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Original Research

Antibiotic resistance profile and adhesion properties of *Lactobacillus crispatus* M247

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Abstract

In an attempt to better characterize the strain M247, a strain of *Lactobacillus crispatus* isolated in 1989 from the faeces of a healthy newborn baby, we have further investigated its antibiotic resistance profile, evaluating also antibiotics not recommended by the EFSA, and analyzing in addition its viability in the presence of a high concentration of boric acid, a molecule commonly use to eradicate antimycotic-resistant fungi from the vagina.

At the same time, we have evaluated the ability of M247 to adhere to vaginal epithelial cells to get a better understanding of its vaginal colonizing properties. According to the obtained results, M247 appears to be insensitive to metronidazole, sulfamethoxazole and boric acid.

Such resistance is not transferrable due to the total absence of mobile elements inside its genome.

Moreover, the M247 strain has been shown to adhere by more than 70% to vaginal epithelial cells, thereby providing a mechanistic explanation of its vaginal colonizing capabilities observed both after local and oral use.

Keywords: Community state type, metronidazole, boric acid, probiotic, vaginosis, vaginitis

Introduction

The human vaginal microbiota seems to constitute five distinct community state types (CSTs), four of which are mostly dominated by a single *Lactobacillus* species (CST I: *Lactobacillus crispatus*; CST II: *L. gasseri*; CST III: *L. iners*; CST V: *L. jensenii*), while CST IV is characterized by a paucity or absence of *Lactobacillus* species and consists of a diverse group of facultative and strict anaerobes, including bacteria associated with bacterial vaginosis (BV) [1].

CST IV seems to predispose women to BV, candidiasis, chlamydia, HPV persistence, *sine causa* infertility and spontaneous preterm birth, especially in the presence of increased vaginal bacterial richness [2]. It can therefore be assumed that the lactobacilli inhabiting the human vagina provide the first line of defense in the female urogenital and reproductive tracts. However, not all lactobacilli are equally protective and recent research indicates that of the four *Lactobacillus*-based CSTs, the vaginal microbiota CST I is most closely correlated with a healthy status, thereby suggesting that *L. crispatus* is a biomarker of a healthy vaginal tract [3]. The healthy role played by *L. crispatus* seems to also affect urinary tract infections. Eight different urotypes (UTs) have been indeed described in humans, all of which are found in both men and women, except for UT 7, which occurs in healthy women only and has a relative abundance of *L. crispatus* [4]. Consequently, probiotics containing *L. crispatus* strains might be useful for prophylactic purposes in these, vaginal and bladder, contexts. *L. crispatus* M247 (IDA: LMG-P-23257) is an extensively investigated strain, originally isolated in 1989 from the faeces of a healthy baby, that is characterized by aggregating, colonizing and anti-inflammatory properties evaluated at the gut level [5–8].

Recent studies have also shown its capability to colonize vaginal tissue after local and/or oral administration [9].

From a medical perspective, in order to avoid a new vaginal infection (so-called recurrence), the use of probiotics should be recommended. Probiotics, generally and as requested by the European Food Safety Authority (EFSA), show an important antibiotic sensitivity profile and their concomitant use with antibiotics is generally considered not dangerous but useless, if not a "no-sense" practice [10]. On the other hand, the use of probiotics characterized by non-transferable, single, antibiotic resistance features could be considered a useful tool to make the colonization process more efficient [11].

Metronidazole, antimycotic drugs, lactic acid and boric acid are among the most common substances used in gynaecology to counteract bacterial and fungal infections [12]. Metronidazole is not included on the panel for antibiotic resistance profiling of a probiotic as mandated by the EFSA [13]. Antimycotic drugs can be considered to be totally ineffective with respect to bacterial strains and there is progressive growth of lactobacilli in media containing lactic acid, although the same cannot be definitively asserted for boric acid.

We have therefore studied a comprehensive panel of antibiotics to evaluate if strain M247 shows a differential sensitivity to these, in addition to boric acid. Finally, in order to gain a better understanding of the vaginal colonization capability observed for M247, we have for the very first time evaluated the in *vitro* vaginal adhesion properties of the strain.

