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Lactobacillus crispatus modulates epithelial cell defense against *Candida albicans* through Toll-like receptors 2 and 4, interleukin 8 and human β-defensins 2 and 3

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ABSTRACT

Lactobacilli are members of the normal mucosal microflora of most animals. Probiotic bacteria, such as Lactobacilli, play a major role in the maintenance of a healthy urogenital tract by preventing the colonization of pathogenic bacteria. The potentially probiotic strain *Lactobacillus crispatus* (ATCC 33820) was investigated for its capacity to influence the innate immune response of HeLa epithelial cells to *Candida albicans*. In addition, its capacity to modulate the toll-like receptor (TLR) expression of HeLa cells was investigated by Western blot. When HeLa cells were pre-treated with the *L. crispatus* and infected with *C. albicans*, the interleukin-8 levels were significantly lower than without pre-treatment. Also, the effect of *L. crispatus* on innate immunity was enhanced by its capacity to increase the effect of human β-defensin 3 against *C. albicans* growth. Pre-treating HeLa cells with *L. crispatus* attenuated the yeast's virulence, as demonstrated by its reduced adhesion and growth on human epithelial cells. Our findings indicated, also, that after contact with *C. albicans*, epithelial cells expressed more TLR2/4 than non-infected cells, whereas pre-treatment with *L. crispatus* downregulated the TLR2/4 expression by epithelial cells stimulated with *C. albicans*. In conclusion, our results show that *L. crispatus* promotes epithelial cell defense against *C. albicans* infection through the involvement of TLR2/4, IL-8 and human β-defensin 2 and 3, thus suggesting a probiotic potential of this *Lactobacillus* as an anti-infective agent against *C. albicans*.

1. Introduction

Candida albicans is the most prevalent human fungal pathogen, with an ability to inhabit diverse host niches and cause disease in both immunocompetent and immunocompromised individuals [1]. It has been demonstrated that *C. albicans* can become virulent, passing from commensal to pathogenic when the host defense system fails, especially in immunosuppressed and immunodeficient patients. *C. albicans* is part of the normal microbial flora found on mucosal surfaces such as those of the oral cavity, gastrointestinal tract and vagina in human beings and domestic animals [2]. Moreover, *Candida* species are of increasing concern as causative agents of fungal biofilm-related infections on prostheses and materials used in dental surgery, otolaryngology and in the urogenital tract [3–5]. The development of new technologies based on the control of *Candida* spp. biofilm growth will be a major breakthrough in medicine that will have a strong impact in clinical practice and prevention [6]. Many lactobacilli are known to inhibit the growth of *Candida* spp. in different ways, such as competition for adhesion sites, or stimulation of different subsets of the immune defense system [7–9].

Fungal infections caused by *Candida* species are increasing, particularly in immunocompromised individuals and in women using new methods of contraception, such as the combined contraceptive vaginal ring [10]. Despite the advantages of these new contraceptives, the balance of the vaginal ecosystem can be disrupted by their use, triggering a predisposition for the development of vulvovaginal candidiasis (VVC) [11]. The high incidence and associated healthcare costs of VVC highlight the need for the development of effective agents for its prevention [12]. Vulvovaginal diseases can lead to persistent infection that may contribute to the development of chronic inflammatory diseases [13].

Lactobacilli are involved in the maintenance of the normal vaginal microbiota because they prevent the overgrowth of pathogenic and opportunistic organisms [14]. They can act through the activation of the immune system, competition with other microorganisms for adherence to the vaginal epithelium and the production of lactic acid and bacteriocins [15]. Co-aggregation of *C. albicans* and Lactobacilli may be of great importance in the

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vaginal microenvironment especially by reducing the adhesion of the yeast to the vaginal epithelium [16]. The protective role that Lactobacilli play in VVC seems to be based on mechanisms of adherence of selected *Lactobacillus* species [17] to the vaginal epithelium, leading to intensive colonization of this surface, with the formation of micro colonies and biofilm. In addition, they control the remaining vaginal microflora by the production of active metabolites [18] including β-defensins and the modulation of toll-like receptor and cytokine IL-8 expressions [19].

Controlling *Candida* pathogenesis involves the human beta defensin family (HBDs) of antimicrobial peptides that display broad-spectrum antimicrobial activity against a large array of pathogens such as the *Candida* species [20]. Although the antifungal activity of HBDs has been characterized by some Authors [21], their exact role in the host defense against fungal infection remains unclear [1].

Several recent studies have highlighted the significant role played by the innate immunity of epithelial cells in preventing *Candida* infection [2]. Primary epithelial cells obtained from various sources [8,9,22] inhibited *C. albicans* growth through cytokine and antimicrobial peptide expression [8,23]. In response to *C. albicans* infection, oral epithelial cells were shown to produce a significant amount of interleukin (IL)-6, IL-8, and TNFα [24], which suggests that cytokines play a role in oral infections [22]. The detection and response to microbial infection by the cells involved, such as epithelial cells, depends on the family of pattern-recognition receptors called toll-like receptors (TLRs) [25], which play an active role in recognizing pathogens of considerable target specificity [26]. Some studies have demonstrated the crucial involvement of TLRs in the recognition of fungal pathogens such as *Candida* [27,28], although little is known regarding the function of these receptors following Candida infection [27]. It has been demonstrated that the Candida-killing of keratinocytes involves TLR2 and TLR4 trough NF-kB activation [29]. Given that Lactobacilli, in particular *Lactobacillus crispatus*, may be more likely to contribute to the maintenance of the normal vaginal ecosystem [30], in this study we evaluated the influence of viable *L. crispatus* (LC) on the adhesion capacity of *C. albicans* in the HeLa cell line and the immunomodulatory effect of *L. crispatus* on the cytokine and HBD production induced by *C. albicans*. In addition, since most studies used viable probiotic bacteria, heat-killed *L. crispatus* was examined in this study. We found that viable *L. crispatus* and, to a lesser extent, heat-killed *L. crispatus* promoted epithelial cell defense against *C. albicans* infection through the involvement of TLR2/4, IL-8 and human β-defensin 2 and 3.

