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THE INFLUENCES OF LACTOBACILLUS CELL-FREE SUPERNATANTS ON GROWTH AND VIRULENCE PROPERTIES OF *CAMPYLOBACTER JEJUNI* IN HUMAN ADENOCARCINOMA (HT-29) CELL CULTURE

LAKTOBASİLLERDEN ELDE EDİLEN HÜCRESİZ SÜZÜNTÜLERİN İNSAN ADENOKARSİNOM (HT-29) HÜCRE KÜLTÜRÜNDE *CAMPYLOBACTER JEJUNİ*'NİN ÜREME VE VİRÜLANS ÖZELLİKLERİ ÜZERİNE ETKİLERİ

Fatma KALAYCI YÜKSEK[1], Defne GÜMÜŞ[1], Varol GÜLER[2], Aysun UYANIK ÖCAL[1], Mine ANĞ KÜÇÜKER[1]

[1]Istanbul Yeni Yüzyıl University, Faculty of Medicine, Department of Medical Microbiology, Istanbul, Turkiye
[2]Istanbul Yeni Yüzyıl University, Faculty of Medicine, Department of Medical Biology and Genetic, Istanbul, Turkiye

ORCID IDs of the authors: F.K.Y. 0000-0002-0028-5646; D.G. 0000-0003-4070-6924; V.G. 0000-0002-8726-330X; A.U.Ö. 0000-0001-5255-0133; M.A.K. 0000-0001-8978-219X

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ABSTRACT

Objective: Lactobacilli are the most commonly used probiotics. We examined the influence of cell-free supernatants (CFSs) of *Lactobacillus acidophilus* (La), *L. fermentum* (Lf), *L. plantarum* (Lp) and *L. rhamnosus* (Lr) on growth, adhesion and invasion of *Campylobacter jejuni* 81116 and RM1221 in human adenocarcinoma colon cells (HT-29). We also analyzed the influences of CFSs, *C. jejuni* and their combinations on HT-29 cell viability.

Materials and Methods: Growth and adhesive-invasive bacteria counts were determined using the spectrophotometric method and colony counting method, respectively. We used methyl thiazolyl diphenyl-tetrazolium bromide (MTT) assay for detection of HT-29 cell viability.

Results: During two and four hours of incubation, the growth of RM1221 was significantly decreased ($p<0.0001$) with the effects of the tested CFSs, while the decrease in growth of the 81116 strain was only significant ($p<0.05$) in the presence of La and Lp. All CFSs except La reduced the growth of both *C. jejuni* isolates at 24 hours of incubation. The adhesion of *C. jejuni* 81116 was significantly ($p<0.0001$) reduced in the presence of all CFSs. La and Lr statistically significantly ($p<0.05$ and $p<0.005$, respectively) reduced the adhesion of *C. jejuni* RM1221. Invasion of *C. jejuni* strains was shown not to be affected in presence of all CFSs. *C. jejuni* and each CFSs were found to influence the Human colon adenocarcinoma cells (HT-29) viability differently.

ÖZET

Amaç: Laktobasiller en yaygın kullanılan probiyotiklerdendir. Çalışmamızda, *Lactobacillus acidophilus* (La), *L. fermentum* (Lf), *L. plantarum* (Lp) ve *L. rhamnosus* (Lr)'nin hücresiz süzüntülerinin (CFS) *Campylobacter jejuni* suşlarının (81116 ve RM1221) üremesi, adezyonu ve invazyonu üzerine etkilerini inceledik. Aynı zamanda, CFS'lerin, *C. jejuni* suşlarının ve CFS+*C. jejuni* kombinasyonlarının HT-29 hücre canlılığındaki etkilerini araştırdık.

Gereç ve Yöntem: Üreme ile adezif ve invazif bakterilerin sayıları, sırasıyla, spektrofotometrik ve koloni sayma yöntemleri ile belirlenmiştir. Çalışmamızda, Metil tiazolil difeni-tetrazolium bromid (MTT) deneyini, hücrelerin (HT-29) canlılığını belirlemede kullandık.

