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Lysozyme as a cotreatment during antibiotics use against vaginal infections: An in vitro study on *Gardnerella vaginalis* biofilm models

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Summary. Bacterial vaginoses are frequent in women, most of them involving *Gardnerella vaginalis*. In more than 50% of the cases, usual antibiotic treatments are not capable of eliminating completely the infection, leading to recurrent vaginosis. In addition to the appearance of antibiotic resistance, recurrence can be due to the development of a biofilm by *G. vaginalis*. In vitro experiments on *G. vaginalis* biofilms showed that the biofilm protected bacteria from the antibiotic clindamycin. Also, recombinant human lysozyme (rhLys) was able to both degrade biofilms and prevent their formation. This degradation effect persisted whenever other vaginal commensal or pathogenic microorganisms were added to the culture and on each tested clinical biofilm-producing strain of *G. vaginalis*. The co-administration of rhLys and clindamycin or metronidazole improved both antibiotics' efficiency and lysozyme-driven biofilm degradation. The comparison of both clindamycin and metronidazole antibacterial spectra showed that metronidazole was preferable to treat vaginosis. This suggests that human lysozyme could be added as an anti-biofilm cotreatment to vaginal antibiotherapy, preferably metronidazole, against *Gardnerella vaginalis* infection in vivo. [**Int Microbiol** 19(2): 101-107 (2016)]

Keywords: *Gardnerella vaginalis* · recombinant human lysozyme · clindamycin · metronidazole · biofilms in pathogens

It has been estimated that more than women 300 million around the world suffer from urogenital infections, including bladder, kidney, vagina, urethra, periurethra, and cervix infections [15,18]. During vaginal infections, the commensal saprophytic vaginal microbiota is replaced by a pathogenic microbiota [14]. Bacterial vaginoses are frequent [12] and most of them involve, or could even be triggered by, *Gardnerella vaginalis* [19], a bacterium capable to produce a biofilm. The usual treatment is antibiotherapy, mainly with clindamycin or metronidazole [2]. However, after antibiotic treatment, more than 30% of the patients suffer from recurrent infections after 3–6 months (and more than 50% after 12 months) [5]. This is due, probably, to the inability of antibiotics to efficiently reach bacteria embedded inside the biofilm. Biofilms have indeed been shown to be able to trap antibiotics on their matrix components [3,16], reducing their availability inside the biofilm and thus their efficiency. This can be one of the mechanisms causing antibiotic resistance. In addition, bacteria growing inside biofilms can be in a quiescent state, less sensitive to antibiotic treatment [11]. After the treatment, residual living bacteria can therefore grow again and originate a new infection burst. Additionally, antibiotherapy might also kill all the pathogenic bacteria inside the biofilm, leaving intact the biofilm matrix, which could serve

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as a nest for new pathogens. Enzymatic targeting of biofilms by lysozyme has been reported to degrade biofilms produced by certain bacterial species [10,20], but could have opposite effects on other biofilms [21]. In the present work, we analyzed the use of recombinant human lysozyme as a potential *G. vaginalis* biofilm-degrading treatment in co-administration with antibiotics (clindamycin or metronidazole) in order to reduce the amount of biofilm biomass and to improve antibiotic efficiency.

Material and methods

Strains, culture conditions and biofilm formation. The following bacterial strains were purchased from the BCCM/LMG Bacteria Collection (Gent, Belgium): *Lactobacillus crispatus* (LMG12005), *Lactobacillus gasseri* (LMG13134), *Lactobacillus jensenii* (LMG6414), *Lactobacillus iners* (LMG18913), *Gardnerella vaginalis* (LMG14333 and LMG7832), *Prevotella bivia* (LMG6452), *Bacteroides vulgatus* (LMG17767), *Peptostreptococcus tetradius* (LMG14264) and *Escherichia coli* (LMG2092). The *Candida albicans* strain was purchased from the ATCC (ATCC10231). Prof. Pierrette Melin, from the Medical Microbiology Laboratory, University Hospital of Liege, Belgium, provided us with 9 clinical strains of *G. vaginalis* isolated from vaginal samples and coded Gv 1 to Gv 9. Lactobacillus species are commensal, other species are pathogenic.

