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Probiotic potential of novel Brazilian *Lactobacillus crispatus* strains

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ABSTRACT. *Lactobacilli* are predominant bacterial species colonizing the vaginal surfaces of healthy women, where they play a protective role against opportunistic and polymicrobial infections such as Bacterial Vaginosis (BV). Several *Lactobacillus* species, especially *L. crispatus*, have been prospected for probiotic applications due to their potential antimicrobial and anti-inflammatory capacity. In the last decade, several genomic studies have investigated the genetic factors of *L. crispatus* strains for identifying novel probiotic strains and evaluating their application for improving human or animal health. This mini-review highlights the primary genes associated with *l. crispatus* protective mechanisms identified, in our previous work, on four novel strains isolated from healthy Brazilian women of reproductive age. Among the probiotic features discussed, the role of a pyruvate oxidase-encoding gene, lactate synthesis-related enzymes, bacteriocins genes, and genomic islands is reviewed, and the following steps to confirm their activity are pointed out.

Key words: *Lactobacillus*; Antimicrobial; Bacterium; Antimicrobial peptide

INTRODUCTION

First identified in 1894 by a German physician named Doderlein, *lactobacilli* strains have been reported as the dominant bacterial species that colonize the vaginal epithelium from women of reproductive age [1]. *Lactobacillus crispatus* is the most frequently isolated microorganism and plays an essential role in protecting the host from the overgrowth of potential pathogenic bacteria naturally found in the vagina, which causes polymicrobial synergistic infections known as bacterial vaginosis (BV). These pathogenic microorganisms may include *Enterococcus faecalis*, *Gardnerella vaginalis*, *Prevotella bivia*, and other species [2]. Due to the protective role of the species, *L. crispatus* strains are considered good candidates for probiotic use. Genomic studies have helped identify genetic factors associated with beneficial properties for screening probiotic features in several strains. This short review carried out a brief discussion focused on the main protective mechanisms and associated genes previously identified in four *L. crisptaus* strains [3] isolated from healthy Brazilian women.

LITERATURE REVIEW

Hydrogen peroxide antimicrobial activity

Vaginal *lactobacilli* are aero tolerant anaerobes and may produce hydrogen peroxide (H_2O_2) *in vitro* when cultivated under aerobic conditions, such as aeration [1]. H_2O_2 is mainly formed in carbon and energy metabolism by oxidases, including pyruvate oxidase, lactate oxidase, and NADH oxidases. Increased metabolite levels are generally associated with species lacking the H_2O_2-scavenging enzymes, such as catalase, which leads to its accumulation in the cell and supernatant [4]. Studies have been suggesting that H_2O_2 producing *Lactobacillus* strains present antimicrobial activity against BV-related pathogens, such as *G. vaginalis*, *P. bivia*, and *Candida albicans*, which H_2O_2 physiological concentrations (0.05 to 1.0 mM) play an important role but only in addition to other substances produced by the bacterium [5]. Nonetheless, association studies have shown a positive correlation between H_2O_2 producing strains of *Lactobacillus sp.* and healthy vagina clinical outcomes, suggesting this metabolite as a possible marker for probiotic strains. In the study of Almeida et al., four strains of *L. crispatus* presented a pyruvate oxidase-encoding gene, which was also isolated from healthy women of reproductive age in Brazil. Although those strains may be considered as potential probiotics, further experiments will be required to evaluate the amount of H_2O_2produced and antimicrobial activity. It has also been suggested that H_2O_2 may be involved in anti-inflammatory effects on the host cells by activating the peroxisome proliferator-activated receptor γ (PPAR-γ), which plays a crucial role in the regulation of intestinal inflammation and homeostasis. In this context, proof-of-concept studies should be carried out in vivo using animal models for gastrointestinal inflammatory diseases [4]. Other studies support that H_2O_2 mediated inhibition can be neutralized through the activity of catalase by some pathogens [6,7]. Therefore, investigating the antimicrobial activity caused by acidity, proteases, and the production of bacteriocin compounds is also important.

Bacteriocins and other antimicrobial peptides

To promote the vaginal ecosystem restoration, besides organic acids and hydrogen peroxide, *Lactobacillus crispatus* presents antagonistic activity resulting from other antimicrobial compounds production, for example, bacteriocins [8]. Bacteriocins are ribosomal synthesized antimicrobial peptides produced by different bacterial species, whose primary function is to kill and inhibit the growth of other bacteria. These antimicrobial compounds act through the formation of pores in the cytoplasmic membrane of the target bacteria, which, after altering the permeability of the membrane,

increasing the flow of transmembrane ions and causing a drop in intracellular pH, inhibiting enzymatic processes and leading the cell to death [9].

