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MILK MODULATES *CAMPYLOBACTER* INVASION INTO CACO-2 INTESTINAL EPITHELIAL CELLS

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Raw milk is a recognized source of *Campylobacter* outbreaks, but pasteurization is an effective way to eliminate the causative agent of Campylobacteriosis. Whereas breastfeeding is protective against infectious diseases, consumption of formula milk is thought to be not. However, in relation to *Campylobacter,* such data is currently unavailable. Although both pasteurized and formula milk are pathogen free and prepared in a quality controlled manner, the effect they have on the virulence of *Campylobacter* species is unknown. Here, we studied the effect of cow, goat, horse, and formula milk on *Campylobacter* invasion into intestinal epithelial Caco-2 cells, a pathogenic feature of this bacterial species, using a gentamicin exclusion invasion assay. We found that all milk products modulated the invasion of *Campylobacter* species into the Caco-2 cells in a dose-dependent manner. Control experiments showed that the milks were not toxic for the Caco-2 cells and that the effect on invasion is caused by heat labile (e.g., milk proteins) or heat stable (e.g., sugar/lipids) components depending on the *Campylobacter* species studied. This *in vitro* study shows for the first time that pasteurized and formula milk affect the invasion of *Campylobacter.* We recommend a prospective study to examine whether pasteurized and formula milk affect Campylobacteriosis.

Keywords: *Campylobacter,* pasteurized, formulated, milk, invasion, Caco-2

Introduction

Mammalian milk and milk products are not only a rich and cheap source of nutrients [1, 2] but, in its untreated form, also a potential source of pathogens [3–5]. The bacterial pathogens include different bacterial species that are able to induce severe diarrhea [6, 7], that is often followed by post-infectious diseases, such as listeriosis, Reiter's syndrome, reactive arthritis, and the Guillain–Barré syndrome [8–12]. One of the bacterial species, the zoonotic human pathogen *Campylobacter,* is linked with large outbreaks caused by drinking unpasteurized milk [13–15]. *Campylobacter* species are the causative agents of Campylobacteriosis and are mainly cultured from stools of children (<5 years of age), teenagers, and the elderly (>65 years of age) [16]. To prevent infections by these potential contaminants in milk, including *Campylobacter*, milk is routinely pasteurized or subjected to even more stringent heating (UHT). In contrast, it is unknown whether the pasteurized milk modulates bacterial pathogenicity and, to our knowledge, no studies to date have addressed the effects of pasteurized milk in relation to *Campylobacter* invasion.

Human breast milk is thought and shown in a number of studies to harbor health benefits for infants [17–19]. Secondly, breastfeeding is correlated with decreased infections and child mortality when compared to formula milks [20], which may, in addition to breastfeeding, be linked to the socio-economic status of the parents [21]. Although breastfeeding is considered to be the most ideal feeding method for infants potentially protecting against gastrointestinal pathogens [22, 23], such as *Campylobacter* [24], there are several reasons why parents may choose to use formula milks [25, 26]. Pro- and prebiotics have been added to formula milks to enhance health benefits, but to receive European Food Safety Administration (EFSA)-backed approval for these claims, more randomized double blind studies are required [27]. Formula milks have occasionally been linked to outbreaks caused by *Enterobacter sakazakii* [28] and *Salmonella* spp. [29], but to our knowledge, it has not been studied whether formula milks

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can reduce or enhance intestinal epithelial invasion of bacterial pathogens, such as *Campylobacter*.

Therefore, the objective of this study was to assess whether pasteurized and formula milk modulate the invasion of *Campylobacter* species into intestinal epithelial cells.

