Inhibition of *Candida albicans* morphogenesis by chitinase from *Lactobacillus rhamnosus* GG

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Lactobacilli have been evaluated as probiotics against *Candida* infections in several clinical trials, but with variable results. Predicting and understanding the clinical efficacy of *Lactobacillus* strains is hampered by an overall lack of insights into their modes of action. In this study, we aimed to unravel molecular mechanisms underlying the inhibitory effects of lactobacilli on hyphal morphogenesis, which is a crucial step in *C. albicans* virulence. Based on a screening of different *Lactobacillus* strains, we found that the closely related taxa *L. rhamnosus*, *L. casei* and *L. paracasei* showed stronger activity against *Candida* hyphae formation compared to other *Lactobacillus* species tested. By exploring the activity of purified compounds and mutants of the model strain *L. rhamnosus* GG, the major peptidoglycan hydrolase Msp1, conserved in the three closely related taxa, was identified as a key effector molecule. We could show that this activity of Msp1 was due to its ability to break down chitin, the main polymer in the hyphal cell wall of *C. albicans*. This identification of a *Lactobacillus*-specific protein with chitinase activity having anti-hyphal activity will assist in better strain selection and improved application in future clinical trials for *Lactobacillus*-based *Candida*-management strategies.

*Candida albicans* is one of the most prevalent fungal pathogens, causing both superficial mucosal candidosis and life-threatening invasive infections¹. Under normal circumstances, *C. albicans* lives as a commensal on human mucosal surfaces, but can shift to a pathogenic lifestyle after fungal adhesion and overgrowth, followed by tissue invasion and mucosal infection.² This process is enabled by hyphal morphogenesis, which implies the reversible transition between unicellular yeast cells and the filamentous hyphal growth form. The hyphal cell wall is more rigid due to higher levels of chitin and is decorated with other (glyco)proteins compared to the cell wall of unhyphalized yeast cells³. These characteristics enable the hyphae to penetrate epithelial tissues, damage endothelial cells and provoke an inflammatory response, making hyphal morphogenesis crucial for the virulence of *C. albicans*⁴–⁸.

*Candida* infections are traditionally treated with antifungal compounds such as azoles, but resistance to azoles is rising and worrisome⁹. In recent years, the concept of targeting virulence factors instead of pathogen viability has become increasingly popular¹⁰. The shift of *C. albicans* to hyphal growth forms is a prime example of such a virulence process to target. This shift has been linked to disturbances in the human microbiota and a decreased ability of the commensal microbiota to control *Candida* infections¹¹. Due to this key role of the commensal microbiota, the potential of probiotics such as lactobacilli to remodel the composition and/or activity of the microbiota is increasingly explored for application in the vaginal tract¹²–¹⁴, the oral cavity of elderly¹⁵–²¹, and the gastro-intestinal tract of preterm neonates and children²²–²³. However, clinical trials that assess such interventions have not shown a uniform efficacy of the probiotic *Lactobacillus* strains applied. In addition, it was reported that some *Lactobacillus* taxa still occur in high numbers in women suffering from vulvovaginal candidosis²⁴. To better understand the molecular basis of the efficacy of *Lactobacillus* strains against *C. albicans*, it is important to identify the probiotic *Lactobacillus* factors that are able to inhibit *Candida* virulence.

Up to now, mechanistic investigations into the anti-*C. albicans* activity of lactobacilli have mainly focused on their *in vitro* growth-inhibitory capacity, which have generally revealed antimicrobial molecules present in

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the supernatant, including lactic acid and H₂O₂. These molecules are however produced widespread by lactobacilli and thus cannot explain differences between Lactobacillus taxa and strains. A few recent studies also described Lactobacillus strains that could interfere with hyphal formation, but effector components remain unidentified. Recently, mechanistic research on probiotics has – at least partly – shifted from strain-specific properties to effector molecules that are more conserved over whole taxa, since such core effector molecules have broader application potential for probiotic screening and mechanistic understanding. In this study, we aimed to identify anti-C. albicans hyphae factors of Lactobacillus, by first performing a thorough screening of various strains followed by detailed biochemical analysis of the active molecules.

**Results**

**Selected Lactobacillus strains show strong hyphae-inhibitory activity.** First, we aimed to compare the anti-Candida activity between different Lactobacillus taxa. Since hyphal morphogenesis is the most important virulence factor of C. albicans, we focused on the effect of lactobacilli on serum-induced hyphal morphogenesis. We selected twenty strains available in-house or in the Belgian Co-ordinated Collections of Micro-organisms, representing the different taxa/phylogenetic groups that have been recently described as being mainly nomadic or vertebrate-adapted. Strains were thus selected from the L. casei group, L. plantarum group, L. reuteri, L. fermentum, L. gasseri, L. jensenii and L. crispatus. The inhibition rates showed large variation among the tested strains, ranging from 91% (L. casei AMBR2) to 14% (L. plantarum WCFS1) (Fig. 1a).

