

Lactobacillus crispatus represses vaginolysin expression by BV associated Gardnerella vaginalis and reduces cell cytotoxicity *Anaerobe* (2018) 50:60–63

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Keywords: Bacterial vaginosis, *G. vaginalis*, *L. crispatus*, vaginolysin, sialidase

Abstract

Using a chemically-defined medium simulating genital tract secretions, we have shown that pre-adhering *Lactobacillus crispatus* to HeLa epithelial cells reduced cytotoxicity caused by *Gardnerella vaginalis*. This effect was associated to the expression of vaginolysin and was specific to *L. crispatus* interference, as other vaginal facultative anaerobes had no protective effect.

Main text

Lactobacillus crispatus is an important urogenital species that is routinely found in the vagina of healthy women [1, 2], contributing to the maintenance of normal vaginal microbiota, while its absence has been associated with a range of vaginal abnormalities, especially bacterial vaginosis (BV) [3, 4]. BV is the leading vaginal disorder in women of reproductive age worldwide and it has been associated with serious public health consequences, including pelvic inflammatory disease [5], acquisition and transmission of the HIV virus [6] and preterm birth [7]. It has been proposed that *Gardnerella vaginalis*, a facultative anaerobe, is the pathogen responsible for the initiation of BV [8, 9]. This bacteria is capable of adhering to vaginal cells, establish biofilms and induce cytotoxicity on vaginal epithelial cells [10, 11]. However, despite being the most prevalent and virulent species found in BV, *G. vaginalis* can also be a part of the vaginal microbiota in healthy women [12, 13]. Genetic differences among *G. vaginalis* strains may underlie the diverse pathological features and outcomes that have been associated with this species, raising the possibility that distinct pathogenic and non-pathogenic strains or even subspecies exist [14-18]. The *G. vaginalis* diversity might represent a critical turning point in clarifying ecological interactions and virulence factors contributing to symptoms and sequelae of BV [19]. Therefore, understanding the interactions between beneficial lactobacilli and *G. vaginalis* is of extreme importance to help unravel the pathogenesis and progression of this condition.

This study aimed to investigate whether the BV-positive and BV-negative *G. vaginalis* strains differ in their abilities to interact with a cervical epithelial (HeLa) cell monolayer pre-treated with *L. crispatus*, using a functional cytotoxicity model, which represents a significant improvement over our previous study [10], since we used a medium simulating genital tract secretions (mGTS) [20] and epithelial cells were previously covered with *L. crispatus*, mimicking a normal vaginal ecosystem. First, bacterial suspensions of three BV-positive *G. vaginalis* strains (UM067, NCBI accession number: KP996675.1; UM121, NCBI: KP996681.1; UM241, NCBI: KP996683.1), three BV-negative *G. vaginalis* strains (UM016, NCBI: KP996686.1; UM085, NCBI: KP996679.1; UM131, NCBI: KP996676.1) and *L. crispatus* EX533959VC06 were grown in mGTS for 48 h at 37°C with 10% CO₂ (Shel Lab, Cornelius Oregon, USA) [20]. For the cytotoxicity assays, *L. crispatus* suspension, adjusted to 1 × 10⁷ cfu/mL, was added to a monolayer of HeLa cells for 3 h. Afterwards, blind bacterial suspensions, adjusted to the same

concentration, were added to a HeLa cells monolayer pre-adhered with *L. crispatus* for 3 h. Additional controls were conducted to analyze whether *L. crispatus* effects reflect a specie-dependent response or whether other vaginal associated species can exhibit similar effects. Thus, herein, we included the following bacterial species as controls: *Corynebacterium tuberculostearicum* UM137Ct2 (NCBI: KT805279); *Staphylococcus warnerii* UM224Sw (NCBI: KT923488); and *Nosocomiicoccus ampullae* UM121Na (NCBI: KT805272), due to their low cytotoxicity and adhesion levels similar to *L. crispatus* [21]. Cytotoxicity was scored on a 0 to 5 scale [11]. Numeric scores were assigned as follows: 0, no difference between the test and the control; 1, 25% of the cells were rounded; 2, 25–50% of the cells were rounded; 3, 50% of the cells were rounded with partial monolayer disruption; 4, 50% cells were rounded, with extensive disruption of the monolayer; and 5, complete disruption or absence of the monolayer.

