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Key Roles of Human Polymorphonuclear Cells and Ciprofloxacin in *Lactobacillus* Species Infection Control

Narcisa Mandras,[a] Vivian Tullio,[a] Pio Maria Furneri,[b] Janira Roana,[a] Valeria Allizond,[a] Daniela Scalas,[a] Giulio Petronio Petronio,[b,c] Virginia Fuochi,[b] Giuliana Banche,[a] Anna Maria Cuffini[a]

Microbiology Division, Department of Public Health and Paediatrics, University of Turin, Turin, Italy[a]; Microbiology Section, Department of Bio-Medical Sciences, University of Catania, Catania, Italy[b]; BioBIM—Multidisciplinary Interinstitutional BioBank, IRCCS San Raffaele Pisana, Rome, Italy[c]

Lactobacilli have the potential to act as reservoirs of antibiotic resistance genes similar to those found in human pathogens, with the risk of transferring these genes to many pathogenic bacteria. In this study, we investigated the role of human polymorphonuclear cells (PMNs) against *Lactobacillus* spp. both resistant and susceptible to ciprofloxacin (a fluoroquinolone) and the effect of ciprofloxacin on the interaction between PMNs and three *Lactobacillus* spp. with different patterns of susceptibility to this drug. Hence, the primary functions of PMNs, such as phagocytosis and bacterial intracellular killing, against lactobacilli were investigated. The rate of PMN phagocytosis was high for ciprofloxacin-sensitive and ciprofloxacin-resistant strains. The patterns of intracellular killing of ciprofloxacin-sensitive and ciprofloxacin-resistant strains by PMNs underline that PMNs alone were able to kill lactobacilli. The addition of ciprofloxacin to PMNs did not result in a significant increase in the bacterial uptake by phagocytes. On the contrary, ciprofloxacin had a marked effect on PMN intracellular killing, resulting in increased numbers of killed ciprofloxacin-sensitive bacteria in comparison with antibiotic-free controls. Our data show that by itself, the profile of antibiotic resistance does not constitute an intrinsic factor of greater or lesser pathogenicity toward the host. The ability of PMNs to kill a diverse array of bacterial pathogens is essential for human innate host defense, primarily in immunocompromised patients.

Lactobacillus spp., considered nonpathogenic bacteria of the human gastrointestinal and vaginal microbiotas, are widely used as probiotics in dairy products. Moreover, they can positively interact with other microorganisms of the human microbiota (1).

Infections caused by lactobacilli are extremely rare; however, they can spread into the blood and cause serious clinical infections (2, 3). Many investigators have demonstrated that lactobacilli have a high natural resistance to many antimicrobial drugs, such as fluoroquinolones, e.g., ciprofloxacin (CIP) (4–7); our recent studies showed that the mechanism of resistance to fluoroquinolones could be associated with the presence of a NorA-like efflux pump found in ciprofloxacin-resistant (CIP^r) strains of *L. fermentum* isolated from the human oral cavity (8).

For several decades, studies on selection and dissemination of antibiotic resistance have focused mainly on clinically relevant species. The magnitude of the problem is significantly increased by the ability of bacteria to transfer resistance horizontally and by the mounting increase in the overuse and misuse of antibiotics, which has created an enormous selective pressure toward resistant bacteria.

Gene transfer occurs widely *in vivo* between pathogenic and commensal bacteria of the human microbiota, as identical resistance genes are present in several bacterial species from different hosts. Lactobacilli have the potential to act as reservoirs of antibiotic resistance genes similar to those found in human pathogens, with the risk of transferring these genes to many pathogenic bacteria (5, 9, 10). Fortunately, drug resistance does not always correlate with therapeutic clinical failure: the host defense mechanisms play a key role during infection in preventing the triggering and spread of bacterial infection (11).