2. Materials and methods

2.1. Bacterial strains and cell line

L. crispatus cells (ATCC 33820) cultured in MRS broth (Oxoid S.p.A., Milan, Italy) in anaerobic conditions (Gas-Pak system, BBL, Becton Dickinson Biosciences, Milan, Italy) at 37 °C overnight were harvested by centrifugation, washed twice and resuspended in phosphate-buffered saline (PBS) at a concentration of 10^7 colony-forming units (CFU)/ml. Viable cells were counted by plating serial dilutions on MRS agar [31]. Bacteria resuspended in PBS (10^7 CFU/ml) were heat-killed by heat treatment (100 °C/15 min).

C. albicans was inoculated into 10 ml Sabouraud dextrose broth (BD Difco) (Becton-Dickinson) and grown to the stationary phase overnight at 30 °C in a shaking water bath. Following culture, blastoconidia were collected, washed with PBS, counted by means of a hemocytometer and adjusted to10^7 cells/ml.

The HeLa cells, human cervical cancer cells (ATCC CCL-2), were grown in Dulbecco's modified Eagle's minimal essential medium (DMEM) (Sigma, Aldrich). Culture broths were supplemented with 10% (w/v) heat-inactivated fetal bovine serum (Gibco, Germany) and penicillin G (5000 U ml^{-1}). Cultures were incubated in 25 cm^2 tissue culture flasks (Corning Costar, Milan, Italy) at 37 °C in 5% CO_2 until confluence.

2.2. L. crispatus and C. albicans competition assay

The antagonist activity of the Lactobacillus was screened against *C. albicans* using the co-culture method [32]. Briefly, 1 ml portions of viable and heat-killed *L. crispatus* (10^7 CFU/ml) were mixed with 1 ml of *C. albicans* (10^6 CFU/ml) and cultured on the appropriate media. The optical density of the culture medium was measured at 8, 12 and 18 h after incubation at 580 nm. An aliquot of each mixture was spread on Sabouraud dextrose agar medium and the plates were incubated at 37 °C for 18 h, after which the CFU were counted by the spread-plate technique. Assays were performed at least in triplicate, and the data are expressed as the mean ± standard deviation (SD).

2.3. Adhesion assays and growth

The adherence to epithelial cells was examined using HeLa cells following the procedure described by Tallon et al. [33]. In brief, the monolayers of HeLa cells were seeded at a concentration of 1.4×10^4 cells cm^{-2} in 12-well tissue culture plates and incubated at 37 °C in 5% CO_2. The cells were used when they approached confluence, with a change of medium on alternate days. The number of cells was within the range of 5×10^5 to 6×10^5 cells per well.

Overnight cultures of *L. crispatus* were appropriately diluted with DMEM to give a bacterial concentration of approximately 5×10^7 cells ml^{-1}, and 0.5 ml of PBS were added to each well of the tissue culture plate and incubated at 37 °C in 5% CO_2 atmosphere. After 15, 30, 60 and 120 min of incubation, the monolayers were washed three times with PBS, the cells were lysed by adding a 0.05% solution of triton-X100 and the number of viable adhering bacteria was determined by plating serial dilutions on MRS agar plates. The multiplicity of infection (MOI) used in this study was approximately 100–120 (bacteria/cells).

Since *L. crispatus* downregulates *C. albicans* adherence to epithelial cells, we investigated the effect of *L. crispatus* on the growth of *C. albicans*. The growth of *C. albicans* was investigated as described in Section 2.1.

The assays were conducted at least in triplicate, and the data are expressed as the mean ± SD.

2.4. Interference with pathogen adhesion

The effect of *L. crispatus* on the adhesion of *C. albicans* to HeLa cells was assessed as described by Martín et al. [34] with some modifications using a probiotic/pathogen ratio of 10:1. For the exclusion tests, *L. crispatus* and HeLa cells were incubated for 30 min, after which *C. albicans* cells were added and incubation was continued for a further 15, 30, 60 and 120 min. After incubation, the cell monolayers were washed with PBS and aliquots of the cultures were transferred to plates containing S-BHI (Becton-Dickinson) with 20 μg/ml penicillin G (selective for *C. albicans*) to determine the number of viable adhering bacteria. The susceptibility of *L. crispatus* to the antibiotic was confirmed prior to the adhesion assays. The results are representative of three independent experiments, and the data are expressed as the mean ± SD.

2.5. Determination of IL-8 and HBD-2 and HBD-3 proteins by sandwich enzyme-linked immunosorbent assay (ELISA)

To determine the protein levels of the cytokine IL-8 and HBD-2/3, the supernatants obtained from the cultures stimulated with

viable *L. crispatus* or heat-killed *L. crispatus* for 12 h and *C. albicans* for another 18 h, using a ratio of 10:1, or *Candida* alone were collected, centrifuged to separate bacteria and stored at −80 °C until assayed using titerzyme ELISA Kits, according to the manufacturer's instructions (R&D Systems, Minneapolis, MN; Orgenium Laboratories Oy; Phoenix Pharmaceuticals, Inc., respectively). The optical density of the wells was determined using a microplate reader (Eppendorf Biophotometer) set to 450 nm with a wavelength correction set to 540 nm [35].

2.6. Effect of HBD-2 and HBD-3 on C. albicans growth

For the antibacterial assay, the *C. albicans* suspension (10^6 cells) in 200 μl of PBS was incubated with HBD-2 or HBD-3 (20 μg/ml) (Alpha Diagnostic International, San Antonio, TX, USA) for 6 h at 37 °C; (this concentration and time were chosen after preliminary experiments exhibiting the best dose and time/response effect). *C. albicans* cultured without HBD-2 or HBD-3 was used as a negative control. *C. albicans* cultured without HBD-2 or HBD-3 in the presence of 0.5 μg/ml of fungizone (Amphotericin-B, Sigma–Aldrich) was used as a positive control. After incubation period, 50 μl of each culture was diluted in 450 μl of PBS and spread on Sabouraud dextrose agar. The agar plates were then incubated for 18 h at 37 °C and the CFU were counted and plotted as the means ± SD of three different experiments.