Materials and methods

Antibiotic resistance profile

The *L. crispatus* M247 LMG P-23257 resistance to antibiotics profile was assessed according to ISO 10932:2010. Microdilution was performed in LSM 2X broth (90% Iso-Sensitest Broth and 10% MRS Broth, Oxoid, Thermo Fisher Scientific), pH 6.85±0.1. The minimum inhib-

itory concentrations (MICs) were determined for the 19 listed antibiotics: ampicillin, penicillin, clindamycin, linezolid (range, 0.03 to 16 µg/ml), vancomycin, ciprofloxacin (range, 0.25 to 128 µg/ml), neomycin, gentamicin, streptomycin (range, 0.5 to 256 µg/ml), kanamycin (range, 2 to 1024 µg/ml), erythromycin, quinupristin-dalfopristin (range, 0.016 to 8 µg/ml), tetracycline, chloramphenicol, rifampicin, rifaximin, trimethoprim (range, 0.125 to 64 µg/ml), metronidazole and sulfamethoxazole (range, 0.5 to 256 µg/ml) (for all antibiotics, Sigma-Aldrich). Furthermore, boric acid, a substance routinely used for treatment of BV, was tested by microdilution method within the range of 16 to 10,000 µg/ml. *Lactobacillus paracasei* ATCC 334 antibiotic resistance profiles were determined as quality reference controls. The antibiotic resistance profiles of *L. crispatus* M247 LMG P-23257 were evaluated on the basis of European standards such as guidance from the EFSA. To determine the sensitivity or the resistance of *L. crispatus* M247, a comparison between the MIC values obtained for each antibiotic and the breakpoints described for the corresponding genus or species by the EFSA was taken into consideration; in this evaluation, the reference was the *Lactobacillus acidophilus* group. Briefly, the microdilution test consists of these steps: preparation of all antibiotic dilutions and precoating of microplates with 100 µl of the different concentrations; the sub-cultivation of tested and reference strains by smear for 72 hours at 37°C under anaerobic conditions.

Some colonies from a single plate were picked up and resuspended in tubes containing 3 ml of a sterile saline solution. The bacterial suspensions were compared to the McFarland standard No. 1 and the turbidity was spectrophotometrically confirmed with absorbance readings ranging between 0.16–0.2 at a wavelength of 600 nm; these absorbance values correspond to 3×10^8 colony forming units (CFU/ml).

Bacterial suspensions were diluted by 500 times in the LSM 2X medium and 100 µl was seeded in the microplates. Microplates were incubated for 48 hours at 37°C under anaerobic conditions and read to assess the sensitivity or resistance to the antibiotic by means of the presence/absence of visible growth.

Adhesion tests

Two adhesion tests were carried out to evaluate the colonizing abilities of *L. crispatus* M247 LMG P-23257 within the intestinal and vaginal tracts. Two different human epithelial cell lines, HT29-MTX and HeLa (Sigma-Aldrich), respectively derived from human colon and cervix, were selected to perform the experiments. The HT29-MTX and HeLa cell lines were routinely cultured in Dulbecco's Modified Eagle Medium (DMEM), High Glucose + 10% decomplemented foetal calf serum (FCS), with 50 µg/ml di L-glutamine at 37°C with 5% CO_2 (all sera and media from Euroclone). Two days before the adhesion test, human cells were rinsed with Hank's balanced salt solution, treated with trypsin, counted and diluted at a concentration of 2.5×10^5 cells/ml. 1 ml of these solutions was seeded in 24-well plates. Two wells for each cell line were seeded to perform the test in duplicate. The 24-well plates were incubated for 48 hours at 37°C with 5% di CO_2 until cells reached confluence. The day before the test *L. crispatus* M247 LMG P-23257 was inoculated in MRS Broth (Oxoid, Thermo Fisher Scientific) and cultured for 24 hours at 37°C under a microaerophilic atmosphere. The day of the adhesion test, the wells seeded with the human cell lines were checked for confluence and washed with Hank's balanced salt solution and incubated for 1 hour with 875 µl of DMEM, High Glucose + 1% decomplemented FCS at 37°C with 5% CO_2.