Bulgular: İki ve dört saatlik inkübasyonlarda, tüm CFS'ler RM1221 suşunun, La ve Lp CFS'leri ise, 81116 suşunun üremesini anlamlı düzeyde azaltmıştır (sırasıyla, $p<0,0001$, $p<0,05$). Tüm CFS'ler (La hariç) 24 saatlik inkübasyonda her iki suşun da üremesini baskılamıştır. *C. jejuni* 81116'nin adezyonu tüm CFS'lerin varlığında istatistiksel olarak anlamlı düzeyde baskılanmıştır ($p<0,0001$). *C. jejuni* RM1221'nin adezyonu La ve Lr süzüntüleri varlığında istatistiksel olarak anlamlı düzeyde baskılanmıştır (sırasıyla, $p<0,05$ ve $p<0,005$). Suşların invazyon özellikleri süzüntülerin varlığında etkilenmemiştir. İnsan kolon adenokarsinom (HT-29) hücrelerinin canlılığı hem *C. jejuni*'nin ve hem de her bir CFS'nin varlığında farklı yönlerde etkilenmiştir.

Corresponding author/İletişim kurulacak yazar: fatma.kalayci@yeniyuzyil.edu.tr

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Conclusion: Our results suggest that CFSs have suppressive effects on the growth and adhesive properties of *C. jejuni* in a time-dependent manner. The viability of HT-29 depends on incubation time and which strain is tested.

Keywords: *C. jejuni*, lactobacilli, growth, adhesion, invasion, cell viability

Sonuç: Sonuçlarımız CFS'lerin *C. jejuni*'nin üreme ve adezyonunu temas süresine bağlı olarak baskıladıklarını göstermektedir. HT-29 hücrelerinin canlılığı inkübasyon süresi ve incelenen suşa bağlı olarak etkilenmiştir.

Anahtar Kelimeler: *C. jejuni*, laktobasiller, üreme, adezyon, invazyon, hücre canlılığı

INTRODUCTION

Probiotics are known as living microorganisms that provide advantages for the host's health by stimulating the immune system, competing with pathogens for receptor binding and production of acids, bacteriocins, bio-surfactants and hydrogen peroxide to inhibit pathogens (1–4). In recent years, many studies have reported the roles of probiotics not only as supportive therapeutics but also as an alternative treatment method for infectious diseases, gastrointestinal tract diseases and control of oral health. Lactobacilli are known as the most commonly used probiotic microorganisms (5-7). Although they are known to have inhibitory effects on pathogens, the effects of their products have not been investigated extensively on *Campylobacter jejuni*.

C. jejuni, a foodborne pathogen, causes gastroenteritis and can be responsible for post-infectious complications in humans (8, 9). Adhesion-invasion mechanisms of the bacterium are very important during infectious processes and are related to cell death and the distribution of mucosal barriers in the host (10, 11). The increase of antibiotic-resistant strains is an important problem that leads to public health concerns and an economic burden (8, 9). Therefore, alternative and supportive options need to be considered. There are studies that present promising results to cope with stages of infection using the antagonist relationship between probiotic microorganisms and pathogens (12-20).

This study aimed to investigate the influence of lactobacilli cell-free supernatants (CFSs) on the growth and virulence properties (adhesion and invasion abilities) of two *C. jejuni* strains (81116 and RM1221) in the human adenocarcinoma cells (HT-29), mimicking host conditions. The study also examined the effects of *C. jejuni* strains and CFSs of lactobacilli, together or separately, on the viability of HT-29 cells.

MATERIALS AND METHODS

Bacteria and preparing of cell-free supernatants

Two *Campylobacter* strains *(C. jejuni* 81116 and RM1221) were kindly provided by Dr. György Schneider, (University of Pécs, Hungary). *C. jejuni* strains were grown in Brucella broth (BB) (Besimik, Turkiye) under microaerophilic conditions at 37°C for 48 hours.

Lactobacilli (*Lactobacillus acidophilus* ATCC 314-La, *L. fermentum* ATCC 9338-Lf, *L. plantarum* ATCC 14917-Lp and *L. rhamnosus* ATCC 53103-Lr), which are commonly sold in pharmacies and markets, were examined (21, 22).

De-Man Rogosa-Sharpe (MRS) broth (Conda, Spain) was used on growth of Lactobacilli under anaerobic conditions at 37°C for 24 hours. Following overnight cultivation of *Lactobacillus* strains, supernatants were collected via centrifugation at 4000 rpm for 30 minutes at 4°C, then filtered with 0.2 µm pore size filters (12, 23).