2.7. Western blot analysis of the TLR2/4 expression in HeLa cells treated with L. crispatus and challenged with C. albicans

The aim of these experiments was to determine whether treatment of HeLa cells with *L. crispatus* changed the way in which HeLa cells responded to the *C. albicans* strain. HeLa cells (1×10^5 cells/well) were treated with culture medium (negative control) or bacterial suspensions containing 1×10^6 CFU/ml of *L. crispatus* or 1×10^6 heat-killed *L. crispatus* at a ratio 1:10 HeLa cells to bacterial cells. After 12 h incubation at 37 °C, the cells were washed twice with PBS and 1×10^5 CFU/ml suspension of *C. albicans* was added and incubated for another 18 h. Cell supernatants were collected, centrifuged to separate bacteria and frozen (−80 °C) for later quantification of the IL-8 cytokine and HBD-2/3 protein levels. At the end of the experiments, the cells were collected and the expression of TLR2/4 protein was determined by Western blot analysis with specific antibodies directed for TLR2/4 (Santa Cruz Biotechnology, CA), as described by Paolillo et al. [36].

Protein loading was checked by reprobing the membranes with tubulin (Santa Cruz Biotechnology, Inc.) to show that the protein levels had not changed. Semiquantitative analysis of Western blot results was performed by densitometric measurements (NIH image V 1.6 software). The optical density was normalized according to the house-keeping gene tubulin.

To exclude LPS contamination, Gram-positive bacteria and *C. albicans* were tested by a turbidimetric Limulus amoebocyte lysate assay. The limit of detection of this assay was 1 pg/ml LPS *Escherichia coli*, and the endotoxin was found to be non-detectable in all samples tested [37]. The results are representative of three independent experiments, and the data are expressed as the mean ± SD.

2.8. TLR2/TLR4-blocking experiments

For blocking experiments, cultured HeLa cells were grown on six-well plates and pre-treated with the monoclonal antibodies anti-human TLR2 and TLR4 (TL2.1 10 μg/ml; TL4.1 10 μg/ml; Santa Cruz Biotechnology, CA) or isotype matched control-purified mouse IgG 2a MAbs (10 μg/ml; Santa Cruz Biotechnology, CA) for 1 h and then stimulated with *L. crispatus* for 12 h and infected with *C. albicans* for another 18 h. The culture media were then collected and analyzed for IL-8 and HBD-2/3 by ELISA.

2.9. Statistical analysis

All the experiments in this study were performed three times. Experimental values are given as means ± SD. The statistical significance of the differences between the control values and the test values was evaluated using a one-way ANOVA. The data were analyzed using the statistical toolbox of Excel software (Microsoft Corp., Seattle, WA). Values of $P < 0.05$ were considered statistically significant.

3. Results

3.1. L. crispatus-related growth inhibition of C. albicans

The antagonist effect of *L. crispatus* against *C. albicans*, was assessed in co-culture assays and compared with the growth ability of *C. albicans* in the same culture medium. A decrease in the cell division rate of the yeast tested was observed from 8 h of co-incubation (Fig. 1). When *C. albicans* in the mixed suspension was counted over a longer period of time, the activity of the yeast was detected between 12 and 18 h of incubation. In no case was there an effect of *C. albicans* against the Lactobacilli.

3.2. Adherence and interference assays

As the ability of bacteria to adhere to different tissues is a factor in colonization, the adhesiveness with the different exposure times of viable and heat-killed *L. crispatus* to epithelial cells was investigated. The results demonstrated that, after 30 min viable *L. crispatus* adhered strongly to the epithelial cells (Fig. 2A; 89 ± 5.1%), whereas heat-killed *L. crispatus* showed a lesser adhesion to HeLa cells (67 ± 3.9%), suggesting that structures sensitive to heat may be involved in adherence mechanisms. After the incubation time of 60 and 120 min, no further significant increase was observed in viable or heat-killed *L. crispatus*.

In addition, the effect of viable and heat-killed *L. crispatus* on the adhesion of *C. albicans* to epithelial HeLa cells was investigated. Inhibition values were calculated as a percentage of adherent

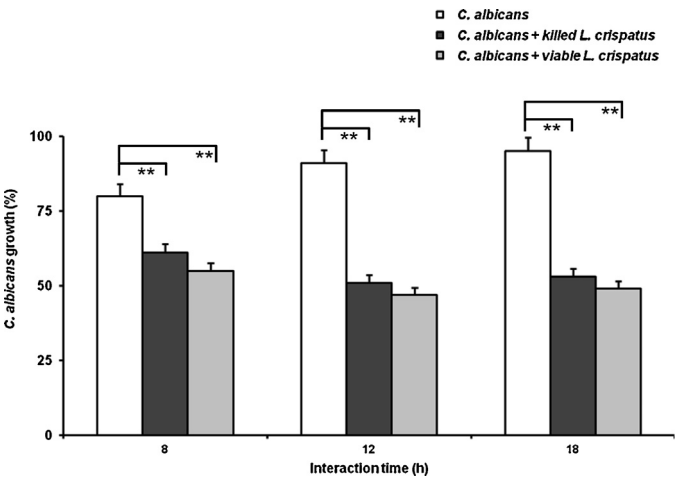

Fig. 1. Effect of *L. crispatus* on *C. albicans* growth. *C. albicans* (1×10^5 CFU/ml) was incubated with or without viable or heat-killed *L. crispatus* (1×10^6 CFU/ml) and the CFU was quantified after 8, 12 and 24 h of incubation by spreading on Sabouraud dextrose agar. The data are means ± SD of three independent experiments. The asterisks indicate a statistically significant difference between *C. albicans* grown in the presence of viable or heat-killed *L. crispatus* versus *C. albicans* alone. $^{**}P < 0.01$.