Furthermore, we also quantified the expression levels of vaginolysin (*vly*) and sialidase (*sld*) transcripts in three different conditions: *G. vaginalis* planktonic cells (pre-infection) (*i*); after *G. vaginalis* infection on a monolayer of HeLa cells (post-infection in HeLa) (*ii*); and after *G. vaginalis* infection on a monolayer of HeLa cells pre-treated with *L. crispatus* (post-infection in HeLa with Lc) (*iii*). Briefly, total RNA of these three different conditions was extracted using an ExtractME RNA Bacteria & Yeast kit (Blirt S.A., Poland) with minor changes, as optimized before [22]. Quantitative PCR (qPCR) was prepared by mixing together 5 μ L of iQ SYBR green supermix (Bio-Rad, Hercules, CA, USA), 2 μ L of 1:100 diluted cDNA, 0.5 μ L of 5 μ M Forward and Reverse primes and water up to 10 μ L. Primer sequences for target genes are listed in our previous studies, as follows: *16sRNA* (Fw and Rv) [23]; *vly* (Fw2 and Rv2) and *sld* (Fw1 and Rv1) [10]. Normalized gene expression was determined by using the delta C_t method ($E^{\Delta C_t}$), a variation of the Livak method, where $\Delta C_t = C_t$ (reference gene) - C_t (target gene) and E stands for the reaction efficiency experimentally determined. At least three biologic replicates of each condition were performed. The data were analyzed using the *t*-test or A-NOVA with the statistical software package GraphPad Prism version 6 (GraphPad Software Inc., La Jolla, CA, USA). *P*-values of less than 0.05 were considered significant.

Our results highlighted that BV-positive *G. vaginalis* strains were able to induce more extensive damages on the HeLa monolayer than BV-negative strains (Fig. 1a), supporting our previous data [10]. Remarkably, when *L. crispatus* was pre-adhered to HeLa, cytotoxicity effect of all *G. vaginalis* was significantly reduced ($p < 0.05$).

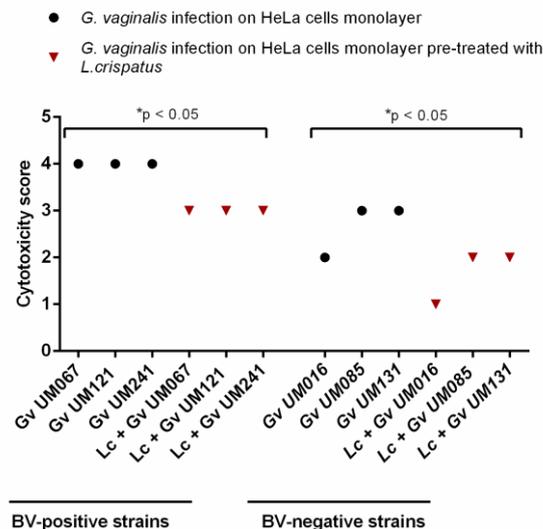


Fig. 1A | Differential cytotoxicity profile of *G. vaginalis* (Gv) isolated from women with BV (BV-positive strains) and without BV (BV-negative strains). Cytotoxicity score of BV-positive and BV-negative Gv strains, in two different conditions: Gv infection on a monolayer of HeLa cells and on a monolayer pre-coated with *L. crispatus* (Lc). *Values are significantly different between the 2 groups of Gv strains under the same conditions (one-way ANOVA, $p < 0.05$).