Cell culture

Human colon adenocarcinoma cells (HT-29) were used in our experiments and specific cell culture conditions as previously defined (13, 15).

HT-29 cells were seeded in 96-well microplates for bacterial growth and cell viability assay, seeded in 24-well plates for invasion and adhesion experiments. To provide a confluent monolayer cell culture, density was adjusted as approximately 5×10^4 cells for 24-well and 1×10^4 cells for 96-well plates. Plates were incubated at 37°C, under 5% CO_2 conditions for 24 hours.

Infection of HT-29 cells with *C. jejuni*

The overnight cultures of *Campylobacter* isolates were prepared in Brucella broth at 37°C. For infection of HT-29 cells, a suspension of approximately 10^9 CFU/mL of each strain was used.

Before inoculation of *C. jejuni*, Dulbecco's Modified Eagle Medium (DMEM) containing antibiotics was replaced with antimicrobial solution-free DMEM and CFSs were added into each well (20 µL in each 96-well plates and 50 µL in each 24-well plates). The plates were incubated for one hour at 37°C.

HT-29 cells were inoculated with *C. jejuni* and microaerophilic conditions were provided for incubation (as seen below). All assays were performed three times.

Bacterial growth

Bacteria were incubated for two, four and 24 hours to investigate the alterations of growth in the presence/absence of CFSs. The influence of each CFSs was detected by measuring the changes in absorbance at 600 nm. Bac-

terial growth in cell culture with CFSs was compared to cell culture without CFSs (as negative control).

Bacterial adhesion and invasion
HT-29 cells were incubated with *C. jejuni* for three hours at 37°C under microaerophilic conditions. The effect of each CFS on bacterial adhesion and invasion was determined by comparing colony counts (as CFU/mL) from cell lysates of HT-29 grown in the presence/absence of CFSs. We determined colony counts of adhesive and invasive bacteria as described previously (24, 25).

Bacterial adhesion
Phosphate buffer saline (PBS) was used to wash the wells three times to remove unbound bacteria after incubation for three hours. Then, the cells were lysed using 500 µl 1% Triton X-100 for 10 minutes at 37°C under 5% CO_2 conditions. Following the homogenization and inoculation of cell lysates on Mueller–Hinton agar (MHA) (Spesera, Turkiye) supplemented with sheep blood (5% defibrinated), media was incubated at 37°C for 48 hours under microaerophilic conditions.

Bacterial invasion
After bacterial inoculation and three hours incubation, the wells were washed with PBS three times. Then, a medium supplemented with 300 ng/ml gentamicin was added to each well for killing non-invasive (extracellular) bacteria. Microaerophilic conditions were provided for incubation of the plates and they were incubated for two hours at 37°C. The lyses of HT-29 cells were provided using Triton X-100 as mentioned above. For detection of invasive bacteria, the homogenized cell lysates were inoculated on MHA (Spesera, Turkiye) supplemented with sheep blood and incubated for 48 hours under microaerophilic conditions at 37°C.

Viability of Human adenocarcinoma colon cells (HT-29)
A methyl thiazolyl diphenyl-tetrazolium bromide (MTT) assay was used for detecting cell viability. The effects of CFSs and *C. jejuni*, together and separately, on the viability of HT-29 were investigated. Experimental conditions were prepared as mentioned above and HT-29 cells were incubated for 24 and 48 hours under microaerophilic conditions.

Following incubation, the wells were washed with PBS three times to remove residue. Then, a fresh culture medium was added. According to Mosmann, MTT was prepared (12 mM, Neofrox 3580 MTT) and added into each well. The HT-29 cells were incubated at 37°C for four hours under microaerophilic conditions (26).

After incubation, the media were aspirated to remove the content from the wells. Then, Dimethyl sulfoxide (DMSO) was added into each well. The plates were incubated at room temperature for 10 minutes to dissolve the formazan crystals into a colored solution. Lastly, absorbance values were measured at 540 nm via the spectrophotometric method.

The cell viability of HT-29 was investigated by comparing the absorbance values of dissolved formazan crystals produced by HT-29 cells in the presence/absence of CFSs or *C. jejuni*, separately and together.

