# Inhibitory activity of vaginal lactobacilli towards *Candida* spp.

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## Introduction and purpose.

The homeostasis of the vaginal ecosystem results from complex interactions and synergies among the host and different microorganisms that colonize the vaginal mucosa. Healthy vaginal microbiota is generally dominated by *Lactobacillus* spp.

These bacteria form a critical line of defense against potential pathogens by producing antimicrobial compounds, or through competition for adherence to the vaginal epithelium. For the positive effects of lactobacilli on the health of female genital tract there is an increasing interest for their use in probiotic formulations for the prophylaxis and therapy of several vaginal disturbances.

Vulvovaginal candidiasis (VVC) is a common infection compromising the quality of life of many women. *Candida* infection affects 70-75% of women at least once during their lives, 40-50% of them experience at least one recurrence, and about 5-8% of these women suffer from recurrent VVC. *Candida albicans* is the most frequent etiologic agent of VVC. Many lactobacilli are known to protect from *Candida* infection but the mechanisms underlying antifungal activity are still not clearly understood.

The present study aims to isolate vaginal lactobacilli from healthy women and to evaluate their ability to counteract *Candida* infections, focusing on hydrogen peroxide generation, lactic acid production and antimicrobial supernatant fluids activity. A major potential application of this study concerns the identification of active *Lactobacillus* strains to propose as probiotics for prophylaxis and/or adjuvant therapy of VVC.

## Methods.

***Isolation of vaginal lactobacilli from healthy women and taxonomic characterization.*** Fifteen healthy pre-menopausal women (aged 18-45 years old) were recruited for the present study. All volunteers provided a written informed consent in accordance with the Ethics Committee of the University of Bologna. Vaginal swabs (E-swabs, Copan) were self-collected by the women, and immediately processed for lactobacilli isolation.

*Lactobacillus* clones were isolated onto de Man, Rogosa and Sharpe (MRS) and Brain-Heart Infusion (BHI) agar plates (Difco) supplemented with 0.05% L-cysteine in anaerobic conditions. Colonies with different morphologies yielding variable rods were selected for glycerol stock preparation.

Genomic DNA was extracted from the selected strains using DNeasy Blood & Tissue Kit (Qiagen) and DNA was amplified with *Lactobacillus* genus-specific primers Lac1 and Lac2. The positive isolates were taxonomically characterized at the species level by sequencing the 16S ribosomal RNA (rRNA) gene.

***Hydrogen peroxide determination.*** Lactobacilli were tested for their ability to produce $H_2O_2$ by cultivating them onto MRS plates containing 0.25 mg/mL 3,3', 5,5'-tetramethylbenzidine and 0.01 mg/mL of horseradish peroxidase in anaerobic condition for 72 h. Plates were exposed to air and on the basis of the time required for the blue coloration to appear, isolates were scored as low [score 1(>20 min)], medium [score 2 (10-20 min)] and high producing strains [score 3 (<10 min)]. Isolates which did not produce blue coloration were scored as 0.

***$^1$H-NMR analysis.*** $^1$H-NMR spectra were recorded at 298 K with an AVANCE III spectrometer (Bruker) operating at a frequency of 600.13 MHz. The signals were assigned by comparing their chemical shift and multiplicity with Chenomx software data bank.

## Results.

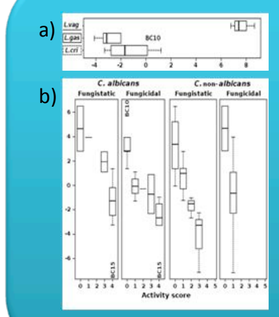
The *Lactobacillus* isolates were taxonomically identified as follows: 8 strains of *L. crispatus* (BC1-BC8), 6 strains of *L. gasseri* (BC9-BC14), and 3 strains of *L. vaginalis* (BC15-BC17). All *Lactobacillus* strains exhibited a good generation of hydrogen peroxide.

When the anti-fungal activity of *Lactobacillus* was assessed, *L. crispatus* supernatants were the most effective, especially versus *C. albicans* and *C. lusitaniae*

| Species | Strain | Accession n. | pH | $H_2O_2$ (score) | Lactate (mM) | Butyrate (mM) |
|---|---|---|---|---|---|---|
| *L. crispatus* | BC1 | AB976542 | 3.93 | 3 | 2.91 | $3.48 \times 10^{-1}$ |
| *L. crispatus* | BC2 | AB976543 | 4.13 | 3 | 6.83 | 0.00 |
| *L. crispatus* | BC3 | AB976544 | 4.21 | 1 | 9.45 | 0.00 |
| *L. crispatus* | BC4 | AB976545 | 3.87 | 1 | 3.32 | $3.54 \times 10^{-1}$ |
| *L. crispatus* | BC5 | AB976546 | 3.70 | 2 | 5.10 | $1.25 \times 10^{-1}$ |
| *L. crispatus* | BC6 | AB976547 | 4.03 | 2 | 7.87 | $8.33 \times 10^{-1}$ |
| *L. crispatus* | BC7 | AB976548 | 3.91 | 1 | 1.42 | $1.35 \times 10^{-2}$ |
| *L. crispatus* | BC8 | AB976549 | 4.08 | 1 | 3.05 | $4.64 \times 10^{-1}$ |
| *L. gasseri* | BC9 | AB976550 | 3.90 | 2 | 4.75 | 0.00 |
| *L. gasseri* | BC10 | AB976551 | 4.54 | 3 | 9.40 | 0.00 |
| *L. gasseri* | BC11 | AB976552 | 4.20 | 3 | $1.46 \times 10^{1}$ | 0.00 |
| *L. gasseri* | BC12 | AB976553 | 4.17 | 3 | 9.47 | 0.00 |
| *L. gasseri* | BC13 | AB976554 | 3.87 | 1 | 1.62 | $1.84 \times 10^{-2}$ |
| *L. gasseri* | BC14 | AB976555 | 4.74 | nd* | $3.63 \times 10^{1}$ | $1.00 \times 10^{-2}$ |
| *L. vaginalis* | BC15 | AB976556 | 3.95 | 1 | $4.74 \times 10^{1}$ | $6.42 \times 10^{-1}$ |
| *L. vaginalis* | BC16 | AB976557 | 4.59 | 1 | $2.44 \times 10^{1}$ | 0.00 |
| *L. vaginalis* | BC17 | AB976558 | 4.28 | 3 | $2.34 \times 10^{1}$ | $3.27 \times 10^{-1}$ |

