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Probiotics, Galacto-oligosaccharides, and zinc antagonize biological effects of enterohaemorrhagic *Escherichia coli* on cultured cells and brine shrimp model

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ABSTRACT

The effect of GOS, Zn, and two probiotic strains (*Lactobacillus plantarum* CIDCA 83114 and *Lactobacillus kefiri* CIDCA 8348) against EHEC biological effects was studied using Vero cells and *Artemia* nauplii to evaluate its toxicity, and Caco 2 cells, to evaluate its adherence.

GOS, Zn, and GOS-Zn decreased the cytotoxic effect of EHEC supernatants on Vero cells. The addition of *L. plantarum* CIDCA 83114 and *L kefiri* CIDCA 8348 did not have a significant protective effect against EHEC supernatants. Only one negative interaction between *L. kefiri* CIDCA 8348 and Zn was observed on Vero cells. In the case of *Artemia* nauplii, an increase in survival was also observed when GOS and GOS-Zn were added.

Zinc was the most effective element to inhibit EHEC adherence to Caco cells. *L. plantarum* CIDCA 83114 and *L. kefiri* CIDCA 8348 were not able to reduce significantly *per se* the adherence of the pathogen to Caco-2/TC7 cells.

The beneficial effect of probiotics, GOS, and Zn against EHEC biological toxicity supports their incorporation into functional foods.

1. Introduction

Functional foods can be defined as edible products claiming potentially positive effects on health beyond basic nutrition. Some functional foods are obtained by adding a particular functional ingredient, such as foods containing probiotic microorganisms or prebiotics (*e.g.*, yogurt, fermented milks and Cheddar cheese) (Denkova, Goranov, Denkova, Teneva, & Kostov, 2016; Gibson et al., 2017; Stanton, Ross, Fitzgerald, & van Sinderen, 2005).

Probiotics are live microorganisms which when administered in adequate amounts confer a health benefit on the host (Reid, 2016). It is known that certain probiotic bacteria have positive impact on gut health by stimulation of the immune response, promoting anti-inflammatory effects or protection against enteric pathogens such as *Salmonella, Shigella, Proteus* and *E. coli* (Johnson-Henry, K C Donato, Shen-Tu, G Gordanpour, & Sherman, 2008; Stober, Maier, & Schmidt, 2010; Denkova, Yanakieva, Denkova, & Mancheva, 2013; Zeng et al., 2017). Enterohaemorrhagic *Escherichia coli* (EHEC) is a food-borne zoonotic pathogen, responsible for outbreaks of bloody diarrhea enterocolitis and hemolytic uremic syndrome. The hemolytic uremic syndrome is an acute renal failure complication associated with Shiga toxin (Nguyen & Sperandio, 2012). The main virulence compounds responsible for disease development are the proteins involved in bacterial adherence and the Shiga toxins. Previous works reported that lactobacilli isolated from kefir grains have the ability to antagonize EHEC infections by inhibiting its adherence to human intestinal cells and dismissing its biological damage resulting from the action of Shiga toxin on cultured cells (Hugo, Kakisu, De Antoni, & Pérez, 2008; Kakisu, Abraham, Farinati, Ibarra, & De Antoni, 2013).

Prebiotics are defined as substrates that are selectively used by host microorganisms, conferring health benefits (Gibson et al., 2017). Galacto-oligosaccharides (GOS) are well-known prebiotic compounds, with a great capacity to increase the number of bifidobacteria and lactobacilli in the gastrointestinal tract, after oral administration (Manderson et al., 2005; Roberfroid, 2007). GOS exhibit different beneficial host effects beyond their role as promoting agents of the growth of bifidobacteria and lactobacilli. Indeed, it has been demonstrated that GOS can increase calcium absorption and promote desirable immune modulation effects, such as an increase in IgA levels (Sangwan, Tomar, Ali, Singh, & Singh, 2015; Whisner et al., 2013).

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Moreover, GOS and other non-digestible carbohydrates exhibit anti-adhesive properties against some intestinal pathogens by mimicking the host receptor sites (Ebersbach, Andersen, Bergström, Hutkins, & Licht, 2012; Shoaf, Mulvey, Armstrong, & Hutkins, 2006).

In turn, zinc is one of the most important trace elements for human health and an essential component of many enzymes. It has a key role in the growth and cell differentiation of rapid turnover tissues, including the immune system and the gastrointestinal tract (Barnett, Hamer, & Meydani, 2011; Brandão-Neto, Stefan, Mendonca, Bloise, & Castro, 1995). Zinc supplements have been used to prevent and treat childhood diseases, such as acute diarrhea, gastroenteritis, pneumonia, and malaria (Black, 1998; Salvatore et al., 2007), providing strong evidence of the importance of this micronutrient. About 20% of the world population could be at risk of zinc deficiency; thus it is of great interest to fortify the food or add Zn as an ingredient in food supplements (Gharibzahedi & Jafari, 2017; Wessells & Brown, 2012).

The combined effect of zinc and probiotics has shown beneficial properties on the nutritional and immunologic status of humans, improving mineral absorption or increasing cellular immune response (Scholz-Ahrens et al., 2007; Shamir, Makhoul, Etzioni, & Shehadeh, 2005). In addition, the combined use of zinc and prebiotics has proved to be efficient in the treatment of acute diarrhea in children when administered as oral rehydration solutions (Passariello et al., 2011).