The ability of professional phagocytes, such as polymorphonuclear cells (PMNs), to ingest and kill microorganisms is central to innate immunity (11), and the current trend of therapy requires the use of antibiotics that potentiate the host's defense mechanisms (12, 13). Hence, the aim of this study was to evaluate (i) the efficacy of PMNs to eradicate different *Lactobacillus* spp. and (ii) the effect of CIP on the interaction between PMNs and three *Lactobacillus* spp. with different patterns of susceptibility or resistance to CIP, in order to evidence potential differences in bacterial susceptibility to phagocytes.

MATERIALS AND METHODS

Bacterial strains. Five human lactobacillus isolates were included in this study (two *Lactobacillus acidophilus*, two *L. gasseri*, and one *L. plantarum* isolate). Lactobacilli were isolated from the vaginal microbiotas of healthy women who gave their informed consent. Bacteria were identified by means of molecular techniques. Briefly, 16S rRNA PCR-restriction fragment length polymorphism (RFLP) and 16S rRNA-ISR-23S rRNA 2-step multiplex PCR were used for this purpose (14, 15). The strains were stored at −80°C in cryovials (Microbank, bioMérieux, Rome, Italy) until use. Then lactobacilli were subcultured on De Man, Rogosa and Sharpe agar (Oxoid, Milan, Italy) to ensure viability and purity.

Antimicrobial activity of ciprofloxacin against *Lactobacillus* spp. Fresh solutions of CIP (Sigma-Aldrich, Milan, Italy), prepared for each experiment, were shown to be free from endotoxin in a standard *Limulus* amebocyte lysate (LAL) assay (BioWhittaker, Walkersville, MD).

The MIC of CIP for the five strains was determined by the broth microdilution method recommended by CLSI document M45-A2 (16), with an inoculum of 10^5 CFU/ml, and an inoculum of 10^7 CFU/ml to

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Address correspondence to Vivian Tullio, vivian.tullio@unito.it.

TABLE 1 Effect of CIP on phagocytosis and intracellular killing of CIP^s (MIC = 0.5 μg/ml) and CIP^r (MIC = 32 μg/ml) *L. acidophilus* by PMNs

Time (min)	% phagocytosis of *L. acidophilus*, mean ± SEM				Survival index of *L. acidophilus*, mean ± SEM (% of bacteria killed)			
	CIP^s		CIP^r		CIP^s		CIP^r	
	Controls	CIP	Controls	CIP	Controls	CIP	Controls	CIP
30	37.3 ± 1.05	40.6 ± 0.09	35.2 ± 0.08	36.2 ± 1.08	1.25 ± 0.09 (75)	1.15[b] ± 0.01 (85)	1.28 ± 0.11 (72[c])	1.30 ± 0.04 (70)
60	40.1 ± 1.11	42.9 ± 0.71	38.0 ± 0.06	40.0 ± 0.76	1.39 ± 0.06 (61)	1.14[a] ± 0.11 (86)	1.26 ± 0.09 (74)	1.21 ± 0.11 (79)
90	42.2 ± 1.14	49.0 ± 0.84	42.2 ± 0.31	45.0 ± 2.48	1.48 ± 0.13 (52)	1.12[a] ± 0.06 (88)	1.31 ± 0.06 (69)	1.26[b] ± 0.1 (74)

[a] Significantly different from the control ($P < 0.01$).
[b] Significantly different from the control ($P < 0.05$).
[c] Percentage of initial bacterial population killed by PMNs.

perform tests with phagocytes. MICs were read after incubating the plates under a 5% CO_2 atmosphere (Genbag CO_2; bioMérieux) (16) at 35°C for 48 h.

PMNs. Blood was drawn from a pool of 72 healthy subjects who gave their informed consent. PMNs were separated from lithium-heparinized venous blood using Ficoll-Paque (Pharmacia S.p.A., Milan, Italy), as previously described in detail (11, 17, 18). Briefly, the viability of PMNs was assayed by trypan blue testing and was greater than 95%. PMNs (10^6 cells/ml) were suspended in RPMI 1640 medium (Gibco Laboratories, Grand Island, NY) and incubated at 37°C in a shaking water bath (150 rpm) before the addition of bacteria (10^7 CFU/ml). In all experiments, the ratio of bacteria to PMNs was 10:1.

