previously [31]. The experiments were carried out in at least triplicate. The mean absorbance of the control wells containing only confluent cell culture without any bacteria was taken as

100%. The percentage of metabolically active cells treated with probiotic bacteria and *Campylobacter jejuni* was then calculated.

Cell culture Assays

Adhesion, Invasion, and Intracellular survival of C. jejuni K49/4 with PSI cl.1 and CLAB cells

Bacterial adhesion tests on PSI cl.1 and CLAB cell monolayers were carried out in 96-well tissue culture plates. After washing the cell monolayers with PBS, each of the probiotic bacteria (approximately 1×10^8 CFU/mL) was added simultaneously with *C. jejuni* K49/4 (2×10^8 CFU/mL) to each well. The control wells were prepared by adding *C. jejuni* K49/4 to the cell monolayers. The cells were incubated at 37 °C in 5% CO₂ for 2 h, to allow adhesion and invasion. After washing with DMEM, DMEM containing 100 µg/mL gentamicin was added, to determine the number of invaded *C. jejuni*. After a 1-h incubation, the monolayers were lysed with 1 mL/L (v/v) Triton X-100, for 5 min and were serially diluted. The intracellular bacteria were determined by plate counting at 3, 9, 24 and 48 h post-infection. The total numbers of adherent and internalised bacteria were determined simultaneously by performing the invasion assay, but without gentamicin treatment. The differences between the numbers of total and intracellular bacteria were calculated as the number of adherent *C. jejuni* cells.

Adhesion, invasion, and translocation of C. jejuni K49/4 using the PSI cl.1 and B10XI polarised cell model

A functional cell model using PSI cl.1 and B10XI cells was developed to determine inhibitory effect of the probiotic bacteria on the *C. jejuni* K49/4 adhesion, invasion and translocation of *C. jejuni* to the basolateral compartment of the well. When cells were confirmed to have reached confluence using TEER ($>1600 \Omega/\text{cm}^2$ for PSI cl.1 cells; $>240 \Omega/\text{cm}^2$ for B10XI cells), the monolayers were washed twice with 100 µL DMEM without antibiotic/supplements, and infection assays were performed by seeding the bacterial inoculum of each probiotic culture (approximately 1×10^8 CFU/mL) together with *C. jejuni* K49/4 (approximately 2×10^8 CFU/mL) in the apical chamber. The total numbers of adhered and invaded *C. jejuni* K49/4 were determined at 3, 17 and 24 h post-infection after lysing the cells by addition of 500 µL TritonX-100 and plating on Columbia agar plates. Bacterial count for infection of cell lines and chosen time intervals were similar as in our previous studies [32,33]. In addition, the numbers of translocated *C. jejuni* K49/4 were determined at the same time intervals. To investigate the effects of *C. jejuni* K49/4 on PSI cl.1 and B10XI cell monolayer integrity following the infection of the cell lines, the TEER was measured at 0, 3, 17, and 24 h post-infection. The TEER of the infected cells was compared to non-infected cells. Furthermore, the effects of co-incubation of probiotics with *C. jejuni* K49/4 were also assessed.

Statistical analysis

To define the effects of lactobacilli on *C. jejuni* K49/4 adhesion, data from triplicate samples from at least three independent experiments were analysed statistically using the Predictive Analytics (PASW) statistics 202 software, version 18.0 (IBM Corp., Armonk, NY, USA), for the significance of any changes in

bacterial numbers. Statistical analyses were performed with unpaired Student's t-tests to estimate the statistical significance. Data are presented as means \pm standard deviations (error bars) of the replicate experiments. All statistical values were considered significant at $P \leq 0.01$.

Abbreviation

TEER transepithelial resistance

Declarations

Ethics approval and consent to participate

Ethics approval was not necessary for this research.

Consent for publication

Not applicable.

Availability of data and materials

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Competing interests

The authors declare that they have no competing interests.

Funding

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Authors' contributions

MŠP, AK, TL, DMT and SSM conceived the study. MŠP, AK and TL performed the experimental work with *Campylobacter jejuni* and cell cultures. DMT and AK helped with the analysis of the obtained results. MŠP, AK, TL, DMT and SSM interpreted the results and MŠP wrote the article. All authors read and approved the final manuscript.

