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Lack of correlation between in vitro antibiosis and in vivo protection against enteropathogenic bacteria by probiotic lactobacilli

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Abstract

Increased resistance to infection is one of the beneficial effects attributed to probiotic microorganisms. This effect may be due to several mechanisms: production of inhibitory substances, blocking of adhesion sites on the intestinal surface, competition for nutrients and stimulation of mucosal and systemic immunity. The present study aimed to investigate the correlation between in vitro and in vivo antimicrobial activity of probiotic lactobacilli. The agar spot test was used to show that twenty *Lactobacillus* strains were able to inhibit the enteropathogenic bacterium *Yersinia enterocolitica*. This inhibition was mainly attributable to a decrease in pH resulting from dextrose fermentation by lactobacilli. The inhibition of *Y. enterocolitica*, *Salmonella enterica* serovar Typhimurium and *Listeria monocytogenes* by two probiotic strains, *Lactobacillus casei* C1 and *Lactobacillus plantarum* C4, was also associated with the pH decrease. However, both strains lacked protective effects in mouse experimental infection models, with the exception of long-lasting pre-treatment with *L. plantarum* C4, which exerted a partial protective effect against *S.* Typhimurium that was attributable to an immunostimulatory mechanism. Our results show that in vitro antibiosis tests do not provide useful information on the probiotic potential of *Lactobacillus* strains.

Keywords: Probiotics; *Lactobacillus*; Antibiosis; Infection

1. Introduction

Probiotics are defined as microorganisms that confer beneficial health effects upon the host when administered live and in adequate amounts [2,8,11,15,18,39]. Commonly used bacterial probiotics are strains of *Lactobacillus* and *Bifidobacterium* species [27,33]. There is a growing body of evidence underpinning the ability of probiotics to exert several beneficial effects: alleviation of symptoms of lactose intolerance, reduction of hypercholesterolemia, prevention and alleviation of bacterial and viral diarrhea, anti-inflammatory activity, reduction of atopic disease symptoms and prevention of carcinogenicity [8,9,20,24,30,34,37,39].

An interesting property of probiotics is in their ability to form a microbial barrier against enteropathogenic bacteria. There are several proposed mechanisms by which probiotics may protect the host from intestinal infections: production of inhibitory substances, blocking of adhesion sites on the intestinal surface, competition for nutrients and stimulation of mucosal and systemic immunity [16,33,36,40]. There is good evidence that probiotic bacteria possess antimicrobial activity against a range of enteropathogens [22,30]. In consequence, the ability to inhibit the growth of indicator bacteria has been included by several authors among the criteria for in vitro selection of probiotic strains [10,12,21,38]. However, it has been recognized that there is a need to assess the correlation

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between in vitro antimicrobial activity and the efficient inhibition of enteropathogens in the intestinal tract [30]. In this paper we studied the role of pH on the inhibition of growth of indicator bacteria by lactobacilli and demonstrated the absence of correlation between in vitro antibiosis and the ability of lactobacilli to protect mice against experimental infections with enteropathogenic bacteria.

2. Material and methods

2.1. Bacterial strains

A total of 20 strains of lactobacilli were isolated from raw goat milk and commercial dairy products (see Table 1 for species distribution). The species identification was performed by conventional phenotypic methods in our laboratory [4]. Strains C1 (*Lactobacillus casei*) and C4 (*Lactobacillus plantarum*) were isolated from commercial fermented milk samples. The *L. casei* strain was defined as probiotic by the manufacturers of the fermented product. Both strains have shown resistance to gastric pH and bile salts and possess immunomodulatory properties. The probiotic profile of *L. plantarum* C4 has been studied in our laboratory: this strain exerted immune restoration in immunocompromised mice [5], showed regulatory action on the intestinal microbiota [13,14] and reduced pro-inflammatory cytokines in a murine model of experimental infection [28]. *Escherichia coli* C17 was isolated from feces of a healthy mouse [4]. *Salmonella enterica* serovar Typhimurium CECT4156 was obtained from the Spanish Type Culture Collection. *Yersinia enterocolitica* IP383 is a serotype O9 strain that carries the virulence plasmid pYV [25]. A virulent isolate of *Listeria monocytogenes* was kindly provided by Dr. De La Rosa (Hospital Virgen de las Nieves, Granada, Spain). Lactobacilli strains were cultured in Lactobacilli MRS agar (Difco Laboratories, Francisco Soria Melguizo, Madrid, Spain) for 24 h at 37 °C. The other strains were grown in trypticase soy agar (TSA, Difco) in the same conditions.

