



MICROBIOLOGY

Novel insights in bacterial vaginosis etiology through genomic approaches

MARCELLE O. ALMEIDA, MARCUS VINICIUS C. VIANA, JANAÍNA C. CERQUEIRA, FLAVIA F. ABURJAILE, ANDREY A.Z. JUNIOR, VASCO AZEVEDO & RODRIGO D.O. CARVALHO

Abstract: Bacterial vaginosis (BV) has been considered as dysbiosis state whose etiology is not fully understood. This condition affects a large number of women of reproductive age and its study has been highly relevant due to the growing association of BV with and gynecological and obstetric complications and diseases, in addition to a greater susceptibility to sexually transmitted diseases, including HIV. The vaginal microbiota composition presents high variability among different ethnic groups of women, although, generally, the prevalence of lactobacilli species has been reported. Several studies suggest they may play a protective role, especially *Lactobacillus crispatus* whose population is typically present in low proportions in women with BV. This review article describes the contributions and limitations of genomic approaches in elucidating protective characteristics and mechanisms associated with colonization and persistence of lactobacilli strains. Although some genetic features were associated with resilience of *L. crispatus* during BV, further studies are required to uncover their functions.

Key words: bacterial vaginosis, genomics, health, *Lactobacillus*.

BACTERIAL VAGINOSIS

The vagina microbiota is characterized by several bacterial species that colonize its mucosal surfaces. Microbial colonization in mammals starts at birth when the fetus leaves the placenta and has contact with the external environment. In human beings, the infant vaginal microbiota is mainly composed of lactobacilli acquired by the maternal microbiota (Wang et al. 2020). However, cesarean delivered infants present a different microbiome formation and may lack commensal species and be colonized by opportunistic bacteria such as *Enterococcus* sp. and *Klebsiella* sp. (Stinson et al. 2018). The genus *Lactobacillus* consists of Gram-positive, homo- or heterofermentative and non-spore-forming

rods, whose primary fermentation process is the production of lactic acid from different sources of sugars such as fructans, starch, or glycogen. However, some strains can produce acetic acids as well. This genus currently has 261 species with relative heterogeneity showing paraphyletic clustering (Zheng et al. 2020).

In adulthood, the vaginal microbiota presents high variability depending on ethnicity, sexual behavior and temporal variation (Wessels et al. 2018). In this context, reproductive-age women may present five major communities state types (CST). The CST I, II, III and V, are dominated by lactobacilli species, where *L. iners*, *L. crispatus*, *L. gasseri* and *L. jensenii* are more frequent, respectively. However, a more equal distribution of anaerobic bacteria

can be found in CST IV, including *Prevotella*, *Megasphaera*, *Gardnerella vaginalis*, *Sneathia* and *Atopobium vaginae*. Moreover, the same subject may present transitions between CST in different moments of life (Ravel et al. 2011, Gajer et al. 2012, Tettamanti Boshier et al. 2020). Studies suggest a symbiotic relationship between the microbiota and the host, in which the hormones stimulate the vaginal epithelia to produce glycogen. In exchange, these lactobacilli create a protective environment against infections or colonization of pathogens and non-indigenous microbes by acidifying the vaginal microenvironment and secretion of antimicrobial components (Tachedjian et al. 2017). Among the protective species, *L. crispatus* is the most strongly associated with gynecological health, accounting for more than 24,5% to 81% of the cervical microbiome and is the most resilient during temporal variation (Gajer et al. 2012, Dols et al. 2016, Pramanick et al. 2019, Van der veer et al. 2019). Some strains are even used as probiotics due to their pathogen-inhibitory properties (Wang et al. 2017, Cohen et al. 2020).

L. iners, also has a high abundance in the vaginal environment, varying between 17 and 84%, is prevalent in 25% asian, 45% white, 22% black and 14% hispanic women (Ravel et al. 2011, Dols et al. 2016, van Houdt et al. 2018). However, human populations bearing this ecological state dominated by *L. iners*, have been considered much more susceptible to the replacement by polymicrobial state rich in strict anaerobic and facultative anaerobic species (van Houdt et al. 2018, Pramanick et al. 2019). Some authors suggest that vaginal commensal species might be replaced by bacteria from the perianal skin and fecal communities (Vahidnia et al. 2015). This group might include *Gardnerella vaginalis*, *Atopobium vaginae*, *Prevotella sp*, *Mycoplasma hominis*, *Leptotrichia amnionii*, *Sneathia*

sanguinegens and other novel taxa belonging to Clostridiales order such as bacterial vaginosis-associated bacterium (BVAB) 1, BVAB2 and BVAB3 (Onderdonk et al. 2016, Lennard et al. 2017).