Phagocytosis and intracellular killing by PMNs. Both phagocytosis of radiolabeled lactobacilli ([^{3}H]uracil; specific activity, 1,270 GBq/mmol; NEN Life Science Products, Milan, Italy) and intracellular bacterial killing by PMNs were investigated by incubating the bacteria and the phagocytes (10:1) in RPMI 1640 medium at 37°C for periods of 30, 60, and 90 min. Phagocytosis and intracellular killing were assessed as previously described (11). Radioactivity was expressed as counts per minute per sample. The percentage of phagocytosis at a given sampling time was calculated as follows: percentage of phagocytosis = (number of counts per minute in PMN pellet/number of counts per minute in total bacterial pellet) × 100 (17). PMN killing values were expressed as the survival index (SI), which was calculated by adding the number of surviving microorganisms at time zero to the number of survivors at time *x* and dividing by the number of survivors at time zero (18). According to this formula, if bacterial killing was 100% effective, the SI would be 1.

CIP influence on PMN phagocytosis and intracellular killing. The influence of CIP on both the phagocytosis and intracellular bacterial killing by PMNs was investigated by incubating the bacteria and the phagocytes (ratio, 10:1) at 37°C in a shaking water bath for periods of 30, 60, and 90 min in the presence of 1× MIC of the drug. Drug-free controls were also included. Phagocytosis and intracellular killing were assessed by the methods described above (18).

Statistical analysis. Each test was performed in quadruplicate, and the results were compared with those obtained with the controls and expressed as the means and standard errors of the means (SEMs) for 10 separate experiments. Statistical evaluation of the differences between test and control results was performed by analysis of variance using Tukey's test. A *P* value of <0.05 was considered significant.

RESULTS

Susceptibility testing showed that one *L. acidophilus* strain and one *L. gasseri* strain were ciprofloxacin sensitive (CIP^s), with MICs of 0.25 and 0.5 μg/ml with inocula of 10^5 and 10^7 CFU/ml, respectively. The other species of lactobacilli (one *L. acidophilus*, one *L. gasseri*, and one *L. plantarum* strain) were ciprofloxacin resistant (CIP^r), with MICs of 16 μg/ml (inoculum of 10^5 CFU/ml) and 32 μg/ml (inoculum of 10^7 CFU/ml).

Tables 1 to 3 summarize the percentages of phagocytosis of CIP^s and CIP^r strains of lactobacilli. The overall data show that the rate of PMN phagocytosis was high for all CIP^s strains (*L. acidophilus* and *L. gasseri*, 37 to 42% and 43 to 49%, respectively) and CIP^r strains (*L. acidophilus*, 35 to 42%; *L. gasseri*, 44 to 47%; and *L. plantarum*, 44 to 45%) in drug-free controls. The addition of 1× MIC of CIP (0.5 μg/ml) to PMNs incubated with CIP^s *L. acidophilus* (Table 1) and *L. gasseri* (Table 2) did not result in a significant increase in the bacterial uptake by PMNs: lactobacilli were phagocytized at a rate similar to that of the drug-free controls (Tables 1 and 2). Also for CIP^r strains, the data show that CIP did not modify PMN bacterial uptake, since percentages of intracellular *L. acidophilus* (36 to 45%), *L. gasseri* (42 to 48%), and *L. plantarum* (45 to 46%) were comparable to those observed in the drug-free controls (35 to 42%, 44 to 47%, and 44 to 45%, respectively) (Tables 1 to 3).