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Additional Material

Additional file 1: Visual scheme of experiment

We assessed the effect of different *Lactobacillus* strains on *C. jejuni* adhesion, invasion and translocation using pig (PSI) and chicken (B1OXI) enterocyte cell lines of non tumorigenic origin.

Figures

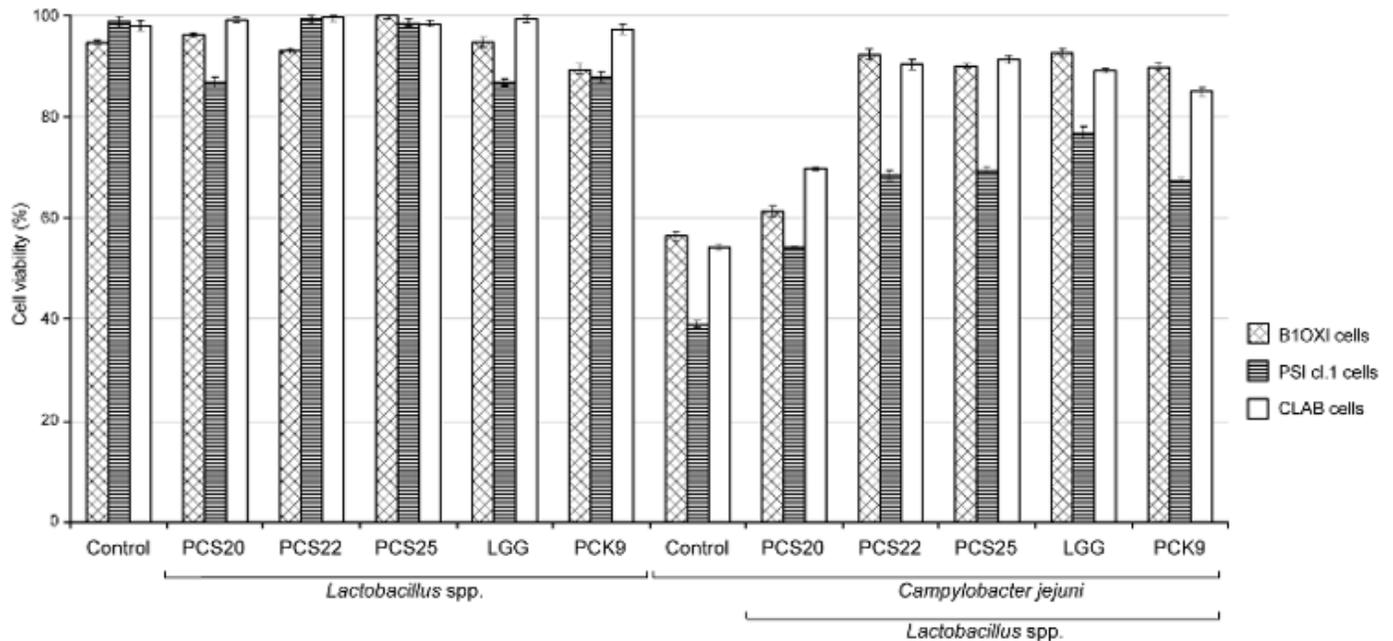


Figure 1

MTT proliferation assay. PSI c1.1, B1OXI and CLAB cells were seeded at 6×10^5 cells/ well. As confluent monolayers, the cells were exposed to the selected probiotic bacteria (1×10^8 CFU/mL) or to the combination of the probiotic bacteria (1×10^8 CFU/mL) and *C. jejuni* (2×10^8 CFU/mL), for 24 h (Figure 1). Error bars indicate the standard deviation from at least three independent experiments. Data are expressed as % cell survival, compared to the control.

