Competitive inhibition of three novel bacteria isolated from faeces of breast milk-fed infants against selected enteropathogens

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Abstract

Numerous *in vitro* and *in vivo* studies conducted using different probiotic micro-organisms have demonstrated their ability to interfere with the growth and virulence of a variety of enteropathogens. The reported beneficial effects of the use of probiotics to complement antibiotic therapy or prevent diarrhoea or gastrointestinal infection in infants have increased in recent years. In the present study, we demonstrated the capacity of supernatants obtained from three novel probiotics (*Lactobacillus paracasei* CNCM I-4034, *Bifidobacterium breve* CNCM I-4035 and *Lactobacillus rhamnosus* CNCM I-4036) isolated from the faeces of breastfed infants to inhibit the growth of enterotoxigenic and enteropathogenic (EPEC) bacteria, such as *Escherichia coli*, *Salmonella* and *Shigella*. To assess their potential antimicrobial activity, the 17 and 24 h cell-free supernatants broth concentrates (10✕) having 1, 2 or 4 % of the three probiotics were incubated with EPEC bacteria strains. After 17 h of co-culture, the supernatants were able to inhibit the growth of *E. coli*, *Salmonella* and *Shigella* up to 40, 55 and 81 %, respectively. However, the inhibitory capacity of some supernatants was maintained or completely lost when the supernatants (pH 3·0) were neutralised (pH 6·5). Overall, these results demonstrated that *L. paracasei* CNCM I-4034, *B. breve* CNCM I-4035 and *L. rhamnosus* CNCM I-4036 produce compounds that exhibited strain-specific inhibition of enterobacteria and have the potential to be used as probiotics in functional foods.

Key words: Bacterial competition; Bifidobacterium breve; enteropathogens; Lactobacillus paracasei; Lactobacillus rhamnosus; probiotics.

Probiotics are defined as living micro-organisms that confer a health benefit to the host when administered in adequate amounts[1]. One of the most frequent health claims for probiotics concerns the putative reduction and prevention of infectious disease in the gastrointestinal tract. The regular intake of probiotic micro-organisms has been demonstrated to prevent several infectious diseases, allergic disorders, diarrhoea and inflammatory diseases, such as inflammatory bowel disease[2].

Bacteria are present in the food, water and environment, promoting gastrointestinal tract susceptibility to different types of infection, which produce a variety of illnesses in human subjects worldwide. *Escherichia coli* strains possess variable mechanisms of pathogenesis, while enterotoxigenic (ETEC) strains produce enterotoxin and enteropathogenic (EPEC) strains that adhere to epithelial cells; the latter type of infection remains a formidable cause of diarrhoeal illnesses. Zoonotic pathogens and those transmitted through faecal–oral contact, such as *Salmonella* and *Shigella*, respectively, constitute an important public health problem, especially in developing countries with substandard hygiene and unsafe water supplies. Antibiotics represent the first line of treatment, but the use of antibiotics causes an imbalance in the complex ecosystem of the human gastrointestinal tract. Thus, therapeutic alternatives to prevent or complement antibiotic therapy are currently being assessed. In this respect, the use of probiotics is a promising tool to prevent EPEC infections.

Several studies have demonstrated antimicrobial activity by lactobacilli and bifidobacteria against gastrointestinal microbial pathogens[3]. The mechanism underlying these effects of probiotics are largely unknown but are likely to be multifactorial, including the reduction of luminal pH, competition for nutritional sources, inhibition of adhesion to

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Abbreviations: EPEC, enteropathogenic; ETEC, enterotoxigenic.

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epithelial cells, stimulation of the host immune system and the production of organic acids, bacteriocin or bacteriocin-like substances[4].

Some probiotics produce metabolites that inhibit the growth of bacteria and fungi[5,6] and have been used to prevent intestinal pathogenic infections, such as those caused by *Salmonella*, *Shigella*, *E. coli*, *Listeria* and *Helicobacter pylori*[7–11]. Due to their reported health benefits and the large number of novel probiotic strains, they have become attractive candidates for incorporation into functional foods and food products.

Among all probiotic bacteria, those isolated from the faeces of exclusively breastfed infants are of special interest due to lactic acid bacteria predominance, and these strains also seem to provide protection against enteric and systemic disorders caused by bacterial pathogens[12,13].