The cells were maintained on Schaedler agar enriched with vitamin K_1 and 5% sheep blood (BioMerieux, Brussels, Belgium) at 37 °C under anaerobic conditions. Biofilms were grown at 37 °C under anaerobic atmosphere in 96-well plates in 100 µl Schaedler broth enriched with vitamin K_3 (BioMerieux) for colony forming units (CFU) counting and for biofilm quantification. Ring-Test strips in 100 µl BHI (BioMerieux) were used for Ring-Test assay to evaluate the ability of strains to form biofilms. E-test Clindamycin (BioMerieux) was used to test antimicrobial resistance according to the manufacturer's instructions.

Antibiotics and lysozyme. The following antibiotics and lysozyme tested in this work were purchased from Sigma-Aldrich (Belgium): clindamycin hydrochloride (C5269), metronidazole (M1547), and recombinant human lysozyme (rhLys) produced in rice (L1667). This lysozyme was selected in an attempt to reduce as much as possible the human immune response [1,17].

Toxicity test on eukaryotic cells (MTS assay). The toxicity of rhLys, clindamycin and metronidazole was tested on the VK2/E6E7 cell line, representative of the vaginal epithelium and obtained from its creator, Prof. Raina Fichorova (Boston, MA, USA) [7]. A total of 20,000 cells in 100 µl per well were cultured in 96-well plates in keratinocyte serum-free medium (Ker-SFM, Life Technologies, Gent, Belgium) as previously described [7]. After 24 h, the culture medium was replaced (100 µl/well) by antibiotics and lysozyme dissolved in culture medium or by fresh culture medium alone (negative control). Toxicity was assessed 24 h later using the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) (MTS) assay (Promega, Brussels, Belgium) according to the manufacturer's instructions.

Biofilm quantification, biofilm immobilization assay and cellular viability of biofilm bacteria. The quantification biofilm formation was performed by using the crystal violet staining assay. Biofilms were fixed in 100 µl pure methanol for 15 min and then were stained in 100 µl 0.1% crystal violet solution (Merck, Belgium) for another 15 min. Excess stain was removed with water. Staining was solubilized from biofilms in 100 µl 33% acetic acid and the absorbance of the liquid was measured at 595 nm wavelength using an Epoch microplate spectrophotometer (BioTek Instruments, Winooski, VT, USA).

Biofilm immobilization assay from BioFilm Control (Saint Beauzire, France), providing data about biofilm cohesion, has been previously described [6]. Additionally, the strips were here coated at 37 °C with 100 µl 3% bovine serum albumin (BSA) in phosphate buffer saline (PBS) for 1 h, and then rinsed. Biofilms were generated as described above, in the presence of the kit magnetic beads. Antibiotics and lysozyme or negative control (culture broth alone) were added (10 µl/well) when required without replacing the broth, and the beads displacement was tested according to the manufacturer's instructions. The counting of viable bacteria from biofilms was performed by counting colony forming units (CFU). Biofilms were generated as described above, then scraped off and resuspended in 200 µl of fresh medium. Suspensions were then plated on Schaedler agar enriched with vitamin K_1 and 5% sheep blood (Becton Dickinson, Belgium) and incubated at 37°C under anaerobic atmosphere for 48 h before CFU counting.

Statistical tests. Statistical analyses were performed on toxicity tests, biofilm immobilization assays and biofilm quantification assays results using 2-way ANOVA followed by Bonferroni's post-test.

Results

Toxicity test on eukaryotic cells (MTS assay). The results of the assessment of potential toxicity of clindamycin, metronidazole and rhLys on the VK2/E6E7 cell line, as a model for the vaginal epithelium, are shown in Fig. 1. The maximal concentrations of the tested antibiotics and lysozyme were selected based respectively on their solubility and on the literature [21]. No detectable toxicity was observed in the presence of metronidazole (tested up to 600 µg/ml), lysozyme (tested up to 100,000 U/ml) or clindamycin up to 200 µg/ml. However, the cell activity was significantly lower in the presence of clindamycin 400 and 600 µg/ml. Upper concentrations limits for this study were as follows: 100 µg/ml for clindamycin (200 µg/ml was considered to be too close to toxic levels), 600 µg/ml for metronidazole, and 100,000 U/ml for lysozyme.