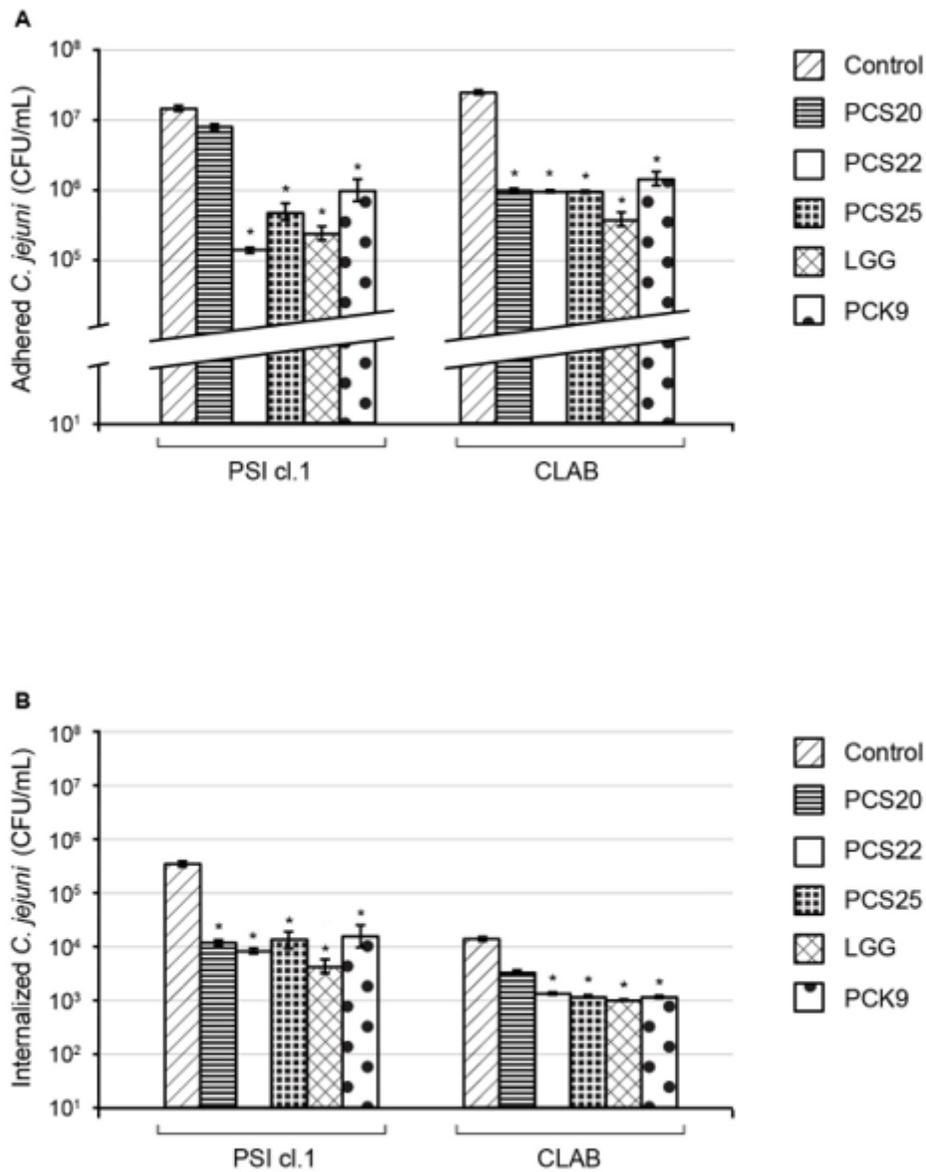
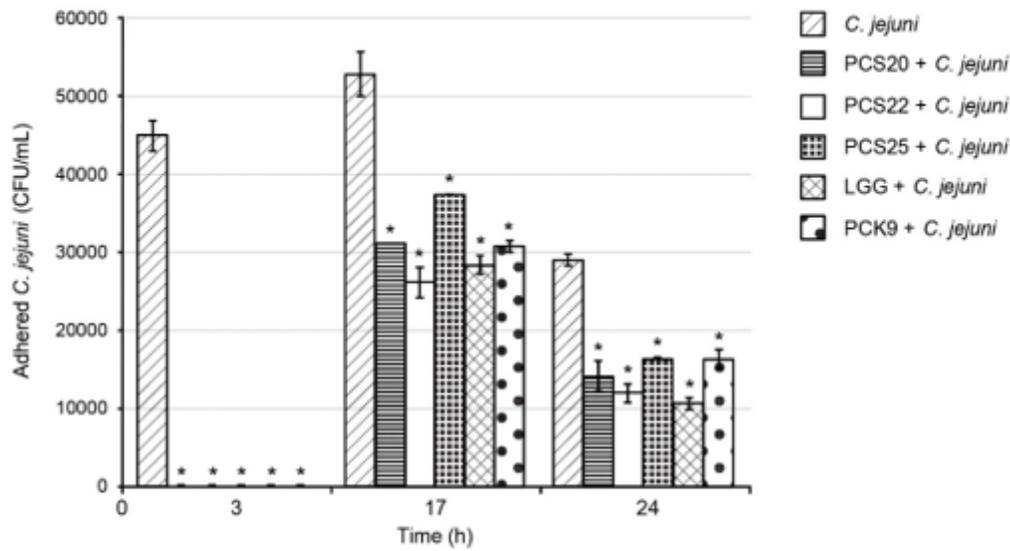