Recently, we reported the selection and characterisation of three probiotic strains (*Lactobacillus paracasei* CNCM I-4034, *Bifidobacterium breve* CNCM I-4035 and *Lactobacillus rhamnosus* CNCM I-4036) isolated from the faeces of breastfed infants[11]. We demonstrated that these strains exhibited probiotic potential, survival in gastrointestinal conditions and adhesion to intestinal cells, and they were also innocuous to human health. Their safety status has been confirmed by sensitivity to antibiotics, toxicology, the absence of undesirable metabolites and acute ingestion studies in immunocompetent and immunosuppressed mice. Furthermore, antimicrobial activity against human rotavirus and *Listeria monocytogenes* has also been described for these probiotics.

The aim of the present study was to demonstrate the ability of *L. paracasei* CNCM I-4034, *B. breve* CNCM I-4035 and *L. rhamnosus* CNCM I-4036 to inhibit the growth of *E. coli* ETEC, *E. coli* EPEC, *Salmonella* and *Shigella*.

Materials and methods

Lactic acid bacteria strains from exclusively breastfed infants

Three novel strains of lactic acid bacteria were isolated from the faeces of breastfed infants, and strains that demonstrated adhesion to intestinal epithelial cells and resistance to gastrointestinal conditions were identified and denominated as *L. paracasei* CNCM I-4034, *B. breve* CNCM I-4035 and *L. rhamnosus* CNCM I-4036, according to a previously described protocol[11]. Briefly, twelve healthy, exclusively breastfed infants, aged 1 month, were selected for the study at the Clinic Hospital of the University of Granada. The present study was conducted according to the guidelines laid down in the Declaration of Helsinki, and all procedures involving human subjects were approved by the Ethical Committee of the University of Granada. Written informed consent was obtained from the parents after a careful explanation of the nature of the study.

Isolation of supernatants from lactic acid bacteria strains

To obtain probiotic bacteria supernatants with 1× or 10× concentrations, the bacterial strains were grown anaerobically for 17 or 24 h at 37°C in Man, Rogosa and Sharpe (lactobacilli) or Man, Rogosa and Sharpe plus cysteine (bifidobacteria) media, respectively.

The supernatants were obtained by centrifugation at 12 000 **g** for 10 min and were then neutralised to pH 6·5 using NaOH (1 M). To obtain the 10× supernatant, the neutralised 1× supernatant was concentrated by freeze-drying. The supernatants were sterilised by filtration through 0·22 μm pore-sized filters and stored at −20°C until further use.

Escherichia coli ETEC, Escherichia coli EPEC, Salmonella and Shigella strains and growth conditions

E. coli ETEC, *E. coli* EPEC, *Salmonella typhimurium*, *Salmonella typhi* and *Shigella sonnei* strains were obtained from the Spanish Type Culture Collection (CECT). We chose the following strains:

E. coli ETEC: CECT 501 and CECT 515; *E. coli* EPEC: CECT 727 and CECT 729; *S. typhimurium* CECT 443 and CECT 4594; *S. typhi*: CECT 725; and *S. sonnei*: CECT 457, CECT 4887[T] and CECT 413.

The *E. coli* and *Salmonella* strains were grown in tryptone soy broth and incubated aerobically for 24 h at 37°C. The *Shigella* strains were grown in nutrient broth and incubated aerobically for 24 h at 37°C.

Activity of lactic acid bacteria supernatants against pathogens

These assays were performed in polystyrene ninety-six-well (volume, 200 μl/well) plates (Maxisorp). Tryptone soy (*E. coli* and *Salmonella*) or nutrient (*Shigella*) broth was inoculated with a 5% (v/v) concentrated microbial cell solution and grown overnight (*E. coli* and *Salmonella*). The supernatants, with or without neutralisation, were added to a final concentration (v/v) of 1 and 4% (*E. coli*, *Salmonella* and *Shigella*) or just neutralised to 2 and 4% (*Shigella*). The ability of each strain to inhibit the pathogenic strains was evaluated by monitoring bacterial growth at 37°C in tryptone soy or nutrient medium in ninety-six-well plates according to the methods of Chenoll et al.[10]. Bacterial growth was analysed at 620 nm using a Multiskan microplate reader (Thermo Fisher Scientific). In each case, the percentage of resistance was calculated by comparing the final optical densities at 620 nm obtained with different concentrations of supernatants with those of the corresponding control samples.

Statistical analysis

Results are expressed as mean and standard deviation. The differences between the mean values for the different treatments with lactic acid bacteria supernatants were analysed using one-way ANOVA. The least significant difference test was used for *a posteriori t* paired comparison of the mean values. The statistical analysis was performed using Statgraphics plus (version 5.1) software (Manugistics).