Protection provided to *Gardnerella vaginalis* by its biofilm (E-Test and CFU counting). Protection against antibiotic provided to bacteria present in the vaginal biofilm was illustrated on *G. vaginalis* (LMG14333) using clindamycin. Using an E-Test, the minimum inhibitory concentration (MIC) of clindamycin for that strain was 0.064 µg/ml. We arbitrarily multiplied that concentration by a 1000-fold factor (64 µg/ml) and applied it on a 24 h *G. vaginalis* biofilm for another 24 h. CFU counting was then performed

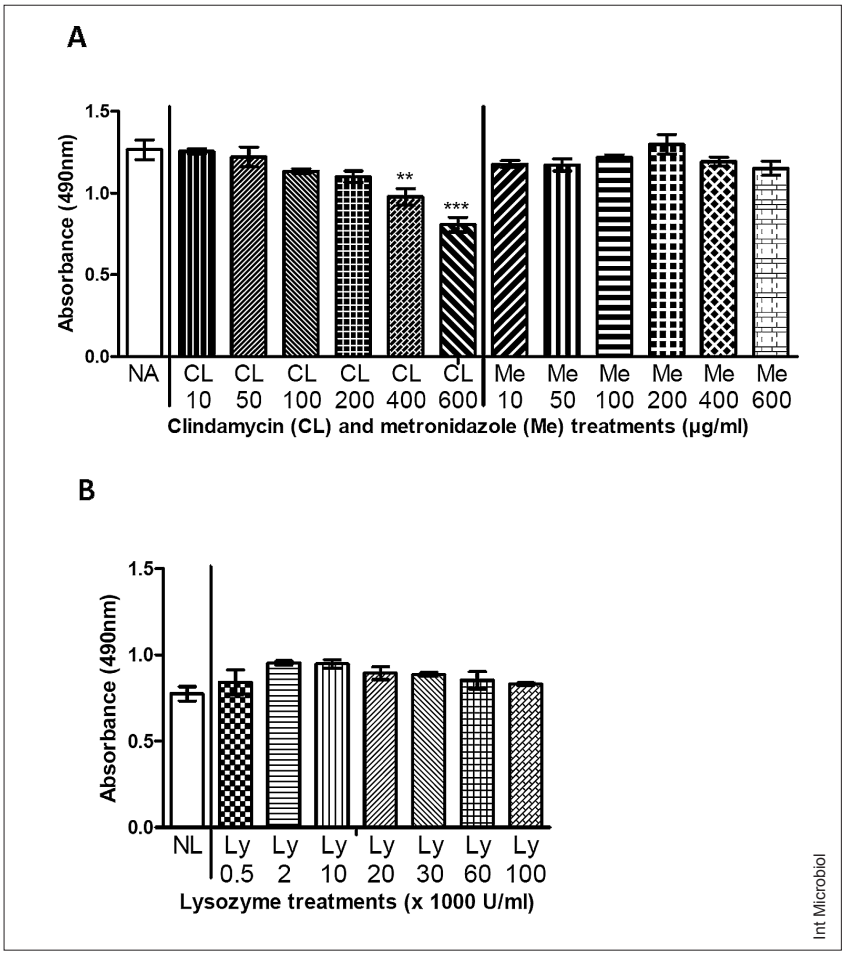

Fig. 1. Cellular activity (MTS assay) of vk2/E6E7 cells after 24 h in the presence of (**A**) dilutions of clindamycin (CL) or metronidazole (Me) (2 h MTS contact), or (**B**) rhLys (Ly) (1 h MTS contact). NA: no antibiotic. NL: no lysozyme. Statistically significant differences from control without antibiotic are represented as follows: **: $P < 0.01$; ***: $P < 0.001$; n = 3.

on bacteria present in the supernatant or included inside the biofilm. Results showed 100% mortality for the cells present in the supernatant while 0.66% of the cells present in the biofilm survived the treatment, when compared to antibiotic-free control.

Anti-biofilm activity of rhLys (biofilm immobilization assay and crystal violet staining). Recombinant human lysozyme rhLys was added (100,000 U/ml) to *G. vaginalis* (LMG7832) cells before biofilm formation or to 24-h biofilm to check its anti-biofilm activity. Results showed that this lysozyme rhLys could both prevent biofilm formation and degrade existing biofilm (Fig. 2).