A possible explanation for this fact could be that *L. crispatus* is blocking *G. vaginalis* adherence [24], indicating that competitive exclusion of this species could be a key role protecting the vagina from invading pathogens [25]. Trying to unravel whether this response was *L. crispatus*-specific, we pre-coated the HeLa monolayer with *C. tuberculostearicum*, *N. ampullae* or *S. warnerii*. Curiously, we verified that the selected bacterial species were not able to reduce the cytopathogenic alterations caused by *G. vaginalis* on the epithelial monolayer (Fig. 1b). This supports the specific role of *L. crispatus* in reducing *G. vaginalis* cytotoxicity. Recent studies have shown that cytoprotective effect of *L. crispatus* seems to be related with stimulation of immune response [26], reduction of cell apoptosis [27], or changes on the physical properties of the plasma membrane in HeLa cells [28].

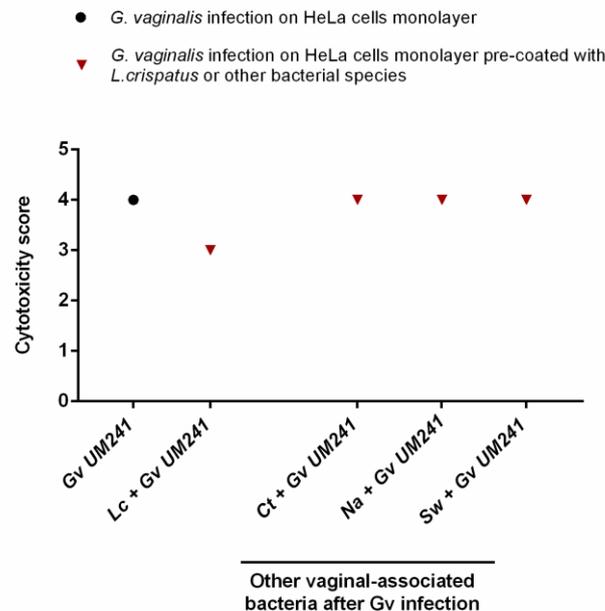


Fig. 1B | Differential cytotoxicity profile of *G. vaginalis* (Gv) isolated from women with BV (BV-positive strains) and without BV (BV-negative strains). Cytotoxicity score after Gv infection on a monolayer of HeLa cells pre-coated with Lc or with other vaginal-associated species: *C. tuberculostearicum* (Ct), *N. ampullae* (Na) and *S. warnerii* (Sw). *Values are significantly different between the 2 groups of Gv strains under the same conditions (one-way ANOVA, $p < 0.05$).

The different cytotoxic activity between BV-negative and BV-positive isolates could be due to a pre-forming toxin produced by *G. vaginalis*, vaginolysin, which is able to induce cell death and is thus a virulence factor [29]. Furthermore, *G. vaginalis* virulence has also been associated to sialidase [30]. This enzyme is known to facilitate the destruction of the protective mucus layer on the vaginal epithelium [30]. Therefore, to compare the cytotoxicity effect and expression levels of *vly* and *sld* transcripts between the two groups of strains, we used a BV-positive *G. vaginalis* UM241 and a BV-negative *G. vaginalis* UM131 strain, which carry both genes of interest (Castro et al. 2015). Our results revealed differences in the expression of both genes, being the transcript levels of *vly* (Fig. 2a) higher when compared to the transcript levels of *sld* (Fig. 2b), similar to what was verified in our previous report [10].

Interestingly, our data also revealed that after post-infection by a BV-positive strain, transcript levels of both genes were significantly higher than in pre-infection (Fig. 2a and Fig. 2b). Remarkably, pre-coating the HeLa monolayer with *L. crispatus* caused a repression of expression in 2.58-fold and 1.89-fold for *vly* and *sld* transcripts, respectively. Regarding the BV-negative strain, the same tendency was observed for *vly* expression between the post- and pre-infection conditions (Fig. 2a). However, no differences were detected in *sld* expression (Fig. 2b). Surprisingly, our findings suggest that no direct association seems to exist between *vly* and *sld* expression by a BV-negative group and the presence of *L. crispatus*, despite the ability of this species to repress the cytotoxic activity of both *G. vaginalis* groups.