Materials and methods

Bacterial strains

Campylobacter jejuni (GB11), *Campylobacter coli* (B18389), *Campylobacter fetus* (B36094), and a *Campylobacter lari* (ATCC35221) isolate were used in this study and were obtained from a GBS patient (GB11) [30], two bacteremia patients (B18389 and B36094) and a water-related strain (ATCC35221), respectively. To minimize *in vitro* passages, the *Campylobacter* strains were recovered from the original (patient-isolated) glycerol stock by culturing on Butzler agar plates (Becton Dickinson, Breda, The Netherlands). A second passage was allowed for optimal vitality before these strains were used in experiments. After recovery, cells were harvested in Dulbecco's modified Eagle medium (DMEM) (Life Technology, Breda, The Netherlands) containing 10% fetal bovine serum (FBS) (Life Technology, Breda, The Netherlands) and 1× non-essential amino acids (NEAA) (Life Technology, Breda, The Netherlands), and densities were adjusted according to the optical density at an $OD_{600\,nm}$ where 1 OD equals 2.5×10^{09} CFU/ml.

Milk and milk products

Pasteurized cow, goat milk, and soy drink were obtained from the grocery store, Oosterhout (NB), The Netherlands. Horse milk (unpasteurized) was obtained from a horse milkier, Strijen, The Netherlands. Instant formula milks: NR 1: instant formula baby milk (0–6 months) Nestlé Standard 1, NR 2: instant formula toddler milk (6–10 months) Nutricia Nutrilon with pronutra Standard 2, NR 3: instant formula toddler milk (6–10 months) Friso standard 2, and NR 4: instant formula toddler milk (12–36 months) Nestlé were obtained from the grocery store, Oosterhout (NB), The Netherlands. NR 1, NR 2, and NR 3 were supplemented with pre- and/or probiotics. Milk oligosaccharide sialyllactose present in cow milk was obtained from FrieslandCampina.

Intestinal epithelial cell line

Caco-2 human intestinal epithelial cells were maintained in DMEM medium (Life technology) supplemented with 10% FBS (Life technology) and 1× NEAA (Life Technology). The cells were routinely grown in a 75-cm^2 flask (Greiner Bio-One, Alphen a/d Rijn, The Netherlands) at 37 °C in a humidified 5% CO^2–95% air incubator (Binder, Tuttlingen, Germany). Confluent stock cultures were washed with Hanks balanced salt solution (HBSS) (Life Technologies, Breda, The Netherlands) and trypsinized with trypsin–EDTA (Lonza, Verviers, Belgium), and 5.0×10^5 cells/ml were seeded in a new 75-cm^2 flask.

Gentamicin exclusion assay

Briefly, *C. jejuni*, *C. coli*, *C. fetus*, and the *C. lari* strain were grown overnight on a Butzler agar plate (Becton Dickinson, Breda, The Netherlands) in an anaerobic jar (Mart, Geldermalsen, The Netherlands) under microaerophilic conditions. *Campylobacter* strains were harvested from the Butzler agar plate and inoculated in a 15-ml tube (Greiner Bio-One) that contained 5 ml of DMEM medium (Life Technologies) + 10% FBS (Life Technologies) and 1× NEAA (Life Technologies) obtaining a final concentration of 1.0×10^8 CFU/ml measured by OD_{600nm}. Milk was diluted in the ratio's DMEM medium + 10% FBS + 1× NEAA–milk; (1:1) for CM0, GM0, HM0, and SJ0; (1:10) for CM1, GM1, HM1, and SJ1; and (1:100) for CM2, GM2, HM2, and SJ2 where CM is cow milk, GM is goat milk, HM is horse milk, and SJ is soy drink. Hundred microliters of the *Campylobacter* suspension was added to 900 μl (DMEM–milk) dilutions and incubated for 10–15 min. For formulated milk, the protocol was followed that was placed on the milk powder box to prepare the milk concentrations that are advised for home usage with the exception that not water, but HBSS (Life Technology) was used as soluble, where after the same dilutions (DMEM–milk) were prepared as for pasteurized milk. After this incubation/infection period, the medium above the differentiated Caco-2 cells in a 12-well plate (Greiner Bio-One) was replaced with 1 ml of this *Campylobacter*–DMEM–milk suspension and incubated for 3–4 h, where after the Caco-2 cells were washed (3×) with HBSS (Life Technologies) and refreshed with DMEM medium (Life Technologies) + 10% FBS (Life Technologies) + 1× NEAA (life Technologies) and 250 μg/ml gentamicin (Becton Dickinson, Breda, The Netherlands), an antibiotic concentration found to efficiently kill the used *Campylobacter* species. After 2 h of exposure to gentamicin (Becton Dickinson), the protocol was used as previously described to visualize the intracellular bacteria [31]. To measure toxicity effects of the milk, the Caco-2 cells were incubated for 4 h with each milk product, then washed three times with HBSS (Life Technologies) and incubated for 5–10 min with 0.4% Trypan blue (Life Technologies) and analyzed under the microscope for cellular uptake of Trypan blue.