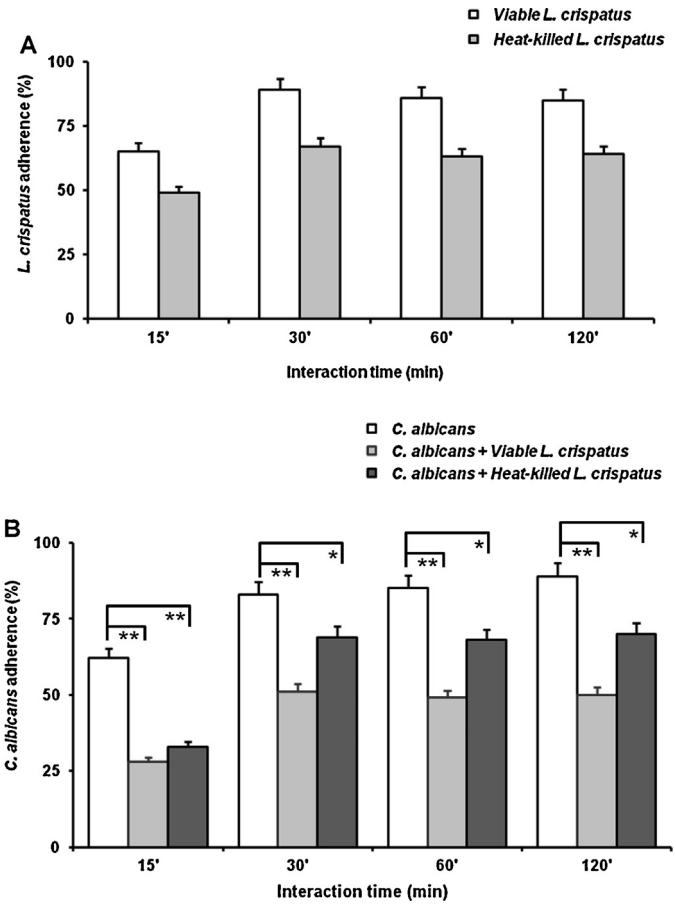

Fig. 2. Adherence of *L. crispatus* (A) or *C. albicans* in presence of *L. crispatus* (B) to HeLa cells. Cultured epithelial cells were treated with *L. crispatus* (1×10^6 CFU/ml) for 15′, 30′, 60′ and 120′. Error bars indicate standard deviations (A). Cultured epithelial cells were treated with viable or heat-killed *L. crispatus* (1×10^6 CFU/ml) for 30′ and infected with *C. albicans* (1×10^5 CFU/ml) for 15′, 30′, 60′ and 120′ (B). The data are expressed as the mean ± SD percentage of adherence in three independent experiments. The asterisks indicate a statistically significant difference between *C. albicans* grown in the presence of viable or heat-killed *L. crispatus* versus *C. albicans* alone. $^*P < 0.05$, $^{**}P < 0.01$.

yeast per HeLa cell. *L. crispatus* significantly reduced the adhesion of *C. albicans* to HeLa cells; in particular, the results indicated that preincubation with viable *L. crispatus* and infection for 30 min led to a reduced percentage of adhesion of *C. albicans* to HeLa cells, while heat-killed *L. crispatus* showed only slightly diminished adhesion of the yeast to HeLa cells (51 ± 3.6% and 69 ± 3.9%, respectively) compared to cells infected with *C. albicans* alone (83 ± 3.8%) (Fig. 2B). At 60 and 120 min of infection, no further changes in the adherence were observed. No morphological changes were observed after adhesion of *C. albicans* to the epithelial cells (Fig. 3).

3.3. *L. crispatus downregulated the effect of C. albicans on IL-8 secretion*

To determine whether *L. crispatus* was able to influence the innate response of HeLa cells to *C. albicans*, the cells pre-treated with medium alone or with suspensions of viable or heat-killed *L. crispatus* were infected with *C. albicans*. The supernatants of the cultures were then collected and assayed for IL-8. Fig. 4A shows that the IL-8 concentrations by HeLa cells cultured with viable or heat-killed *L. crispatus* were slightly higher than the untreated control (HeLa cells treated only with medium) (60 ± 2.4

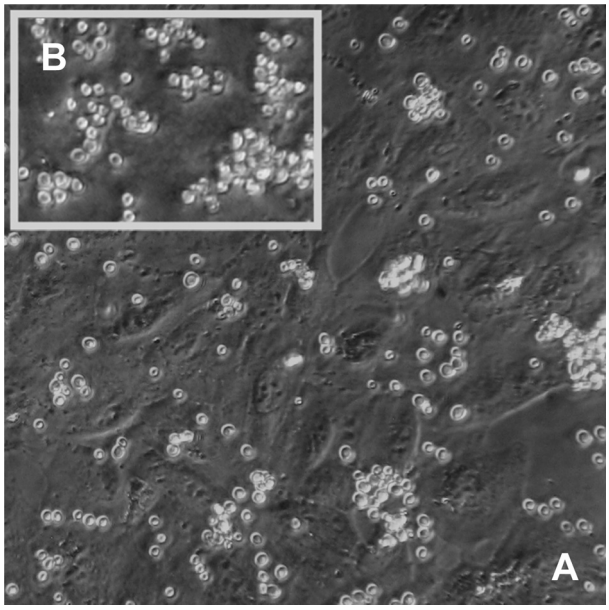

Fig. 3. Representative experiment of HeLa cells infected with *C. albicans* after 18 h of incubation (A) and *C. albicans* alone (B). No morphological change of can be seen (Bright-field microscopy at 400×).

and 122 ± 2.9 pg/ml, respectively versus 48 ± 2.1 pg/ml). When, instead, HeLa cells were stimulated with *C. albicans*, IL-8 was released at a significantly higher level (389 ± 4.0 pg/ml) compared to the untreated control (48 ± 2.4 pg/ml). On the contrary, the cells pre-treated with viable *L. crispatus* for 12 h showed a marked decrease in the IL-8 level in response to *C. albicans* compared to cells treated with *C. albicans* alone (195 ± 3.1 versus 389 ± 4.0 pg/ml). Also, HeLa cells treated with heat-killed *L. crispatus* and infected with *C. albicans* showed a decreased release of IL-8 (250 ± 3.9 pg/ml) compared to cells stimulated with *C. albicans* alone, but to a lesser degree than with viable *L. crispatus*.