Lactic acid has been described as key bioactive metabolite of Lactobacillus, and is also reported to affect C. albicans. Therefore we next measured the concentration of D-lactic acid and L-lactic acid in the supernatant of these strains, after growth into stationary phase. All strains were able to produce lactic acid from glucose, although in different ratios of D- and L-lactic acid (Fig. 1b). The level of inhibitory activity of the tested lactobacilli did not increase with an increasing concentration of either isomer, in fact, the inhibitory activity actually showed a negative correlation with the concentration of D-lactic acid (based on Pearson correlation, p-value < 0.0001 for D-lactic acid).

The five best performing strains in our tests all belonged to the L. casei group (L. rhamnosus, L. casei and L. paracasei, based on a comparative genome analysis-defined taxonomy as proposed in), suggesting an effector molecule that is shared among these taxa.

**The major peptidoglycan hydrolase of L. rhamnosus GG and lactic acid jointly mediate C. albicans hyphae inhibition.** To further elucidate how Lactobacillus can impact hyphal morphogenesis, we first explored whether the contributing L. (para)casei/rhamnosus factors are surface-bound, secreted, or both. L. rhamnosus GG was chosen as model, since this strain is well-characterized at genetic and molecular level.
first compared the effect of live *L. rhamnosus* GG cells on serum-induced hyphal formation to its cell-free culture supernatant, containing solely secreted molecules, and to UV-inactivated or heat-killed *L. rhamnosus* GG cells. Cells treated in both ways should no longer secrete molecules, but in contrast to the heat-killed cells, the surface proteins of the UV-inactivated cells should not be denatured. We showed that the supernatant from *L. rhamnosus* GG inhibited hyphal formation almost completely (97%), whereas the UV-inactivated *L. rhamnosus* GG cells inhibited hyphal formation of *C. albicans* to the same extent as live cells (57% and 51%, respectively) (Fig. 2a). The heat-killed cells, on the other hand, were no longer able to inhibit *C. albicans* hyphal formation. These results thus indicate that the main core *L. rhamnosus*-specific effectors molecules are secreted, but can also be surface-bound or are supplemented by a heat-sensitive cell-bound effector.

Next, we explored the activity of the major documented *L. rhamnosus* GG surface molecules that could have putative hyphae-binding properties due to lectin-sugar interactions. Key candidates for hyphae-binding include the lectin-like protein 1 (Llp1) and 2 (Llp2)\(^\text{43}\), the galactose-rich exopolysaccharides (EPS)\(^\text{44}\) and the major secreted protein 1 (Msp1), which is mannosylated\(^\text{45}\)

Llp1 and Llp2 have been shown to bind to D-mannose and the complex sugar mannan by sepharose-binding and glycan array screening\(^\text{46}\), both of which are present in the outer layer of *C. albicans* cell wall\(^\text{3,6}\). We therefore aimed to explore whether this sugar-binding capacity could also result in interference with hyphal morphogenesis. Treatment with Llp1 and Llp2 did not, however, show a reduction of *Candida* hyphal formation at 50 \(\mu\)g/ml (Fig. 2b), a previously documented active antibacterial concentration\(^\text{43}\). Proteins with lectin-like properties can also be found on the hyphal surface\(^\text{46,47}\), rendering the glycoconjugates on the lactobacillary surface potential interaction partners as well. In agreement with previous results\(^\text{48}\), isolated EPS from *L. rhamnosus* GG was able to inhibit hyphal morphogenesis, but only at a rather high concentration of 200 \(\mu\)g/mL (Fig. 2b). In contrast, the peptidoglycan hydrolase Msp1 from *L. rhamnosus* GG tested here demonstrated a remarkably strong inhibitory activity (Fig. 2c), reducing hyphal morphogenesis with more than 50%, at concentrations as low as 5 \(\mu\)g/mL. To

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**Figure 2.** Inhibition of *C. albicans* hyphae by *L. rhamnosus* GG and its components. Hyphal induction of *C. albicans* (10\(^6\) cells/ml) during co-incubation with (a) live *L. rhamnosus* GG cells, cell-free supernatant (CFS), UV-inactivated cells and heat-killed cells (10\(^8\) cells/ml); (b) the isolated lectin-like proteins Llp1 and Llp2 (50 \(\mu\)g/mL) and purified EPS (200 \(\mu\)g/mL) from *L. rhamnosus* GG; (c) different concentrations of Msp1 from *L. rhamnosus* GG; (d) different concentrations of lactic acid (50% L-lactic acid and 50% D-lactic acid) and (e) the combination of lactic acid (mixed, D-lactic acid and L-lactic acid) and Msp1; (f) Biofilm formation during co-incubation with *L. rhamnosus* GG cell-free supernatant (CFS) (20%), lactic acid (0, 4%) and Msp1 (10 \(\mu\)g/mL), with MRS broth and water as respective controls. The results were normalized to hyphal formation and biofilm formation of *C. albicans* solely. Single and double asterisks indicate respectively p-values below 0.1 and 0.01, compared to *C. albicans* solely.
check whether Msp1 was only inhibiting hyphal morphogenesis and not the viability of C. albicans, we determined the growth capacity of the C. albicans cells after three hours and six hours of hyphal induction in presence of Msp1. This showed that the viability of the C. albicans was not affected during the treatment with Msp1 (Supplementary Fig. S1).