An extra well without cells was inoculated with 875 µl of DMEM medium, one for each seeded well. Meanwhile, the *L. crispatus* M247 LMG P-23257 strain was centrifuged and the pellet washed twice with sterile distilled water and

dosed with the McFarland standard 0.5, which gives an optical density comparable to a bacterial suspension density of 1.5×10^8 CFU/ml.
This suspension was diluted 1:10 in DMEM, High Glucose + 1% FCS and 125 µl was used to inoculate the 4 wells seeded with the human cell lines and the other control wells. The time of contact between the cells and *L. crispatus* M247 LMG P-23257 was 60 min at 37°C with 5% CO_2. The Multiplicity of Infection (MOI) for *L. crispatus* M247 LMG P-23257 and the cell lines was 5:1. After incubation, 1 ml of the control wells was harvested and serially diluted and plated on MRS agar (Oxoid, Thermo Fisher Scientific); for the seeded wells, the medium was removed and the cell monolayers were washed 3 times with 1 ml of Hank's balanced salt solution for 5 min. The 4 seeded wells were inoculated with 100 µl of trypsin and incubated for 5 min at 37°C to break the cell monolayers, and the solutions were recovered with 900 µl of Maximum Recovery Diluent (MRD, Difco, BD). The obtained ml were serially diluted and plated to quantify the adhesive ability of *L. crispatus* M247 LMG P-23257. All plates were incubated for 72 hours at 37°C under anaerobic conditions. Results were analyzed in order to determine the adhesive abilities of *L. crispatus* M247 LMG P-23257 to HT29-MTX and HeLa cell lines. The adhesion percentages were calculated using the following formula: P=(µ/M)×100, where P represents the adhesion percentage of *L. crispatus* M247 LMG P-23257 to the human cell lines HT29-MTX or HeLa; µ represents the vible count of *L. crispatus* M247 LMG P-23257 bonded to the human cell lines HT29-MTX or HeLa expressed as a logarithmic value; and M represents the vital count of *L. crispatus* M247 transformed as a logarithmic value for the wells without human cells.

Results

The antibiotic resistance profiles of *L. crispatus* M247 were assessed for 19 antibiotics and for boric acid. Ten of these antimicrobials were compared to the defined microbiological cut-off values reported in the EFSA guidelines. The *L. crispatus* M247 strain was considered susceptible to a specific antimicrobial when its growth was inhibited at a concentration equal to or lower than the EFSA established cut-off value. The reference for the evaluation of the susceptibility/resistance profile considered was the *L. acidophilus* group. As reported in **Table 1**, *L. crispatus* M247 was susceptible to 10 of the 19 antibiotics tested in compliance with EFSA guidelines and to an additional seven not included in the EFSA panel.
The strain exhibited a significant resistance to metronidazole, sulfamethoxazole and boric acid. It is noteworthy that metronidazole and boric acid are routinely used for the treatment of vaginal infection.

Antibiotic	MIC (µg/ml)	EFSA Guidelines
Gentamicin	1	S
Kanamycin	8	S
Streptomycin	2	S
Tetracycline	0,25	S
Erythromycin	0,03	S
Clindamycin	0.03	S
Chloramphenicol	1	S
Vancomycin	1	S
Ampicillin	1	S
Ciprofloxacin	64	nr
Neomycin	1	/
Penicillin	0,5	/
Quinupristin-dalfopristin	1	/
Linezolid	4	/
Trimethoprim	64	/
Rifampicin	0.5	/
Rifaximin	0.25	/
Metronidazole	>256	/
Sulfamethoxazole	>256	/
Boric acid	10.000	/

Table 1 Minimum inhibitory concentration (MIC) values for L. *crispatus* M247 expressed as µg/ml. First column: antimicrobial substance, second column: MIC values obtained, third column: comparison with the European Food Safety Authority (EFSA) microbiological cut-off values (S: susceptible, nr: not required, /: not mentioned in the EFSA guidelines). The strain was found to be resistant to metronidazole, sulfamethoxazole and boric acid

Adhesion tests for *L. crispatus* M247 were carried out in duplicate for the two selected human cell lines HT29-MTX and HeLa.