A pathological vaginal biofilm contains multiple species. Biofilms were generated by a 50:50 mix of *G. vaginalis* (LMG14333) and each of the following vaginal species: *L. crispatus*, *L. gasseri*, *L. jensenii*, *L. iners*, *Prev. bivia*, *B. vulgatus*, *E. coli*, *Pept. tetradius* and *C. albicans*. Recombinant human lysozyme rhLys 100,000 U/ml was added on 24-h biofilms and biofilm were quantitated 24 h later. Figure 3 shows the differences in the degradation of biofilms of the nine species tested.

The anti-biofilm activity of rhLys 100,000 U/ml was then tested for 24 h on 24-h biofilms produced by 9 clinical strains of *G. vaginalis*. The lysozyme significantly reduced the biomass of 8 out of the 9 biofilms tested (Fig. 4), with great differences among strains. Strain Gv 4 produced very little biofilm compared to the other tested strains and its biomass reduction by this lysozyme did not reach statistical significance. Biomass reduction in our test conditions ranged from 20% to 95%, with a mean of 59% for these 9 strains.

Impact of rhLys on antibiotic efficiency (CFU counting and biofilm immobilization assay). The impact of rhLys on antibiotic efficiency against *G. vaginalis* biofilm was first tested with clindamycin. *Gardnerella vaginalis* (LMG14333) biofilms were generated during 24 h. Various concentrations of clindamycin (0.5, 1, 2, 4, 8, 16, 32

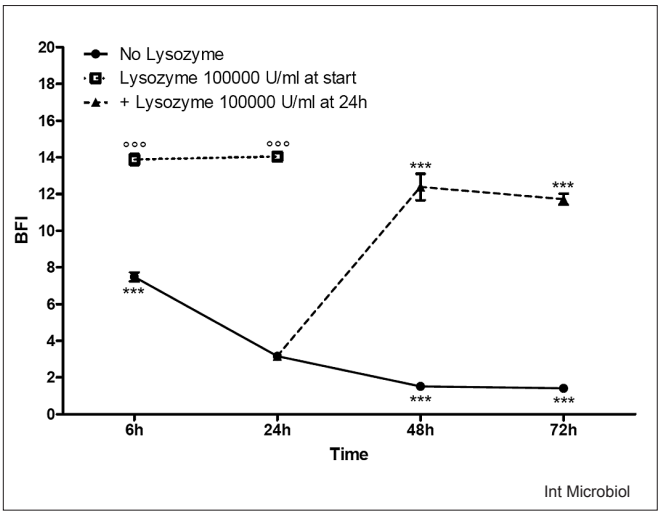

Fig. 2. Anti-biofilm activity (biofilm immobilization assay) of rhLys 100,000 U/ml added before biofilm formation or on a 24-h biofilm produced by *Gardnerella vaginalis*. Lower BioFilm Index (BFI) means stronger biofilm. Statistically significant differences between conditions with and without rhLys at start for each time point are represented as follows: °°°: $P < 0.001$, while differences from 24 h without lysozyme at start are represented as follows: **: $P < 0.01$; ***: $P < 0.001$; n = 3.

and 64 µg/ml) were then added during 24 h with or without lysozyme 20,000 U/ml.

Adding rhLys to clindamycin from 4µg/ml and above reduced the number of remaining CFUs obtained after biofilm homogenization by factors ranging from 4.1-fold to 8.6-fold, providing a more efficient treatment against *G. vaginalis* biofilm than clindamycin used alone.

Clindamycin and metronidazole can both be used to treat vaginoses. In order to check which antibiotic had the most suitable range of activity against vaginal species, each antibiotic was added to 72 h biofilm generated by each of the following species: *L. crispatus, L. gasseri*, *L. jensenii*, *L. iners*, *G. vaginalis* (LMG14333), *Prev. bivia*, *B. vulgatus*, *Pept. tetradius* and *E. coli*.

Table 1 shows first that, at their highest tested concentration, clindamycin killed 100% of the cells of 2 out of 4 pathogenic species, while metronidazole achieved 100% mortality in each of these species. Also, clindamycin (100 µg/ml) killed 100% of the cells of each of the tested commensal species. Some *L. crispatus* and *L. gasseri* cells remained alive and were capable of proliferation after metronidazole 600 µg/ml treatment. *L. iners* and *B. vulgatus* did not survive the 96h culture and produced no CFU. They were not included in Table 1. The yeast *C. albicans* has not been tested here.