| Lactobacilli strains | *C. albicans* 1 | *C. albicans* 2 | *C. albicans* 3 | *C. albicans* 4 | *C tropicalis* | *C. krusei* | *C. parapsilosis* | *C. glabrata* | *C. lusitaniae* |
|---|---|---|---|---|---|---|---|---|---|
| *L. crispatus* BC1 | Fungicidal | Fungicidal | Fungicidal | Fungicidal | Fungistatic | Not active | Not active | Fungistatic | Fungicidal |
| *L. crispatus* BC2 | Fungistatic | Fungicidal | Fungicidal | Fungistatic | Not active | Not active | Not active | Not active | Fungicidal |
| *L. crispatus* BC3 | Fungicidal | Fungicidal | Fungistatic | Fungicidal | Not active | Not active | Not active | Not active | Fungicidal |
| *L. crispatus* BC4 | Fungicidal | Fungicidal | Fungicidal | Fungicidal | Not active | Not active | Not active | Fungistatic | Fungicidal |
| *L. crispatus* BC5 | Fungicidal | Fungicidal | Fungicidal | Fungicidal | Fungistatic | Not active | Not active | Not active | Fungicidal |
| *L. crispatus* BC6 | Fungistatic | Fungicidal | Fungicidal | Fungicidal | Fungistatic | Not active | Not active | Fungistatic | Fungicidal |
| *L. crispatus* BC7 | Fungicidal | Fungicidal | Fungicidal | Fungicidal | Fungistatic | Not active | Not active | Fungistatic | Not active |
| *L. crispatus* BC8 | Fungistatic | Fungicidal | Fungistatic | Fungistatic | Not active | Not active | Not active | Not active | Fungicidal |
| *L. gasseri* BC9 | Fungistatic | Fungicidal | Fungistatic | Fungistatic | Not active | Not active | Not active | Not active | Not active |
| *L. gasseri* BC10 | Not active | Not active | Not active | Not active | Not active | Not active | Not active | Not active | Not active |
| *L. gasseri* BC11 | Not active | Not active | Not active | Not active | Not active | Not active | Not active | Not active | Not active |
| *L. gasseri* BC12 | Fungistatic | Not active | Fungistatic | Fungistatic | Not active | Not active | Not active | Not active | Fungicidal |
| *L. gasseri* BC13 | Fungistatic | Fungistatic | Fungistatic | Fungistatic | Not active | Not active | Not active | Not active | Fungicidal |
| *L. gasseri* BC14 | Fungistatic | Not active | Not active | Not active | Not active | Not active | Not active | Fungistatic | Not active |
| *L. vaginalis* BC15 | Fungicidal | Fungicidal | Fungicidal | Fungicidal | Fungistatic | Not active | Not active | Fungistatic | Fungicidal |
| *L. vaginalis* BC16 | Fungistatic | Not active | Not active | Not active | Not active | Not active | Not active | Not active | Not active |
| *L. vaginalis* BC17 | Fungistatic | Fungicidal | Not active | Fungistatic | Not active | Not active | Not active | Not active | Fungicidal |

We identified 40 molecules mainly belonging to the families of aminoacids, organic acids monosaccharides, ketones and alcohols. A Principal Component Analysis (PCA) was performed on the entire set of metabolites identified in *Lactobacillus* supernatants (see the figure below). Metabolic profiles varied to a greater extent according to the taxonomy. In particular, metabolome of *L. vaginalis* significantly differed from those of *L. crispatus* and *L. gasseri* ($P < 0.05$). The highest metabolic heterogeneity was observed within *L. gasseri*, as demonstrated by the width of the corresponding boxplot. Even fungistatic and fungicidal activities of the vaginal lactobacilli were related to their metabolome. Strains with different activity scores were clearly separated in the vertical direction: the most active strains occupied the lower positions, while the less active strains were placed in the higher areas of the two-dimensional space represented by the biplot.




(a) Box plots representing the distribution of *Lactobacillus* species in relation to the metabolome. Lines within the boxes indicate the median values of the samples groups corresponding to *L. crispatus*, *L. gasseri* and *L. vaginalis* species.

(b) Box plots representing the distribution of fungistatic/fungicidal activity scores towards *C. albicans* and *C.* non-*albican* in relation to the metabolome. Lines within the boxes indicate the median values of the samples groups corresponding to the different activity scores (0-4 for *C. albicans*; 0-5 for *C.* non-*albicans*). Each box represents the interquartile range (25–75thpercentile). The bottom and top bars indicate the 10th and 90th percentiles, respectively. Outlier values are indicated (BC10 and BC15).

## Conclusion.

A major potential application of this study concerns the identification of active *Lactobacillus* strain that could be administered as probiotics for prophylaxis and/or adjuvant therapy of vulvovaginal candidiasis. Further studies are ongoing to elucidate the mechanisms by which lactobacilli exert their protective functions against *Candida*.

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