Results

Pathogen inhibition assays

The *L. paracasei* CNCM I-4034 supernatants were specific in their effects against the pathogenic strains. The not neutralised

17 h supernatant when used at 1 and 4% concentrations inhibited the growth of *S. typhi* CECT 725 by 23 and 41%, respectively. However, when it was neutralised, the inhibitory capacity was lost. The not neutralised 24 h supernatant used at 1 and 4% concentrations inhibited the growth of *S. typhi* CECT 725 by 25 and 23%, respectively; when this supernatant was neutralised, growth was inhibited by 24 and 23%, respectively (Fig. 1(c)). These results suggest that compounds of different nature might be present in the supernatant.

When both not neutralised supernatants were used at 4% concentration, the 17 h supernatant inhibited the growth of *S. typhimurium* CECT 443 and the 24 h supernatant inhibited the growth of *S. typhimurium* CECT 4594 by 29 and 39%, respectively (Fig. 1(a) and (b)). The neutralised supernatants inhibited the growth of *S. sonnei* CECT 413 by 81% (17 h) and 82·10% (24 h) when utilised at 4% concentration and by 32·5% (17 h) when utilised at 2% concentration (Fig. 4(a)). Similar results were observed against *S. sonnei* CECT 4887ᵀ, as the supernatants used at 4% concentration showed 9·2% (17 h) and 20·5% (24 h) inhibition, and those used at 2% concentration showed 9·9% (24 h) inhibition (Fig. 4(b)). Only the 17 h supernatant was effective against *S. sonnei* CECT 457, as

growth was inhibited by 6·3 and 25·9% when utilised at 2 and 4% concentrations, respectively (Fig. 4(c)).

When used at 1 and 4% concentrations, the not neutralised 17 h *B. breve* CNCM I-4035 supernatant was able to inhibit the growth of *S. typhi* CECT 725 by 37 and 46%, respectively, and by 25 and 29%, respectively, when the supernatants were neutralised. Similar results were obtained with the 24 h supernatants at 1 and 4% concentrations, as these showed 34 and 48% inhibition, respectively, when the supernatants were not neutralised and 34 and 15% inhibition, respectively, when the supernatants were neutralised (Fig. 2(c)). The supernatants did not inhibit the growth of *E. coli* EPEC CECT 727 and *E. coli* EPEC CECT 729 (Fig. 2(a) and (b)). However, the neutralised supernatants did inhibit the growth of *S. sonnei* CECT 457 by 19·4% (24 h) when utilised at 4% concentration and by 25·8% (24 h) when utilised at 2% concentration (Fig. 4(f)). The neutralised supernatants were not able to inhibit *S. sonnei* CECT 4887ᵀ and *S. sonnei* CECT 413 (Fig. 4(e) and (d), respectively).