Due to the emergence of new knowledge about the benefits of using probiotics, prebiotics or zinc, their joint administration could be a good strategy for improving their health benefits (Scartoni et al., 2015; Yazar, Güven, & Dinleyici, 2016). However, most previous works have studied probiotics, prebiotics, and zinc as independent therapeutic agents, and on our best of knowledge, the interaction of zinc with probiotics and GOS (synergistic, antagonistic, or neutral effects) on the pathogenicity of *E. coli* EHEC has not been reported yet. Therefore, the aim of this study was to examine the effect of combining zinc, GOS, and two probiotic lactobacilli strains (*L. plantarum* CIDCA 83114 and *L. kefiri* CIDCA 8348) as inhibitors of the biological action of *E. coli* EHEC strain 69160 *in vitro*, using culture cell lines. In addition, *in vivo* studies were conducted using *Artemia salina* (brine shrimp) as an animal model.

2. Materials and methods

2.1. Prebiotic and zinc

A commercial syrup Vivinal® GOS (Friesland Foods, Zwolle, Holland) kindly donated by Friesland Foods Domo, was used in this study. Vivinal® GOS contained ≥44% (w/w) GOS of different degrees of polymerization (DP): 8% of high-molecular-weight oligosaccharides (DP ≥ 5); 10% of tetrasaccharides (DP4); 22% of trisaccharides (DP3); 38% of disaccharides (DP2) and lactose, and 23% of monosaccharides, including glucose and galactose without additives (Tymczyszyn, Gerbino, Illanes, & Gómez-Zavaglia, 2011). GOS were used at a final concentration of 2% (v/v) in the *in vitro* assays (Shoaf et al., 2006) and 0.5% (v/v) in the brine shrimp lethality test. One mmol/L $ZnSO_4.7H_2O$ (J.T. Baker, Mexico) was used in all the experiments (Passariello et al., 2011). The stock solutions of GOS (20% (v/v)) and zinc (50 mmol/L) were prepared in milliQ water, sterilized by filtration through a membrane of 0.22 μm and diluted in the eukaryotic culture medium or the *Artemia salina* medium (see sections 2.3, 2.4 and 2.7).

2.2. Bacterial strains and culture conditions

Lactobacillus plantarum CIDCA 83114 and *Lactobacillus kefiri* CIDCA 8348 isolated from kefir grains (Garrote, Abraham, & De Antoni, 2001) and selected for their probiotic properties (Bolla, Carasi, Serradell, & De Antoni, 2012; Carasi, Trejo, Pérez, De Antoni, & Serradell, 2012; Golowczyc, Mobili, Garrote, Abraham, & De Antoni, 2007; Hugo et al., 2008) were used in this study. *E. coli* O157:H7 strain 69160 was isolated from a clinical case at "Sor Maria Ludovica" hospital (La Plata, Argentina) and tested positive for Shiga toxin (Type II) stx 2, intimin (eae), and hemolysin (hly), as determined by a PCR assay (Hugo et al., 2008; Kakisu et al., 2013). Bacteria were maintained at −80 °C and each strain was cultured twice in liquid medium before the experiments. *L. plantarum* CIDCA 83114 and *L. kefiri* CIDCA 8348 were grown in MRS broth (de Man, Rogosa, & Sharpe, 1960) (Difco Laboratory, Detroit, MI, USA) at 37 °C for 24 h and at 30 °C for 48 h, respectively. *E. coli* was grown statically in trypticase soy broth at 37 °C for 16 h.

2.3. Cell culture conditions

Caco-2/TC7 and Vero cells were grown in Dulbecco's Modified Eagle's Medium (DMEM; Microvet, Buenos Aires, Argentina) supplemented with 15% and 10% v/v, respectively, of inactivated fetal bovine serum (Natocor, Córdoba, Argentina), 1% (w/v) non-essential amino acids, and antibiotics. Cells were seeded at a concentration of 1.25×10^5 cells/well in 24-wells tissue culture plates. Plates were incubated at 37 °C in a humidified 5% CO_2 -95% air incubator. Monolayers were used at recent post-confluence.

2.4. Effect of E. coli supernatant on Vero cells

Crude supernatant of *E. coli* 69160 were prepared from filter-sterilized cultures and used on Vero cells at different dilutions in DMEM according to Hugo et al., 2008. Different dilutions of the spent culture supernatant (from 1/1000 to 1/10) were incubated for 48 h on Vero cell monolayers to study the cytotoxic effect of Shiga toxin. Addition of GOS (2% w/v), $ZnSO_4$ (1 mmol/L), or lactobacilli suspensions (10^7 CFU/mL), as well as the combinations Zn-GOS, Zn-lactobacilli, GOS-lactobacilli, or Zn-GOS-lactobacilli were measured for their inhibitory effects. Chloramphenicol (100 μg/mL) was added to inhibit the growth of the lactobacilli during the experiments. Cell damage was measured by microscopic observation and the extracellular lactate dehydrogenase (LDH) activity.