3.4. *L. crispatus modulated the effect of C. albicans on human β-defensin 2/3 expression*

Epithelial cells play a key role in the host's innate immunity against bacterial and yeast infections by means of an active process that involves various antimicrobial peptides.

As shown in Fig. 4B, the basal level of HBD-2 increased with pre-treatment of *L. crispatus*. In the presence of *C. albicans*, viable or heat-killed *L. crispatus* reduced the HBD-2 expression in the epithelial cells. These results suggest an antagonistic effect between *L. crispatus* and *C. albicans* on the HBD-2 production.

We also investigated HBD-3 production by epithelial cells following stimulation. As shown in Fig. 4C, the epithelial cells produced a low basal HBD-3, which significantly increased when the cells were stimulated with viable or heat-killed *L. crispatus*. *C. albicans* alone also greatly increased the HBD-3 production by epithelial cells. The epithelial cells pre-treated with viable or heat-killed *L. crispatus* and then infected with *C. albicans* showed a greater release of HBD-3, thus showing the efficacy of *L. crispatus* in promoting HBD-3 secretion by epithelial cells against *C. albicans*.

3.5. *HBD-2 and HBD-3 inhibit C. albicans growth*

To elucidate the possible role of antimicrobial peptides HBD-2 and HBD-3 in the host defense of the vaginal tract, we analyzed the direct effect of these two peptides on the growth of *C. albicans*.

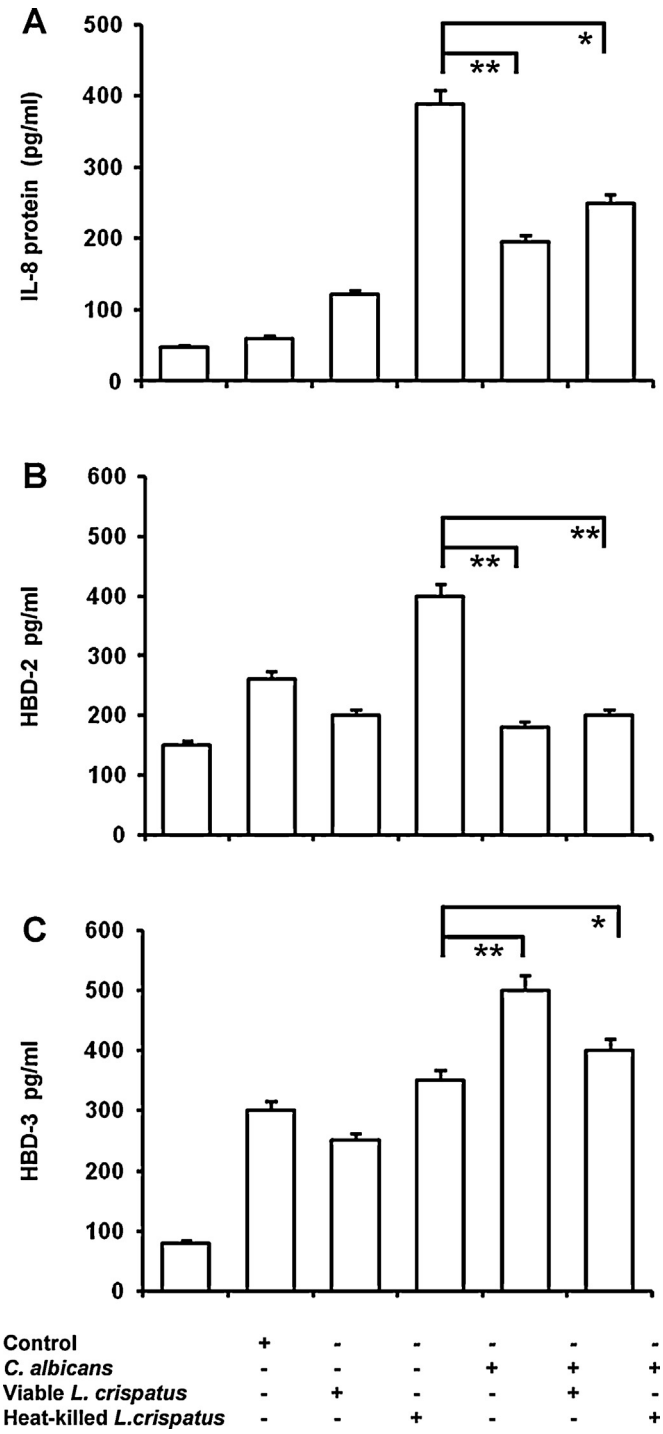

Fig. 4. Production of IL-8 (A), HBD-2 (B) and HBD-3 (C) in HeLa cells after treatment with viable or heat-killed *L. crispatus* (1×10^6 CFU/ml) for 12 h and infected or not with *C. albicans* (1×10^5 CFU/ml). At 18 h post infection, culture supernatants were collected and the IL-8, HBD-2 and HBD-3 secreted were measured by ELISA. The data are means ± SD of three independent experiments. The asterisks indicate a statistically significant difference between *C. albicans* grown in the presence of viable or heat-killed *L. crispatus* versus *C. albicans* alone. $^*P < 0.05$, $^{**}P < 0.01$.

When *C. albicans* was treated for 6 h with 20 μg/ml of HBD-2 or HBD-3 and then spread on Sabouraud dextrose agar, the number of colonies was significantly lower than with the control (untreated *C. albicans*). In particular, the inhibitory effect of HBD-3 on *C. albicans* growth appeared to be more efficient than that of HBD-2 (Fig. 5).