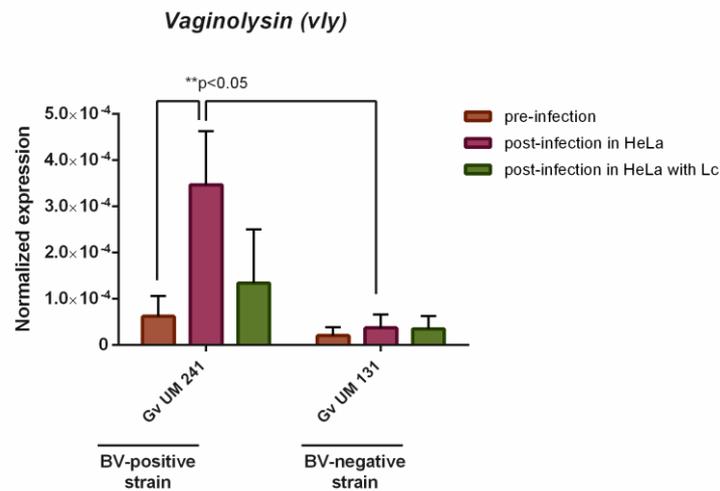


Fig. 2A | Differential transcriptomic profile of *G. vaginalis* (Gv) isolated from women with BV (BV-positive strain) and without BV (BV-negative strain). Vaginolysin (*vly*) transcript levels within Gv planktonic cells (pre-infection), after Gv infection on a monolayer of HeLa cells (post-infection in HeLa) and after Gv infection on a monolayer of HeLa pre-treated with *L. crispatus* (post-infection in HeLa with Lc) were quantified. Results are expressed as normalized expression in relation to 16S rRNA and represented as mean ± SEM. **A specific condition was significantly different in terms of gene expression between a BV-positive and a BV-negative strain (T-test, $p < 0.05$).

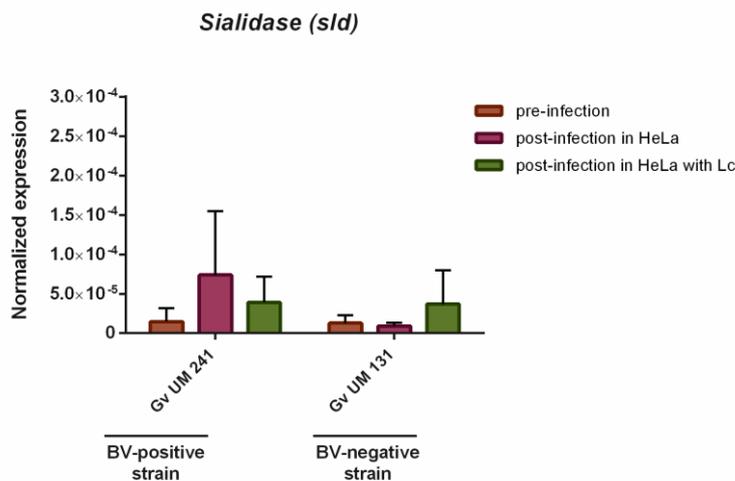


Fig. 2B | Differential transcriptomic profile of *G. vaginalis* (Gv) isolated from women with BV (BV-positive strain) and without BV (BV-negative strain). Sialidase (*sld*) transcript levels within Gv planktonic cells (pre-infection), after Gv infection on a monolayer of HeLa cells (post-infection in HeLa) and after Gv infection on a monolayer of HeLa pre-treated with *L. crispatus* (post-infection in HeLa with Lc) were quantified. Results are expressed as normalized expression in relation to 16S rRNA and represented as mean ± SEM. **A specific condition was significantly different in terms of gene expression between a BV-positive and a BV-negative strain (T-test, $p < 0.05$).