2.2. Agar spot test

The 20 strains of *Lactobacillus* listed in Table 1 were tested for antimicrobial activity. The agar spot test described by [35] was used with some modifications. 24-h cultures were harvested in sterile phosphate-buffered saline (PBS, pH 7.2), adjusted by turbidemetry to obtain about 10^{10} bacteria/ml and spotted (10 µl) onto the surface of Lactobacilli MRS agar and TSA plates that were incubated in anaerobic and aerobic conditions for 24 h at 37 °C to allow the bacterial growth. Target bacteria were *L. plantarum* C4, *L. casei* C1, *Lactobacillus acidophilus* C15, endogenous *L. acidophilus*, endogenous *E. coli*, *S.* Typhimurium CECT 4157, *L. monocytogenes* and *Y. enterocolitica* IP383. Target bacteria were grown at 37 °C for 24 h onto TSA tubes for non-lactic acid bacteria, or Lactobacilli MRS agar tubes for lactic acid bacteria, harvested in PBS and adjusted to about 10^8 bacteria/ml. A 100 µl volume of this suspension was mixed with 10 ml of melted TSA or Lactobacilli MRS agar and poured over the plates on which the producer was grown. The plates were incubated at 37 °C. After 24 h of incubation, the size of inhibition zones was read.

Table 1
Inhibition of *Y. enterocolitica* by 20 *Lactobacillus* strains.

Lactobacillus species	Number of tested strains	Inhibitory strains in the indicated media[a]	
		MRS agar	TSA
Lactobacillus acidophilus	3	3	0
Lactobacillus plantarum	9	9	0
Lactobacillus coprophilus	1	1	0
Lactobacillus lactis	3	3	0
Lactobacillus salivarius	1	1	0
Lactobacillus brevis	1	1	0
Lactobacillus casei	2	2	0

[a] Results were repeated across four experiments.

2.3. Assay of antimicrobial activity in culture supernatants

Further studies on the antimicrobial activity of *L. plantarum* C4 against *L. casei* C1, *Y. enterocolitica* IP383 and *S.* Typhimurium CECT 4157 were performed with culture supernatants. The producer strain was grown aerobically in batch cultures (50 ml) at 37 °C. The following media were used: Lactobacilli MRS broth (Difco), trypticase soy broth (TSB, Difco) and TSB containing 2% dextrose (TSB-2). Culture samples were obtained at the desired time points, bacterial cells were sedimented by centrifugation and supernatants were sterilized by filtration through a 0.22-µm-pore-sized membrane (Millipore Corporation, Bedford, MA, USA). The antimicrobial activity of supernatants was assessed by a semiquantitative assay: 100-µl volumes of TSB were dispensed into wells of a 96-well tissue culture cluster (Costar, Cambridge, Mass.) and twofold serial dilutions of the supernatants were performed. The wells were inoculated with 5 µl of TSB containing about 5×10^5 target bacteria, clusters were incubated at 37 °C for 24 h and bacterial growth was checked by the presence of turbidity and bottom sediments. In some assays, the supernatants were adjusted to pH 7.0 and the remaining activity was assayed. In time course assays, the number of viable lactobacilli was determined by plating 10-µl portions of appropriate dilutions of uncentrifuged samples onto MRS agar and the antimicrobial activity of supernatants was assessed as described above.

2.4. Mouse treatment with probiotic bacteria

Specific-pathogen-free female BALB/c mice aged 6–8 weeks were obtained from the Unit of Animal Experimentation of the University of Granada (Granada, Spain) and housed in groups of four animals under standard barrier conditions in cages with steel grid floors at the University of Granada Animal Care Facility. All animal experiments were conducted in accordance with the guidelines of the European Convention for the Protection of Vertebrate Animals