The *L. rhamnosus* CNCM I-4036 not neutralised 17 and 24 h supernatants inhibited the growth of *S. typhi* CECT 725 by 55 and 29%, respectively, but this inhibition was completely lost
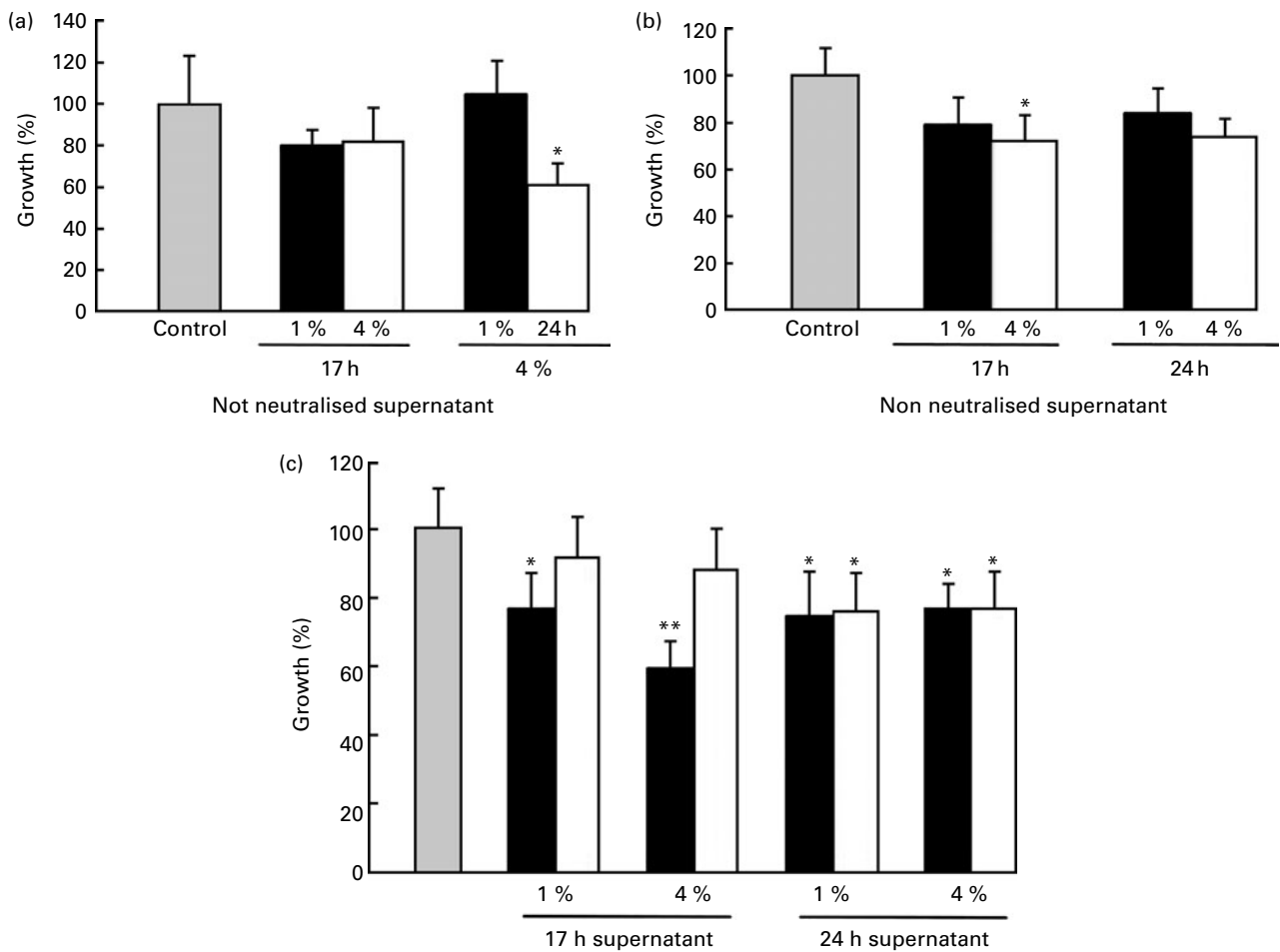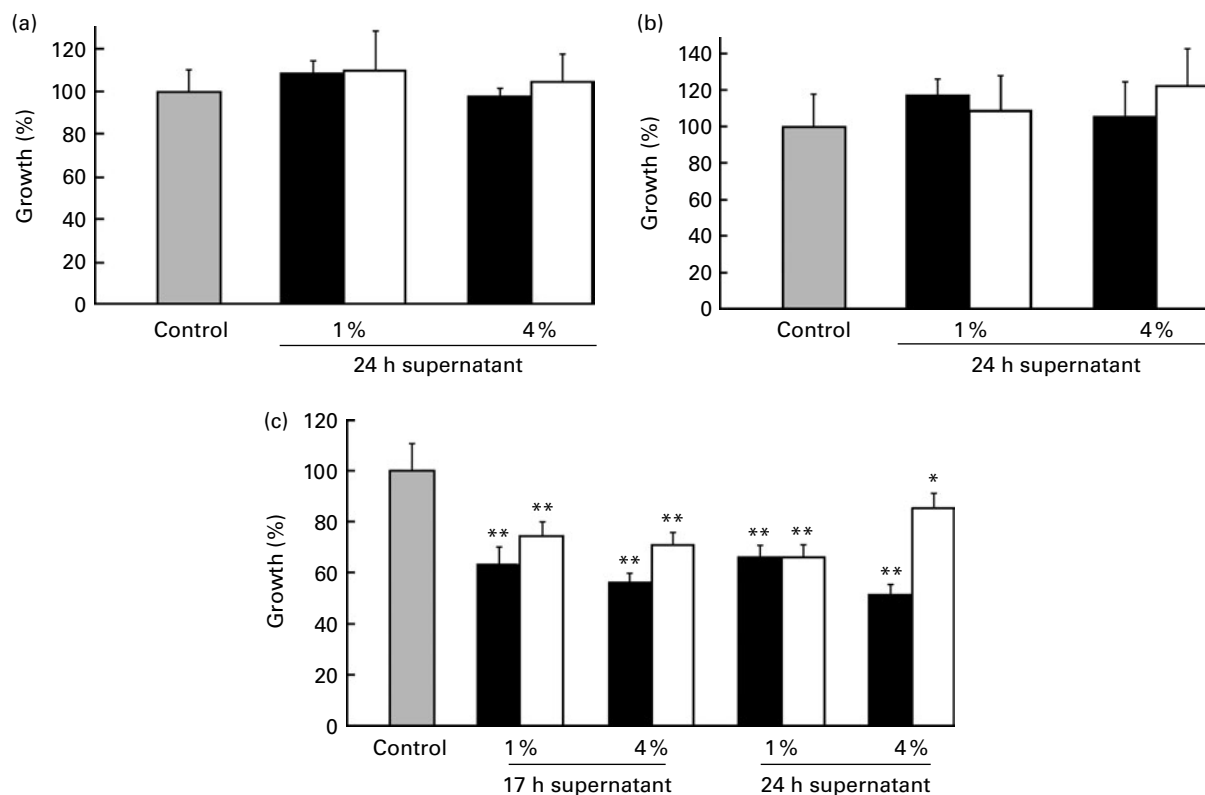