Considering the interest of selecting metronidazole to kill vaginal pathogenic bacteria in biofilms (Table 1), rhLys (100,000 U/ml) was added to metronidazole (600 µg/ml) on a 72 h *G. vaginalis* (LMG7832) biofilm and their impact on biofilm degradation was tested after 24 h using a biofilm immobilization assay. Metronidazole-only and lysozyme-only conditions were added in comparison. Metronidazole-only condition did not show any biofilm degradation (72 h biofilm BFI [BioFilm Index]: 1.45; BFI after 24 h of metronidazole: 1.50). A low BFI means a mechanically resistant biofilm able to block the kit magnetic beads. As expected, the lysozyme-only condition weakened the biofilm (BFI rising up to 7.97). However, lysozyme and metronidazole co-administration degraded the biofilm even more, with a BFI reaching 10.59. These differences were statistically significant ($P < 0.001$).

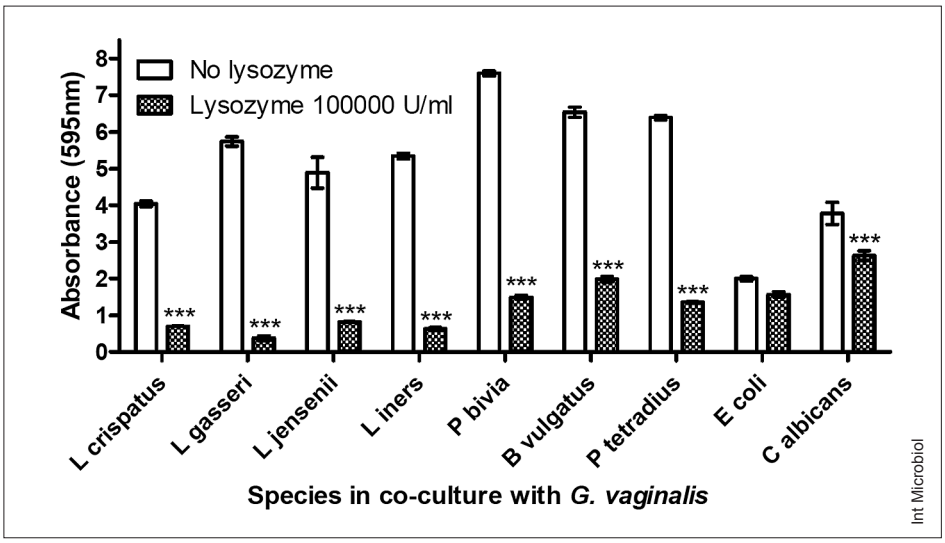

Fig. 3. Anti-biofilm activity (crystal violet staining) of rhLys 100,000 U/ml added on 24 h biofilms produced by a 50:50 mixes of *Gardnerella vaginalis* and each of 9 other vaginal species. Statistically significant differences between conditions with and without lysozyme are represented as follows: ***: $P < 0.001$; n = 3.

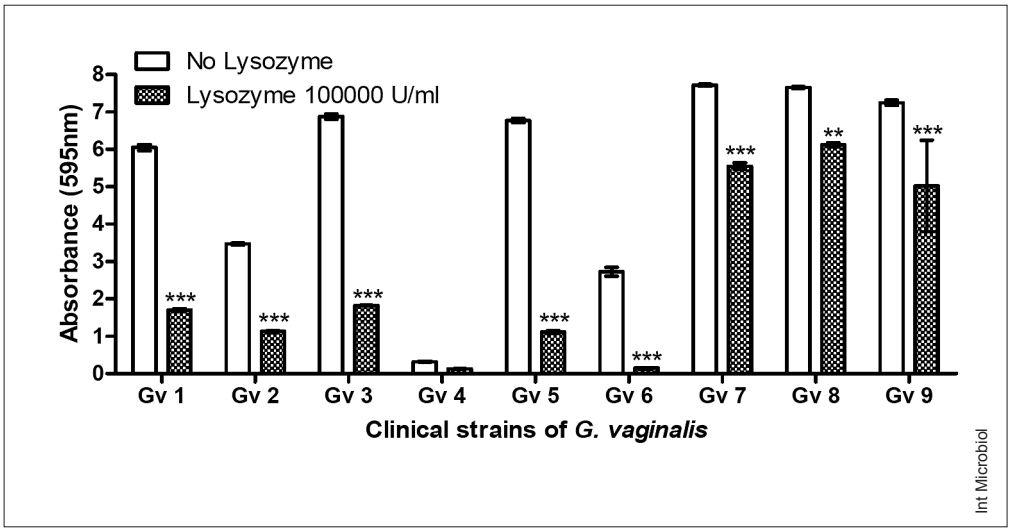

Fig. 4. Anti-biofilm activity (crystal violet staining) of rhLys added during 24 h on 24-h biofilms produced by each of 9 clinical strains of *Gardnerella vaginalis*. Statistically significant differences between conditions with and without lysozyme are represented as follows: **: $P < 0.01$, ***: $P < 0.001$; n = 4.