Statistical analysis

Statistical analysis was performed using Instat software (version 5; GraphPad Software, San Diego (CA), United

States of America). Invasiveness was expressed as the mean number ± the standard error of the mean in CFU per milliliter retrieved from the infected cell line. The Kruskal–Wallis one way ANOVA test that included the Dunn's multiple comparison test was used to calculate statistical differences between untreated versus pasteurized milk or formulated milk or soy drink treated data sets after the gentamicin exclusion assay; a $p < 0.05$ indicated statistical significance.

Results

Although raw milk is a well-recognized source of bacterial contaminants affecting the gastrointestinal tract and beyond, the effect of pasteurized milk on the invasion capacity of gastrointestinal pathogens is poorly studied, and no information for *Campylobacter* species is currently available. In the present study, we analyzed whether pasteurized milk would affect the invasion of *Campylobacter* species into the Caco-2 intestinal epithelial cells and found that pasteurized cow milk significantly reduced the invasion of *C. coli* and *C. fetus*, whereas the effect of pasteurized cow milk on *C. jejuni* and *C. lari* invasion was neglectable *(Table 1A)*.

Compared to pasteurized cow milk, pasteurized goat milk had a more pronounced effect on *Campylobacter* invasion. Whereas the *C. jejuni* strain was found to be significantly enhanced in invasion when exposed to pasteurized goat milk, the opposite was observed for the *C. coli* and *C. fetus* strains; both were significantly reduced in their ability to invade *(Table 1B)*. Pasteurized horse milk is not available in The Netherlands, so we therefore mimicked the pasteurization process in the laboratory for raw horse milk. After exposing all the *Campylobacter* species to the laboratory-prepared pasteurized horse milk, we observed a significant reduction in invasion into the Caco-2 intestinal epithelial cells for all *Campylobacter* species except *C. lari (Table 1C)*.

To exclude possible toxic effects of the milks when added directly onto the epithelial cells, we tested whether the cow, goat, and horse milk were toxic for the Caco-2 intestinal epithelial cells by analyzing the uptake of Trypan blue. In all cases, no enhanced Trypan blue uptake was detected in the Caco-2 cells, indicating that toxicity was not an issue. This is supported by the observation that some *Campylobacter* species invaded the Caco-2 cells in enhanced numbers when exposed to milk products *(Table 1A–1C)*.

Direct toxicity of the milks on *Campylobacter* species was also analyzed. After overnight incubation of the most common *Campylobacter* species (*C. jejuni* and *C. coli*) isolated from Campylobacteriosis patients, no toxic effect was observed when these species were exposed to cow and goat milk *(Fig. 1a)*. In contrast, in the same experiment, horse milk had a slight but not significant toxic effect on