Fig. 1. Effect of 17 and 24 h supernatants of *Lactobacillus paracasei* CNCM I-4034 on growth of *Salmonella typhimurium* (a) CECT 4594, (b) CECT 443 and (c) *Salmonella typhi* CECT 725. Values were significantly different: * $P < 0.05$; ** $P < 0.01$. ▨, Control; ■, not neutralised; □, neutralised.

Fig. 2. Effect of supernatant of *Bifidobacterium breve* CNCM I-4035 on growth of *Escherichia coli* enteropathogenic (a) CECT 729, (b) CECT 727 and (c) *Salmonella typhi* CECT 725. Values were significantly different: * $P < 0.05$; ** $P < 0.01$. ▨, Control; ■, not neutralised; ▢, neutralised.
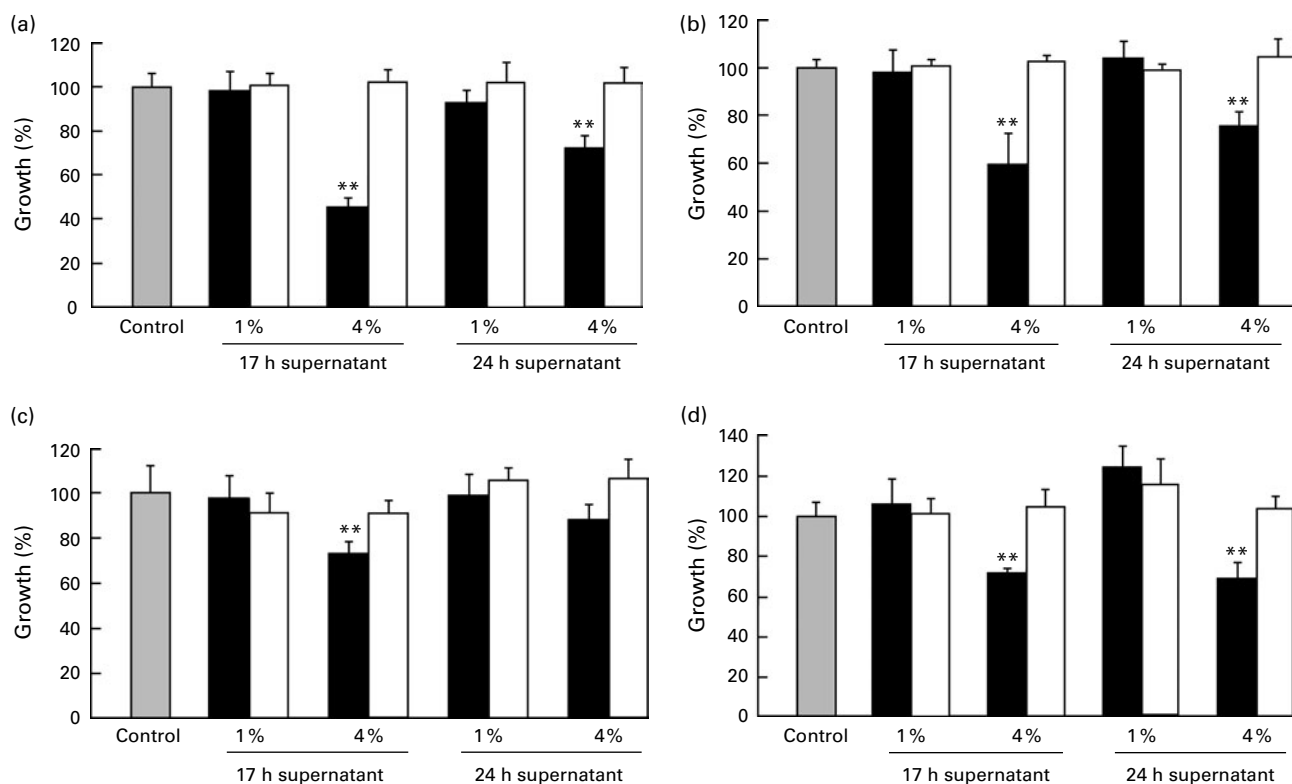
when the supernatants were neutralised (Fig. 3(a)). Similar results were observed against *E. coli* ETEC CECT 501, with 40 and 25 % inhibition, and these 17 and 24 h supernatants also inhibited the growth of *E. coli* ETEC CECT 515 by 29 and 31 %, respectively (Fig. 3(b) and (d)). The growth of *E. coli* EPEC CECT 729 was only affected by the 17 h supernatants used at 4 % concentration (27 % inhibition, Fig. 3(c)). The neutralised supernatants did inhibit the growth of *S. sonnei* CECT 413 by 81 % (17 h) and 82 % (24 h) when utilised at 4 % concentration, and 16·3 % (24 h) inhibition was observed when utilised at 2 % concentration (Fig. 4(g)). The supernatants were slightly effective against *S. sonnei* CECT 4887[T], with minor inhibitory percentages of 29·1 % (24 h) when utilised at 4 % concentration (Fig. 4(h)), and against *S. sonnei* CECT 457, showing 33·8 % (24 h) inhibition when the supernatant was utilised at 4 % concentration and 16 % (24 h) inhibition when utilised at 2 % concentration (Fig. 4(i)).

