RESEARCH LETTER

# Exploring the antimicrobial properties of vaginal *Lactobacillus crispatus* against preterm birth-associated bacteria

**Emmanuel Amabebe[1], Noopur Bhatnagar[1], Nitin Kamble[2], Steven Reynolds[3] and Dilly O Anumba[1]**

[1]Department of Oncology and Metabolism, University of Sheffield, Sheffield, UK
[2]School of Dentistry, University of Sheffield, Sheffield, UK
[3]Department of Infection, Immunity and Cardiovascular Disease, University of Sheffield, Sheffield, UK

**Correspondence should be addressed to D O Anumba: d.o.c.anumba@sheffield.ac.uk**

## Lay summary

The need to develop new treatments to prevent unprompted premature delivery before 37 weeks of pregnancy remains pressing and unmet. Bacteria (*Lactobacillus* species) that promote vaginal health produce biochemical compounds that prevent the growth of microbes such as *Gardnerella vaginalis*. Overgrowth of *G. vaginalis* can cause vaginal infection with smelly discharge and increase a woman's risk of sexually transmitted infections and premature delivery. In this study, we examined how normal health-promoting (*L. crispatus*) and potentially harmful (*G. vaginalis*) vaginal bacteria interact in a laboratory setting. This was in order to observe natural and effective agent(s) from *L. crispatus* that can hinder the growth of *G. vaginalis* and accompanying immune response. We observed that *L. crispatus* clears *G. vaginalis* by itself and with several biochemical compounds that it produces. Such biochemical compounds can be developed into treatment for vaginal infections and premature delivery due to infection and inappropriate immune response.

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The need to develop novel therapies to prevent spontaneous preterm birth (sPTB <37 weeks) remains pressing and unmet. PTB affects about 15 million births annually. It is the largest direct cause of death in children <5 years, with associated debilitating morbidity in surviving infants (Suff *et al.* 2019). Majority (70%) of PTBs occur spontaneously with significant contribution from ascending genital tract infections and inflammation (Romero *et al.* 2014). A dysbiotic vaginal microbiota can lead to an infectious inflammation with the release of proinflammatory cytokines such as IL-1, IL-6, RANTES that may initiate the pathway to delivery prematurely (Amabebe & Anumba 2018, Amabebe *et al.* 2018, 2019). *Lactobacillus* species (e.g. *Lactobacillus crispatus*) that promote vaginal health produce molecules that prevent the growth of microbes such as *Gardnerella vaginalis* that are associated with common genital tract infections such as bacterial vaginosis (BV) and sPTB (Amabebe & Anumba 2018, 2022).

In order to explore the antimicrobial properties of vaginal *L. crispatus* against BV and sPTB-associated bacteria, we examined the interaction of *L. crispatus* and *G. vaginalis in vitro* to explore for natural and effective agent(s) from *L. crispatus* that inhibit the growth of *G. vaginalis* and probable associated inflammatory response.

Both *L. crispatus* (ATCC 33820, De Man, Rogosa and Sharpe) and *G. vaginalis* (ATCC 14019, brain heart infusion) were grown on their respective media and then co-incubated (1:5) on Columbia agar (CA, enriched with 5% horse blood or serum) under anaerobic condition: 80% $N_2$, 10% $CO_2$, and 10% $H_2$ at 37°C for 48 h. Three different preparations of *L. crispatus*: bacterial suspension (ULc), centrifuged not filtered (supernatant with fewer cells, UCLc), and filtered

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supernatant from UCLc (cell-free supernatant, FLc) were co-incubated with *G. vaginalis* separately on CA. *L. crispatus* alone cultured on CA was used as control. After incubating for 48 h, bacterial growth was confirmed by Gram staining (Fig. 1A). Samples were extracted from the agar plates using a wet sterile Dacron swab and suspended in 600 µL phosphate buffered saline before analysis. Bacterial protein and metabolite expression were determined by liquid chromatography-mass spectrometry and proton NMR spectroscopy ($^1$H-NMR).