The patterns of intracellular killing toward CIP^s and CIP^r *L. acidophilus*, *L. gasseri*, and *L. plantarum* strains by PMNs are shown in Tables 1 to 3. In the drug-free controls, all CIP^s *L. acidophilus* and *L. gasseri* strains were killed by PMNs in ranges from 52 to 75% and 60 to 76%, respectively, within 90 min of observation (Tables 1 and 2). Likewise, PMNs were able to kill the CIP^r strains, over the whole observation period, in ranges between 69 and 74% (*L. acidophilus*), 39 and 51% (*L. gasseri*), and 55 and 73% (*L. plantarum*) (Tables 1 to 3).

TABLE 2 Effect of CIP on phagocytosis and intracellular killing of CIP^s (MIC = 0.5 μg/ml) and CIP^r (MIC = 32 μg/ml) *L. gasseri* by PMNs

Time (min)	% phagocytosis of *L. gasseri*, mean ± SEM				Survival index of *L. gasseri*, mean ± SEM (% of bacteria killed)			
	CIP^s		CIP^r		CIP^s		CIP^r	
	Controls	CIP	Controls	CIP	Controls	CIP	Controls	CIP
30	49.3 ± 3.45	43.3 ± 0.05	47.2 ± 0.78	45.2 ± 1.08	1.24 ± 0.09 (76)	1.19[b] ± 0.01 (81)	1.61 ± 0.15 (39[c])	1.46[b] ± 0.14 (54)
60	48.6 ± 1.79	41.2 ± 2.01	45.9 ± 0.49	48.0 ± 0.09	1.27 ± 0.06 (73)	1.24 ± 0.11 (76)	1.49 ± 0.09 (51)	1.47 ± 0.09 (53)
90	43.3 ± 3.14	47.1 ± 1.04	44.6 ± 0.71	42.8 ± 1.21	1.40 ± 0.13 (60)	1.21[a] ± 0.06 (79)	1.54 ± 0.09 (46)	1.28[a] ± 0.07 (72)

[a] Significantly different from the control ($P < 0.01$).
[b] Significantly different from the control ($P < 0.05$).
[c] Percentage of initial bacterial population killed by PMNs.

TABLE 3 Effect of CIP on phagocytosis and intracellular killing of CIP^r *L. plantarum* (MIC = 32 μg/ml) by PMNs

Time (min)	% phagocytosis, mean ± SEM		Survival index, mean ± SEM (% of bacteria killed)	
	Controls	CIP	Controls	CIP
30	44.2 ± 0.28	46.2 ± 3.11	1.45 ± 0.06 (55[b])	1.40 ± 0.06 (60)
60	45.2 ± 0.19	46.3 ± 0.39	1.27 ± 0.06 (73)	1.25 ± 0.06 (75)
90	44.5 ± 0.71	45.2 ± 1.11	1.36 ± 0.11 (64)	1.21[a] ± 0.11 (79)

[a] Significantly different from the control ($P < 0.01$).
[b] Percentage of initial bacterial population killed by PMNs.

The presence of CIP at 1× MIC increased significantly the number of killed CIP^s *L. acidophilus* (85 to 88%) and *L. gasseri* (76 to 81%) organisms, in comparison with antibiotic-free controls, until 90 min of incubation ($P < 0.01$/$P < 0.05$) (Tables 1 and 2); 0.1× MIC CIP enhanced significantly the intracellular microbicidal activity against CIP^r *L. acidophilus* compared with the controls: during the 90-min period, the intracellular bacterial load was reduced by 74% ($P < 0.05$) (Table 1). The intracellular load of CIP^r *L. gasseri* was reduced by 72% and that of *L. plantarum* by 79% ($P < 0.01$) (Tables 2 and 3).

DISCUSSION

Quinolones and fluoroquinolones are broad-spectrum chemotherapeutic drugs effective on a wide range of microorganisms, characterized by complete oral bioavailability and high tissue diffusion. In particular, CIP has a broad antibacterial spectrum, with bactericidal activity toward Gram-positive and Gram-negative aerobes (5, 19); it accumulates inside the PMNs and remains active, leading to a significant reduction in the number of intracellular bacteria, such as *Aggregatibacter actinomycetemcomitans* (formerly *Actinobacillus actinomycetemcomitans*) (20).

