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Pre-treatment with probiotics prolongs survival after experimental infection by multidrug-resistant *Pseudomonas aeruginosa* in rodents: An effect on sepsis-induced immunosuppression

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ABSTRACT

Based on several randomised clinical studies indicating benefit from oral probiotic intake for the prevention of hospital-acquired infections in critically ill patients, this study aimed to explain the mechanism of action of probiotics for the prevention of lethal experimental infection by multidrug-resistant (MDR) *Pseudomonas aeruginosa*. Experiments using an *Escherichia coli* strain susceptible to all antimicrobials were also conducted. C57BL/6 mice were pre-treated intraperitoneally with sterile water for injection or *Lactobacillus plantarum*. Survival was recorded and mice were sacrificed for measurement of apoptosis and tissue bacterial overgrowth and for isolation and culture of splenocytes for cytokine production. Experiments were repeated after pre-treatment with a commercial preparation of four probiotics (*L. plantarum*, *Lactobacillus acidophilus*, *Saccharomyces boulardii* and *Bifidobacterium lactis*; LactoLevure®). Peripheral blood mononuclear cells (PBMCs) of healthy volunteers were stimulated by heat-killed *P. aeruginosa* following pre-treatment with medium or probiotics. Pre-treatment with *L. plantarum* significantly prolonged survival after challenge by either MDR *P. aeruginosa* (66.7% vs. 31.3%; $P = 0.026$) or *E. coli* (56.0% vs. 12.0%, $P = 0.003$). Survival benefit was even more pronounced when mice were pre-treated with LactoLevure®. Tissue bacterial outgrowth and apoptosis of white blood cells and splenocytes were not altered. TNFα and IL-10 production by splenocytes of mice pre-treated with probiotic was increased and IFNγ production was decreased. Pre-treatment with LactoLevure® restored production of IL-17. Stimulation of human PBMCs after probiotic pre-treatment was accompanied by reduced gene expression of *SOCS3*. The results suggest that the protective effect of probiotics is mediated through prevention of sepsis-induced immunosuppression.

1. Introduction

Hospital-acquired infections remain the most important complications of patients hospitalised in intensive care units (ICUs). Several double-blind randomised trials have been published throughout the last years indicating that oral administration of probiotics can decrease the incidence of these infections [1–8]. Despite their clinical benefit, the mechanism of action of probiotics has not been analysed in these studies. Moreover, most of these studies are characterised by great heterogeneity due to the different species of probiotics administered to patients. It appears that the benefit from probiotics depends greatly on the type of administered probiotic. Some studies have used preparations of single probiotic species and others have used a mixture of probiotics.

Probiotics are living micro-organisms widely found in nutritional supplements. With regard to their mechanisms of action, they are thought to act either by modulation of the intestinal microecology so as to prevent bacterial translocation into the systemic circulation or by modifying the immune response of the host [9].

The current study goes well beyond previously published animal models and aims to provide clear-cut answers on the mechanism of prevention of severe infections by multidrug-resistant (MDR) *Pseudomonas aeruginosa* following pre-treatment with probiotics. MDR *P. aeruginosa* is a common pathogen in patients hospitalised in the ICU. This study also aimed to challenge the findings of previous

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randomised clinical trials, some testing one single probiotic species and others testing a mixture of probiotics. To this end, the study was conducted in two steps: a first step evaluating the efficacy of pre-treatment with *Lactobacillus plantarum*, which was contained in all probiotic preparations administered in randomised trials of critically ill patients [1–8]; and a second step evaluating the effect of a commercially available preparation of four probiotics.

2. Animals and methods

2.1. Experimental animals

A total of 309 male C57BL/6 outbred mice weighing 20–25 g and aged 7–9 weeks (Hellenic Pasteur Institute, Athens, Greece) were used. Experiments were performed in the Laboratory for Experimental Medicine of ATTIKON University General Hospital (Athens, Greece). Following acclimatisation, mice were kept in metal cages with a constant rotation rate of 70 air-changes per hour. Mice were fed standard chow (type 4rf 18) and were allowed water ad libitum. Mice were kept in an isolated room with a controlled temperature of 24 °C and a day/night cycle of 06:00 h:18:00 h. The study was approved by the Veterinary Directorate of the Prefecture of Athens, Greece.

2.2. Study design

One bloodstream isolate of *P. aeruginosa* and another of *Escherichia coli* from patients with severe sepsis were used for this study. The studied isolate of *P. aeruginosa* was resistant to piperacillin/tazobactam, ceftazidime, imipenem, meropenem, ciprofloxacin and amikacin, whereas the studied *E. coli* isolate was susceptible to all antimicrobials. Sensitivity was determined after measuring the minimum inhibitory concentration (MIC) of each antimicrobial followed by interpretation according to Clinical and Laboratory Standards Institute (CLSI) criteria.

Single colonies were incubated in Mueller–Hinton broth (Oxoid Ltd., London, UK) for 30 min for *E. coli* and 6 h for *P. aeruginosa* in a shaking water-bath at 37 °C. The different times of incubation were adapted to the time needed by each species to reach logarithmic growth. The inoculum was then diluted to 1×10^8 CFU/mL using a 0.5 McFarland standard and was centrifuged. The bacterial pellet was diluted in water for injection (WFI) and was used for the experiments. In the first set of experiments, mice were assigned into four groups and interventions were performed under light ether anaesthesia.