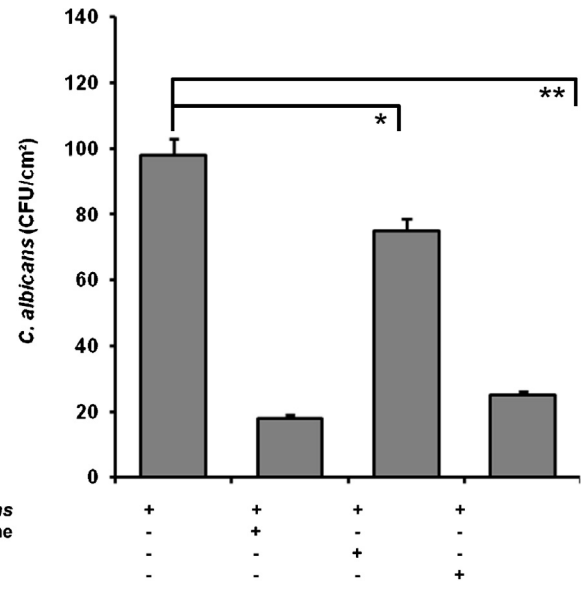

Fig. 5. Representative histogram of *C. albicans* grown in presence of HBD-2 or HBD-3. The cultures were incubated with 20 μg/ml HBD-2 or 20 μg/ml HBD-3 for 6 h and then seeded on Sabouraud dextrose agar and incubated for 18 h. The data are presented as the values obtained for *C. albicans* grown in the presence of the HBDs compared to *C. albicans* alone. The results are the means ± SD of three separate experiments. The asterisks indicate a statistically significant difference between *C. albicans* grown in the presence of the HBDs versus *C. albicans* alone. $^*P < 0.05$, $^{**}P < 0.01$.

3.6. *L. crispatus* modulated the TLR2/4 expression in HeLa cells following contact with *C. albicans*

Epithelial cells are a major interaction site between the host and the environment. The host's line of defense at this interface is therefore crucial to controlling infection. Epithelial cells are involved in the control of infections through different pathways including specific receptors such as TLRs [19,22].

We investigated the effect of viable or heat-killed *L. crispatus* on the modulation of the TLR2/4 expression in HeLa cells challenged or not with *C. albicans*. The cells were collected and the expression of TLR2/4 proteins was determined by Western blot. The expression levels of TLR2 (Fig. 6A) were enhanced by *L. crispatus*, and to a lesser extent the TLR4 levels (Fig. 6B). When HeLa cells were pre-treated with viable *L. crispatus* and infected with *C. albicans*, the TLR2/4 expressions decreased compared to the expression in HeLa cells cultured with *C. albicans* but not pre-treated with *L. crispatus*. The cells pre-treated with heat-killed *L. crispatus* and infected with *C. albicans* showed a lesser downregulation of the TLR2/4 expressions.

3.7. TLR2/TLR4-mediated IL-8, HBD-2 and HBD-3 production after *L. crispatus* treatment and *C. albicans* infection

To determine the role of the TLR2/4 receptors on the IL-8, HBD-2 and HBD-3 production induced by *L. crispatus* treatment and *C. albicans* infection, the cells were preincubated for 60 min with a TLR2/4-neutralizing antibody or an IgG2a isotype control antibody and then stimulated with viable or heat-killed *L. crispatus* for 12 h and infected with *C. albicans* for another 18 h. The cell culture supernatants were collected and assayed by ELISA for the IL-8 and HBD-2/3 production (Fig. 7).

Pre-treatment with the anti-TLR2 and anti-TLR4 antibodies, but not the isotype control antibody, inhibited the *C. albicans*-induced IL-8 (Fig. 7A) and HBD-2/3 expression (Fig. 7B and C) in HeLa cells.