Statistical analysis
The significant differences between experimental conditions and control conditions were calculated. Results were analyzed with two-way ANOVA followed by Dunnett's multiple comparisons test for growth alterations. One-way ANOVA followed by Dunnett's multiple comparisons test was performed for adhesion and invasion results. Alterations of cell viability were detected by two-way ANOVA followed by Dunnett's and Sidak's multiple comparisons test. The significant differences between experimental conditions and control conditions were evaluated. All results were presented as mean±SD. Differences with p values less than 0.05 were accepted as indicative of statistically significant differences.

RESULTS

Bacterial growth
After incubation for two and four hours, CFSs of La and Lp significantly reduced ($p=0.046$ and $p=0.039$, respectively for two hours and $p=0.043$ and $p=0.029$, respectively, for four hours) the growth of *C. jejuni* 81116 strain; all CFSs decreased significantly ($p<0.0001$) the growth of *C. jejuni* RM1221 (Figure 1a, Figure 1b).

According to the 24 hour incubation results, the growth *C. jejuni* 81116 strain was statistically significantly decreased ($p=0.0034$ for Lf, $p<0.0001$ for Lp and $p=0.0025$ for Lr) in the presence of all CFSs except for La. It was found that all CFSs were shown to decrease ($p<0.0001$) the growth of *C. jejuni* RM1221 statistically significantly. Furthermore, the most effective inhibition was seen for *C. jejuni* RM1221 in the presence of each CFSs at 24 hours incubation (Figure 1b).

Bacterial adhesion
The adhesion of *C. jejuni* 81116 was found to be significantly reduced ($p<0.0001$) in the presence of all CFSs. La and Lr CFSs' were found to significantly reduce ($p=0.0068$, and $p=0.026$, respectively) the adhesion of *C. jejuni* RM1221 (Figure 2).

Bacterial invasion
The effects of all CFSs on bacterial invasion were found statistically insignificant ($p>0.05$) for both *C. jejuni* strains (Figure 3).

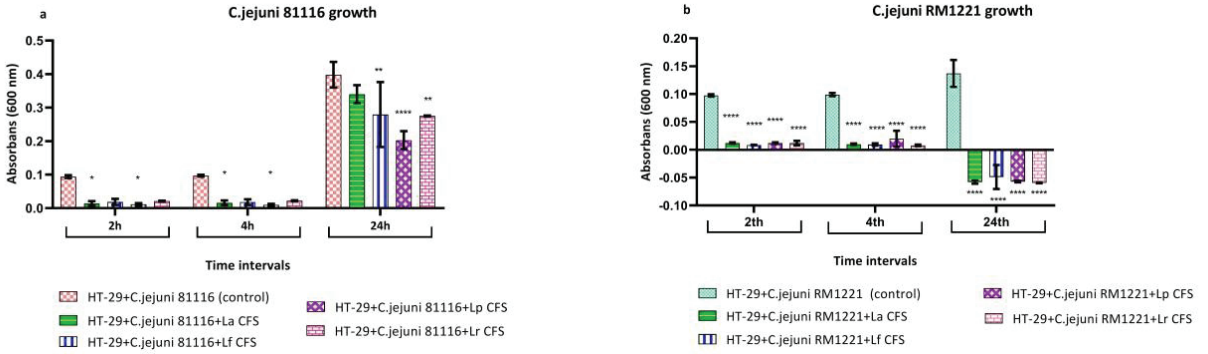

Figure 1: The influences of CFSs on the growth of *C. jejuni*

The growth of *C. jejuni* grown in HT-29 with and without CFSs were examined using two way ANOVA followed by Dunnett's multiple comparisons test.

*, **, ****: Significance levels were as p<0.05, p<0.005 and p<0.0001, respectively.