Although the production of lactic acid by the lactobacilli could not really explain the observed variation in anti-hyphal activity between different Lactobacillus strains (Fig. 1), we also exogenously added lactic acid in this screening to quantify its contribution to the antihyphal activity of L. rhamnosus GG. Lactic acid as such, at naturally occurring culture supernatant concentrations (1%, a combination of D- and L-lactic acid in a 1:1 ratio), also reduced morphogenesis by approximately 50% (Fig. 2d).

Since the supernatant showed very strong activity and since Msp1 and lactic acid are major components of the supernatant, we next investigated whether Msp1 could act synergistically with lactic acid. The combination of lactic acid at a lower concentration than present in the supernatant (0.5%) and Msp1 (2 µg/ml) was shown to decrease hyphal formation more than 94%, a level of inhibition comparable to the cell-free supernatant, indicating this combination contained the main effectors conferring the anti-hyphal activity to L. rhamnosus GG (Fig. 2e). Since we observed a negative correlation between D-lactic acid production and hyphal inhibition and since the best-performing strains mainly produced L-lactic acid, we compared the synergistic effect of mixed lactic acid on Msp1 activity to both isomers separately. Remarkably, this comparison showed no differences between the isomers (Fig. 2e).

Hyphal morphogenesis is tightly linked to biofilm regulation of C. albicans49, we therefore next investigated whether L. rhamnosus GG could also inhibit C. albicans biofilm formation. This experimental set-up revealed that the supernatant of L. rhamnosus GG was able to decrease biofilm formation of C. albicans. The two main components of the supernatant, lactic acid and Msp1, separately also showed anti-biofilm activity, however no clear synergistic effect was observed with the concentrations of lactic acid and Msp1 tested (Fig. 2f).

Mutant analysis of L. rhamnosus GG supports key role for Msp1.

As mutual interactions between the individual molecules on the lactobacillary surface might strengthen or attenuate the anti-hyphal activity of individual purified molecules, we performed additional experiments with specific L. rhamnosus GG isogenic mutants available from our previous research (see Materials and methods section)42. This complementary approach also allowed us to study molecules that could not be purified to a sufficient level.

Mutant analysis confirmed that the presence or absence of the EPS layer and lectins does not play a crucial role in the anti-hyphal activity of L. rhamnosus GG cells, as shown in Fig. 3a. Previous research showed the importance of the SpaCBA pili and their fucose and mannose residues in L. rhamnosus GG interactions with host cells and glycoconjugates, such as intestinal mucus50,51, of which structural homologs might be present on the hyphal surface. These complex, heteropolymeric SpaCBA pili themselves are difficult to purify50–52, therefore we included the isogenic spaCBA mutant of L. rhamnosus GG in the mutant analysis. This showed that the presence or absence of these SpaCBA pili did not play a significant role in the anti-hyphal activity of L. rhamnosus GG (Fig. 3a).

Due to the central role of Msp1 in bacterial growth and cell separation, an isogenic knock-out mutant is not available in L. rhamnosus GG33. However, the dltD mutant is an interesting generic surface mutant of L. rhamnosus GG, because the lipoteichoic acids are no longer D-alanylated, resulting in dramatic shifts in surface charge and association with surface proteins and other molecules41. Remarkably, the hyphal morphogenesis of C. albicans was almost completely abolished by L. rhamnosus GG dltD mutant cells. To explore whether this could also be explained by the activity of Msp1, we checked whether Msp1 stayed more associated with the surface of dltD mutant cells after secretion than in the wild-type cells. Fluorescently labelled anti-Msp1 antibodies showed that Msp1 was indeed a twofold less secreted in the supernatant of the dltD mutant (Fig. 3b) and appeared to be present in higher concentration on the surface of these mutant cells (Fig. 3c). This thus probably resulted in a higher bio-availability of Msp1 in experiments using the dltD mutant cells as compared to wild-type cells. The consequential comparison between the effects of the supernatant from L. rhamnosus GG wild-type and dltD mutant on hyphal morphogenesis showed that the lower secretion of Msp1 in the supernatant of the dltD mutant indeed resulted in a significantly lower inhibition (p = 0.0001) (Fig. 3d).