The transition from the healthy condition of the microbiota to the ecological alteration of this environment is called dysbiosis. These shifts in the microbial community have a pivotal role in the onset and maintenance of bacterial vaginosis (BV). This infirmity affects 23 to 29% of women of reproductive age and can be associated with gynecological or obstetric complications and diseases such as pelvic inflammatory disease and increased susceptibility to HIV (Bautista et al. 2016, Peebles et al. 2019). However, the etiology of BV and the molecular mechanisms have not been wholly understood despite decades of research.

KNOWN MECHANISMS AND HYPOTHESIS

Currently, two main hypotheses are used to explain the depletion of *Lactobacillus*: (i) the incompetent lactobacilli strains hypothesis, in which there is insufficient production of antimicrobials and metabolites responsible for environment acidification (Turovskiy et al. 2011); (ii) the phage hypothesis, where bacteriophages from the microbiota or the external environment could be responsible for the lysis of vaginal lactobacilli (Turovskiy et al. 2011, Jung et al. 2017).

The incompetent lactobacilli

Lactic acid is an essential metabolite responsible for maintaining normal pH (4.5) in the vagina of reproductive women and has antimicrobial, antiviral and immunomodulatory properties. Some strains of lactobacilli can generate larger amounts of lactic acid, due to increased fermentation of glucose and other sugars generated through amylase breakdown

of glycogen (Tachedjian et al. 2017). Microbiomes predominantly constituted by *L. crispatus* tend to present lower pH than communities dominated by *L. jensenii*, *L. gasseri* and *L. iners*. Interestingly, a study has found a positive correlation between high levels of this carbohydrate and *L. crispatus* increased population, but not for *L. iners* (Vaneechoutte 2017). This could be explained by the complex nutritional demands of this bacterium as it grows best on nutrient-rich media, but not in De Man, Rogosa and Sharpe (MRS) medium, generally used for *Lactobacillus* (Falsen et al. 1999).

Moreover, lactobacilli activity, including lactate production, can be affected by the menstrual cycle stages as the secretion of glycogen by epithelium cells is regulated by sexual hormones like estrogen in the vagina (Wessels et al. 2018). Prospective studies suggest that some subjects may present *L. iners*-dominated communities during menses, whereas *L. crispatus* may dominate in periods between menses, although this cannot be generalized once other CST can be frequently found as well (Gajer et al. 2012, Petrova et al. 2015). Another fact corroborating with *L. iners* low lactic acid production is their ability to produce only L-lactate. At the same time, *L. crispatus* strains can produce both L- and D- isomeric forms. In this context, it seems that D-lactate has a more significant protective role against pathogens (Witkin et al. 2013). In addition, *L. iners* strains have been found to produce other metabolites such as succinate, which may contribute to increased pH (Tachedjian et al. 2017). Intriguingly, a comparison of *L. crispatus* strains of lactobacilli-dominated (healthy) and BV-associated microbiomes found no correlation of L- and D-lactic acid production *in vitro* (Abdelmaksoud et al. 2016).

The protective role of vaginal lactobacilli has also been attributed to the secretion of

antimicrobials *in vitro*, such as hydrogen peroxide (H_2O_2). However, this mechanism *in vivo* has not been considered as critical but complementary to other protective factors like lactic acid activity (Tachedjian et al. 2018). Early cross-sectional studies conducted in western countries have shown that 59 - 79% of vaginal lactobacilli strains isolated from healthy subjects are H_2O_2 -producers while 13 - 23% of strains isolated from patients affected with BV were able to produce this antimicrobial compound, suggesting that the late group of women may be predominantly colonized by less protective lactobacilli strains (Nagy et al. 1991, Hillier et al. 1992). Other studies suggest the insufficient protection by *L. iners* strains against vaginal colonization by other species have revealed they produce less or no hydrogen peroxide at all (Tachedjian et al. 2017).

Parasitism mechanisms via bacteriocins and other peptides involved in adherence, biofilm formation, or epithelial barrier regulation, produced by specific strains of *Lactobacillus* sp., have been suggested in the context of BV protection, although the molecular characterization of these peptides have been poorly explored by conventional microbiological or molecular biology approaches (Dover et al. 2008, Maldonado-Barragán et al. 2016, Vaneechoutte 2017, Anton et al. 2018). Since these genes may present high variability and functional differences from strain-to-strain, high standard genomic sequencing strategies may provide insights on those mechanisms.

Phages lysogeny

Bacteriophages are abundant in vaginal *Lactobacillus* sp., including *L. crispatus* (Damelin et al. 2011). Although most of these viruses are temperate, they may excise from the chromosome, replicate and lyse bacterial cells whenever they enter the lytic cycle. Following this hypothesis, phages may have a crucial role

in maintaining vaginal lactobacilli community population and dynamics, in which their excision could occur naturally or be induced by stress conditions such as DNA-damaging agents (Baugher et al. 2014).