Table 1. Cow, goat, or horse milk affects *Campylobacter* invasion

A) Strain	Untreated[a]	Cow milk CM0[b]	Cow milk CM1[c]	Cow milk CM2[d]	*p* value
GB11 *(C. jejuni)*	361,429 ± 57,463	291,143 ± 38,695	329,857 ± 59,386	224,286 ± 31,309	Not significant
B18389 *(C. coli)*	323,200 ± 46,414	52,200 ± 12,496*	18,6400 ± 84,350	210,000 ± 28,142	*$p = 0.0055$
B36094 *(C. fetus)*	13,000 ± 2,176	3,020 ± 1,018*	7,000 ± 1,957	14,960 ± 3,460	*$p = 0.0077$
35221 *(C. lari)*	2,840 ± 349	5,520 ± 1,163	4,460 ± 1,634	2,640 ± 331	Not significant
B) Strain	**Untreated**	**Goat milk GM0[a]**	**Goat milk GM1[b]**	**Goat milk GM2[c]**	***p* value**
GB11 *(C. jejuni)*	225,714 ± 22,161	668,000 ± 200,219*	294,857 ± 57,497	216,143 ± 26,425	*$p = 0.0387$
B18389 *(C. coli)*	695,714 ± 204,321	103,571 ± 18,145*	362,714 ± 131,920	423,286 ± 140,877	*$p = 0.0034$
B36094 *(C. fetus)*	8,160 ± 987	2,480 ± 545*	4,320 ± 821	7,020 ± 1,485	*$p = 0.0119$
35221 *(C. lari)*	2,100 ± 123	4,200 ± 768	3,280 ± 611	1,680 ± 292	Not significant
C) Strain	**Untreated**	**Horse milk HM0[a]**	**Horse milk HM1[b]**	**Horse milk HM2[c]**	***p* value**
GB11 *(C. jejuni)*	233,333 ± 21,012	21,333 ± 2,539*	88,333 ± 6,741	183,333 ± 26,866	*$p = 0.0002$
B18389 *(C. coli)*	237,833 ± 28,517	46,833 ± 11,912*	173,333 ± 15,342	213,333 ± 13,920	*$p = 0.0013$
B36094 *(C. fetus)*	16,317 ± 3,775	2,783 ± 593*	9,033 ± 2,124	14,983 ± 2,334	*$p = 0.0041$
35221 *(C. lari)*	1,617 ± 596	767 ± 145	1,400 ± 383	1,667 ± 552	Not significant

[a,b,c,d] See *Materials and Methods* for the experimental details. Numbers show the colony forming units per milliliter ± standard error of the mean. Experiments for each condition and each *Campylobacter* species were independently repeated five times.
*Shows statistical differences using the Kruskal–Wallis one way ANOVA test that included the Dunn's multiple comparison test

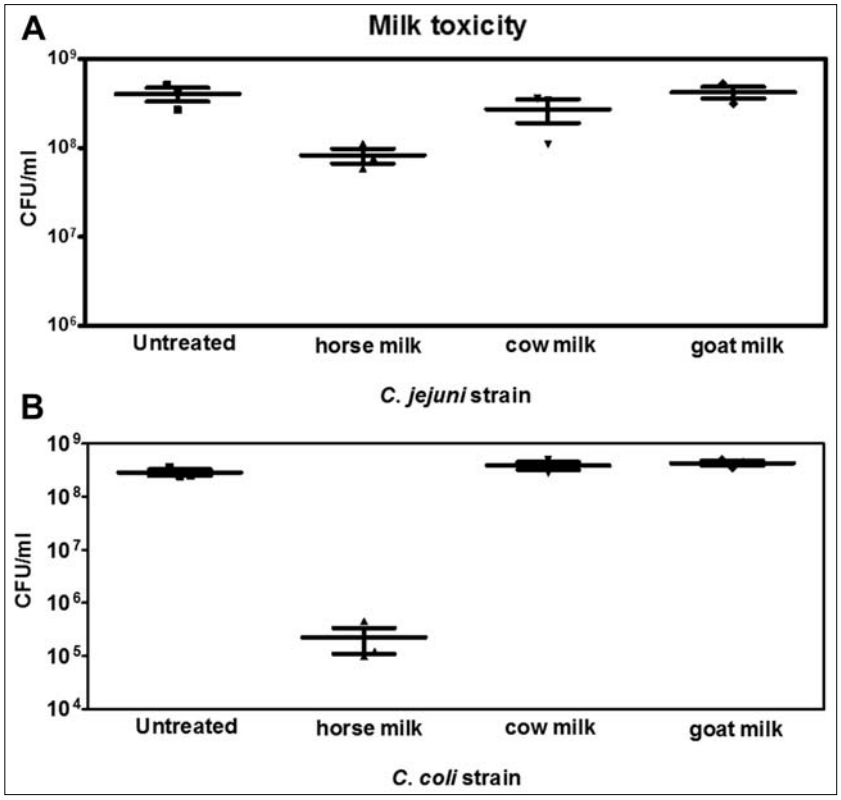

Fig. 1. Horse milk is toxic for *C. coli*. Milk susceptibility of A) *C. jejuni* or B) *C. coli* to horse, cow, or goat milk determined by incubating overnight at 37 °C in a microaerophilic environment 1.0 × 10^9 CFU *Campylobacter* bacteria in 1 ml DMEM medium + 10% FBS + 1× NEAA–milk (1:1) ratio. Scatter plot shows the mean and standard error of the mean of three independent experiments