2.5. LDH assay

The effect of EHEC supernatant on cell permeability was also measured by the lactate dehydrogenase assay. LDH activity was measured in the cell-free supernatant using a commercial kit (Wiener Laboratory, Rosario, Argentina). The method is based on the following reaction scheme:

$$\text{L}-\text{ Lactate} + \text{NAD}^+ \xrightarrow{\text{LDH}} \text{Pyruvate} + \text{NADH} + \text{H}^+$$

The rate of NADH formation is proportional to the LDH catalytic activity and is determined by registering the absorbance at 340 nm. The lactate dehydrogenase activity in the supernatant of the treated cells was calculated as the percentage of the maximum cytotoxic effect obtained by EHEC supernatant. The effect of lactobacilli, GOS, and zinc without supernatant was measured as a negative control.

The cytotoxic dose-response effect was fitted according to equation (1):

$$\%\ \text{Cytoxicity} = \text{Cytotoxicity}_{max}/(1 + 10^{(\text{LogEC50} - [\text{SN}]).\beta}) \quad \text{Equation 1}$$

where Cytotocity_{max} is the maximum cytotoxic effect, LogEC50 is the logarithm of the dose producing 50% of the cytotoxic effect, [SN] is the concentration of supernatant expressed as (1/dilution) $\times 10^3$, and β is the slope of the plot. The concentration of toxin was expressed as 1/dilution $\times 10^3$ assayed from 1 to 100, where the lowest value corresponds to dilution 1/1000 (highest dilution) and the highest value to dilution 1/10.

2.6. Optical microscopy

Vero cells were cultured on round glass coverslips (Assistent,

Glaswaren fabric KarlHetch KG, Sondheim, Germany). After 48 h incubation with the EHEC supernatant, the monolayers were washed with PBS, fixed with paraformaldehide 1% (w/v) and stained with May-Grunwald Giemsa. Coverslips were mounted and observed using a Leica optical microscope (Leica DM500, Wetzlar, Germany) equipped with a digital camera.

2.7. Brine shrimp lethality test

The toxicity of the filtered *E. coli* EHEC supernatant was tested at different dilutions using *Artemia salina* as an animal model. Artemia, a brine shrimp, was purchased as dormant eggs (cysts) (Aquapex-Artemia Pro® Orniex Portugal). The cysts were hatched in artificial seawater, prepared by dissolving a commercial marine salt (2% w/v) in distilled water and incubating with air bubbling at 28 °C–30 °C for 36 h with a proper light source. Then, the cysts were hatched and grown to become nauplii, which were used for toxicity experiments. For this purpose, nauplii were transferred into a 24-well culture plate containing 2.5 mL of artificial seawater with antibiotics (Anti-anti™, Gibco, USA) to avoid contamination. Different dilutions of EHEC supernatant, either alone or combined with GOS, Zn, or GOS-Zn, were added to each well and incubated for further 24 h. Ten nauplii were used in each well and three replications were used for each condition. The final concentrations of GOS and Zn in these sets of experiments were 0.5% v/v and 1 mmol/L, respectively. A series of control tests were run in parallel, containing different dilutions of culture broth with or without GOS, Zn, and GOS-Zn. After 24 h, nauplii survivors were counted using a dissection microscope, and the percentage of the survival of each dose was calculated.

2.8. Association of EHEC with Caco-2/TC7 cells

Bacteria cultures were centrifuged (5000xg, 5 min) and pellets were suspended in DMEM with no antibiotics and no fetal bovine serum. The lactobacilli-*E. coli* ratio used was 1:1 (1×10^7 CFU/mL). Monolayers of Caco-2/TC7 cells were washed twice with phosphate buffer saline and then pre-incubated for 1 h with the lactobacilli suspensions, GOS (2% w/v), $ZnSO_4$ (1 mmol/L), and the combinations Zn-GOS, Zn-lactobacilli, GOS-lactobacilli, or Zn-GOS-lactobacilli. Afterward, *E. coli* suspensions were added and further incubated at 37 °C for 1 h. After incubation, the monolayers were washed three times with phosphate buffer saline and lysed with sterile distilled water containing 0.1% v/v of Triton X100 at 37 °C for 30 min. Cell suspensions were mechanically disrupted by 6 passages using a syringe with a 22 G needle. The number of associated bacteria was determined by plating appropriate dilutions on nutrient agar. The association of *E. coli* alone was measured as a control.

2.9. Statistical analysis

All experiments were carried out on duplicate samples using three independent cultures of bacteria. The relative differences were reproducible independently of the cultures used. The non-linear fit of Log Dose response from the program GraphPad Prism 5 was used (GraphPad Software Inc., San Diego, CA, 2007). Analysis of variance (ANOVA) was carried out using the statistical program STATISTIX 8 Software (Analytical Software, Tallahassee, FL, USA). Means were compared by Dunnett's test for multiple comparisons and the difference was considered significant when $p < 0.05$.

3. Results

Shiga toxins produce a non-reversible cytopathic effect on Vero cells due to the adherence of the specific receptor to the Shiga toxin (globotriaosylceramide Gb3Cer) (Karmali, Gannon, & Sargeant, 2010). The Shiga toxin is known to act in target cells by inhibiting the protein synthesis, thus leading to the cell death by apoptosis or pyroptosis mechanisms (Lee et al., 2016; Platnich et al., 2018). To quantify the loss of cell viability in Vero cells treated with EHEC supernatant, the release of LDH was determined (Fig. 1). Lactate dehydrogenase is a cytoplasmic enzyme present in all living cells. Its presence in the cell culture supernatant is an indication of cell lysis. A sigmoidal fitting dose response behavior was observed between the supernatant dilution and the cell damage (LDH release) (Fig. 1). The more diluted the supernatant (expressed as concentration from 1/dilution $\times 10^3$), the lower their cytotoxicity. The presence of GOS, Zn, and GOS-Zn in the medium led to a dramatic increase in the EC50, indicating a competitive effect (Fig. 1 and Table 1). After the analysis of the dose-response curves of the LDH assay, the 1/50 dilution of the EHEC supernatant was selected for further *in vitro* experiments. At this supernatant dilution, the differences among treatments were maximum.