The combination of the approach using either mutants or isolated molecules thus further demonstrated the key role for Msp1 in the anti-hyphal activity of L. rhamnosus GG. This finding is in agreement with the fact that the other tested strains from the L. casei group showed strong activity (Fig. 1a), since Msp1 has been shown to be conserved among - at least a part of - the L. casei group42, while the other studied molecules are rather specific for the strain L. rhamnosus GG.

Msp1 shows chitinase activity, independent of its glycosylation state.

We subsequently aimed to explore the interaction between Msp1 and Candida cells in more detail. First, we compared the binding to hyphal cells between L. rhamnosus GG, as a strong anti-hyphal strain, and L. plantarum WCFS1, being one of the least effective strains tested previously (Fig. 1a). These strains belong to the limited number of Lactobacillus strains whose main peptidoglycan hydrolases have been thoroughly characterized33,53. Both their major peptidoglycan hydrolases have been shown to be localized at the poles of the Lactobacillus cells, but they differ in hydrolytic activity and glycosylation state: Msp1 has documented N-acetylglucosaminidase activity53 and appears to be glycosylated with mannose residues85, while Acm2 from L. plantarum WCFS1 was identified as an endo-β-acetylgalactosaminidase88 and appears to be glycosylated with N-acetylgalactosamine residues86. We first explored whether these dissimilarities are reflected in a different interaction of the Lactobacillus strains with the hyphae. Microscopic inspection of C. albicans hyphae after induction in presence of L. rhamnosus GG revealed that the Lactobacillus poles appeared to be the main contact point with the hyphal cells (Fig. 4a, right panel). In contrast to L. rhamnosus GG, L. plantarum WCFS1 cells did not appear to closely interact with the hyphae (Fig. 4a, left panel), suggesting that the close binding of L. rhamnosus GG poles to the hyphae is important for its
anti-hyphal activity. Counting the attached and unattached Lactobacillus cells in three different repeats showed that 60 ± 6% of the L. rhamnosus GG bound to the hyphae, while none of L. plantarum WCFS1 did (data not shown).

To explore whether the binding between Msp1 and C. albicans hyphae could indeed be due to their sugar-lectin interactions, as suggested above, we next investigated the activity of non-glycosylated Msp1. After chemical deglycosylation, the level of hyphal inhibition showed to be similar to native (glycosylated) Msp1 (Fig. 4b), indicating that another mechanism probably underlies the anti-hyphal activity of Msp1.

Despite their different origin, chitin from C. albicans and peptidoglycan from L. rhamnosus GG show some structural similarities due to the presence of N-acetylglucosamine residues in both their backbones. Because of this, and because of the close contact between the Lactobacillus poles and the hyphae, we hypothesized that Msp1 might be able to use chitin, the main polymer of the hyphal cell wall, as a substrate. Based on assays with chitin-azure, we found that Msp1 is indeed able to break down chitin, to the same extent as a commercially available chitinase from Streptomyces griseus (Fig. 4c). Finally, we determined whether a chitinase inhibitor would be...
able to restore *C. albicans* hyphal morphogenesis. Bisdionine C, a known chitinase inhibitor, partially reversed the inhibitory effects of Msp1 on hyphal morphogenesis (Fig. 4d), further substantiating the chitinase activity as basis for the anti-hyphal capacity of Msp1.

**Discussion**

In the present study, we showed that certain *Lactobacillus* taxa can inhibit hyphal morphogenesis of *C. albicans* more efficiently than others. More specifically, we demonstrated that the major secreted protein and main peptidoglycan hydrolase of *L. rhamnosus* GG, Msp1, is the key effector and can reduce hyphal formation by its chitinase activity, especially in combination with lactic acid, another important metabolite of lactobacilli.

Our findings on the complete inhibition of hyphal formation by the supernatant from *L. rhamnosus* GG is in line with previous observations on the effect of *L. rhamnosus* LR32 supernatant on hyphae density in *C. albicans* biofilms. Moreover, a comparison between live cells and both UV-inactivated and heat-killed cells provided novel insights into the underlying molecular mechanism, as the effectors need to be structurally intact, but not necessarily actively secreted during the hyphal induction.

The multilayered cell wall of *C. albicans*, existing of an inner layer of chitin and β-glucans and an outer layer of mannans and (glycosylated) proteins, offers several potential target sites for the binding with lactobacillary factors. We tested different secreted and surface-bound molecules from *L. rhamnosus* GG, which are represented in the schematic overview in Fig. 5. By combining the results on anti-hyphal activity of purified molecules with these of different mutant strains, we found the combination of Msp1 and lactic acid to be the key effectors and synergistically abolish hyphal morphogenesis.