*L. crispatus* produced a zone of inhibition (ZoI) on the media containing *G. vaginalis* that was more profound with ULc compared to UCLc and FLc samples (Fig. 1A). Proteins involved in carbohydrate fermentation – lactate dehydrogenase, α-glucosidase, pyruvate kinase, enolase, fructose-bisphosphate aldolase, and phosphoglycerate mutase (Fig. 1B); as well as antimicrobial, defence and survival/tolerance activities – bacteriocin helveticin J, S-layer protein, pyruvate oxidase, universal stress protein, and conserved protein with bacterial Ig like domain were observed in the ZoI but not in the *Gardnerella* region. These proteins were of *L. crispatus* origin and only observed in ULc. Furthermore, metabolites with antimicrobial, anti-inflammatory, and antioxidant activities, that is, allicin were observed in the ZoI only (ULc and UCLc), while lactate and N-acetylneuraminate were higher in ZoI (ULc and FLc) compared to the corresponding *Gardnerella* regions.

*L. crispatus* inhibits the growth of *G. vaginalis in vitro* induced by antimicrobial and anti-inflammatory agents. Although the cell-free supernatant of *L. crispatus* also inhibited the growth of *G. vaginalis*, both *L. crispatus* and its by-products are required for more potent inhibition of *G. vaginalis*. That is, *L. crispatus* showed potential to inhibit a dysbiotic vaginal microbiota and infectious inflammation that may initiate the pathway to sPTB. Lactic acid, bacteriocin, S-layer protein, hydrogen peroxide ($H_2O_2$) produced by metabolism of pyruvate to acetyl phosphate by pyruvate oxidase (Fig. 1B), and other antimicrobial proteins and metabolites are involved. The eubiotic, antimicrobial, prebiotic, and anti-inflammatory actions of some of these compounds especially lactic acid, $H_2O_2$ (Fig. 1B), and bacteriocins have been reported.