- Sham operation: mice were administered 0.3 mL of WFI by intraperitoneal (i.p.) injection followed 48 h later by another i.p. injection of 0.3 mL of WFI.
- Group A: mice were administered 3×10^7 CFU/mouse of *L. plantarum* by i.p. injection followed 48 h later by i.p. injection of 0.3 mL of WFI. *Lactobacillus plantarum* was provided in a powder format (Smart Intermed, Athens, Greece) and was reconstituted with WFI.
- Group B: mice were administered 0.3 mL of WFI by i.p. injection followed 48 h later by an i.p. challenge with 3×10^7 CFU/mouse of *P. aeruginosa* in a volume of 0.3 mL.
- Group C: mice were administered 3×10^7 CFU/mouse of *L. plantarum* by i.p. injection followed 48 h later by an i.p. challenge with 3×10^7 CFU/mouse of *P. aeruginosa* in a volume of 0.3 mL, exactly as described for groups A and B.

Mice were then transferred to their cages and paracetamol suppositories were administered every 12 h to reduce suffering. Survival was recorded every 12 h for a total of 7 days starting from the day of bacterial challenge. Some animals were sacrificed 6 h after bacterial challenge. For animal sacrifice, mice were injected intramuscularly with 100 mg/kg ketamine hydrochloride. One midline abdominal incision was then performed under aseptic conditions. Following entrance into the peritoneal cavity, the intestines were displaced to the left and blood was sampled from the lower vena cava under aseptic conditions. Blood was placed in heparin-coated tubes for determination of blood cell apoptosis. In addition, specimens of liver, lower lobe of the right lung and right kidney, and the entire spleen were excised and placed in separate sterile containers.

After being weighed under sterile conditions, tissue specimens were homogenised with 1 mL of Mueller–Hinton broth using a tissue grinder and were diluted four consecutive times 1:10 in sodium chloride 0.9%. Then, 0.1 mL of each dilution was plated onto MacConkey agar (Becton Dickinson, Cockeysville, MD) and plates were incubated for 24 h at 35 °C. Cultured microorganisms were identified by the API20E and API20NE systems (bioMérieux, Marcy-l'Étoile, France) and the number of colonies was counted and multiplied by the corresponding dilution factor. Results were expressed as $\log_{10}$ CFU/g. The minimum detection limit was 10 CFU/g.

For measurement of the rate of apoptosis, 50 μL of whole blood was placed in plastic tubes. Red cell lysis was performed using a ready-made lysing solution (VersaLyse Solution; Beckman Coulter, Miami, FL). After washing with ANNEXIN binding buffer (Immunotech, Marseille, France), white blood cells (WBCs) were incubated for 15 min in the dark at 4 °C with the protein ANNEXIN-V (ApoScreen; Cell Lab, Beckman Coulter) labelled with the fluorochrome fluorescein isothiocyanate (absorbance 515 nm) and propidium iodide in Texas red (electron coupled dye, absorbance 617 nm; Invitrogen, Carlsbad, CA) and were analysed after passage through an FC500 flow cytometer (Beckman Coulter) with separate gating for each WBC subpopulation by their characteristic forward and side scattering. Unstained cells were used as negative controls. Cells staining positive for ANNEXIN-V and negative for propidium iodide were considered apoptotic.

Spleens were transferred into 1 mL of RPMI 1640 (Biochrom, Berlin, Germany), gently squeezed and passed through a sterile filter (250 mm, 12 × 13 cm; Alter Chem Co., Athens, Greece). Isolated splenocytes were collected and counted in a Neubauer chamber after exclusion of dead cells by trypan blue staining. Splenocytes were incubated in the wells of a 24-well plate at a density of 5×10^6 mL^{-1} in RPMI 1640 supplemented with 2 mM of glutamine (Biochrom), 10% foetal bovine serum (FBS) (Biochrom), 100 U/mL penicillin G and 0.1 mg/mL streptomycin (Sigma, St Louis, MO) at 37 °C in 5% CO_2. Splenocytes were stimulated for 24 h, 48 h or 5 days in the presence or absence of 10 ng/mL of lipopolysaccharide (LPS) of *E. coli* O55:B5 (Sigma), 5 μg/mL Pam_3Cys-SKKK (EMC Microcollections, Tübingen, Germany), 5 μg/mL phytohaemagglutinin (PHA) (EMC Microcollections) or 5×10^5 CFU/mL of heat-killed *Candida albicans*. Following incubation, plates were centrifuged and the supernatants were collected. Concentrations of tumour necrosis factor-alpha (TNFα), interferon-gamma (IFNγ), interleukin (IL)-1β, IL-6, IL-10, IL-17 and IL-22 in supernatants were measured using an enzyme immunoassay (R&D Inc., Minneapolis, MN). The lower limit of detection was 15 pg/mL for TNFα, 8 pg/mL for IL-10 and 16 pg/mL for each of IL-1β, IL-6, IL-17, IL-22 and IFNγ. Apoptosis of isolated splenocytes was also measured, as described above.

In the second set of experiments, mice were assigned into two groups and interventions were performed under light ether anaesthesia.

- Group D: mice were administered 0.3 mL of WFI by i.p. injection followed 48 h later by an i.p. challenge with 3×10^7 CFU/mouse of *E. coli* in a volume of 0.3 mL.

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