

Short communication

Lactobacillus crispatus represses vaginolysin expression by BV associated Gardnerella vaginalis and reduces cell cytotoxicity

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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.

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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

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1×10^7 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 tuberculoostearicum* 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 ($p < 0.05$). 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. tuberculoostearicum*, *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].

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

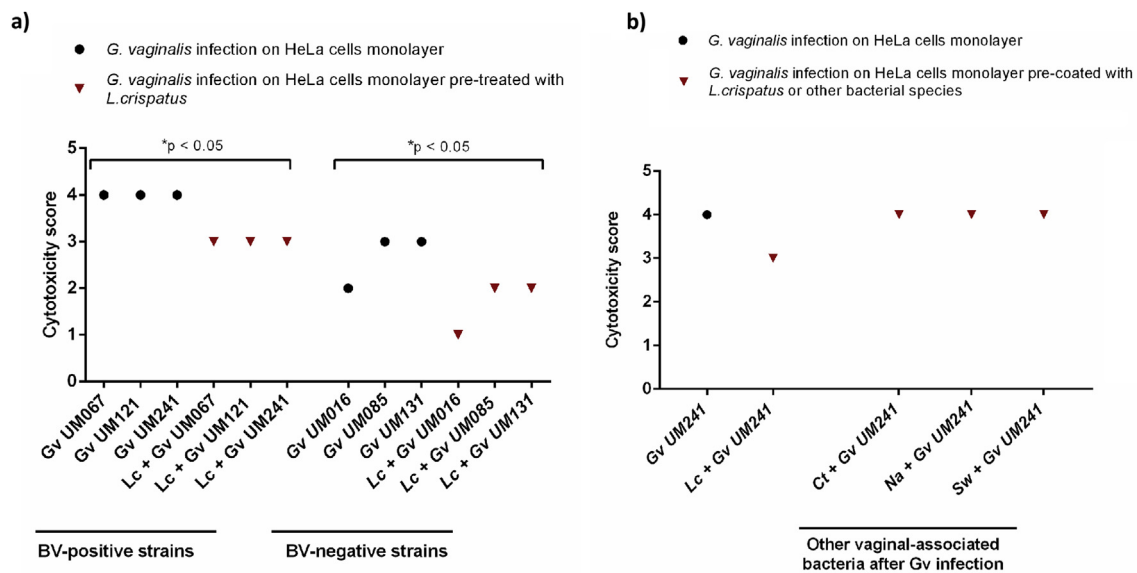


Fig. 1. Differential cytotoxicity profile of *G. vaginalis* (Gv) isolated from women with BV (BV-positive strains) and without BV (BV-negative strains). **a)** Cytotoxicity score of BV-positive and BV-negative Gv strains, in two different conditions: Gv infection on a monolayer of HeLa cells and on a monolayer pre-coated with *L. crispatus* (Lc); **b)** cytotoxicity score after Gv infection on a monolayer of HeLa cells pre-coated with Lc or with other vaginal-associated species: *C. tuberculoostearicum* (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, $p < 0.05$).

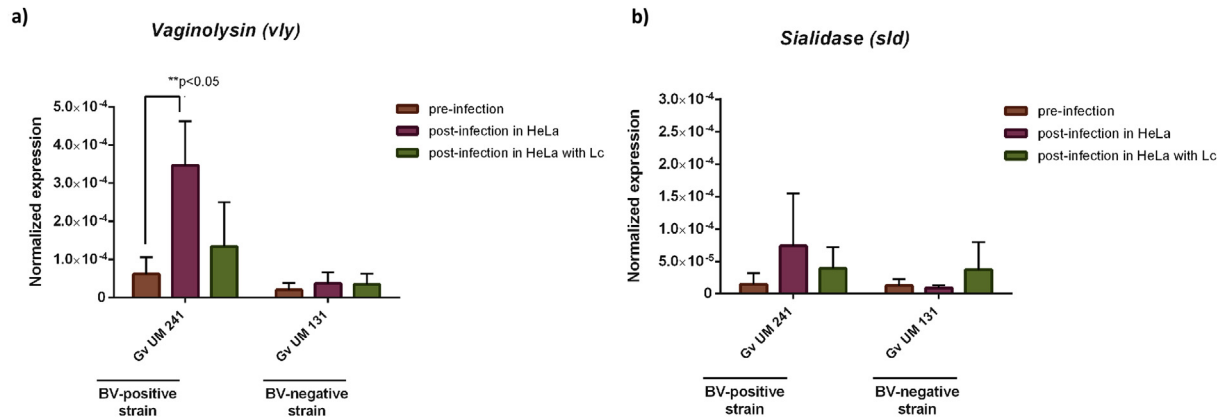


Fig. 2. Differential transcriptomic profile of *G. vaginalis* (*Gv*) isolated from women with BV (BV-positive strain) and without BV (BV-negative strain). **a)** Expression of vaginolysin (*vly*) and; **b)** sialidase (*sld*) genes by *Gv* isolates. Transcript levels within *Gv* planktonic cells (pre-infection), 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 \pm SEM. **A specific condition was significantly different in terms of gene expression between a BV-positive and a BV-negative strain (T-test, $p < 0.05$).

a BV-positive strain, transcript levels of both genes were significantly higher than in pre-infection (Fig. 2a and b). 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.

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]. A limitation of our study was the use of only a representative *Lactobacillus* species, correlated with healthy vaginal microflora, and we did not explore how other lactobacilli would interact with either *G. vaginalis* or other bacterial species found in BV. 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.

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