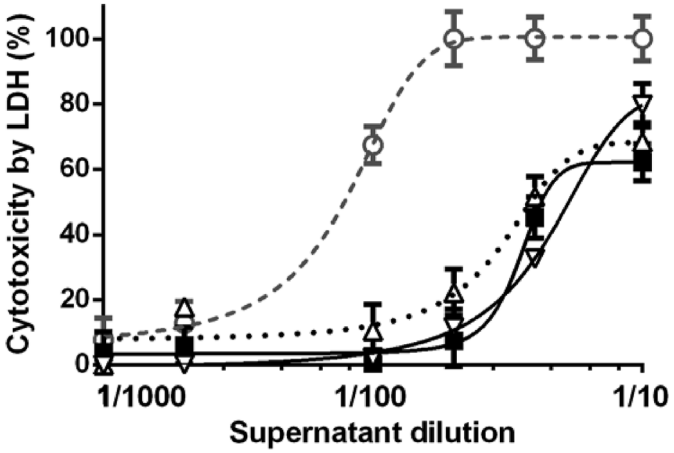

Fig. 1. Cytotoxic effect of EHEC supernatant on Vero cell. Cells were incubated with different dilutions of EHEC supernatant during 48 h and the cytotoxic effect was measured by the PI uptake (A) and the LDH assay (B). Vero Cells + EHEC supernatant (○), cells in the presence of GOS (■), cells in the presence of Zn (△), and cells in the presence of GOS-Zn (▽). Solid and dashed lines correspond to the dose-response fitting according to equation (1).

In addition, the microscopic action of EHEC supernatant containing Shiga toxin was measured on Vero monolayers in the presence or absence of GOS and Zn (Fig. 2). When the cells were incubated in presence of supernatant of EHEC cultures, a noticeable detachment of the monolayers together with morphological changes (rounded cells instead of the typical spindle shape of Vero cells) was observed (Fig. 2 B), thus denoting great damage on Vero cells. When GOS, Zn, or both compounds were added, the cytotoxic effect decreased (Fig. 2C–E).

Then, the effect of adding probiotic *L. plantarum* CIDCA 83114 and *L. kefiri* CIDCA 8348 was investigated, together with GOS, Zn, and GOS-Zn, on Vero cells exposed to EHEC supernatant (Fig. 3). *L. plantarum* CIDCA 83114 and *L kefiri* CIDCA 8348 did not have a significant protective effect against EHEC supernatant. The incorporation of *L. plantarum* CIDCA 83114 to GOS, Zn, or GOS-Zn did not modify the protection effect observed in the absence of lactobacilli (Fig. 3). As observed in *L. plantarum* CIDCA 83114, the incorporation of *L kefiri* CIDCA 8348 to GOS and GOS-Zn showed no additional protective effect. Surprisingly, the combination of Zn and *L. kefiri* CIDCA 8348 had a negative effect, enhancing Vero cell damage. However, this antagonistic effect disappeared when Zn was incorporated together with GOS (GOS-Zn).

To evaluate the toxic effect of EHEC supernatant *in vivo*, a brine shrimp model was used (Fig. 4). EHEC supernatant were toxic for *Artemia* nauplii, causing the loss of their viability in a dose-dependent manner. The incubation with the GOS and GOS-Zn combination in the presence of EHEC supernatant showed a protective effect, increasing the survival of nauplii. Indeed, EHEC supernatant showed an EC50 of

Table 1
Maximum cytotoxic effect of EHEC supernatant on Vero cells.

Parameters	EHEC supernatant	GOS	Zn	GOS-Zn
Maximum Cytotoxicity (%)	100.00 ± 0.37(a)	61.75 ± 1.45(c)	67.90 ± 3.90(c)	82.50 ± 1.18(b)
EC50	7.87 ± 0.75(c)	35.06 ± 1.36(b)	29.95 ± 5.97(b)	43.84 ± 1.45(a)
B	0.147 ± 0.030	0.081 ± 0.029	0.045 ± 0.029	0.025 ± 0.005
R^2	0.9997	0.9936	0.9544	0.9993
Dilution corresponding of EC50	1/127	1/28.5	1/33.4	1/22.8

EC50: Logarithm of the dose producing 50% of cytotoxic effect.
β: Hill slope (of the fitting parameters from Fig. 1, obtained using Equation (1)).
Different letters represent significantly different media values ($p < 0.05$).

0.15, and this value was twice as high in the presence of GOS and GOS-Zn (Table 2). The increase in the EC50 value showed the same competitive effect of GOS and GOS-Zn on EHEC supernatant observed in the *in vitro* assays (Tables 1 and 2). The *in vitro* assays showed a similar protective effect of GOS, Zn, and GOS-Zn against the cell damage induced by EHEC supernatant. However, the protective effect of Zn was not observed using the brine shrimp model.