Discussion

Nowadays, it is admitted that biofilms can act as a protective shielding for bacteria [4,13]. This can occur following several mechanisms, such as trapping antibiotics before they can reach their target [16] or keeping bacteria in quiescent state in some parts of the biofilm [9]. We found that biofilms produced by *G. vaginalis* were able to protect the bacteria against clindamycin, an antibiotic frequently used to treat vaginoses, allowing a fraction of the cells to survive an antibiotic administration equal to 1000× the MIC. In vivo, this phenomenon could trigger recurrent vaginoses. Highly recurrent vaginoses could be due to bacteria forming very protective biofilms. In addition, even when 100% of the bacteria are killed by the antibiotic treatment, the persistence of the residual matrix of the biofilm can be a favorable soil for bacteria recolonization [8]. Therefore targeting the biofilm itself is crucial to reduce vaginosis recurrences. Several techniques used in our study, including the biofilm immobilization assay, demonstrated the interest of using lysozyme to fragilize and degrade *G. vaginalis* biofilms. We showed that recombinant human lysozyme could reduce both

Table 1. Comparative activity spectrum of clindamycin and metronidazole administered during 24-h on 72-h biofilms generated by commensal and pathogenic vaginal strains

Species	CTRL	Clindamycin (µg/ml)			Metronidazole (µg/ml)		
		0.1	10	100	0.1	10	600
Lactobacillus crispatus	20,000[a]	15,168	1,672	0	20,000[a]	20,000[a]	19,472
Lactobacillus gasseri	20,000[a]	67	0	0	20,000[a]	4,462	12,428
Lactobacillus jensenii	15	21	0	0	6,198	0	0
Gardnerella vaginalis	15,216	153	0	0	5,516	7	0
Prevotella bivia	5	0	0	0	3,424	0	0
Peptostreptococcus tetradius	13,126	13,624	3,840	150	7,208	0	0
Escherichia coli	20,000[a]	20,000[a]	20,000[a]	20,000[a]	20,000[a]	20,000[a]	0

[a]20,000 CFUs is the counting limit. Therefore, it must be read as "at least 20,000 CFUs".

cohesion and biomass of pre-existing *G. vaginalis* biofilms and that it could also prevent biofilm formation. A recent study supports these findings [10].

In vivo vaginal biofilms may contain many species [22]. By using in vitro experiments, we modeled multi-species biofilms, each model originated by mixing *G. vaginalis* with one other microorganisms selected for its presence in pathological or non-pathological vagina. The biofilms so obtained were dependent on the species involved, and each of them was degraded by the recombinant human lysozyme

We also tested rhLys on clinical strains of *G. vaginalis* that presented great heterogeneity in biofilm production. The lysozyme degraded the biofilm produced by each clinical strain, its efficiency depending on the tested strain, which could be due to differences in the matrix constituting the biofilms.

We found that the biofilm produced by *G. vaginalis* was able to protect this species against clindamycin. Therefore, the combined use of an antibiotic (clindamycin or metronidazole) with rhLys was tested, and the bactericidal and biofilm degradation effects were greater than when lysozyme or antibiotic were tested alone. The comparison of both antibiotics regarding their toxicity against eukaryotic cells and on their spectrum of activity against vaginal species, revealed that metronidazole was a better choice than clindamycin. Regarding biofilm degradation, the increased destructive effect obtained when adding metronidazole to lysozyme might not only be due to the destruction of the existing biofilm but also to a lower production of new biofilm matrix due to the death of bacteria producing it. Co-administration of both molecules could therefore be highly recommended as a treatment of *G. vaginalis*-based vaginoses, helping not only to treat an isolated vaginosis but also to prevent recurrent vaginoses. A combined lysozyme-metronidazole toxicity test on epithelial would then be advisable as part of the treatment validation steps.

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Competing interests. None declared.

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