Discussion

Probiotics display important characteristics that benefit human health. Although knowledge of the potential mechanisms underlying the effects of probiotics against enteropathogens is largely unknown, these mechanisms are likely multifactorial. Important mechanisms that have been shown to underlie these antagonistic effects include the reduction of luminal pH, competition for adhesion sites and nutritional sources,

secretion of antimicrobial substances, toxin inactivation and immune stimulation[3,14]. Several previous reports have indicated that lactic acids, organic acids, bacteriocins, proteases, peroxides and exopolysaccharides exert antibacterial and antifungal effects[3,5,15]. In addition, three novel probiotics (*L. paracasei* CNCM I-4034, *B. breve* CNCM I-4035 and *L. rhamnosus* CNCM I-4036) were previously demonstrated to resist low pH and high bile salt concentrations and to adhere to the colon[11]. Moreover, a safety evaluation of these probiotics, including their antibiotic resistance patterns, an assessment of certain metabolic activities (D-lactate production, bile salt deconjugation and amine biogen production) and *in vivo* acute ingestion profiles (immunosuppressed and immunocompetent mice), was also performed. Taken together, these previous results demonstrated that these probiotics were able to inhibit some strains of *L. monocytogenes* and the infection of human cells with rotavirus *in vitro*[11].

The results of the present study demonstrated that supernatants from cultures of the three novel probiotics *L. paracasei* CNCM I-4034, *B. breve* CNCM I-4035 and *L. rhamnosus* CNCM I-4036 inhibited the growth of EPEC bacteria in a strain-specific manner. Tsai *et al.*[6] showed a similar effect of three *Lactobacillus* strains against *E. coli* ETEC during a 20 h incubation, and similar results have also been obtained using organic acids[16,17]. For *Bifidobacterium* strains, the production of different compounds with inhibitory capacity against enteropathogen strains has also been described[18−20].

Fig. 3. Effect of supernatant of *Lactobacillus rhamnosus* CNCM I-4036 on growth of (a) *Salmonella typhi* CECT 725, (b) *Escherichia coli* ETEC CECT 501, (c) *E. coli* EPEC CECT 729 and *E. coli* ETEC CECT 515. ** Value was significantly different ($P < 0.01$). ▨, Control; ■, not neutralised; □, neutralised.