The action of GOS, Zn, and GOS-Zn on the adherence of EHEC to Caco-2/TC7 cells was also studied (Fig. 5). In the absence of probiotic lactobacilli, the presence of Zn or GOS-Zn significantly reduced the adherence of EHEC to Caco-2/TC7 cells. Indeed, in the presence of Zn, there was a 2.5 log reduction in EHEC adherence with respect to the control whereas in the presence of GOS, there was a ~1 log reduction but it was not significant.

Fig. 5 also depicts the adherence of EHEC to Caco-2/TC7 in the presence of probiotic *L. plantarum* CIDCA 83114 and *L. kefiri* CIDCA 8348 strains. *L. plantarum* CIDCA 83114 and *L. kefiri* CIDCA 8348 were not able to reduce significantly *per se* the adherence of the pathogen to

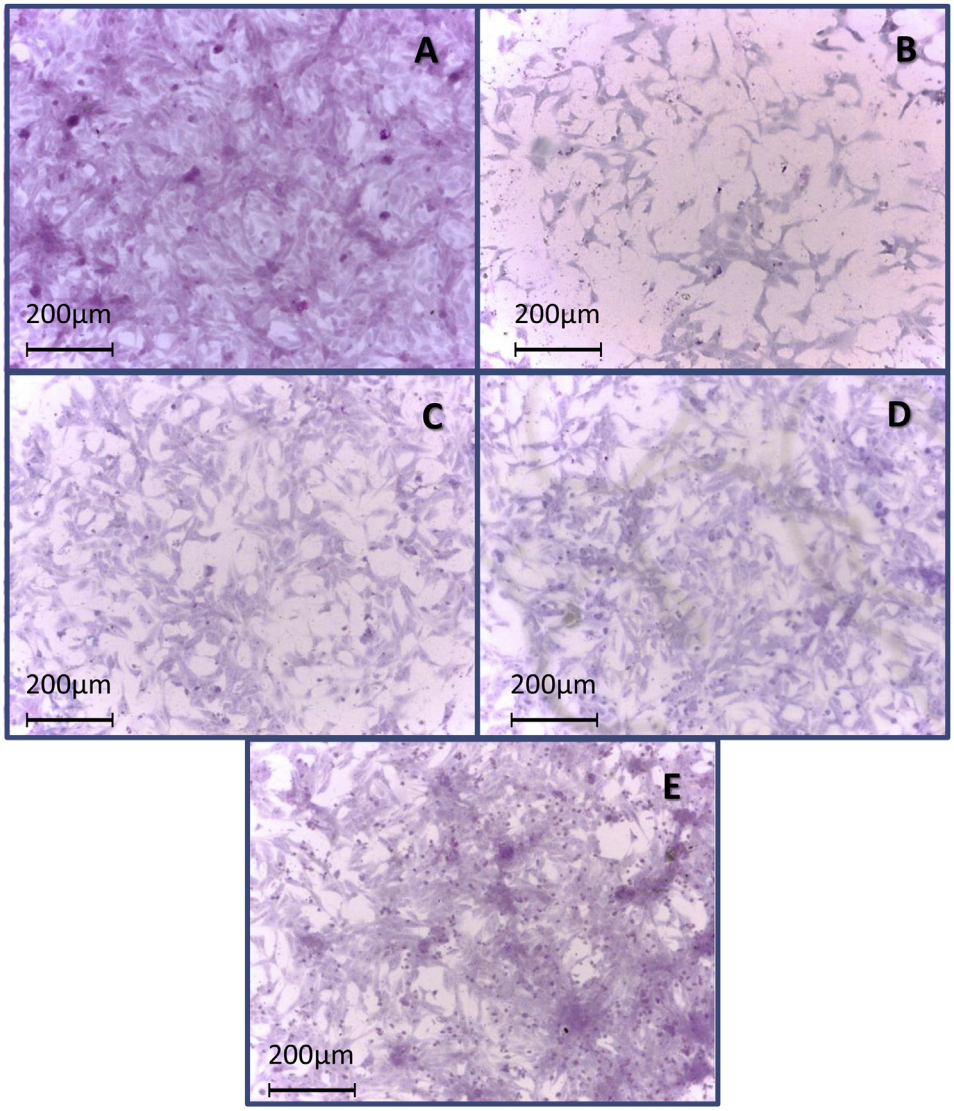

Fig. 2. Microscopic image of Vero cells incubated with EHEC supernatant containing Shiga toxin (dilution 1/50) at 37 °C for 48 h. (A) Control cells (incubated with DMEM), (B) cells incubated with EHEC supernatant (containing Shiga toxin), (C) cells incubated with GOS and EHEC supernatant, (D) cells incubated with Zn and EHEC supernatant, (E) cells incubated with GOS-Zn and EHEC supernatant.