Taking in consideration our novel findings and our previous observations [10, 24] we underline the importance of *L. crispatus*, since it seems to possess some factors that can trigger protective mechanisms against BV-positive *G. vaginalis* strains [25]. However, there is no doubt that a refined genomic characterization of the *G. vaginalis* strains might allow a better knowledge of the molecular mechanisms behind the different patterns of cytotoxicity.

Acknowledgements

This work was supported by Portuguese National Funds (FCT) under the scope of the strategic funding of UID/BIO/04469/2013 unit and COMPETE 2020 (POCI-01-0145-FEDER-006684). JC, and MER acknowledge the financial support of individual Grants SFRH/BD/93963/2013, and SFRH/BPD/95401/2013 respectively. NC is an Investigador FCT.

References

- [1] R.F. Lamont, J.D. Sobel, R.A. Akins, S.S. Hassan, T. Chaiworapongsa, J.P. Kusanovic, et al. The vaginal microbiome: new information about genital tract flora using molecular based techniques. *BJOG* 118 (2011) 533-49, <http://dx.doi.org/10.1111/j.1471-0528.2010.02840.x>.
- [2] J. Ravel, P. Gajer, Z. Abdo, G.M. Schneider, S.S. Koenig, S.L. McCulle, et al. Vaginal microbiome of reproductive-age women. *Proc. Natl. Acad. Sci. U S A* 108 Suppl 1 (2011) 4680-7, <http://dx.doi.org/10.1073/pnas.1002611107>.
- [3] J. M. Macklaim, A.D. Fernandes, J.M. Di Bella, J.A. Hammond, G. Reid, G.B. Gloor. Comparative meta-RNA-seq of the vaginal microbiota and differential expression by *Lactobacillus iners* in health and dysbiosis. *Microbiome* 1 (2013) 12, <https://doi.org/10.1186/2049-2618-1-12>.
- [4] H. Verstraelen, R. Verhelst, G. Claeys, E. De Backer, M. Temmerman, M. Vaneechoutte. Longitudinal analysis of the vaginal microflora in pregnancy suggests that *L. crispatus* promotes the stability of the normal vaginal microflora and that *L. gasseri* and/or *L. iners* are more conducive to the occurrence of abnormal vaginal microflora. *BMC Microbiol.* 9 (2009) 116, <http://dx.doi.org/10.1186/1471-2180-9-116>.
- [5] H. Sharma, R. Tal, N.A. Clark, J.H. Segars. Microbiota and pelvic inflammatory disease. *Semin. Reprod. Med.* 32 (2014) 43-9, <http://dx.doi.org/10.1055/s-0033-1361822>.
- [6] P. Mirmonsef, L. Krass, A. Landay, G.T. Spear. The role of bacterial vaginosis and trichomonas in HIV transmission across the female genital tract. *Curr. HIV Res.* 10 (2012) 202-10, PMID: PMC3788616.
- [7] A.S. Dingens, T.S. Fairfortune, S. Reed, C. Mitchell. Bacterial vaginosis and adverse outcomes among full-term infants: a cohort study. *BMC Pregnancy Childbirth* 16 (2016) 278, <http://dx.doi.org/10.1186/s12884-016-1073-y>.