The *L. paracasei* CNCM I-4034 17 h supernatants showed a differential effect against *S. typhi* CECT 725, as inhibition was observed when the supernatants were not neutralised, but these effects were lost upon neutralisation. A similar finding demonstrated that when both *Lactobacillus* supernatants were used against *S. typhimurium* CECT 4594, *S. typhimurium* CECT 443 and *E. coli* EPEC CECT 729, only the supernatant that was not neutralised was able to inhibit the growth of EPEC bacteria when utilised at 4% concentration. These results are consistent with those described in the study by Mauch *et al.*[15], who found that the activity of the compounds produced by *Lactobacillus brevis* PS1 was higher at low pH values, i.e. pH 5, and that this effect was partially diminished after proteolytic treatment, indicating the production of organic acid and proteinaceous compounds[5]. In another study, the total loss of antimicrobial activity at pH 7 suggested that organic acids were involved[21]. At low pH values, organic acids exist in non-dissociated forms, which facilitate their penetration into the hydrophobic cell membranes of bacteria. Several reports have also shown that the major groups of inhibitory compounds produced by the probiotics include lactic acid, volatile acids and bacteriocins[5,15,21–23].

The *B. breve* CNCM I-4035 supernatants showed a strain-specific effect on *S. typhi* CECT 725, as growth was inhibited in all cases. The nature of compounds produced by bifidobacteria differs from organic acid[18]; these substances, which are present in the supernatant, are highly effective against *S. typhimurium* and *E. coli*, and this effect has been attributed to the production of low-molecular-weight lipophilic molecules[19].
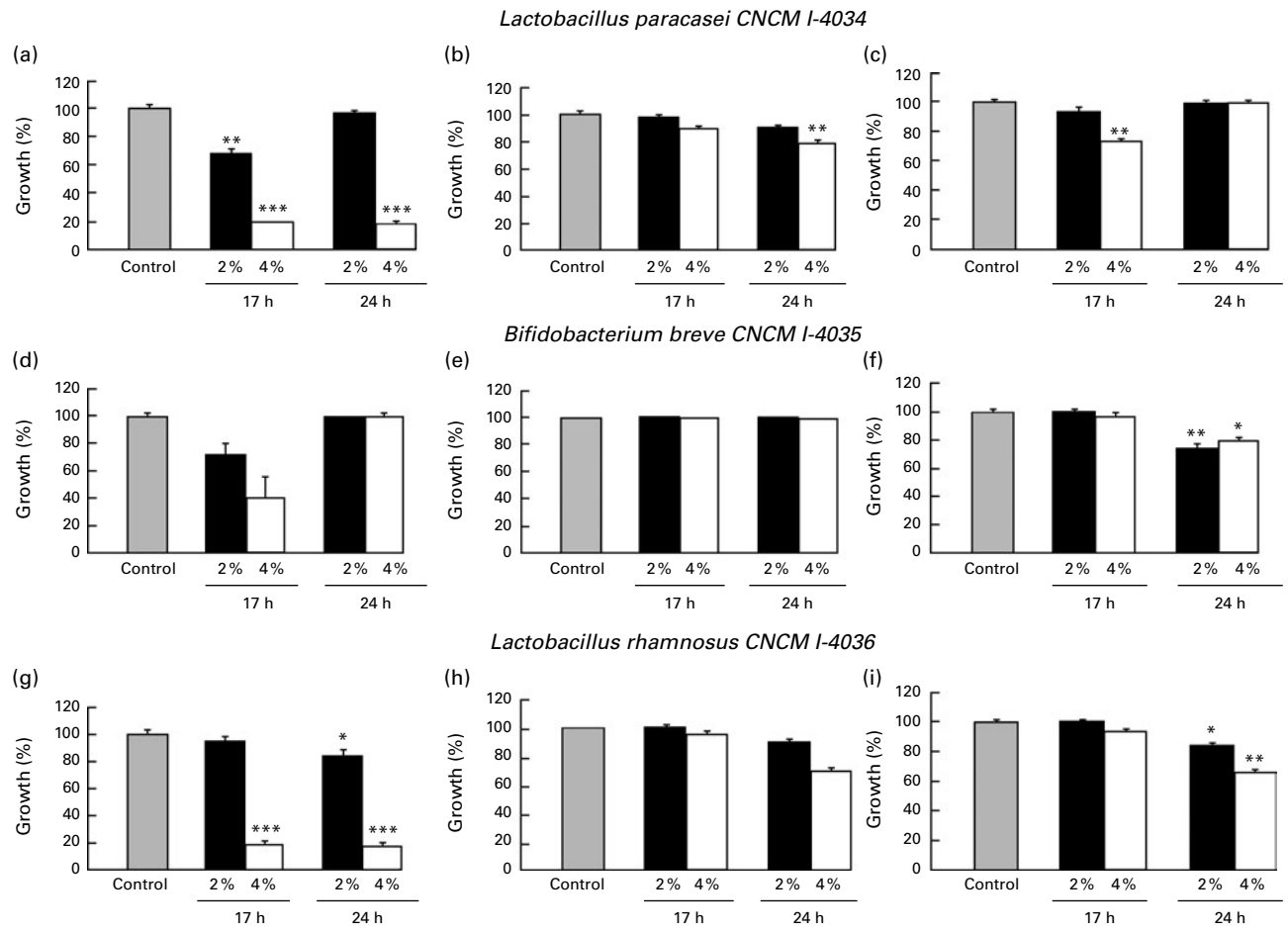
Some authors have attributed the production of organic acid to antimicrobial mechanisms[24,25]. In addition, Fukuda *et al.*[20] found that the acetate produced by bifidobacteria improves intestinal defence against *E. coli* EHEC.