The degrading effects of chitinases on a yeast cell wall were first described for the fungus *Trichoderma viride*, but they have to the best of our knowledge not yet been described for bacterial peptidoglycan hydrolases nor for any lactobacillary protein. Although chitin and peptidoglycan share some structural similarities, namely the presence of N-acetylglucosamine residues in their backbones, the previously studied peptidoglycan hydrolase activity of Msp1 was not shown to involve these bounds. Msp1 was shown to carry γ-D-glutamyl-L-lysyl-endopeptidase activity, cutting between the D-glutamine and L-lysine residues in the peptide stem in bacterial peptidoglycan. This peptidoglycan hydrolase activity was also shown to be conserved among a number of the tested *L. casei* group strains and was also found in *L. casei* BL23. Unfortunately, structural information on peptidoglycan

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**Figure 4.** Enzymatic activity of Msp1. (a) Microscopic images of *L. rhamnosus* GG (left) and *L. plantarum* WCFS1 (right) after incubation with *C. albicans* hyphae. Arrows indicate sites where the poles of lactobacilli seem to interact with the hyphae. Representative images are shown. (b) Chemical deglycosylation of Msp1 does not influence its anti-hyphal activity. (c) Msp1 can break down chitin-azure, a chitin derivative. As a control, the sodium acetate buffer was used. (d) The chitinase inhibitor, Bisdionine C, can prevent hyphal inhibition partially. The results on hyphal inhibition were normalized to hyphal formation of *C. albicans* solely. Asterisks indicate p-values below 0.05, double asterisks indicate p-values below 0.01, compared to *C. albicans* solely.
and lysine were also observed in the peptide stem of L. fermentum in vivo, however, D-glutamine share its chitinase activity with other types of peptidoglycan hydrolases. In L. fermentum, D-glutamine in an acid environment, although we could not observe a difference in synergistic effect with Msp1 for its acid tolerance, the proportion of chitin in the hyphal cell wall has been shown to be even more increased in an acid environment. Thirdly, although we could not observe a difference in synergistic effect with Msp1 between D- and L-lactic acid at the tested concentrations, the presence of L-lactic acid, the main isomer in the supernatant of L. casei, has been shown to result in glucan masking on the hyphal surface, potentially making the hyphae more sensitive to the chitinase activity of Msp1.

The chitinase activity of Msp1 might also be relevant for non-hyphae producing Candida species, as it has been described that the chitin levels are elevated in C. glabrata during infections in a murine colitis model. In addition, in Crohn’s disease patients, both an increase in C. glabrata and a decrease in Firmicutes was found to characterize the gut microbiota.

Lactobacilli and their specific properties are often evaluated at strain-level. Thanks to the approach in this study, we could suggest that this C. albicans hyphae inhibitory activity is possibly present in most strains belonging to the L. casei group due to their specific peptidoglycan structure and accompanying hydrolyses. In this way, the study gives additional indications that probiotic mechanistic research should not only be performed on strain-level to find core properties. The effects of Lactobacillus strains expressing this specific type of peptidoglycan hydrolase and whether they show stronger potential as anti-C. albicans than others, should of course still be substantiated with in vivo evidence. Although it is difficult to explain the mixed results of clinical trials based on our findings in hindsight, since often the applied Lactobacillus strains are not specified to strain- or species level or a probiotic mixture and different formulations were often used, L. casei group strains were used in a number of clinical trials with positive outcomes. Treatment with L. rhamnosus GR-1 (formulated in gelatin capsules) improved symptoms in women suffering from vulvovaginal candidosis and L. rhamnosus HS111 (formulated as dry powder in capsules) contributed to a significant reduction of Candida infection in the oral cavity. In contrast, a clinical trial assessing Shirota on L. casei Candida HS111 (formulated as dry powder in capsules) contributed to a significant reduction of candidiasis with negative results actually investigated Candida viability rather than virulence, which would not be affected by the hyphal-inhibitory activity of the lactobacilli. Of course, when evaluating a Lactobacillus strain or species for its anti-Candida potential, other factors than inhibition of hyphae should be considered. For example, L. plantarum CMPG5300 did not show high

Figure 5. A schematic representation of the proposed mode of action underlying the anti-C. albicans activity of L. rhamnosus GG and the other possible, investigated targets. On the surface of L. rhamnosus GG, several potential interaction partners for components on the cell wall of C. albicans cells can be found. The potential interactions between C. albicans and L. rhamnosus GG surface components that were tested in this manuscript are indicated with black arrows. In the hyphal cell wall, the proportion of chitin is much higher than in unhyphened cells (a), which makes the polymer available for the hydrolytic activity of Msp1 (c). Subsequent contact with Msp1 causes degrading and destabilizing the hyphal cell wall (d). The size proportions between C. albicans and the lactobacilli are not respected for clarity.