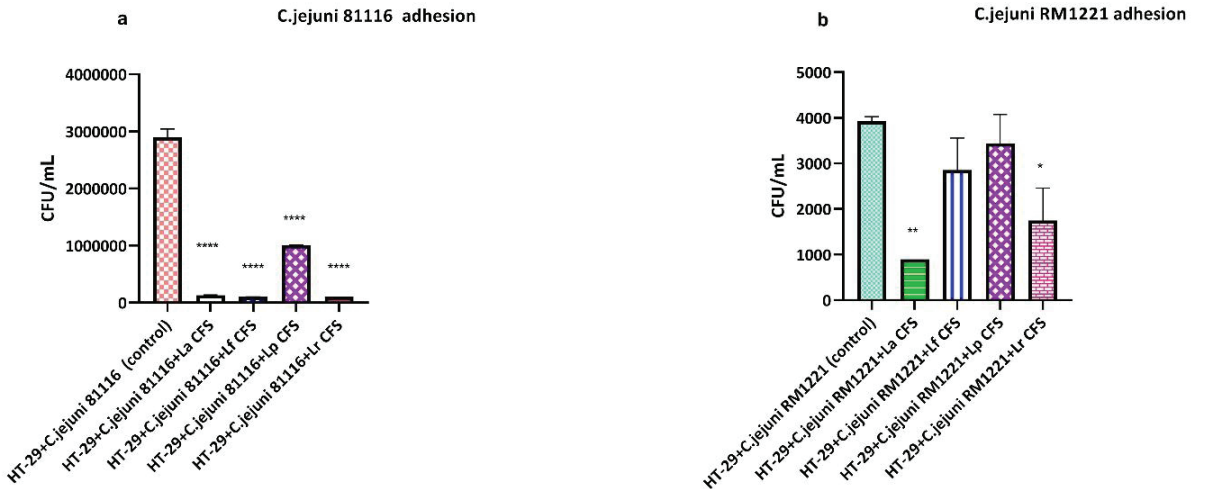

Figure 2: The influences of CFSs on adhesion of *C. jejuni*

The adhesion of *C. jejuni* in HT-29 with/without CFSs was analyzed using one-way ANOVA followed by Dunnett's multiple comparisons test.

*, **, ****: Significance levels were as p<0.05, p=0.0068 and p<0.0001, respectively.

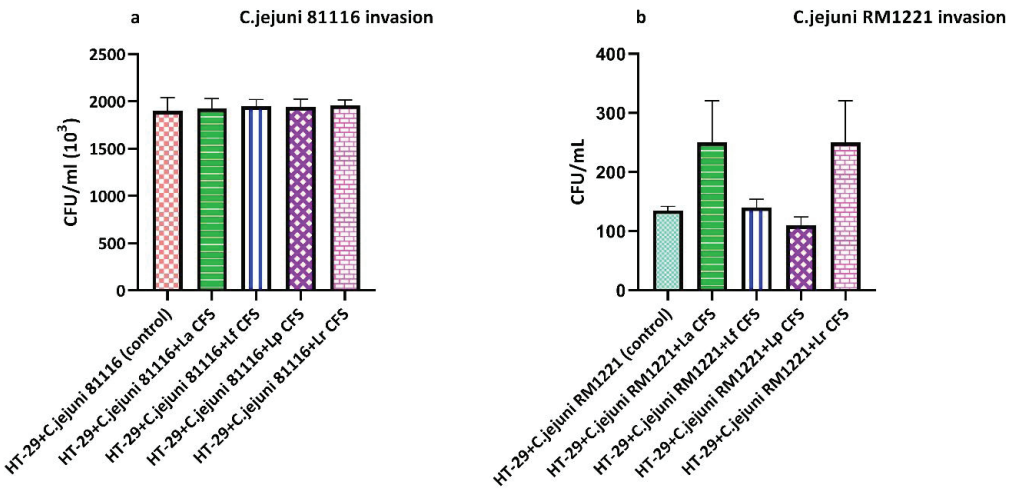

Figure 3: The influences of CFSs on the invasion of *C. jejuni*

The invasion of *C. jejuni* in HT-29 with/without CFSs was analyzed using one-way ANOVA followed by Dunnett's multiple comparisons test.

Viability of HT-29 cells

According to the 24 hour incubation results, the viability of HT-29 cells infected with *C. jejuni* RM1221 increased ($p<0.0001$). The HT-29 cell viability was shown to be decreased significantly ($p<0.0001$) by each CFSs (Figure 4).

After 48 hours, the HT-29 cell viability was significantly reduced in the presence of CFSs (Lf- $p=0.001$, Lp- $p<0.0001$ and Lr- $p=0.0005$) and *C. jejuni* RM1221 ($p=0.0097$), separately (Figure 4).

The influence of *C. jejuni* 81116 on HT-29 cell viability was found to be insignificant ($p>0.05$) on both at 24 and 48 hours (Figure 4).