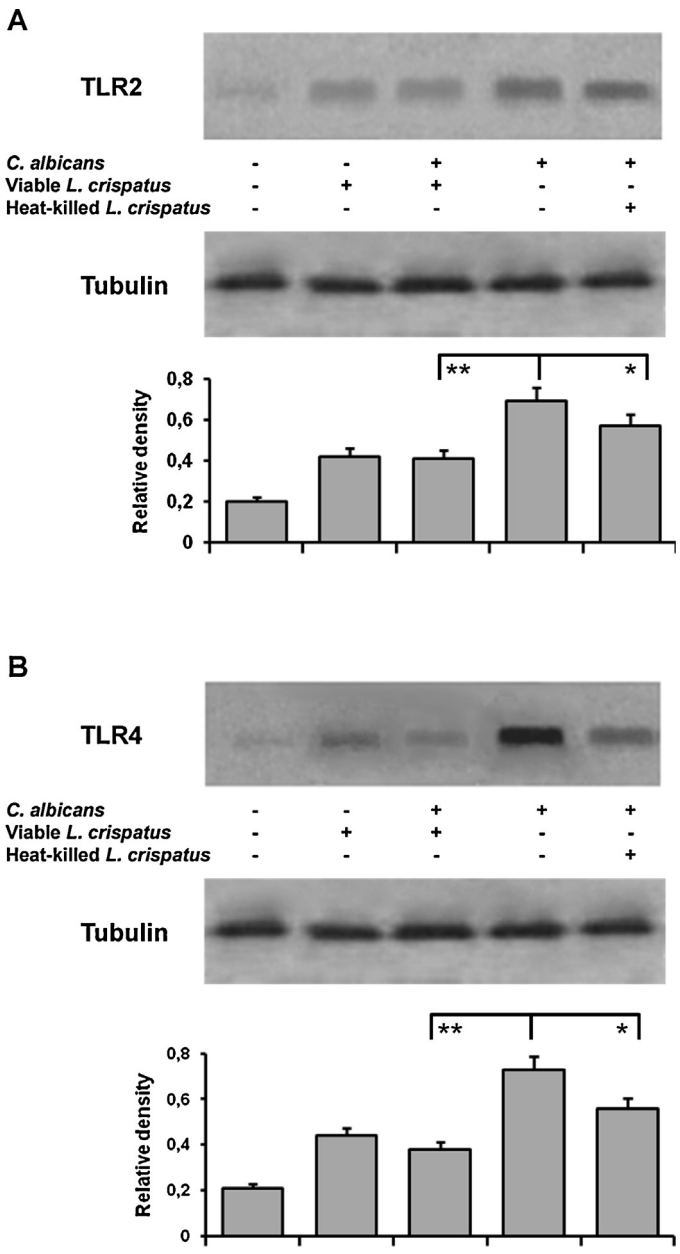

Fig. 6. Western blot of the TLR2 (A) and TLR4 (B) expressions in HeLa cells 12 h after exposure to viable or heat-killed *L. crispatus* (1×10^6 CFU/ml) followed by *C. albicans* (1×10^5 CFU/ml) infection for 18 h, using antibodies directed against TLR2 and TLR4. Line 1, untreated cells; line 2, HeLa cells stimulated with viable *L. crispatus*; line 3, HeLa cells stimulated with viable *L. crispatus* and infected with *C. albicans*; line 4, HeLa cells infected with *C. albicans*; line 5, HeLa cells stimulated with heat-killed *L. crispatus* and infected with *C. albicans*. Densitometric analysis of expression of TLR2 (A) and TLR4 (B). Tubulin was used as an internal control. The results are representative of three independent experiments, and the data are expressed as the mean ± SD. The asterisks indicate a statistically significant difference between *C. albicans* grown in the presence of viable or heat-killed *L. crispatus* versus *C. albicans* alone. $^*P < 0.05$, $^{**}P < 0.01$.

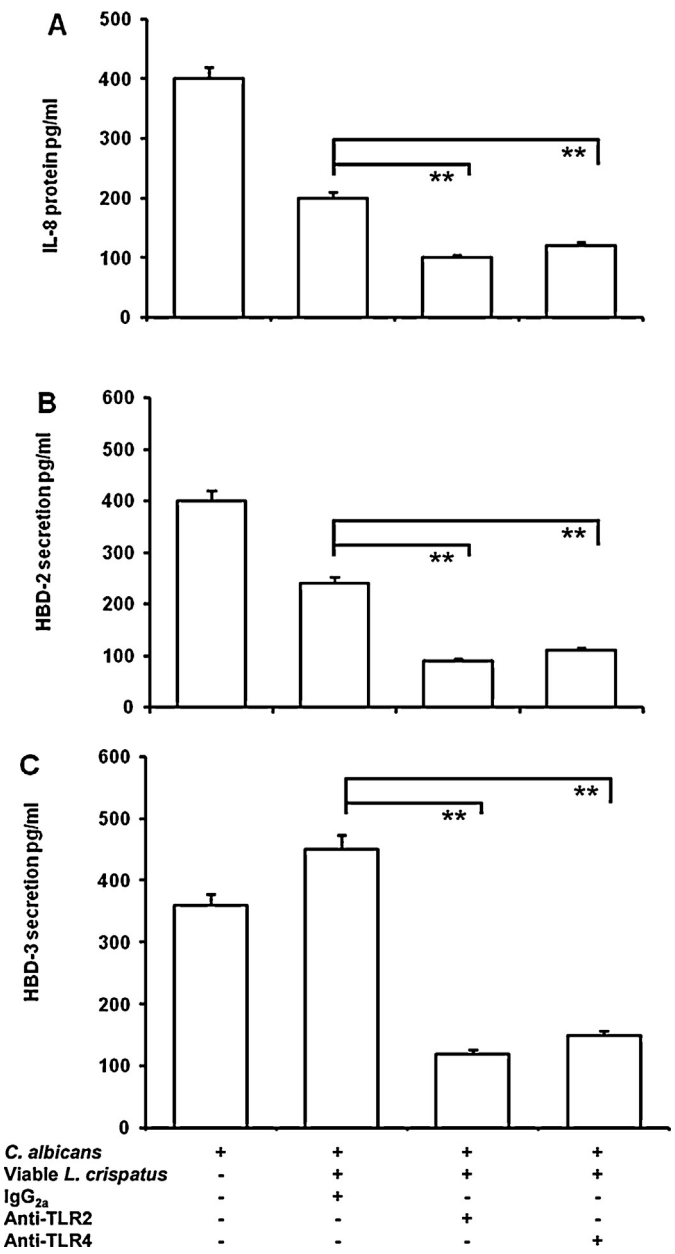

Fig. 7. Responsiveness of HeLa cells to *L. crispatus* is TLR2/4-dependent. HeLa cells were preincubated with 10 μg/ml anti-TLR2, anti-TLR4 or with IgG2a isotype control antibody for 1 h and then stimulated with *L. crispatus* (1×10^6 CFU/ml) for 12 h and infected with *C. albicans* (1×10^5 CFU/ml) for another 18 h. The supernatant medium was analyzed for secretion of IL-8 (A), HBD-2 (B) or secretion HBD-3 (C) by ELISA. The data are means ± SD of three independent experiments. The data are presented as TLR2/TLR4 antibody treatment compared to the isotype control antibody results. The asterisks indicate a statistically significant difference between HeLa cells pre-treated with TLR2/TLR4 MAbs and infected with *C. albicans* and HeLa cells treated with isotype-matched control-purified mouse IgG2a MAbs and infected with *C. albicans*. $^*P < 0.05$, $^{**}P < 0.01$.

4. Discussion

One of the great challenges of the research on the physiopathogenesis of diseases is to match the experimental conditions in vitro as much as possible with the in vivo conditions, which are often much more complex [10,38].