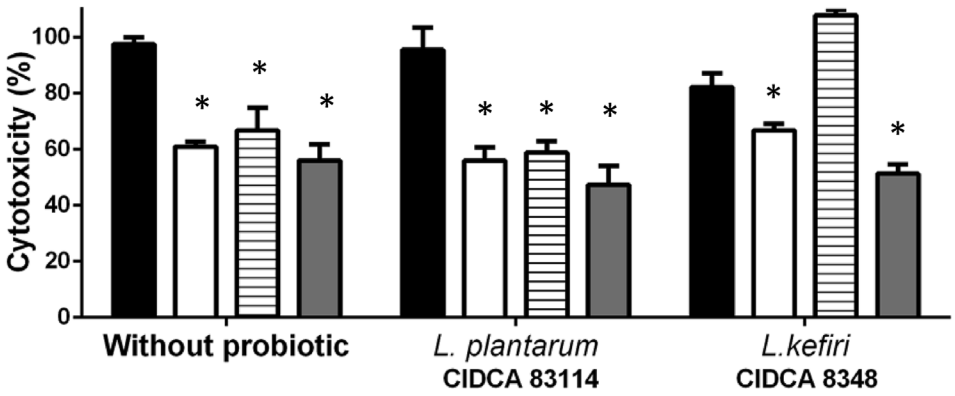

Fig. 3. Cytotoxic effect (%) of EHEC supernatant (dilution 1/50) on Vero cells, in the presence of GOS (white bars), Zn (striped white bars), GOS-Zn (gray bars), and Control (black bars), in the absence or presence of *L. plantarum* CIDCA 83114 and *L. kefiri* CIDCA 8348. Data are shown as mean ± SD. One-way ANOVA followed by a Dunnett's test were used for multiple comparisons. (*) denote statistically significant differences with respect to the control (without lactobacilli, GOS and Zn) ($p < 0.05$).

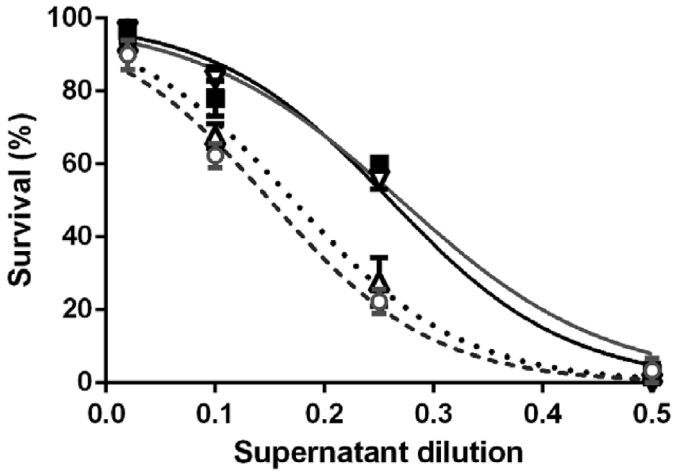

Fig. 4. Cytotoxic effect of EHEC supernatant on *Artemia salina*. The percentage of survival of *Artemia* nauplii after the incubation with different dilutions of EHEC supernatant during 24 h was represented. *Artemia* nauplii + EHEC supernatant (○), *Artemia* nauplii + EHEC supernatant in the presence of GOS (■), *Artemia* nauplii + EHEC supernatant in the presence of Zn (△), and *Artemia* nauplii + EHEC supernatant in the presence of GOS-Zn (▽). Solid and dashed lines correspond to the dose-response fitting according to equation (1).

Caco-2/TC7 cells. The addition of lactobacilli strains together with GOS or GOS-Zn had a neutral effect on EHEC adherence with respect to GOS or GOS-Zn alone. The addition of lactobacilli strains together with Zn showed a slight increase in EHEC adherence with respect to Zn alone (Fig. 5). As shown in Fig. 3, when GOS-Zn and *L. kefiri* were added together, this negative effect disappeared, and adherence decreased to values similar to Zn alone.

4. Discussion

Although the physiopathological mechanism of the genesis of diarrhea and the supra-intestinal clinical manifestations of *E. coli* EHEC infection have been extensively studied, the antagonistic effect of probiotics and prebiotics is not completely known yet (Olano-Martin, Williams, Gibson, & Rastall, 2003; Rolfe, 2000; Walsham et al., 2016). Bacterial adherence and Shiga toxin production are the main processes involved in EHEC pathogenesis. This study investigated the capacity of GOS, Zn, probiotics, and their combinations to prevent the deleterious effects of EHEC supernatant (due to the presence of Shiga toxins) and inhibit the adherence of EHEC to intestinal cells.

In the *in vitro* models using Vero culture cells, GOS, and Zn were shown to play an essential role in reducing the cytotoxic action of EHEC supernatant (Figs. 1–3). It was reported that Zn is able to increase the trans-epithelial electric resistance of enterocyte monolayers infected with EHEC and reduce the translocation of Shiga toxin through them (Crane, Broome, Reddinger, & Werth, 2014). It is also known that, in growth conditions, Zn can specifically inhibit the expression of Shiga toxin by blocking the regulation of its production. In addition, Zn is more potent and efficient than other metals such as iron, manganese, and nickel in reducing the production of Shiga toxin (Crane et al., 2014; Uemura, Katsuge, Sasaki, & Goto, 2017). Taking into account that our experiments were done with EHEC supernatant (Figs. 1–3), the protective effect of Zn could have occurred in the interaction between the Shiga toxin and Vero cells rather than in the production of the toxin.

In the case of GOS, no studies about their capacity to dismiss Shiga toxin effects have been reported. The mechanism of action could be related to the interaction of the galactose units of GOS with the Shiga toxin. In fact, the globotriaosylceramide-3 receptor of the Shiga toxin is a ceramide trihexose formed by the alpha linkage of galactose to lactosyl-ceramide (Nguyen & Sperandio, 2012). The galactose units of GOS may bind to part of the Shiga toxin present in the EHEC supernatant, avoiding its arrival at globotriosylceramide-3 receptors on the surface of eukaryotic cells. In addition, other studies showed that GOS fractions could mimic the ganglioside GM1 cellular receptor and interact with cholera toxin (Sinclair, de Slegte, Gibson, & Rastall, 2009).