According to the 24 hour incubation results, we found that La, Lp and Lr CFSs statistically significantly decreased the viability of *C. jejuni* 81116 infected HT-29 cells ($p=0.01$, $p=0.001$ and $p=0.0036$, respectively). According to the results of the 48 hour incubation, the viability of HT-29 cells infected with *C. jejuni* 81116 was found to be significantly decreased ($p=0.008$, $p=0.001$, respectively) in the presence of La and Lf CFSs; however, Lr was found to significantly ($p<0.0001$) increase the viability of infected HT-29 cells (Figure 5a).

According to the results of the 24 hour incubation, it was found that all CFSs statistically significantly ($p<0.0001$) reduced the viability of *C. jejuni* RM1221 infected HT-29 cells. After 48 hours of incubation, we found that CFSs of La, Lf ($p<0.0001$) and Lp decreased (p: 0.0004) the viability of *C. jejuni* RM1221 infected HT-29 cells (Figure 5b).

DISCUSSION

Our study showed inhibitory effects of CFSs, obtained from the *Lactobacillus* species on *C. jejuni* isolate in human adenocarcinoma (HT-29) cell culture, mimicking host conditions. Numerous studies have reported that *L. acidophilus, L. gasseri, L. fermentum, L. johnsonii, L. reuteri, L. crispatus, L. paracasei, L. plantarum,* and *L.salivarius* or their cell-free supernatants repress the growth of the *Campylobacter* species (15, 27-32). Consistent with previous results, our findings showed that at the first four hours of incubation, the growth of *C. jejuni* RM1221 was reduced by all lactobacilli CFSs, while *C. jejuni* 81116 was only significantly reduced by La and Lp CFSs in HT-29 cell cultures. After 24 hours, all CFSs reduced the growth of both *C. jejuni* RM1221 and 81116 strains, except CFSs of La, which did not alter the growth of *C. jejuni* 81116.

It is well known that adhesion is one of the most important stage for the colonization of colon cells by microbes. Previous studies have found that *L. acidophilus, L. casei, L. rhamnosus* and *L. plantarum* decreased the adhesion of *C. jejuni* strains while *L. rhamnosus* and *L. salivarius* did not prevent the adhesion of *C. jejuni* (15, 29, 33-36). In our study, we found that all CFSs decreased the adhesion of *C. jejuni* 81116 to HT-29 cells while the adhesion of *C. jejuni* RM1221 strain was found to be decreased in the presence of only CFSs of La and Lr. Although anti-adhesive properties of CFSs are shown to be strain-specific, we may conclude that lactobacillus strains have an inhibitory effect on the adhesion of *C. jejuni* strains in general.

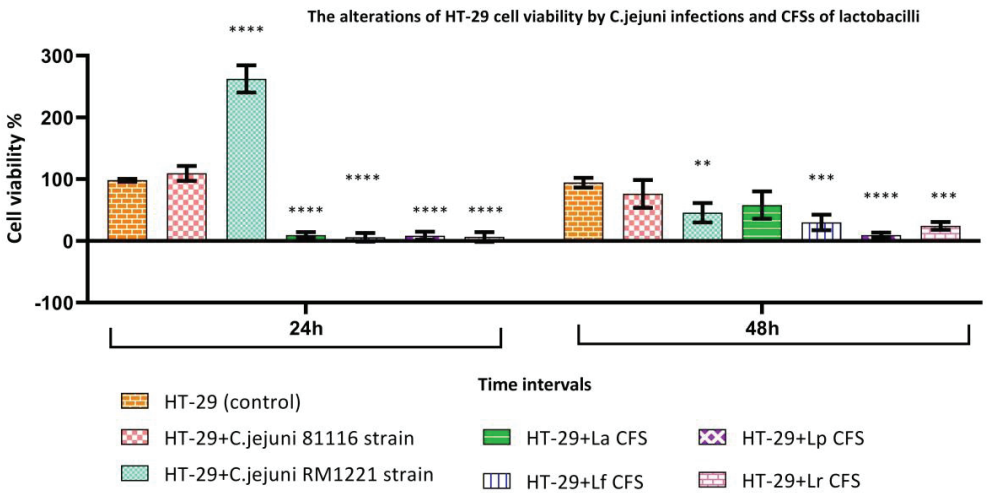

Figure 4: The influences of CFSs and *C. jejuni* infections, separately on HT-29 cell viabilities

The cell viabilities with/without CFSs were analyzed using two-way ANOVA followed by Dunnett's multiple comparisons test
,*,****: Significant at $p=0.0097$, $p\leq0.001$ and $p<0.0001$ levels, respectively.