- [8] J.R. Schwebke, C.A. Muzny, W.E. Josey. Role of *Gardnerella vaginalis* in the pathogenesis of bacterial vaginosis: a conceptual model. *J. Inf. Dis.* 210 (2014) 338-43, <http://dx.doi.org/10.1093/infdis/jiu089>.
- [9] A. Machado, N. Cerca. Influence of Biofilm Formation by *Gardnerella vaginalis* and other anaerobes on bacterial vaginosis. *J. Infect. Dis.* 212 (2015) 1856-61, <http://dx.doi.org/10.1093/infdis/jiv338>.
- [10] J. Castro, P. Alves, C. Sousa, T. Cereija, A. Franca, K.K. Jefferson, et al. Using an *in-vitro* biofilm model to assess the virulence potential of bacterial vaginosis or non-bacterial vaginosis *Gardnerella vaginalis* isolates. *Sci. Rep.* 5 (2015) 11640, <http://dx.doi.org/10.1038/srep11640>.
- [11] J.L. Patterson, A. Stull-Lane, P.H. Girerd, K.K. Jefferson. Analysis of adherence, biofilm formation and cytotoxicity suggests a greater virulence potential of *Gardnerella vaginalis* relative to other bacterial-vaginosis-associated anaerobes. *Microbiology* 156 (2010) 392-9, <http://dx.doi.org/10.1099/mic.0.034280-0>.
- [12] A.A. Aroutcheva, J.A. Simoes, K. Behbakht, S. Faro. *Gardnerella vaginalis* isolated from patients with bacterial vaginosis and from patients with healthy vaginal ecosystems. *Clin. Infect. Dis.* 33 (2001) 1022-7, <http://dx.doi.org/10.1086/323030>.
- [13] R.J. Hickey, L.J. Forney. *Gardnerella vaginalis* does not always cause bacterial vaginosis. *J. Infect. Dis.* 210 (2014) 1682-3, <http://dx.doi.org/10.1093/infdis/jiu303>.
- [14] C.J. Yeoman, S. Yildirim, S.M. Thomas, A.S. Durkin, M. Torralba, G. Sutton, et al. Comparative genomics of *Gardnerella vaginalis* strains reveals substantial differences in metabolic and virulence potential. *PloS one* 5 (2010) e12411, <http://dx.doi.org/10.1371/journal.pone.0012411>.
- [15] M.D. Harwich, Jr., M.G. Serrano, J.M. Fettweis, J.M. Alves, M.A. Reimers, G.A. Buck, et al. Genomic sequence analysis and characterization of *Sneathia amnii* sp. nov. *BMC Genomics* 13 Suppl 8 (2012) S4, <http://dx.doi.org/10.1186/1471-2164-13-S8-S4>.
- [16] M. Janulaitiene, V. Paliulyte, S. Grinceviciene, J. Zakareviciene, A. Vladisauskiene, A. Marcinkute, et al. Prevalence and distribution of *Gardnerella vaginalis* subgroups in women with and without bacterial vaginosis. *BMC Infect. Dis.* 17 (2017) 394, <http://dx.doi.org/10.1186/s12879-017-2501-y>.
- [17] S.V. Balashov, E. Mordechaj, M.E. Adelson, S.E. Gyax. Identification, quantification and subtyping of *Gardnerella vaginalis* in noncultured clinical vaginal samples by quantitative PCR. *J. Med. Microbiol.* 63 (2014) 162-75, <http://dx.doi.org/10.1099/jmm.0.066407-0>.