LDH release (Fig. 1) suggests that zinc, GOS, and GOS-zinc protected Vero cells in a similar way. At higher dilution of supernatant, all of them acted as competitive inhibitors, thus significantly increasing the EC50 (Fig. 1 and Table 1). Additionally, at lower dilutions of supernatant, a significant decrease in the maximum toxicity was observed in all conditions, indicating that there could be alternative, non-competitive protection.

In this study, the protective capacity of two lactobacilli strains, *L.*

Table 2
Artemia salina survival after EHEC supernatant exposure.

Parameters	EHEC supernatant	GOS	Zn	GOS-Zn
EC50	0.15 ± 0.01 (b)	0.27 ± 0.03(a)	0.17 ± 0.01 (b)	0.26 ± 0.02 (a)
β	−5.74 ± 0.77	−4.63 ± 1.20	−5.76 ± 0.88	−5.38 ± 1.05
R^2	0.991	0.975	0.989	0.990

EC50: Logarithm of the dose producing 50% of cytotoxic effect.
β: Hill slope (of the fitting parameters from Fig. 4, obtained using Equation (1)).
Different letters represent significantly different media values ($p < 0.05$).

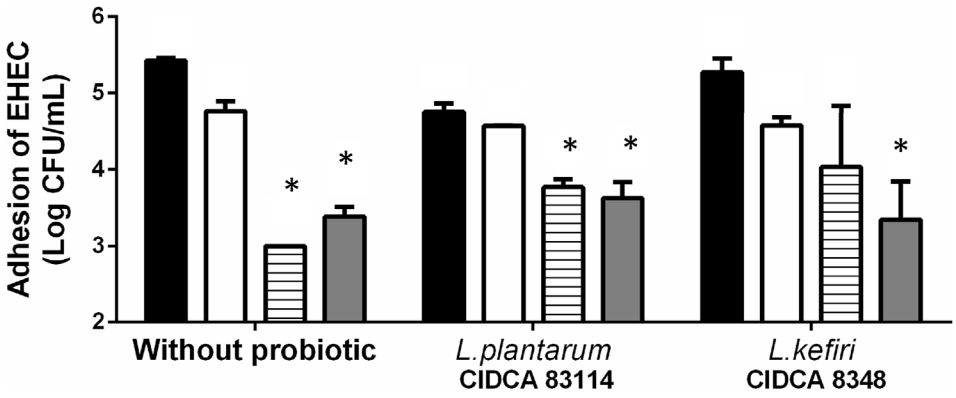

Fig. 5. Adherence of EHEC to Caco-2/TC7cells in the presence of GOS (white bars), Zn (striped white bars), GOS-Zn (gray bars), and Control (black bars), in the absence or presence of *L. plantarum* CIDCA 83114 and *L. kefiri* CIDCA 8348. Data are shown as mean ± SD. One-way ANOVA followed by a Dunnett's test were used for multiple comparisons. (*) denote statistically significant differences with respect to the control (without lactobacilli, GOS and Zn) ($p < 0.05$).

plantarum CIDCA 83114 and *L. kefiri* CIDCA 8348, against the Shiga toxin present in the EHEC supernatant (Fig. 3) was also measured. It has been reported that *L. plantarum* CIDCA 83114 protects Vero cells against Shiga toxin effects, and this protection was ascribed to the toxin biosorption on the cell wall (Kakisu et al., 2013). However, in this study, no significant protective effect was observed for *L. plantarum* CIDCA 83114, probably due to the higher concentration of Shiga toxin in the EHEC supernatant.

The interaction between lactobacilli, GOS, and Zn with Shiga toxin showed that in general no additional effect of lactobacilli was observed, except for *L. kefiri* CIDCA 8348 and Zn. In the presence of Zn, a negative interaction with *L. kefiri* CIDCA 8348 was shown (Fig. 3). This negative effect was also registered in the adherence experiments (Fig. 5). This negative effect can be ascribed to the biosorption of Zn to the surface of *L. kefiri* CIDCA 8348, with the concomitant reduction in the concentration of Zn in the medium. *L. kefiri* CIDCA 8348 is an aggregative strain covered by S-layer proteins, whose capacity to remove heavy metals from the growth media has been extensively reported (Gerbino, Carasi, Araujo-Andrade, Tymczyszyn, & Gómez-Zavaglia, 2015; Gerbino et al., 2012; Gerbino, Carasi, Mobili, Serradell, & Gómez; Zavaglia, 2015).

The cytotoxic effect of the EHEC supernatant using the brine shrimp assay was also measured in an *in vivo* model. The brine shrimp assay is a rapid (24 h), cost-effective, and simple animal model, used for evaluating the toxicity of a variety of compounds (Brix, Cardwell, & Adams, 2003; Rajabi, Ramazani, Hamidi, & Naji, 2015; Xu et al., 2015). To the best of our knowledge, this is the first time that *Artemia salina* has been used to evaluate the toxicity of EHEC supernatant, thus representing an important contribution of this study. Shiga toxins have a very specific receptor in human cells and the usual animal models that are susceptible to their action are mammals such as mice, rabbits, and pigs (Mohawk & O'Brien, 2011).