Composition and accompanying hydrolysis activity in Lactobacillus is quite limited. In L. plantarum WCFS1, the hydrolase responsible for cell septation (Acm2) was identified as an endo-β-acetylglucosaminidase, and in L. gasseri DSM 20243, the major peptidoglycan hydrolase was shown to have N-acetylmuramidase activity. Microscopic examination and anti-hyphae experiments with L. plantarum WCFS1 indicate that Msp1 does not share its chitinase activity with other types of peptidoglycan hydrolases. In L. fermentum, however, D-glutamine and l-lysine were also observed in the peptide stem. This indicates that L. fermentum strains might have a similar peptidoglycan structure and possibly a similar main peptidoglycan hydrolase activity as L. rhamnosus GG, which could explain that the inhibition level of L. fermentum AMBV1 was almost to the same extent as some L. casei strains. Yet, the exact enzymatic activity remains to be substantiated in follow-up studies.

In light of the observed chitinase activity of Msp1, a number of factors could explain the synergistic effects with lactic acid. Firstly, Msp1, as a hydrolase, has an acidic pH optimum. Secondly, while C. albicans is known for its acid tolerance, the proportion of chitin in the hyphal cell wall has been shown to be even more increased in an acid environment. Thirdly, although we could not observe a difference in synergistic effect with Msp1 between D- and L-lactic acid at the tested concentrations, the presence of L-lactic acid, the main isomer in the supernatant of L. casei group strains, has been shown to result in glucan masking on the hyphal surface, potentially making the hyphae more sensitive to the chitinase activity of Msp1.

The chitinase activity of Msp1 might also be relevant for non-hyphae producing Candida species, as it has been described that the chitin levels are elevated in C. glabrata during infections in a murine colitis model. In addition, in Crohn’s disease patients, both an increase in C. glabrata and a decrease in Firmicutes was found to characterize the gut microbiota.
hyphal formation inhibition rates (30%) but has previously been shown to co-aggregate with *C. albicans* and may in this way inhibit *C. albicans* adhesion and contribute to disease prevention65. Depending on the niche, other factors may play a role for applying lactobacilli as an anti-*Candida* therapy, such as the epithelial adhesion of *L. rhamnosus* GG to the gastro-intestinal tract by its SpaCBA pilis36 and *L. rhamnosus* GR-1 to the vaginal mucosa by its Llp1 lectin46. Additional aspects of clinical trials will also influence the outcome, such as the production or formulation, including encapsulation, of the probiotics72 and organisation of clinical trials, including randomisation and the inclusion of control groups.64,69

In conclusion, our data demonstrate that selected *Lactobacillus* taxa show stronger *C. albicans* hyphae inhibition activity than others, especially the taxa belonging to the *L. casei* group. These taxa appear to owe this inhibitory activity to their major peptidoglycan hydrolase, breaking down the main polymer of the hyphal cell wall, chitin. The identification of the peptidoglycan hydrolase as a core probiotic property helps to unravel the complex interactions between probiotic bacteria and *Candida* species, and can assist in the selection of proper probiotic strains for use as potential probiotics in patients with *Candida* infections or at risk for frequent recurrences of it.

**Materials and Methods**

**Microbial strains and culture conditions.** *Lactobacillus* strains (Table 1) were grown at 37 °C without agitation in de Man, Rogosa and Sharpe (MRS) broth (Difco, Erembodegem, Belgium). *C. albicans* SC5314 was grown in yeast extract peptone dextrose (YPD) broth (Carl Roth, Karlsruhe, Germany) at 37 °C and with continuous shaking70.

The in-house *Lactobacillus* isolates were taxonomically characterized to the species level by sequencing the 16S ribosomal RNA gene. Briefly, the complete 16S rRNA gene (1.5 kb) was amplified with the universal 27 F and 1492 R primers and sequenced. The obtained sequences were compared with reference 16S rRNA gene sequences by BLAST analysis at the National Center for Biotechnology Information (NCBI) website (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

The in-house *Lactobacillus* isolates were collected during a clinical study (Nr 20040719) that was reviewed and approved by the ethical committee of regional hospital of Tienen (Belgium) and all patients gave their explicit consent before sampling.

**Inhibition of hyphal formation in *C. albicans.*** Hyphal growth of *C. albicans* was induced by supplementing YPD broth with 10% heat inactivated fetal bovine serum (FBS) (Thermo Fischer, Asse, Belgium), while incubated with or without lactobacilli (10^8 CFU/ml) or purified molecules. After 3 hours of incubation, at least a hundred yeast cells and/or hyphae in four biological repeats were counted microscopically and the ratio of hyphae to yeast cells was calculated.

**Viability of *C. albicans.*** The viability of *C. albicans* during hyphae formation and hyphae-inhibitory treatments was checked by quantifying the viable plate count at 3 and 6 hours of incubation with the macrodilution method on YPD agar.