Intriguingly, African and North American black women cohorts, which are frequently affected with BV, have revealed a high rate of *Lactobacillus* sp. lysogeny in the vaginal environment (Turovskiy et al. 2011). This positive correlation was also confirmed later by another study where lysogeny was found in *L. crispatus* in 77 % of isolates from BV, from which it was possible to detect viral particles when stimulated with stressful agents such as mitomycin C (Damelin et al. 2011). Cigarette chemicals, which is considered a risk factor for BV, have also been associated with phage lysogeny in the same context (Pavlova & Tao 2000). Another study has demonstrated that CRISPR-related genes are upregulated in *L. iners* during BV condition, suggesting this activity could be involved in response to a phage enriched vaginal environment (Macklaim et al. 2013). However, contradictory results have also been obtained in a recent study, where lactobacilli strains isolated from both healthy or BV conditions presented a high number of prophage content, with similar lysogeny activity (Abdelmaksoud et al. 2016). Other studies have raised important questions regarding horizontal gene transfer, which may contribute to increased variability in bacterial genomes and the incorporation of undesirable genes (Chibani-Chennoufi et al. 2004, Baugher et al. 2014).

In this context, the use of comparative genomic methods may offer a good alternative to perform massive characterization of phage genes found in several strains from *Lactobacillus*-dominated versus BV-associated bacterial communities to investigate their role in the maintenance or development of BV.

USE OF COMPARATIVE GENOMICS IN THE INVESTIGATION OF BV

Partial 16S rRNA gene sequences have been used to determine the species composition of vaginal bacterial communities. As mentioned above, these analyses were essential to characterize the major CSTs and the dynamic shifts that different ethnic groups of women may present overtime under an ecological perspective (Ravel et al. 2011). This review describes the main studies focused on investigating functional properties by comparing complete genomes of lactobacilli strains.

L. crispatus is a biomarker for vaginal health, and some strains are used as probiotics against BV (Almeida et al. 2019, Cohen et al. 2020). Due to its association with health promotion, its genetic diversity and mechanisms involved in the probiotic effects of *L. crispatus* have been investigated using genomics data and *in vitro* experiments (Ojala et al. 2014, Abdelmaksoud et al. 2016, France et al. 2016, Van der veer et al. 2019, Pan et al. 2020, Petit & Read 2020, Zhang et al. 2020).

Genomics of *L. crispatus* isolated from the vagina

Comparative genomics with a specific focus on vaginal isolates investigated the phylogeny, gene content, horizontal gene transfer, prophages, metabolism, and positive selection within strains (Ojala et al. 2014, France et al. 2016, Pan et al. 2020, Petit & Read 2020, Zhang et al. 2020). A comparison of strains from different hosts found that human vaginal isolates have a unique CRISPR Type II-A system, and a complete trehalose metabolism operon. However, this carbohydrate is not known yet as a carbon source in the human urogenital tract. In this context, a higher sequence similarity for a glycogen hydrolase (glgX) has been found in

vaginal lactobacilli, suggesting a niche-specific feature to metabolize the highly abundant glycogen in this host site (Pan et al. 2020). A comparison of isolates from feces and vagina found a higher abundance of genes that could confer adaptations for vaginal colonization such as those related to acidic and oxidative stress, pullulanase genes (*pulA*) for glycogen breakdown, and enzymes involved in improving competitive advantage by destroying the cell wall of pathogenic bacteria (Zhang et al. 2020).

Comparison of BV and healthy conditions

Two studies compared isolates from lactobacilli-dominated and BV-associated microbiota, providing insights about the genotypes associated with these conditions (Abdelmaksoud et al. 2016, van der Veer et al. 2019). The analysis *in silico* of 18 strains found no correlation between the number of specific bacteriocin genes, the number of prophages, insertion sequences and phylogroups. In the same study, analysis *in vitro* was carried out to investigate L- and D-lactic acid production associated with phage induction, but no positive correlation was observed. The only features of lactobacilli-dominated isolates presenting a positive correlation with the healthy condition were two transposases genes and three hypothetical proteins. Furthermore, seven genes were found only in BV-associated isolates. Five of these genes seem to be involved in cellobiose transport, and the other two are a putative glucosidase and a hypothetical protein (Abdelmaksoud et al. 2016).