L. rhamnosus CNCM I-4036 inhibited the growth of enteropathogens by 10–50% when supernatants that were not neutralised were used; however, these effects were diminished upon neutralisation. De Keersmaecker *et al.*[26] proposed that the production of lactic acid may be responsible for the antimicrobial effect of *L. rhamnosus* against *S. typhimurium*. However, we cannot disregard the possibility that this inhibitory effect was due to the added production of organic acids and bacteriocins that remain active in an acidic pH.

The extent of the culture incubation may also serve to concentrate the inhibitory substances, which suggests that the concentration of the supernatant after fewer hours of culture may not be high enough to inhibit the growth of enteropathogens. For example, *L. paracasei* CNCM I-4034 24 h supernatants exhibited an inhibitory effect against *S. typhi* CECT 725, which was not affected by neutralisation; however, a different result was observed with the 17 h supernatants.

The antimicrobial activity of probiotics against a wide range of pathogenic micro-organisms has been observed. *S. sonnei* is an enteroinvasive pathogen that induces the inflammatory destruction of the intestinal epithelium, leading to acute recto-colitis and lethal complications[27]. Some authors have demonstrated the inhibition of *S. sonnei* using *Lactobacillus* supernatants that were not neutralised[21,28,29]. Zhang *et al.*[28] described the inhibition of *S. sonnei* using five different

Fig. 4. Effect of 17 and 24 h neutralised supernatants of *Lactobacillus paracasei* CNCM I-4034, *Bifidobacterium breve* CNCM I-4035 and *Lactobacillus rhamnosus* CNCM I-4036 on growth of *Shigella sonnei* (a, d, g) CECT 413, (b, e, h) CECT 4887[T] and (c, f, i) CECT 457. Values were significantly different: * $P<0.05$, ** $P<0.01$; *** $P<0.001$. ▨, Control; ■, 2%; □, 4%.

supernatants from *Lactobacillus* strains, although this capacity was completely lost when the supernatants were neutralised. These results suggest that the action of organic acids is important and that bacteriocins are not involved in the antimicrobial activity of probiotics. However, different results were obtained with the strains tested in the present study, as the highest percentages of growth inhibition with *L. paracasei* CNCM I-4034 and *L. rhamnosus* CNCM I-4036 against *S. sonnei* CECT 413 ($>80\%$) were obtained with neutralised supernatants, which indicates that substances other than organic acids were involved. *B. breve* CNCM I-4035 did not inhibit *S. sonnei* CECT 4887[T] and *S. sonnei* CECT 413. Low percentages of inhibition ($<25\%$) were observed against *S. sonnei* CECT 457, and other studies have demonstrated similar results for bifidobacteria strains[30].

A mixture of organic acids and proteinaceous compounds may affect the growth of EPEC bacteria in a strain-specific manner. Furthermore, due to the reduction in pH, this antimicrobial activity may be attributed to the presence of non-dissociated forms of acids. However, neutralised supernatants were shown to both inhibit and stimulate the growth of enteropathogens in a strain-specific manner, although the role of additional substances is possible. Taken together, the

present results demonstrated that the inhibitory effects of probiotic supernatants were highly strain specific, and similar results have been previously described by other authors[31,32].

In conclusion, in the present study, we showed that supernatants harvested at different culture times from three novel probiotic strains can inhibit the growth of selected strains of *E. coli*, *Salmonella* and *Shigella*. These results suggest that these probiotic strains may produce substances that are either permissive or harmful to enteropathogens, depending on the neutralisation, concentration and culture time of the supernatants. These compounds could be organic acid and/or bacteriocins and are strain specific. Reactome analysis are ongoing to determine the nature of these substances.

Acknowledgements

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