**Inhibition of *C. albicans* biofilm development.** The inhibiting effects on *C. albicans* biofilms were assessed as described previously by71. Briefly, 8 × 10^4 *C. albicans* cells were added to the wells of a 96 well plate, together with the samples (supernatant, lactic acid, Msp1) or controls (MRS or H_2O). After incubation for 24 h at 37 °C, the biofilms were washed twice and then 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (90 µl, 1 mg/ml) (Sigma Aldrich) and phenazine methosulphate (10 µl, 0.2 mg/ml) (Sigma Aldrich) were added to the wells. After a second incubation (37 °C, 30 minutes, in the dark), the absorbance at 492 nm was measured using a Synergy HTX multi-mode reader (Biotek, Drogenbos, Belgium).

**UV-inactivation and heat-killing of lactobacilli.** After two washing steps, lactobacilli were UV-inactivated by three repeats of 15 minutes of UV irradiation, and heat-killed by incubating 20 minutes at 80 °C. Inactivation was confirmed by plating on MRS agar.

**Preparation of cell-free supernatant.** Overnight cultures of lactobacilli were grown without agitation in MRS medium at 37 °C. Cell-free supernatant was prepared by centrifuging the culture at 2000 × g for 10 min at 4 °C and then filtering through 0.2 µm filters (VWR, Haasrode, Belgium).

**D- and L-lactic acid production.** After overnight incubation, cell-free supernatant was obtained by centrifugation (10 min, 2000 × g, 4 °C) and filter sterilization through 0.2 µm filters. The concentration of D- and L-lactic acid was measured with the commercially available kit from R-Biopharm (Darmstadt, Germany).

**Isolation of Llp1 and Llp2 from *L. rhamnosus* GG.** The Llp1 and Llp2 proteins from *L. rhamnosus* GG were isolated as described before40. Briefly, the production of the recombinant protein was induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) in recombinant E. coli BL21 cells expressing the lectins (CMPG10708 and CMPG10709). After incubation (25 °C, shaking), the pellets were suspended in non-denaturing lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl and 20 mM imidazole) and sonicated to release the soluble recombinant lectins from the cells. Afterwards, the lectins were purified using affinity chromatography with a HisTrap™ HP column (GE Healthcare) and size exclusion chromatography with a Highload™ 16/60 column packed with a matrix of Superdex™ prep grade (GE Healthcare).

**Isolation of Msp1 from *L. rhamnosus* GG.** Msp1 was purified by cationic exchange chromatography as described previously45,52. Briefly, the culture supernatant was loaded onto SP Sepharose High Performance (GE Healthcare), equilibrated with 60 mM lactate buffer (pH 4.0). Lactate buffer containing ascending NaCl
concentrations (100–1000 mM) was used to elute bound protein. Fractions containing Msp1 were identified using SDS-PAGE and concentrated using Vivaspin filters (MW cut off 10,000) (Sartorius Stedim biotech GmbH, 37070 Goettingen, Germany).

Deglycosylation of Msp1. Msp1 was chemically deglycosylated by trifluoromethanesulphonic acid (TFMS) method (−20 °C, for 30 minutes), as described before73. After treatment, the proteins were extensively dialyzed and analyzed by SDS-PAGE.

Chitinase activity of Msp1. First, the chitinase activity of Msp1 was investigated based on breakdown of chitin-azure (Sigma), as described previously74. Further confirmation was based on inhibition of chitinase activity by 2.5 mM Bisdionine C (Sigma), as described previously75.

Indirect immunofluorescence using light microscopy. Anti-Msp1 rabbit antiserums were used on wild-type and dltD mutant cells. Anti-rabbit IgG antibodies conjugated with Alexa Fluor 488 were used to visualize Msp1 localization on the cells. Samples were visualized with a Zeiss Axio Imager Z1, equipped with an AxioCam MRm Rev.3 monochrome digital camera. The samples were imaged with a ‘Plan-Neofluar’ 100x/1.3 Oil Ph3 objective. Images were analysed with the supplied AxioVision Rel.4.6 software making overlays of phase-contrast and fluorescent images.

ELISA. The protein concentration in the cell-free supernatant of L. rhamnosus GG and CMPG5540 was determined using bicinchoninic acid (BCA) protein assay. The wells of a 96-well ELISA plate (Greiner, Bio-one) were coated overnight with supernatant (0.5 µg/mL), after lyophilization and resolving in PBS, or Msp1 (at different concentration, standard curve) at 37 °C. Afterwards, the wells were washed three times with PBS/T (PBS with 0.05% Tween 20), 250 µL PBS/T with 25% solution of skimmed milk was added, followed by a 1 hour incubation at 37 °C to block aspecific binding. Next, the wells were washed three times with PBS/T and each well was then filled with 100 µL of Msp1 antiserum diluted 1:2000 in PBS/T and incubated (37 °C, 90 min). Alkaline phosphate-conjugated goat anti-rabbit immunoglobulin G (IgG, Sigma) was diluted 1:3000 in PBS/T and added to each well (100 µL) before incubation (37 °C, 1 h). After incubation (30 min, 37 °C) of the bound antibodies with 150 µL of p-nitrophenyl phosphate (1 mg/mL in 1 M Tris-HCl, pH 9.8) (Sigma) per well, the absorbance (405 nm) of each well was read with a Synergy MX microtiter plate reader (Biotek Instruments).