Based on their primary structures, molecular weights, post-translational modifications, and genetic characteristics, bacteriocins are categorized into 3 main classes: (i) Class I, also named lantibiotics, includes small-sized (<5 kDa), cationic and hydrophobic peptides, such as nisin, lactocin); (ii) Class II includes unmodified, small in size (<10 kDa), heat-stable and cationic hydrophobic peptides. Depending on its mode of action, this class is subdivided into class IIa (such as pediocin PA1 and leukocidin) and class IIb (such as plantaricin A and enterocin X); and finally, (iii) Class III, which includes large (>30 kDa), hydrophilic and heat-poor peptides, such as helveticin J and enterolysin) [9].
In *Lactobacillus crispatus* genomes, several genes encoding bacteriocins were previously identified using the BAGEL (Bacteriocin genome mining tool) webserver. Almeida et al. identified genes encoding bacteriocins Helveticin J, Enterolysin A, and Penocin A and other genes associated with bacteriocins when studying the genome four *L. crispatus* strains (CRI4 CRI8 CRI10 CRI17) isolated from the vagina of healthy women of Brazil. In other strains of *L. crispatus* (VMC1, VMC2, VMC3, VMC4, VMC5, VMC6, VMC7, VMC8), in addition to multiple genes encoding putative Enterolysin A and Helveticin-J, genes encoding for Thermophilin A, Durancin Q, Coagulin A, and Staphylococcin C55β bacteriocins were also identified [10]. Thus, the production of these antimicrobial compounds reveals to be a promising strategy for bacterial vaginosis treatment and can contribute to the urogenital health of the host.

Lactic acid synthesis

Lactic acid is the primary substance produced by *lactobacilli* which helps to protect against vaginal infections. Its primary mechanism of action relies on pH reduction to under 4.5, reaching values less than 3.5 when the vaginal microbiota is dominated by *lactobacillus sp*., protecting against a wide variety of infections from non-indigenous pathogens [11]. Lactic acid is produced by *lactobacillus sp*. in two isomeric forms, D and L-lactic acid, and the proportion of this production is species and strain-dependent [10]. Species such *L. iners* does not have the genes to produce D-lactic acid and produce lower concentrations of L-lactic acid when compared to *L. crispatus* and *L. gasseri*, while *L. jensenii* is only able to produce the D-form [11].

L. crispatus is a potent producer of lactic acid and other bacteria inhibitor compounds. In a manner that the vaginal microbiome highly colonized by *L. crispatus* is less likely to develop bacterial vaginosis [10]. Lactic acid is produced by the enzymes D and L-lactate dehydrogenase, its genes present in different proportions among different *lactobacilli* species. *L. crispatus* encodes two genes for the L a form of the enzyme and one for the D form [3].

A cohort study evaluated the vaginal microbiome of two hundred fifty-five pre-menopausal nonpregnant women found that 20% presented *L. crispatus* dominant microbiome and that these women were less likely to be colonized by *Candida sp*. This study also showed that, *in vitro*, *L. crispatus* cell-free supernatant presents a pH around 4.0 and high levels of lactic acid, inhibiting *Candida albicans* growth [12]. Another study isolated 135 *lactobacilli* from reproductive women's vaginal microbiome. From these, 56% corresponded to *L. crispatus* [13]. From these strains, a selection was tested against *E. coli*, *C. glabrata*, and *Gardnerella vaginalis* using the modified agar spot method, with *L. crispatus* demonstrating the highest activity against all three species when compared to other *Lactobacillus* species [13]. These results show that *lactobacilli* colonization is essential to maintain vaginal health, especially if this colonization is *L. crispatus* dominated

Insights of genomic islands

Genomic islands (GEIs) are key elements from the bacterial accessory genome as they can be responsible for the acquisition of novel genes conferring a fitness benefit in specific habitats [14]. Investigating GEIs regions in *L. crispatus* strains provides essential insights into the evolution, lifestyle adaptation, and metabolic diversity of the species, which may help screen probiotic strains.

CONCLUSION

The four genomes of *L. crispatus* strains previously isolated by Almeida and colleagues (2021) present nine putative GEIs for the strains CRI4 and CRI17 and 14 for CRI8 and CRI10. Each GEIs presents an approximate size of 15.477,222 bp, and together they correspond to 7% of the total genome size from *L. crispatus*. Most gene content in the GI is related to phage integration, bacterial metabolism, and survival, such as type II-A CRISPR-associated proteins and thioredoxin family protein. Moreover, further studies are suggested to investigate potential genes associated with resistance to antibiotic drugs or virulence for safety issues.

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