C. jejuni, whereas a toxic effect on *C. coli* was more pronounced *(Fig. 1b)*.

Since the milk products had significant effects on the invasion of *Campylobacter* species, we further examined whether the cause of this effect should be sought in a protein-based (heat sensitive) or more sugar/lipid-based (heat insensitive) mechanism. After heat inactivating the milk (cow, goat, and horse) for 15 min at 85 °C, we observed for *C. jejuni* and *C. coli* that, depending on the milk and the strain used, the effect on invasion was protein *(C. jejuni)* or sugar/lipid-dependent *(C. coli) (Table 2)*.

We further analyzed whether the effect the cow, goat, or horse milk caused on the invasion of *Campylobacter* species was mammalian milk-specific or could also be induced by other similar products, for example soy drink, which is a milk mimicking product directly generated from

Table 2. Heat inactivation of pasteurized milk reveals that for *C. jejuni* the invasion process is milk protein and for *C. coli* more carbohydrate/lipid dependent

Strain	Untreated[a]	Cow milk CM0[b]	Cow milk CM1[c]	Cow milk CM2[d]	*p* value
GB11 *(C. jejuni)*	226,833 ± 23,340	264,000 ± 20,265	263,000 ± 36,134	281,000 ± 66,586	Not significant
B18389 *(C. coli)*	248,333 ± 22,416	90,750 ± 7,587*	139,500 ± 9,674	185,500 ± 29,182	*$p = 0.0018$
		Goat milk GM0[b]	Goat milk GM1[c]	Goat milk GM2[d]	*p* value
GB11 *(C. jejuni)*		370,500 ± 42,898	258,500 ± 58,300	234,000 ± 26,192	Not significant
B18389 *(C. coli)*		36,750 ± 5,662*	71,500 ± 7,240**	178,500 ± 11,117	*$p = 0.0006$; **$p = 0.0108$
		Horse milk HM0[b]	Horse milk HM1[c]	Horse milk HM2[d]	*p* value
GB11 *(C. jejuni)*		207,500 ± 50,914	258,000 ± 56,868	196,000 ± 31,284	Not significant
B18389 *(C. coli)*		45,250 ± 4,151*	131,000 ± 15,351	229,000 ± 42,814	*$p = 0.0024$

[a,b,c,d] See *Materials and Methods* for the experimental details. The *p* values of this table need to be compared to the *p* values of *Table 1*. If the significant effect of the milk on the invasion is lost, then the invasion is dependent on milk proteins; when it remains, the effect on invasion is carbohydrate/lipid dependent. Numbers show the colony forming units per milliliter ± standard error of the mean. Experiments for each condition and each *Campylobacter* species were independently repeated five times.
[*,**] Shows statistical differences using the Kruskal–Wallis one way ANOVA test that included the Dunn's multiple comparison test