Figure 2

Campylobacter jejuni (2×10^8 CFU/mL) adhesion to (A) and invasion into (B) PSI cl.1 and CLAB pig intestine epithelial cells, after 2 h co-culture without or with probiotic bacteria (as indicated; concentration of 1×10^8 CFU/mL). Error bars indicate the standard deviation from at least three independent experiments. Data are expressed as means \pm SD. * $P \leq 0.01$, versus control.

A: PSI cl.1 cells



B: B10XI cells

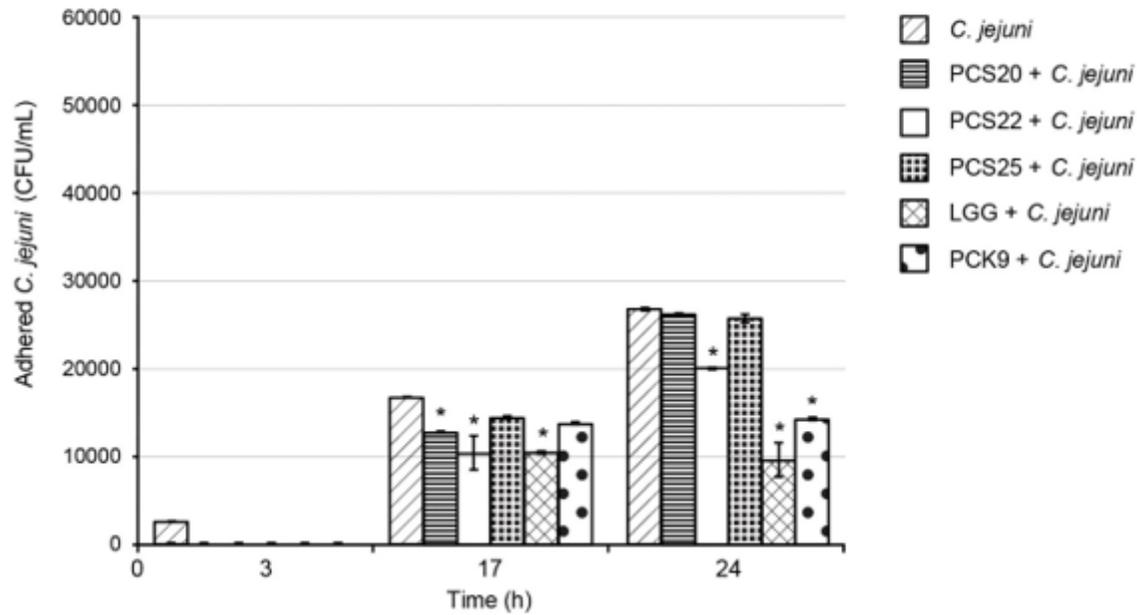
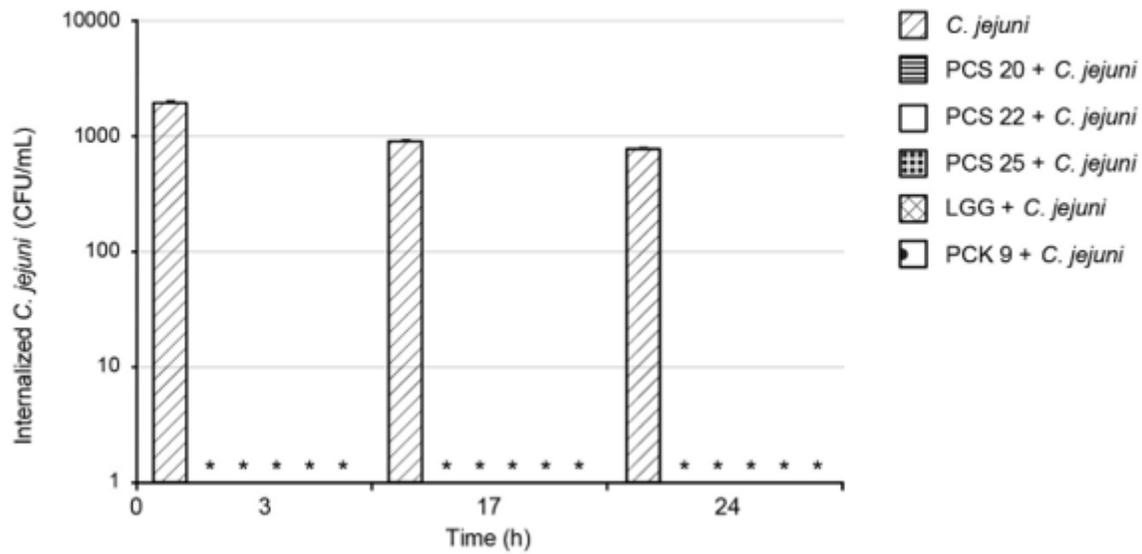