To investigate whether *L. crispatus* possesses the capacity to influence immune responses by microorganisms such as *C. albicans*, experiments were performed after preincubation of HeLa cells with *L. crispatus* followed by infection with *C. albicans*. We evaluated the influence of *L. crispatus* on the adhesion capacity of *C. albicans* to human epithelial HeLa cells and on the modulation of the cytokine release. The results obtained demonstrated that *L. crispatus* inhibited *C. albicans* growth and adherence to HeLa cells. Various Authors have demonstrated the in vivo and in vitro inhibition of *Candida* growth by the *Lactobacillus* strains [10,34], but the exact mechanism of this growth inhibition has not been fully elucidated. Several mechanisms for *L. crispatus* inhibition of *C. albicans* invasion have been suggested. For example, *L. crispatus* competes with

pathogenic bacteria for adhesion to host cells, inhibits the growth of pathogenic and non-pathogenic bacteria by the metabolic products (antibacterial activity), and modulates the host immune response [39]. The present study links TLR2/4 activation in epithelial cells to *L. crispatus* and *C. albicans* infection using a human epithelial cell model. Western blot showed that HeLa cells expressed TLR2 and TLR4 and that the expression was modulated by *L. crispatus* and *C. albicans*, suggesting that these cells may respond to the yeast as well as to *L. crispatus* through the TLRs, in accordance with the data that confirm the involvement of the TLR (2, 4, and 6) pathway in the interaction between the host and Candida species including *C. albicans* [40]. In this work we demonstrated that the induction of IL-8 and HBD2/3 by *C. albicans* and *L. crispatus* appears to be mediated by the TLR2/4 pathway, as its production is inhibited by anti-TLR2/4 blocking. *L. crispatus* diminished *C. albicans* activity, thereby indicating its highly promising antifungal properties. The downregulating effect may suggest, therefore, a novel mechanism with *L. crispatus* acting as an anti-inflammatory molecule through TLR2/4. Further studies are required to elucidate the role of TLR2 and TLR4 in controlling *C. albicans* infection [40], and the molecular mechanism related to this anti-inflammatory effect.

The adhesion and interaction mechanisms between *C. albicans* and epithelial cells are well known, but the data available relate to studies performed specifically between keratinocytes and fungal infection [41]. Bahri et al. [19] hypothesized that *Candida famata* sensing by human epithelial cells may be through specific receptors such as TLRs. To this regard, our study demonstrates an increase in the TLR2/4 mRNA expression following interaction between *C. albicans* and HeLa epithelial cells. TLR4 appears to be the major receptor for LPS, an essential part of the outer cell membrane of Gram-negative bacteria [42]. In contrast, the yeast cell wall component zymosan activates cells via TLR2 [43]. Interestingly, both TLR2 and TLR4 are involved in the recognition of fungal pathogens such as *C. albicans* or *Aspergillus fumigatus*, suggesting that the same pathogens may activate distinct signaling pathways simultaneously [29,44,45]. Pre-treatment with *L. crispatus* followed by *C. albicans* infection downregulated the expression of these TLRs from the targeted epithelial cells. It is well known that TLRs play a critical role as they recognize pathogens and activate the adaptive immune response [19]. In *C. albicans* infection the fact that TLR2 and TLR4 can differentially induce the production of cytokines and β-defensins implies that a single microorganism can involve different TLRs to activate distinct host defense mechanisms, suggesting that TLR2 and TLR4 can induce different intracellular signals [46]. Several studies have reported that epithelial cells are involved in innate immunity against bacterial and yeast infections via an active inflammatory process that involves different mediators, such as IL-6, IL-8 and TNF-α [47]. Given the biological role of epithelial cells and the role of IL-8 as a pro-inflammatory cytokine, we showed that the IL-8 production in response to *C. albicans* can be modulated by pre-treatment with *L. crispatus*, suggesting that *L. crispatus* promotes the host's defense against *C. albicans*.

Preincubation of HeLa cells with *L. crispatus* resulted in decreased immune activation by *C. albicans* with regard to the IL-8 production. Anti-*Candida* activity has been demonstrated in both oral and vaginal epithelial cells, suggesting a local immune response to the infection [48]. Extensive evidence in the literature has demonstrated cytokine and chemokine release from both oral and vaginal epithelial cells, confirming that on contact with *C. albicans*, epithelial cells initiate an inflammatory response leading to tissue irritation, a common feature of both oral and vaginal candidiasis [47,49]. Suppression of inflammatory cytokines may be of strong therapeutic potential, since these cytokines are known to be involved in chronic vaginal inflammation [50].

Epithelial cells also protect the host against *C. albicans* infection through antimicrobial peptides such as HBDs [22]. The induced expression of HBD-2/3 in response to infective and pro-inflammatory stimuli is an immediate and dynamic response by the host epithelium to potential infection, and the mechanism by which this occurs has been the subject of much investigation. Our study demonstrates that *L. crispatus* promotes HBD-2 and HBD-3 production by epithelial cells. Pre-treatment with *L. crispatus* followed by *C. albicans* infection brought about a significant inhibition of HBD-2 and a significant increase in HBD-3. These results, therefore, suggest that *L. crispatus* promotes the host defense through HBD-3 and not HBD-2. To confirm this hypothesis, we investigated the effect of exogenous HBD-2/3 on *C. albicans* viability/growth and found that, in particular, HBD-3 significantly reduced *C. albicans* viability and growth, which suggests the involvement of these peptides in controlling *C. albicans* growth/pathogenesis by epithelial cells [40]. It is important observe that HBD-2 shows a lesser involvement in this antifungal defense, suggesting the presence of pathways involving HBD-2 that are different from other HBDs [40]. It is reasonable to suppose that HBD-2 might show a different effect in relation to the microorganism studied [40] and to the tissue involved [51].

Interestingly, we demonstrated that heat-killed *L. crispatus* contributed to the host immune defense against *C. albicans* infection, but to a lesser degree. The heat-labile components of *L. crispatus*, such as proteins and/or glycoproteins, may be responsible for their adhesion and/or a lesser release of bacteriocins or acid compounds, which are inhibitory to many pathogens [52].

In conclusion, our data show that *L. crispatus* modulated the host pro-inflammatory responses against *C. albicans* through different pathways involving IL-8 and HBD-2/3. These findings also suggest that the vaginal epithelium is more than a simple physical barrier to protect against invading pathogens, but, on the contrary, it may be an efficient player in the innate host defense.

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