From the literature, lactobacilli seem to be intrinsically resistant to fluoroquinolones, and resistance to CIP appears to be a natural trait in many species of *Lactobacillus*, such as *L. plantarum* (5, 19).

In the present study, *L. acidophilus* and *L. gasseri* (both CIP^s and CIP^r) and *L. plantarum* (CIP^r) were used to study the functions of PMNs against these commensal bacteria and to evaluate the possible modulating activity of CIP on PMNs in eradicating these lactobacilli with a different antibiotic susceptibility.

Our data showed that the presence of CIP did not result in a significant increase in the bacterial uptake by PMNs: CIP^s and CIP^r lactobacilli were phagocytized at a rate that was similar to that of the drug-free controls, underlining that the fluoroquinolone did not adversely affect PMN functionality (Table 1 to 3).

In contrast, the results for the intracellular killing demonstrated the ability of phagocytes to kill intracellular bacteria, even CIP^r bacteria, in the absence of drugs. Our data suggest that lactobacilli appear to be effectively "under the control of PMNs" that prevent their proliferation, limiting the elevated risk of their possible uncontrolled dissemination, mainly in immunocompromised subjects. Our data are in agreement with those of Cai et al., who reported the ability of *Lactobacillus* spp. to elicit differential immunomodulatory effects on immune cells by proinflammatory and regulatory cytokine production (21). However, the reason why lactobacilli are killed so effectively by PMNs is not well defined. Dziarski et al. reported that bacteria of low pathogenicity, such as *Bacillus subtilis*, *Micrococcus luteus*, and *L. acidophilus*, are much more easily killed or removed from the organism by the immune system than more pathogenic bacteria (*Staphylococcus aureus*, *Escherichia coli*, and *Proteus vulgaris*). These bacteria are able to evade some of the host killing mechanisms, inhibiting, for example, PGRP-S (peptidoglycan recognition proteins), a protein highly expressed by PMNs with a direct antibacterial effect against Gram-positive bacteria of low pathogenicity (22).

It is known that PMNs kill phagocytized bacteria by several mechanisms, of which the oxidative type are the most common. However, in immunocompromised patients, the oxidative microbicidal mechanisms could be compromised and scarcely operating, inducing a higher risk of infection and contributing consequently to antimicrobial treatment failure (18, 23).

Under the experimental conditions where CIP was present in the culture medium together with phagocytes and CIP^s and CIP^r *L. acidophilus* or CIP^s and CIP^r *L. gasseri*, we observed a significant further increase in the intracellular killing by PMNs in comparison with the drug-free controls ($P < 0.01$) (Tables 1 and 2). These data confirm that CIP and probably other fluoroquinolones (such as moxifloxacin, prulifloxacin, pefloxacin, and sparfloxacin) can help the host defense in the outcome of infections because they accumulate and remain active inside PMNs (20, 24–26).

It is known that CIP has poor activity against *L. plantarum* (4, 27). Our results showed that CIP was able to positively influence the host-bacterium interaction, resulting in an enhancement of human PMN functions against the CIP^r *L. plantarum* strain (Table 3), showing that, by itself, the profile of antibiotic resistance does not constitute an intrinsic factor of greater or lesser pathogenicity toward the host.

The ability of PMNs to kill different arrays of bacterial pathogens is essential for human innate host defense (28). In fact, CIP showed immunomodulatory activity, as it is able to enhance the killing of intracellular lactobacilli, if they move from their usual anatomical district, thus contributing to the eradication of possible infection. This feature can be interesting in immunocompromised individuals, where the synergistic effect of CIP with human PMNs is useful to counteract the proliferation and the possible dissemination of commensal lactic acid bacteria, confirming that antibiotic resistance *in vitro* does not always imply a failure of antimicrobial treatment.

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