Figure 3

Effects of the probiotic bacteria on adhered *C. jejuni* using polarised pig PSI cl.1 (A) and chicken B10XI (B) intestine epithelial cells, after 2 h co-culture without and with putative probiotic bacteria. Error bars indicate the standard deviation from at least three independent experiments. Data are expressed as means \pm SD. * $P \leq 0.01$, versus control.

A: PSI cl.1 cells



B: B10XI cells

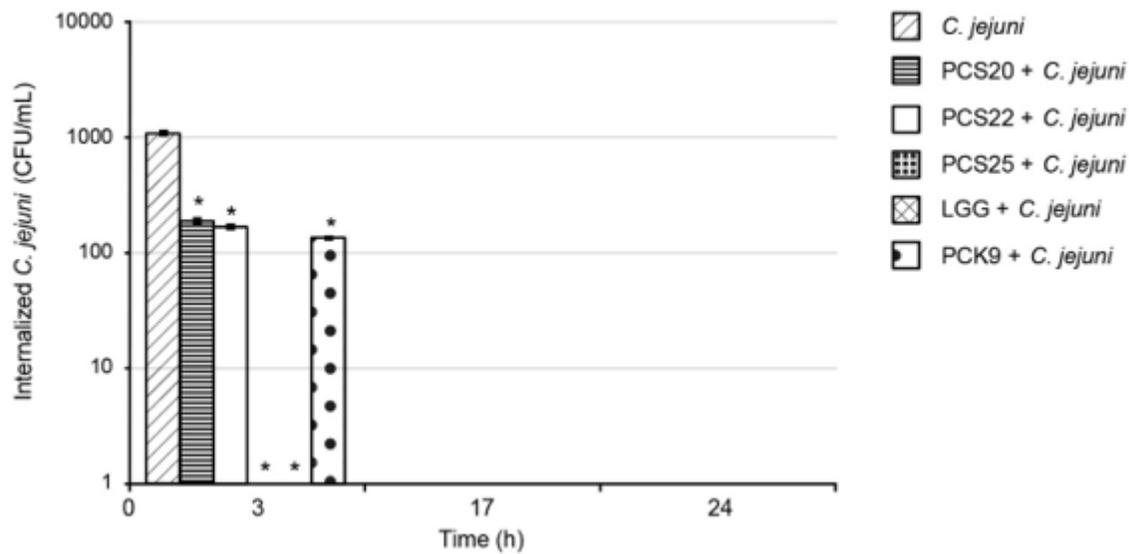
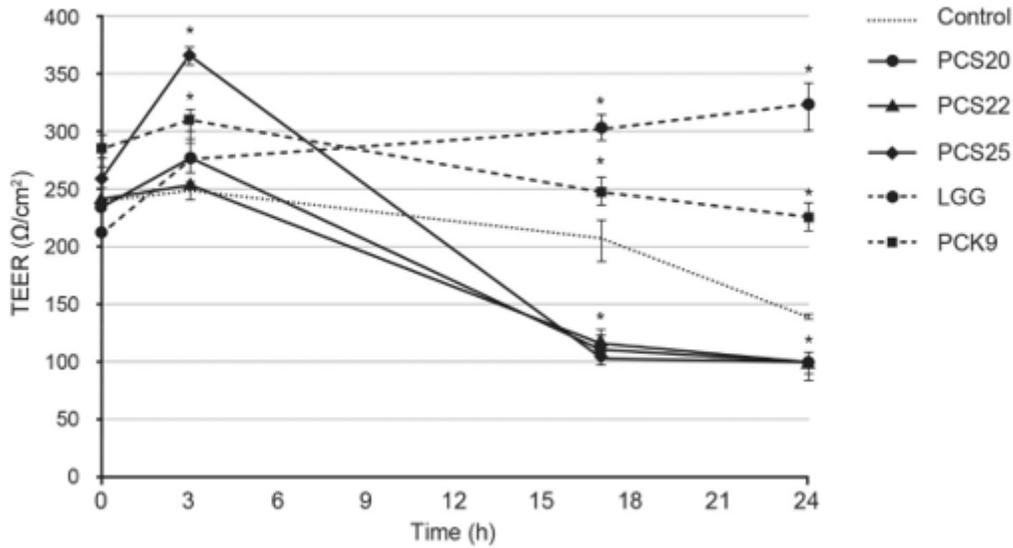