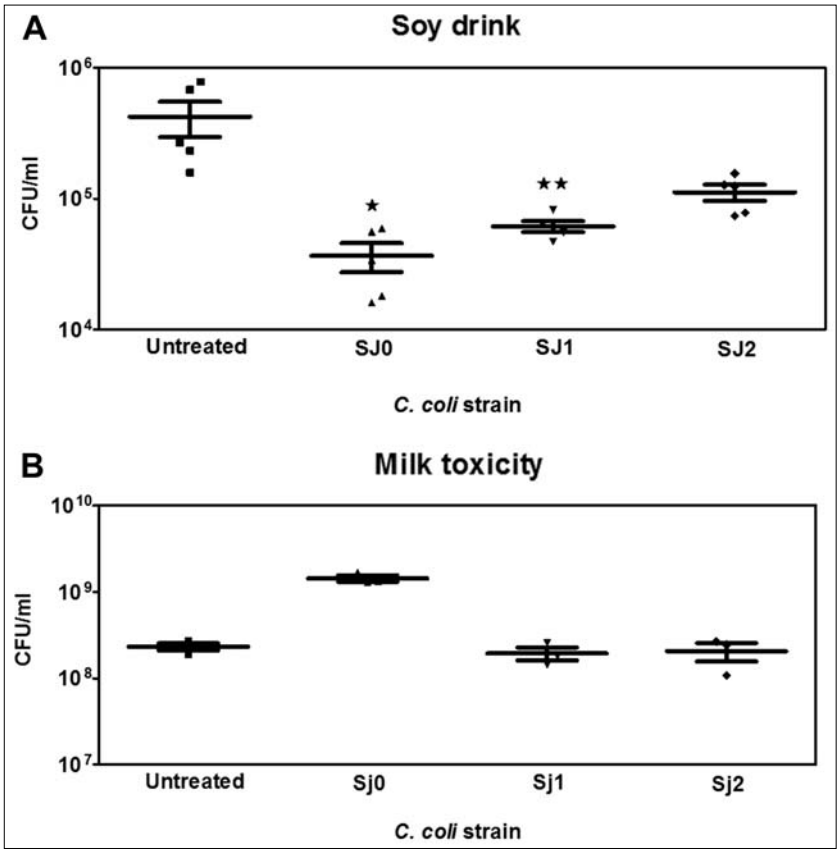

Fig. 2. Soy drink significantly reduces the invasion of *C. coli* into Caco-2 intestinal epithelial cells. A) Soy drink was analyzed on its ability to block the invasion of *Campylobacter* species and was found to significantly reduce the invasion of *C. coli* only at SJ0 and SJ1 ($p < 0.05$) using the Kruskal–Wallis Dunn's multiple comparison test. Scatter plot shows the mean and standard error of the mean of five independent experiments. B) Soy drink was not found to be toxic for *C. coli* after incubating overnight at 37 °C in a microaerophilic environment 1.0×10^9 CFU *C. coli* bacteria in 1 ml DMEM medium + 10% FBS + 1× NEAA–soy drink (1:1) ratio. Scatter plot shows the mean and standard error of the mean of three independent experiments

plants. In contrast to the cow, goat, or horse milk, the soy drink did not have an effect on the invasion of *Campylobacter* species analyzed in this study, with one exception. *C. coli* was significantly altered in invasion when exposed to soy drink *(Fig. 2a)*. After exposing the *C. coli* strain to soy drink overnight, we observed that the soy drink was not toxic for this *Campylobacter* strain *(Fig. 2b)*.

Industrially made milk-based formulas were also tested, and these specifically reduced the invasion of the *C. coli* and *C. fetus* strain into the Caco-2 cells (*Table 3A, 3B* and *3D*). In contrast, *C. jejuni* was only inhibited in invasion by instant formula milk NR 3 and the *C. lari* strain was not affected in its ability to invade the Caco-2 cells by any of the four formula milks tested *(Table 3A–D)*.

Discussion

To our knowledge, this is the first study showing that *Campylobacter* invasion into Caco-2 intestinal epithelial cells is modulated by pasteurized and formula milks. In literature, only one other study describes the effect of pasteurized milk on the virulence of a bacterial pathogen [32]. In this study, the virulence of *Listeria monocytogenes* was enhanced when this bacterial species was incubated in pasteurized milk at 4 °C [32]. For formula milk, only three studies have been performed in relation to the effect these types of milk have on *Escherichia coli* invasion into epithelial cells. In one study, it was shown that formula milk was less protective against *E. coli* invasion when compared to human breast milk [33], which was further confirmed in an *in vivo* study showing that specifically the human milk oligosaccharides are protective [34]. From the control experiments performed in this study related to the strains involved in Campylobacteriosis, it was revealed that, depending on the *Campylobacter* species analyzed, the effect on invasion could be related to protein *(C. jejuni)* or a more heat insensitive carbohydrate or lipid based effect *(C. coli)*. The predominant cow's milk oligosaccharide sialyllactose, however, had no effect on *Campylobacter* invasion (results not shown), suggesting a role of milk lipids in the case of *C. coli*.