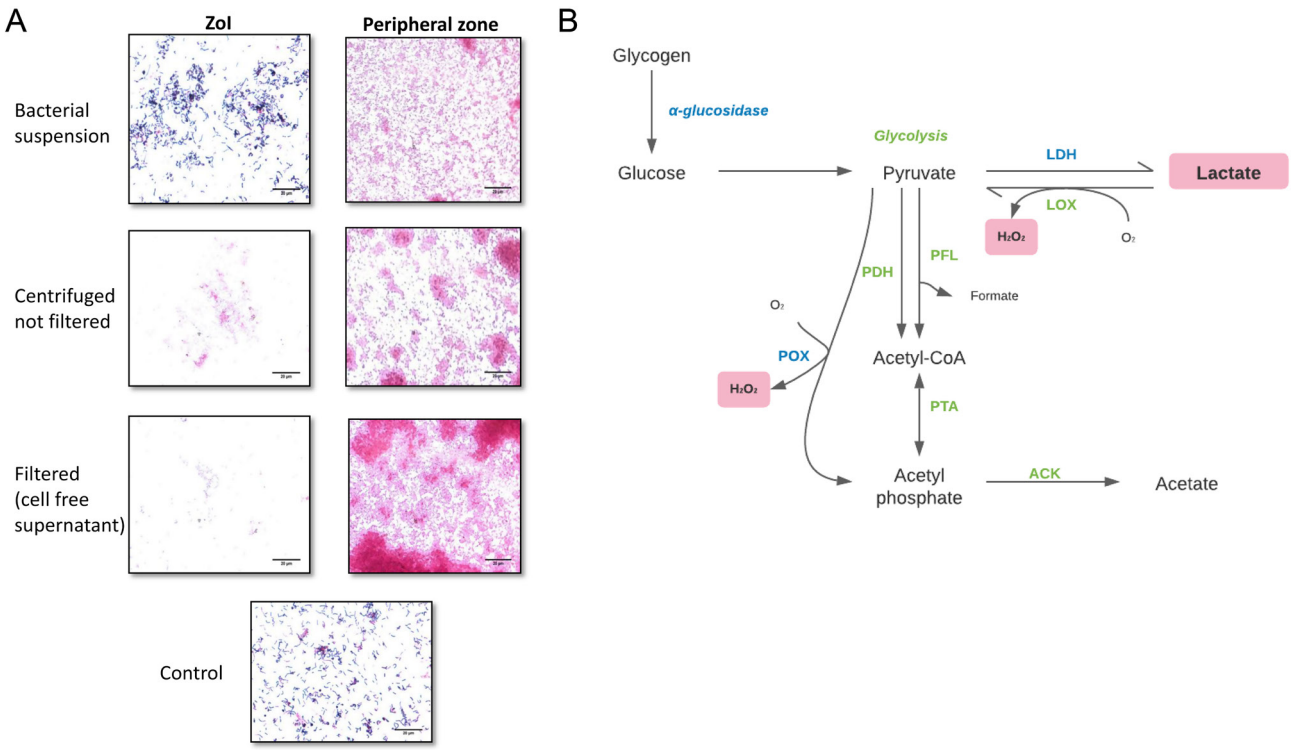


**Figure 1** *Lactobacillus crispatus* inhibits growth of *Gardnerella vaginalis*. (A) Gram staining showing zone of inhibition (ZoI, Gram-positive rods – *L. crispatus* morphotypes, and its by-products) and *peripheral zone* (small Gram-negative cocci – *G. vaginalis* morphotypes). (B) Production of lactate and hydrogen peroxide ($H_2O_2$), the two most widely studied antimicrobial metabolites of lactobacilli. Three different preparations of *L. crispatus*: bacterial suspension in De Man, Rogosa and Sharpe media, centrifuged not filtered (supernatant with fewer cells), and filtered supernatant from centrifuged sample (cell-free supernatant) were co-incubated with *G. vaginalis* separately on Columbia agar (CA, enriched with 5% horse blood or serum). *L. crispatus* was cultured alone on CA as control. After incubation for 48 h, bacterial growth was confirmed by Gram staining. ACK, acetate kinase; LDH, lactate dehydrogenase; LOX, lactate oxidase; PDH, pyruvate dehydrogenase; PFL, pyruvate formate lyase; PTA, phosphotransacetylase; POX, pyruvate oxidase.

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The growth profile of *G. vaginalis* in the ZoI may have been affected by the presence of lactic acid that contribute to a reduction in pH and enhances the antimicrobial activities of $H_2O_2$ that induces oxidative stress, and bacteriocins that are bactericidal (Kalyoussef *et al.* 2012, Amabebe & Anumba 2018, Happel *et al.* 2020, Klotz *et al.* 2020). The pH of the bacterial growth media (pH ~7.3) is different from that of the normal vaginal milieu (pH 3.5–4.5) (Amabebe & Anumba 2018), and this may have impacted the findings and implications of this study. Therefore, subsequent experiments should involve the co-incubation of the species in a single medium such as an artificial vaginal fluid (pH 4.1 adjusted) that simulates the normal vaginal microenvironment. Further investigation of the antimicrobial proteins and metabolites can contribute to the formulation of effective preventive and/or therapeutic interventions against infection-inflammation-associated sPTB. Their antimicrobial or anti-inflammatory properties can be tested singly and in combination. Lastly, the antimicrobial properties of other *Lactobacillus* spp. against common vaginal pathogens co-incubated with vaginal epithelial cells can also be explored.

**Declaration of interest**

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this letter.

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**Author contribution statement**

E A, N K, and D A designed the study. N B, E A, N K, and S R performed the experiments. N B and E A analysed the mass spectrometry data. S R and E A processed and analysed the $^1$H-NMR data. All authors contributed to writing the manuscript led by E A. All authors read and approved the final manuscript for submission.

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