Figure 4

Effects of probiotic bacteria on internalized *C. jejuni* using polarized pig PSI cl.1 (A) and chicken B10XI (B) intestine epithelial cells, after 2 h co-culture without and with probiotic bacteria. Error bars indicate the standard deviation from at least three independent experiments. Data are expressed as means \pm SD. * $P \leq 0.01$, versus control.

A: B10XI cells



B: B10XI cells

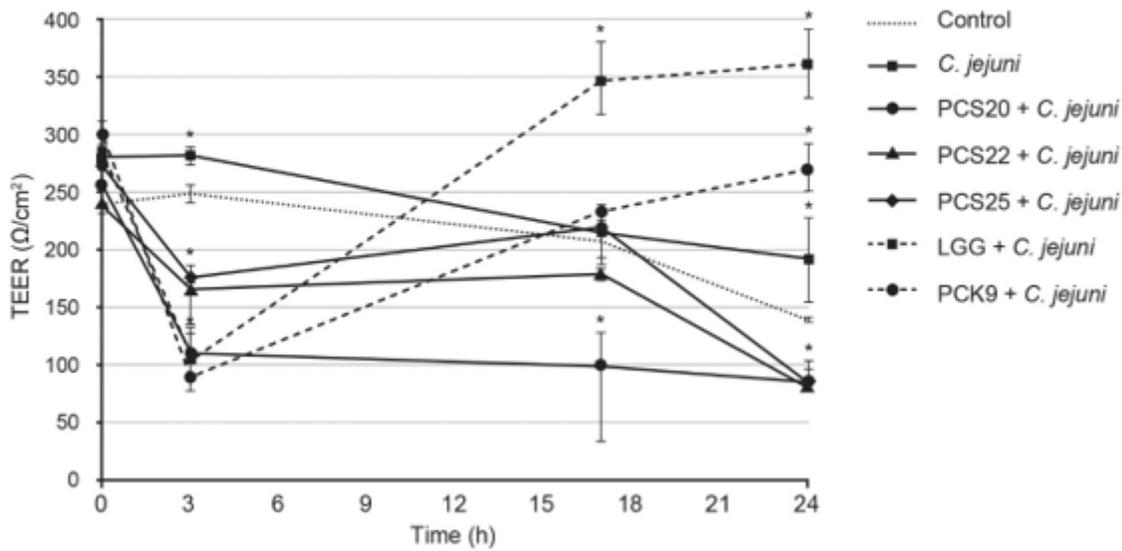
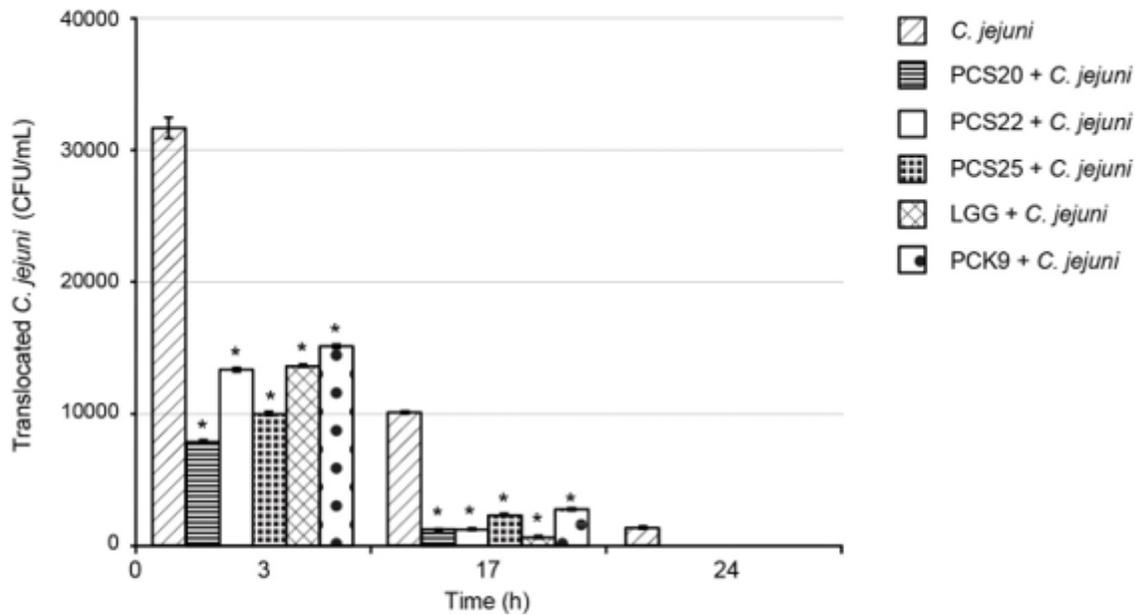