However, it was surprisingly observed that nauplii were susceptible to EHEC supernatant containing Shiga toxin. Indeed, nauplii decreased their viability in the presence of EHEC supernatant in a dose-dependent manner, and the incubation with GOS and GOS-Zn reduced their mortality at 0.27 and 0.26 supernatant dilutions, respectively (Fig. 4). It was recently reported that *Artemia* naupli was sensible to the toxic lectin MytiLec-1 from the Mediterranean mussel (*Mytilus galloprovincialis*) (Hasan et al., 2019). This lectin has a specific binding property to Gb3, the same receptor of Shiga toxins. It was shown that the lectin accumulates in the intestinal tract of nauplii larvae, thus indicating the presence of the Gb3 at this level. In this sense we hypothesize that the Shiga toxin contained in EHEC supernatant bound to the digestive tract of *Artemia* nauplii interrupting the functions of gut cells and the presence of GOS prevent these damage. The brine shrimp model confirmed the protective effect of GOS and GOS-Zn previously observed *in vitro*. The protective effect of Zn against EHEC supernatant was not seen *in* Brine Shrimp model probably due to high marine salts concentrations present in the *Artemia* culture.

Regarding the effects of GOS and Zn on EHEC adherence to intestinal cells, GOS were reported to avoid the adherence of enteropathogenic *E. coli* to Caco-2/TC7 and Hep-2 cells (Shoaf et al., 2006). It was shown that the chain length and/or the nature of sugar linkage are important factors because galactose itself has no effect on the reduction of EHEC adherence (Shoaf et al., 2006). In the conditions assayed, GOS were able to reduce the adherence of EHEC to Caco-2/TC7 cells to a low extent, when used alone or in combination with *L. plantarum* CIDCA 83114 or *L. kefiri* CIDCA 8348 (Fig. 5).

Furthermore, the addition of Zn led to a significant decrease in the adherence of EHEC to Caco-2/TC-7 cells, both alone and in combination with GOS. The presence of probiotic bacteria partially eliminated this effect (Fig. 5). Supraphysiologic concentrations of Zn (0.1–0.4 mmol/L) are known to have deleterious effects on EHEC and other enteric bacteria (Bratz et al., 2013; Zhang, P Carlson, R Schneider, & Duhamel, 2001). However, the viability of *E. coli* EHEC was not affected by GOS or Zn in the conditions assayed (data not shown). Crane, Byrd, and Boedeker (2011) reported an inhibiting effect of Zn on the adherence of EHEC to Hela cells. The interaction of *E. coli* EHEC with the enterocyte surface involves the adherence of intimin and more than 10 fimbrial or afimbrial adhesins (Mcwilliams & Torres, 2014). Zinc is known to inhibit the expression of certain EHEC adhesins such as EspA and EspB (Crane et al., 2011). In the system used, Zn probably acted at this level, by blocking the initial steps of the adherence.

The main result was that the combination of Zn and GOS (GOS-Zn) is capable of protecting the eukaryotic cell from EHEC adherence and the effect of EHEC supernatant (containing Shiga toxin) on their own. It is noteworthy that the amount of zinc needed for this protective effect is at supra-physiologic doses, meaning that zinc is acting in a drug-like fashion. The incorporation of probiotic bacteria to GOS, Zn, or GOS-Zn exhibited no additional effect with regard to the individual compounds. A negative interaction was observed only in the mixture of *L. kefiri* CIDCA 8348 and Zn, but this antagonistic effect was overcome by the combination GOS-Zn-probiotic. The *Artemia* toxic model confirmed *in vivo* the protective role of GOS and GOS-Zn against EHEC supernatant.

5. Conclusions

The *in vitro* and *in vivo* effects of Zn, GOS and GOS-Zn on EHEC supernatants have been thoroughly investigated. The cytotoxic assays with GOS, Zn and GOS-Zn led to increase the values of EC50 on Vero cells treated with EHEC supernatants. Neither *L. plantarum* CIDCA 83114 nor *L. kefiri* CIDCA 8348 have a significant protective effect against EHEC supernatants. The *in vivo Artemia* model showed an increased survival of nauplii when GOS and GOS-Zn were added. In addition, zinc was the most effective element to inhibit EHEC adherence to Caco cells, and none of the two probiotic strains were able to reduce significantly *per se* the adherence of EHEC to Caco-2/TC7 cells.

These results greatly support the formulation of functional foods containing probiotic bacteria with the addition of GOS and Zn. Besides

the well-known beneficial effects of probiotic bacteria, the incorporation of GOS and Zn could increase their health benefits by protecting the host against the pathogenic action of EHEC, inhibiting its adherence and dismissing its cytotoxic damage.

CRediT authorship contribution statement

Esteban Gerbino: Conceptualization, Funding acquisition, Data curation, Formal analysis, Investigation, Methodology, Supervision, Writing - original draft. **Florencia Ghibaudo:** Methodology, Investigation. **E. Elizabeth Tymczyszyn:** Conceptualization, Data curation, Formal analysis. **Andrea Gomez-Zavaglia:** Conceptualization, Visualization, Supervision, Writing - review & editing. **Ayelen A. Hugo:** Conceptualization, Investigation, Methodology, Data curation, Writing - original draft.

Declaration of competing interest

The authors declare that they have no competing interests.

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