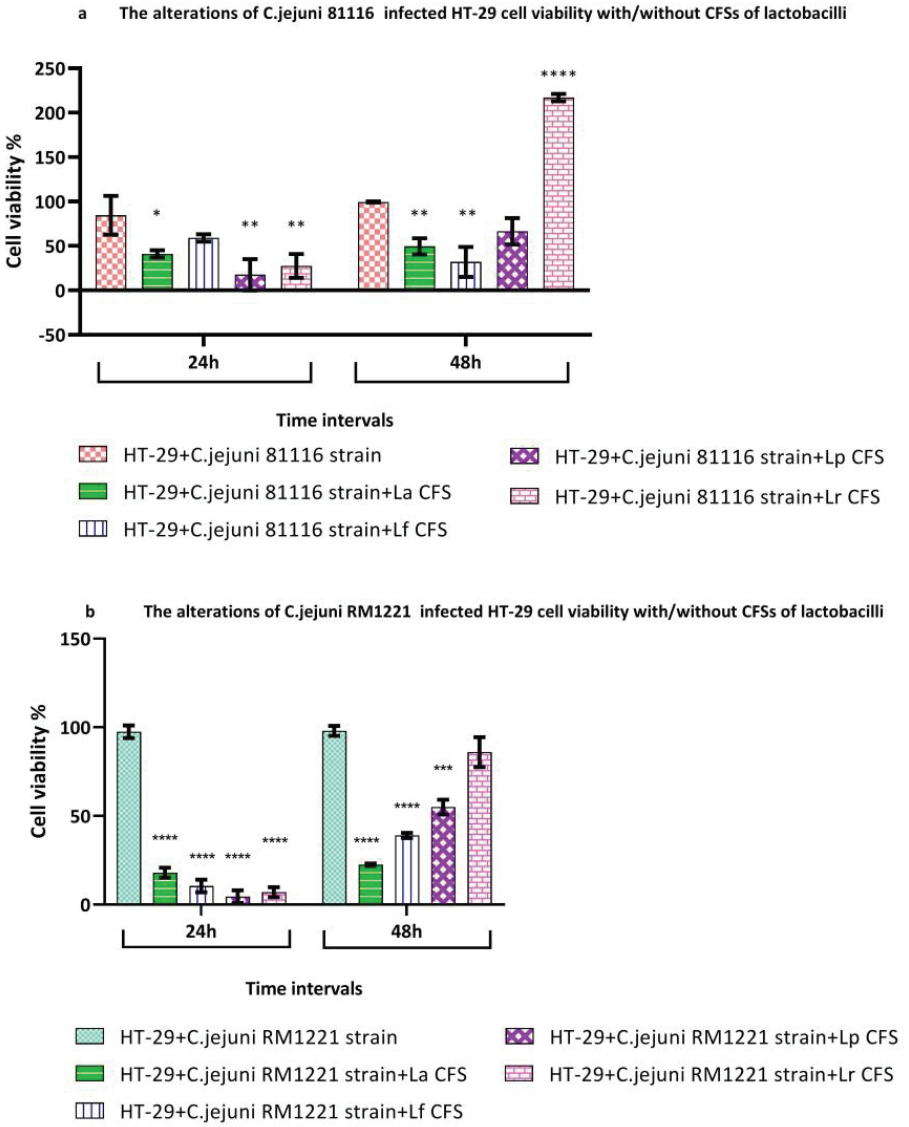

Figure 5: The influences of CFSs and *C.jejuni* infection co-presence on HT-29 cell viabilities

The cell viabilities with/without CFSs were analyzed using two-way ANOVA followed by Sidak's and Dunnett's multiple comparisons tests, respectively

*, **, ***, ****: Significant at p=0.01, p≤0.008, p<0.0005 and p<0.0001 levels, respectively.