Figure 5

Transepithelial electrical resistance (TEER). PS1c.1.1 (not shown) and B10XI cells were seeded at 6×10^5 cells/well, as functional cell models using 12-well Transwell plates. As confluent monolayers, cells were exposed to the selected probiotic bacteria (1×10^8 CFU/mL) and *C. jejuni* (2×10^8 CFU/mL) for 2 h. Error bars indicate the standard deviation from at least three independent experiments. Data are expressed as means \pm SD. * $P \leq 0.01$, versus control.

A: PSI cl.1 cells



B: B10XI cells

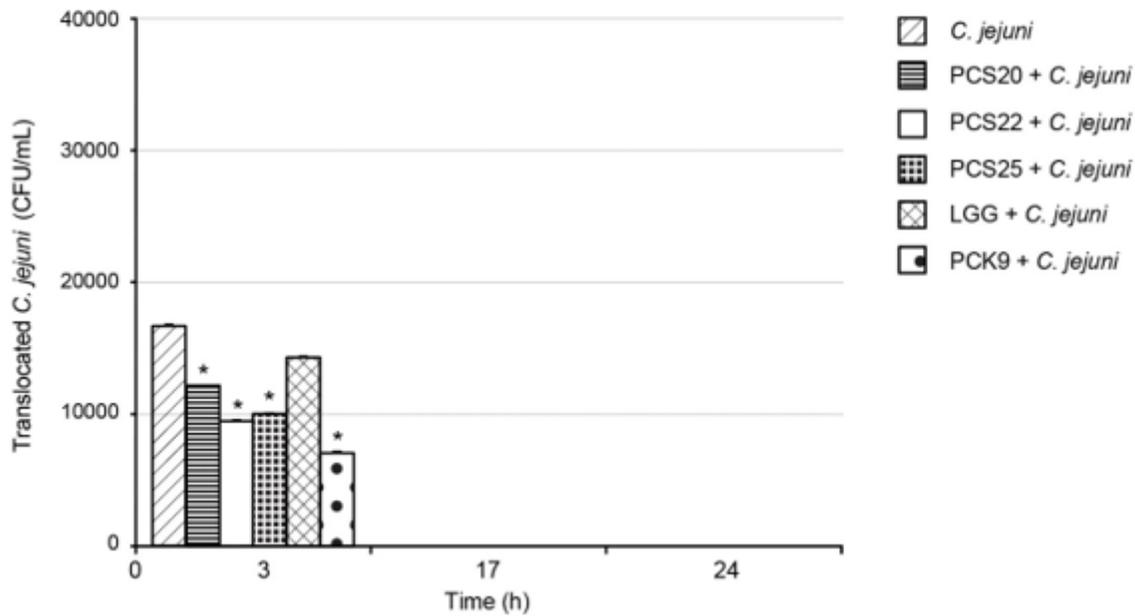


Figure 6

Translocation of *C. jejuni* K49/4 (2×10^8 CFU/mL) through polarised PSI cl.1 (A) and B10XI cells (B). Error bars indicate the standard deviation from at least three independent experiments. Data are expressed as means \pm SD of bacteria detected in the basolateral chamber of the transwells. * $P \leq 0.01$, versus control.

Supplementary Files

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