Analysis of 28 *L. crispatus* strains did not reveal differences in the number of accessory and unique genes, distribution of cluster of ortholog groups (COG), or inhibition of *Neisseria gonorrhoeae in vitro*, biofilm formation, auto-aggregation, organic acid production, carbohydrate metabolism or growth on glycogen. The slow growth on glycogen in some strains from

both health conditions was associated with a deletion in the signal peptide of an extracellular pullulanase type I gene sequence. Within the BV-associated strains, three transposases were more abundant, and three gene fragments of an extra glycosyltransferase were more frequent. The latter was hypothesized to be involved in rapidly adapting and diversifying surface glycans by phase variation, evading the host immune system, and allowing persistence at a low-level during BV. These fragments were highly expressed in the vaginal samples of two women who were responsive to treatment for BV (van der Veer et al. 2019). In short, no correlation was found between vaginal health and *L. crispatus* lactic acid production, number and specificity of bacteriocins, prophages, number or accessory and unique genes, and phylogenetic groups, carbohydrate metabolism, inhibition of *Neisseria gonorrhoeae* growth, biofilm formation, auto-aggregation, organic acid production, carbohydrate metabolism and growth on glycogen. A specific glycosyltransferase could help in persistence during the adaptation and diversification of surface glycans to evade the host immune system (Abdelmaksoud et al. 2016, van der Veer et al. 2019). The results of those studies suggest mechanisms involved in the persistence of *L. crispatus*, rather than prevention of BV. Different factors could complicate the identification of the prevention mechanisms. One hypothesis is that different strains present heterogeneous genetic repertoire involved in metabolism, adherence, and inhibition of pathogens. Possibly, BV-associated samples also present several genes in common with isolates from healthy subjects, which would prevent them from being detected as exclusive. Another one is differences in gene expression (Abdelmaksoud et al. 2016). Besides genetics factors from the microbial community, host-associated factors might contribute to

the pathogenesis of BV, such as ethnicity, age, sexual behaviors, use of contraceptive method, use of antibiotics, diet, stress and smoking (Abdelmaksoud et al. 2016, Barrientos-Durán et al. 2020). In relation to microbial genetic factors, new studies could investigate variation in sequence, positive selection (Anisimova & Liberles 2012), gene expression and regulatory networks (Parise et al. 2020), and the function of hypothetical proteins detected in previous studies (Abdelmaksoud et al. 2016). One important limiting factor of comparative genomics of isolates is that it does not survey the microbial community in a comprehensive manner. Culture independent methods such as whole metagenome sequencing may provide a more precise genetic characterization of dominating-taxa such as *L. crispatus* at Sub species resolution and tracking dynamic changes throughout the time (Berman et al. 2020).

CONCLUSIONS

The genomic studies of *L. crispatus* strains have shown to be useful in characterizing some of the genetic factors involved in the protective mechanisms previously suggested by *in vitro* studies. While the comparison of genomic sequences among isolates from healthy and BV conditions has brought novel insights of genes involved of persistence during BV, rather than the prevention of it. However, many of those genes need further investigation as their function remains unknown. Once the occurrence of BV can be influenced by a variety of *in vivo* factors, we suggest that future genomic studies of *L. crispatus* should focus on unbiased surveys such as whole genome direct sequencing from vaginal microbiome samples, and should be complemented with gene expression analysis.

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MARCELLE O. ALMEIDA¹

<https://orcid.org/0000-0002-8557-3865>

MARCUS V.C. VIANA^{1,2}

<https://orcid.org/0000-0002-7017-6437>

JANAÍNA C. CERQUEIRA¹

<https://orcid.org/0000-0003-3410-1419>

FLAVIA F. ABURJAILE^{1,3}

<https://orcid.org/0000-0002-1067-1882>

ANDREY A.Z. JUNIOR^{4,5}

<https://orcid.org/0000-0002-3046-4565>

VASCO AZEVEDO¹

<https://orcid.org/0000-0002-4775-2280>

RODRIGO D.O. CARVALHO^{1,4}

<https://orcid.org/0000-0003-0216-7173>

¹Universidade Federal de Minas Gerais, Departamento de Genética, Ecologia e Evolução, Instituto de Ciências Biológicas, Av. Presidente Antônio Carlos, 6627, Pampulha, 31270-901 Belo Horizonte, MG, Brazil

²Universidade Federal do Pará, Departamento de Genética, Instituto de Ciências Biológicas, Rua Augusto Corrêa, 01, Guamã, 66075-970 Belém, PA, Brazil

³Universidade Federal de Pernambuco, Departamento de Genética, Av. Prof. Moraes Rego, 1235, Cidade Universitária, 50670-901 Recife, PE, Brazil

⁴Sechenov First Moscow State Medical University, Institute of Molecular Medicine, Bol'shaya Pirogovskaya Ulitsa, 19c1, 119991 Moscow, Russia

⁵Lomonosov Moscow State University, Belozersky Institute of Physico-Chemical Biology, Ulitsa Leninskiye Gory, 1c40, 119992 Moscow, Russia

Correspondence to: **Vasco Azevedo**

E-mail: vasco@icb.ufmg.br

Author contributions

MOA conceived the study and wrote the manuscript; MVCV wrote the manuscript; JCC wrote the manuscript, FA wrote and revised the manuscript, AAZJ wrote the manuscript; VA wrote the manuscript and supervised the study; RDOC wrote the manuscript and supervised the study. All authors read and approved the final version.