As reported in **Table 2**, the strain displayed significant adhesive properties, with values of 75% and 72% for HT29-MTX and HeLa cells, respectively.

HT29-MTX cells

Wells without cells				Seeded wells				Adhesion %
-3	-4	CFU/ml	Log	-2	-3	CFU/ml	Log	
153	14	1,5E+05	5,2	76	5	7,4E+03	3,9	75
147	16	1,5E+05	5,2	84	6	8,2E+03	3,9	76

HeLa cells

Wells without cells				Seeded wells				Adhesion %
-4	-5	CFU/ml	Log	-2	-3	CFU/ml	Log	
65	8	6,6E+05	5,8	160	22	1,7E+04	4,2	72
69	9	7,1E+05	5,9	171	20	1,7E+04	4,2	72

Table 2 Adhesion percentages obtained for *L. crispatus* M247 on HT29-MTX and HeLa cells. *L. crispatus* M247 displayed an adhesion ability of 75% and 72%, respectively, for HT29-MTX and HeLa cells. CFU = colony forming unit

Discussion

M247 is one of the few *L. crispatus* strains that have been properly documented for human use. A good colonizer of the human gut, where it exerts strong anti-inflammatory effects, and the human vagina, where it combats dysbiosis, the strain can be used orally and/or topically to restore the normal vaginal microbiota, contributing to the construction of a stable *L. crispatus*-based community state type. Clearly, its vaginal colonizing properties could be enhanced by the simultaneous use of a molecule limiting the growth of other bacterial species inhabiting the vaginal consortium but not affecting its own vitality. In an attempt to highlight this possibility, we have thoroughly investigated the susceptibility of the M247 strain to various antibiotics and to boric acid. We have observed that the M247 strain shows a relevant resistance to metronidazole, sulfamethoxazole and boric acid. Being metronidazole-resistant seems to be quite important for probiotics which are medically used to maintain a healthy vaginal microbiota. Metronidazole is, in fact, commonly used to treat BV. Since BV seems to occur more frequently in women with a vaginal consortium lacking in lactobacilli (CST IV) and/or containing lactobacilli other than *L. crispatus* (CST II, III and V), the administration of a colonizing *L. crispatus* is a possible tool to prevent infection. In this regard, the co-administration of an *L. crispatus* as M247 (a metronidazole-resistant strain) along with metronidazole should give a further colonizing advantage to M247 which, after administration, will enter into a "less crowded" consortium where all of the metronidazole-sensitive pathogenic and commensal bacteria have been removed by metronidazole. Of course, the total absence of mobile elements in the M247 genome highlights the impossibility of making such a resistance transferrable to other bacterial species, pathogens included, and make the human use of M247 quite safe [14]. In addition, boric acid is a molecule that is commonly used in gynaecology to treat vaginal infections. Particularly effective in cases of antifungal-resistant candidiasis, the confirmation of the resistance of M247 to this molecule supports the co-administration of boric acid and M247, to favour M247 colonization even while attempting to eradicate candida from the vagina.

Last, but not least, we have also investigated the adhesive properties of M247 with respect to vaginal cells. Previous studies had already demonstrated the adhesivity of *L. crispatus* M247 to enterocytes [6]. Such a finding provided a further possible explanation for the observed gut-colonizing properties of the strain. Similar findings, demonstrating the capability of M247 to strictly adhere to vaginal cells, providing a mechanistic explanation of the observed vaginal colonization, were still lacking. With our study we have demonstrated that strain M247

can adhere to vaginal cells with a percentage adhesion higher than 70%.

Conclusions

From our results:

1) the strain M247 was demonstrated to be resistant to metronidazole, sulfamethoxazole and boric acid;
2) on this basis it should be co-administered with metronidazole or with boric acid to more effectively eradicate vaginal pathogens, bacterial and fungal respectively, and to facilitate M247 vaginal colonization;
3) the herewith demonstrated adhesivity capabilities of M247 provide a mechanistic explanation of its activity in colonizing the vaginal epithelium.

Conflict of Interest

FDP is a member of the Pharmextracta Scientific Board; AB has recently been a Pharmextracta consultant; VS, SS, DC and ME all work within the research group of AAT.

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