Furthermore, invasion is another important stage during the infectious process. Although there are a limited number of studies investigating the effects of lactobacilli and their products on the invasion of *C. jejuni*, it appears that their effects are commonly defined as repressive. According to previous findings, *L. helveticus, L. acidophilus, L. paracasei, L. rhamnosus, L. lactis, L. gasseri* and *L. salivarius*, decrease the invasion of *C. jejuni,* but *L. rhamnosus* does not exhibit the same effect (10, 15, 37). Consistent with Wine et al., we showed that CFSs did not alter the invasion of two *C. jejuni* strains tested (37).

In our study we did not analyze which mechanisms were responsible for the inhibition of adhesion and invasion processes. However, previous studies have proposed that probiotics could exclude and/or displace the pathogens in a competitive way (15, 37).

The influence of CFSs, *C. jejuni* and their combinations on HT-29 cell viability were also analyzed in our study. Many studies have reported that lactobacilli and their products affect the host cell viabilities (PSI cl.1, B1OXI, CLAB, Caco-2, HOB, HT-29, HeLa, AGS, MCF-7 and CF cell lines) (13, 34, 39-41). While Pogačar et al. showed that

L. plantarum and *L. rhamnosus* strains did not have any cytotoxic effects on pig and chicken epithelial cells at 24 hours of incubation, Kalaycı-Yüksek et al. reported that CFSs of La, Lf and Lp decreased the viability of HOB cells for three hours of incubation (13, 34). Consistent with earlier research the viabilities of AGS, MCF-7, HT-29 and HeLa cells were found to be gradually reduced depending on incubation and concentration of *L. acidophilus* CFS (13, 41). We found in our study that all CFSs, except La, decreased the viability of HT-29 cells at both incubation periods. Presumably, the effects of lactobacilli and/or their products on cell viability may be related to their acidic pH.

It has been shown that *C. jejuni* causes a cytotoxic effect on pig and chicken epithelial cells (34). However, Bouwman et al. have shown that different *C. jejuni* strains did not induce any cytotoxicity on macrophages. Interestingly, our results have shown that the effect of *C. jejuni* infection on the viability of HT-29 cells is strain-dependent. While *C. jejuni* 81116 did not affect the viability of HT-29 cells at both incubation periods, *C. jejuni* RM1221 increased at 24 hours. However, HT-29 cell viabilities were found to be decreased if the exposure was prolonged to 48 hours. We assume that cell viability is associated with exposure time, types of infected cell lines, and biological properties of *C. jejuni* strains tested.

Moreover, we investigated the effects of CFSs in combination with *C. jejuni* infection on host cell viability. *L. plantarum* and *L. rhamnosus* were shown to have protective effects on the viabilities of *C. jejuni* infected pig and chicken epithelial cells at 24 and 48 hours of incubation. It has also been shown that different lactobacilli combinations decreased the cell viabilities which were infected with *C. jejuni* at both 24 and 48 hours of incubation (34). Consistent with these findings, in our study, CFSs of La, Lp and Lr decreased the viability of HT-29 cells infected with *C. jejuni* 81116 at 24 hours of incubation. However, at 48 hours of incubation, the viability was increased in the presence of Lr CFSs. Furthermore, CFSs of Lf acted as a suppressive on the viability of *C. jejuni* 81116 infected-HT-29 cells. Similar results were observed on the HT-29 cell viabilities infected with *C. jejuni* RM1221 at 24 hours incubation. All CFSs decreased HT-29 cell viability, but suppressive effects of Lr disappeared when incubation was prolonged to 48 hours. It is clear that the influence of CFSs on the viability of infected HT-29 cells is strain-dependent.

In conclusion, the present study demonstrated that CFSs obtained from the *Lactobacillus* species showed inhibitory effects on *C. jejuni* growth and adhesive properties in cell culture. Furthermore, in this study, we found that CFSs have a suppressive effect on the viability of infected/non-infected HT-29 cells which may be related to the acidic properties of CFSs. Although our results showed that the inhibitory effects of CFSs vary depending on exposure time and strains, it is possible to suggest that their inhibitory effects on the biology of *Campylobacter* infections may be taken into consideration.

However there were some limitations in our study. Further clarity is needed as to which inhibitory products of lactobacilli have the most effective roles on pathogens. Also, molecular aspects could identify the mechanisms which are affected during these interactions. In this frame, our findings provide preliminary insights for *in vivo* future studies to focus on the identification of these inhibitory roles of lactobacilli.

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