- [18] J. Castro, N. Cerca. BV and non-BV associated *Gardnerella vaginalis* establish similar synergistic interactions with other BV-associated microorganisms in dual-species biofilms. *Anaerobe* 36 (2015) 56-9, <http://dx.doi.org/10.1016/j.anaerobe.2015.10.008>.
- [19] J.J. Schellenberg, M.H. Patterson, J.E. Hill. *Gardnerella vaginalis* diversity and ecology in relation to vaginal symptoms. *Res. Microbiol.* S0923-2508 (2017) 30060-8, <http://dx.doi.org/10.1016/j.resmic.2017.02.011>.
- [20] R.L. Stingley, H. Liu, L.B. Mullis, C.A. Elkins, M.E. Hart. *Staphylococcus aureus* toxic shock syndrome toxin-1 (TSST-1) production and *Lactobacillus* species growth in a defined medium simulating vaginal secretions. *J. Microbiol. Methods* 106 (2014) 57-66, <http://dx.doi.org/10.1016/j.mimet.2014.08.002>.
- [21] P. Alves, J. Castro, C. Sousa, T.B. Cereija, N. Cerca. *Gardnerella vaginalis* outcompetes 29 other bacterial species isolated from patients with bacterial vaginosis, using in an *in vitro* biofilm formation model. *J. Infect. Dis.* 210 (2014) 593-6, <http://dx.doi.org/10.1093/infdis/jiu131>.
- [22] A. França, A.I. Freitas, A.F. Henriques, N. Cerca. Optimizing a qPCR Gene expression quantification assay for *S. epidermidis* biofilms: a comparison between commercial kits and a customized protocol. *PloS one* 7 (2012) e37480, <http://dx.doi.org/10.1371/journal.pone.0037480>.
- [23] J. Castro, A. França, K.R. Bradwell, M.G. Serrano, K.K. Jefferson, N. Cerca. Comparative transcriptomic analysis of *Gardnerella vaginalis* biofilms vs. planktonic cultures using RNA-seq. *NPJ Biofilms and Microbiomes* 3 (2017) 3, <http://dx.doi.org/10.1038/s41522-017-0012-7>.
- [24] J. Castro, A. Henriques, A. Machado, M. Henriques, K.K. Jefferson, N. Cerca. Reciprocal interference between *Lactobacillus* spp. and *Gardnerella vaginalis* on initial adherence to epithelial cells. *Int. J. Med. Sci.* 10 (2013) 1193, <http://dx.doi.org/10.7150/ijms.6304>.
- [25] T. Ojala, M. Kankainen, J. Castro, N. Cerca, S. Edelman, B. Westerlund-Wikstrom, et al. Comparative genomics of *Lactobacillus crispatus* suggests novel mechanisms for the competitive exclusion of *Gardnerella vaginalis*. *BMC Genomics* 15 (2014) 1070, <http://dx.doi.org/10.1186/1471-2164-15-1070>.
- [26] X.X. Niu, T. Li, X. Zhang, S.X. Wang, Z.H. Liu. *Lactobacillus crispatus* modulates vaginal epithelial cell innate response to *Candida albicans*. *Chin. Med. J.* 130 (2017) 273-9, <http://dx.doi.org/10.4103/0366-6999.198927>.
- [27] E. Motevaseli, M. Shirzad, S.M. Akrami, A.S. Mousavi, A. Mirsalehian, M.H. Modarressi. Normal and tumour cervical cells respond differently to vaginal lactobacilli, independent of pH and lactate. *J. Med. Microbiol.* 62 (2013) 1065-72, <http://dx.doi.org/10.1099/jmm.0.057521-0>.
- [28] N. Calonghi, C. Parolin, G. Sartor, L. Verardi, B. Giordani, G. Frisco, et al. Interaction of vaginal *Lactobacillus* strains with HeLa cells plasma membrane. *Benef. Microbes* 8 (2017) 625-33, <http://dx.doi.org/10.3920/BM2016.0212>.
- [29] S.E. Gelber, J.L. Aguilar, K.L. Lewis, A.J. Ratner. Functional and phylogenetic characterization of vaginolysin, the human-specific cytolysin from *Gardnerella vaginalis*. *J. Bacteriol.* 190 (2008) 3896-903, <http://dx.doi.org/10.1128/JB.01965-07>.
- [30] L. Hardy, V. Jaspers, M. Van den Bulck, J. Buyze, L. Mwambarangwe, V. Musengamana, et al. The presence of the putative *Gardnerella vaginalis* sialidase A gene in vaginal specimens is associated with bacterial vaginosis biofilm. *PLoS One* 12 (2017) e0172522, <http://dx.doi.org/10.1371/journal.pone.0172522>.