### Table 1. Bacterial strains used in this study.

| Strain             | Reference   | Description          | Characteristics                                                                 |
|--------------------|-------------|----------------------|---------------------------------------------------------------------------------|
| L. rhamnosus GG    | ATCC 53103  | Wild-type            | Intestinal isolate                                                             |
| L. rhamnosus CMPG5531 | ATCC 57 | weE mutant of L. rhamnosus GG | Lacks long, galactose-rich exopolysaccharides and shows increased exposure of SpaCBA pili |
| L. rhamnosus CMPG5540 | ATCC 54 | dltD mutant of L. rhamnosus GG | Lacks D-alanylation of lipoteichoic acid and increased exposure of certain surface proteins |
| L. rhamnosus CMPG5537 | ATCC 56 | spaCBA mutant of L. rhamnosus GG | Lacks expression of spaCBA pili                                               |
| L. rhamnosus CMPG10701 | ATCC 53 | lfp1 mutant of L. rhamnosus GG | Lacks expression of Lfp1 lectin                                                |
| L. rhamnosus CMPG10706 | ATCC 55 | lfp2 mutant of L. rhamnosus GG | Lacks expression of Lfp2 lectin                                                |
| L. rhamnosus GR-1 ATCC 5582 | ATCC 59 | Wild-type            |                                                                                  |
| L. casei AMBR2     | ATCC 52036  | Wild-type            |                                                                                  |
| L. casei ATCC 393   | ATCC 52040  | Wild-type            |                                                                                  |
| L. paracasei ATCC 334 | ATCC 52042 | Wild-type            |                                                                                  |
| L. pentosus KCA1   | ATCC 5746  | Wild-type            |                                                                                  |
| L. pentosus ATCC 8041 | ATCC 5747 | Wild-type            |                                                                                  |
| L. plantarum WCFS1 | ATCC 5749  | Wild-type            |                                                                                  |
| L. plantarum CMPG5300 | ATCC 5751 | Wild-type            |                                                                                  |
| L. reuteri RC-14 ATCC 55845 | ATCC 5755 | Wild-type            |                                                                                  |
| L. reuteri AMBY38   | In-house    | Wild-type            | Vaginal isolate                                                                 |
| L. fermentum AMBV1  | In-house    | Wild-type            | Vaginal isolate                                                                 |
| L. gasseri AMBV2    | In-house    | Wild-type            | Vaginal isolate                                                                 |
| L. gasseri AMBV10   | In-house    | Wild-type            | Vaginal isolate                                                                 |
| L. gasseri AMBV28   | In-house    | Wild-type            | Vaginal isolate                                                                 |
| L. gasseri AMBV47   | In-house    | Wild-type            | Vaginal isolate                                                                 |
| L. jensenii AMBV103 | In-house    | Wild-type            | Vaginal isolate                                                                 |
| L. crispatus LMG12004 | BCCM 84  | Wild-type            |                                                                                  |
| L. crispatus AMBV6  | In-house    | Wild-type            | Vaginal isolate                                                                 |
| L. crispatus AMBV104 | In-house   | Wild-type            | Vaginal isolate                                                                 |
Statistics. Shapiro-Wilk normality test (GraphPad Prism 7.02, CA, USA) was used to determine whether the data are normally distributed. Statistical significance between conditions was estimated by one-way ANOVA and Tukey’s multiple comparisons test.

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Acknowledgements
We would like thank the members of the Lebeer lab of Applied Microbiology and Biotechnology (UAntwerp), especially Hanne Tytgat, Ingmar Claes, Ilke De Boeck, Stijn Wittouck, Marianne van den Broek, and Irina Spacova for their contributions to the manuscript. We would also like to thank Geert Schoofs from the Rega Institute (KU Leuven) for his help with the purification of Msp1. We acknowledge financial support from the Flanders Innovation and Entrepreneurship Agency (IWT-SBO ProCure project IWT/50052) and the Research Foundation Flanders (project FWO-SB 1S17916N).

Author Contributions
C.N.A. and S.L. designed the research and wrote the manuscript. C.N.A., E.O. and M.I.P. performed the experimental work. S.L. and D.V. guided part of the work and S.L., D.V., E.O., M.I.P., G.G.G.D., P.C., P.D. reviewed the manuscript. S.L. managed the project and the funding acquisition.

Additional Information
Supplementary information accompanies this paper at https://doi.org/10.1038/s41598-019-39625-0.

Competing Interests: The authors declare no competing interests.

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