Future studies are required to elucidate the exact mechanism on how the invasion of *Campylobacter* species is modulated by milk products. Overall, we propose that a prospective study is required to examine whether pasteurized and formula milk have clinically relevant effects on the prevention of Campylobacteriosis as was previously done for human milk oligosaccharides [35].

Table 3. Formula milk affects *Campylobacter* invasion

A) Strain	Untreated[a]	IF (NR1) 0[b]	IF (NR1) 1[c]	IF (NR1) 2[d]	*p* value
GB11 *(C. jejuni)*	209,231 ± 39,782	277,500 ± 108,503	385,000 ± 84,113	355,000 ± 83,317	Not significant
B18389 *(C. coli)*	986,778 ± 234,378	33,250 ± 947*	112,250 ± 30,877	720,000 ± 63,770	*$p = 0.0024$
B36094 *(C. fetus)*	52,010 ± 12,718	7,750 ± 1,539	11,350 ± 3,513	32,550 ± 9,045	Not significant
35221 *(C. lari)*	16,175 ± 4,586	8,200 ± 2,165	9,725 ± 2,438	8,475 ± 2,019	Not significant
B) Strain	Untreated[a]	IF (NR2) 0[b]	(NR2) 1[c]	(NR2) 2[d]	*p* value
GB11 *(C. jejuni)*	189,867 ± 31,976	950,00 ± 28,120	134,286 ± 31,151	117,429 ± 25,574	Not significant
B18389 *(C. coli)*	721,667 ± 78,418	77,667 ± 11,982*	220,000 ± 79,639	385,667 ± 133,610	*$p = 0.0006$
B36094 *(C. fetus)*	23,517 ± 3,805	5,717 ± 1,109*	9,617 ± 2,210	20,017 ± 4,722	*$p = 0.0123$
35221 *(C. lari)*	8,567 ± 4,066	8,667 ± 2,225	7,583 ± 2,857	7,050 ± 2,753	Not significant
C) Strain	Untreated[a]	(NR3) 0[b]	(NR3) 1[c]	(NR3) 2[d]	*p* value
GB11 *(C. jejuni)*	189,286 ± 34,201	45,800 ± 11,517*	174,000 ± 38,026	164,000 ± 34,728	*$p = 0.0136$
B18389 *(C. coli)*	455,000 ± 288,474	51,000 ± 28,572*	195,000 ± 112,222	244,000 ± 138,672	*$p = 0.0045$
B36094 *(C. fetus)*	27,000 ± 14,107	4,575 ± 1,799	12,850 ± 6,182	32,700 ± 15,605	Not significant
35221 *(C. lari)*	4,900 ± 829	1,825 ± 875	4,975 ± 1,276	6,075 ± 1,572	Not significant
D) Strain	Untreated[a]	(NR4) 0[b]	(NR4) 1[c]	(NR4) 2[d]	*p* value
GB11 *(C. jejuni)*	208,000 ± 67,186	245,600 ± 102,167	246,800 ± 136,111	187,200 ± 45,911	Not significant
B18389 *(C. coli)*	640,200 ± 308,015	338,400 ± 245,829	340,400 ± 267,671	352,200 ± 214,988	Not significant
B36094 *(C. fetus)*	29,350 ± 3,427	5,100 ± 1,300*	15,967 ± 4,013	24,883 ± 7,420	*$p = 0.0024$
35221 *(C. lari)*	26,220 ± 5,905	20,400 ± 3,655	22,400 ± 5,776	17,880 ± 4,744	Not significant

[a,b,c,d] See *Materials and Methods* for the experimental details. Numbers show the colony forming units per milliliter ± standard error of the mean. Experiments for each condition and each *Campylobacter* species were independently repeated five times.
*Shows statistical differences using the Kruskal–Wallis one way ANOVA test that included the Dunn's multiple comparison test

Conclusion

The present study is the first to show that pasteurized and formula milk are able to modulate the invasion of *Campylobacter* species into human intestinal epithelial cells.

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