

# Dynamic Bacterial Growth Modulation in Structurally Distinct and Functionally Tuneable Agarose Hydrogels

Corresponding Author: Dr Jérôme Charmet

**This file contains all editorial decision letters in order by version, followed by all author rebuttals in order by version.**

Version 0:

Decision Letter:

**\*\* Please ensure you delete the link to your author homepage in this email if you wish to forward it to your coauthors \*\***

Dear Dr Charmet,

Thank you again for submitting your manuscript "Dynamic Bacterial Growth Modulation in Structurally Distinct and Functionally Tuneable Agarose Hydrogels" to Communications Materials. We have now received reports from 4 reviewers and, based on their comments, we have decided to invite a revision of your work. You will find the reviewers' reports below. While they find your work of interest, they have raised important points which must be addressed in a revised manuscript.

In particular, we ask that you address the key points on more effectively connecting the findings of the manuscript to the stated motivation and the points raised by Reviewers 2/3 on how hydrogel pore size and 3D architecture affect your findings.

To allow us to move forward with your work, we also ask that you edit your manuscript according to the attached table.

**Please read this document carefully as we will be unable to further assess your revised paper until these important points are addressed.**

Please outline all revisions made in the right-hand column and return the completed table with your updated manuscript files as a Related Manuscript file.

**When resubmitting, please also include:**

- A point-by-point response to the reviewers' comments. If you are unable to address specific reviewer requests or find any points invalid, please explain
- A clean version of your revised manuscript with no mark-ups
- A marked-up version of your paper with all changes highlighted in a different colour

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**We hope to receive your revised paper within six weeks, but we understand that the revisions may take longer. Please let us know if you find that the revision process will take substantially more time.**

We are committed to providing a fair and constructive peer-review process. Please do not hesitate to contact me if you have any questions or would like to discuss these revisions further. We look forward to seeing the revised manuscript and thank

you for the opportunity to review your work.

Best regards,

Steven Caliri, PhD  
Editorial Board Member  
Communications Materials  
orcid.org/0000-0002-7506-3079

#### **Reviewers' comments:**

Reviewer #1 (Remarks to the Author):

##### Comments

##### 1. Interpretive Hierarchy of Findings

The conclusion that “bacterial growth is primarily shaped by stiffness and water loss” is well supported in general, but Gram-positive inhibition in unsubstituted hydrogels constitutes an important exception. The Discussion section should more clearly stratify conclusions by bacterial species group to avoid oversimplification. Currently, this major finding is addressed in only a single paragraph of approximately five lines (lane 333-338), which is insufficient to match the significance of the discovery. Expanding this section with dedicated discussion for each species would strengthen the interpretation and impact.

##### 2. Statistical Synthesis and Effect Size Reporting

While a four-way ANOVA is performed, the link between stiffness/water retention and bacterial growth is shown in supplementary Figure S21-22. Bringing key quantitative effect sizes or correlation coefficients into the main text would make the mechanistic argument more robust.

##### 3. Mechanistic Depth for Gram-Positive Inhibition

The electrostatic repulsion hypothesis is convincingly demonstrated via cytochrome C rescue. However, potential contributions from cell wall structure, biofilm initiation capacity, or hydrophobicity differences should be more thoroughly discussed, even if results are negative.

##### 4. Nutrient-Hydrogel Interaction Mechanisms

The observation that certain media (e.g., M2) soften gels and improve water retention is intriguing. Please expand the discussion on possible physicochemical mechanisms, e.g., ionic strength effects, protein-polysaccharide binding, that could underpin these media-induced changes.

##### 5. Experimental Reproducibility of Inoculation Method

The inoculation approach may introduce variability in bacterial penetration depth and distribution. Clarify whether depth and force were standardized (e.g., fixed-depth needles) and whether this was validated for reproducibility.

##### 6. Presentation Suggestions

At the end of the Introduction, authors typically state the purpose of the study, the critical problem it addresses, and provide a brief overview of the approach and main findings. In the current manuscript, this section reads more like a conclusion rather than a concise statement of study objectives. I recommend revising it to clearly articulate the research aim, the specific gap this work seeks to fill, and a succinct summary of the key results.

In the Results section, most of the text is written in the past tense, which is appropriate for describing experimental findings. However, in the second paragraph beginning on Page 5, line 158, the sentences shift to the present tense. For consistency and clarity, please revise this section to maintain the past tense throughout.

Figure 7 is information-dense. Consider annotating key trends directly on the figure or using arrows to guide interpretation. Simplifying colors or breaking complex panels into multiple sub-figures could improve reader comprehension.

Reviewer #2 (Remarks to the Author):

This manuscript by Dsouza et al. provides a multi-parametric study that explores the effects of different physicochemical parameters of agarose hydrogels on bacteria growth using four different bacterial species. Observations are made on how hydrogel stiffness, water loss, electrostatic repulsion, and hydrogel stiffening from media impact bacteria growth.

The paper gives a fresh perspective on hydrogel properties that can impact bacterial growth, as most researchers only consider growth media/cellular metabolism for plate cultures. The study is systematic, experiments are well-organized and logical, reproducible, the static analysis appears to be well done, and conclusions are generally supported by the data. The materials and cells used are not novel – but since agarose is a very common and widely used material, the findings could be broadly useful to other researchers.

However, there is (1) a poor connection to applications enabled from the findings and (2) a general lack of discussion as to why specific trends are observed. These are weaknesses that dampened this reviewer's enthusiasm for the paper. More specifics are described below.

##### Major weaknesses:

- The goals / main findings of the manuscript are disconnected from the stated motivation to better understand infection, persistence, and antibiotic resistance. It is unclear how the results in this paper advance the understanding of bacteria

behavior in any of these scenarios.

- The advantages of using (un)substituted agarose hydrogels, and agarose hydrogels as a whole, are not discussed in the introduction.
- Data presented in Figures 3-5 feel repetitive with minimal discussion as to why increased hydrogel stiffness causes higher water loss and why hydrogel stiffness and water loss enhance bacteria growth.
- The dramatic increase in Gram-positive bacteria growth area across all growth media in unsubstituted hydrogels (0.2%, Figure 2A) should be discussed further.
- Literature values of similar systems with hydrogel mechanical properties/bacteria growth should be given where possible. Contact angle and storage modulus data should be available given the common use of agarose hydrogels.
- Controlling growth with varying polymer concentration is not specifically discussed in much detail.
- With inoculating bacteria within the hydrogel as a stab, pore size will play a large role in growth across different bacterial species. It was unclear whether the observed growth occurs on the hydrogel exterior, within the hydrogel, or both a combination of the two. The paper does not distinguish between the two environments. Bacteria growth will behave differently when fully confined in the hydrogel versus when bacteria are at the hydrogel exterior, and perhaps there are differences in bacteria penetration into the hydrogel at larger pore sizes / lower agarose concentration.
- The authors use Fig. S10 to discuss US hydrogels, but Fig. S10 only appears to show S hydrogels, according to the caption. This makes the discussion in lines 165-171 rather confusing.

Minor weaknesses:

- Fig. 1 – Flagella and motility – not really addressed in paper but listed in Fig. 1.
- Line 179 – Incorrect reference to supplementary figures.
- Why was 37 C used for all growth experiments? E. coli grows optimally at 37 C, but other bacteria such as P. fluorescens, grow optimally at lower temperatures.
- Lines 247-253. A figure that provides more detailed chemical structures in S and US hydrogels would make for easy understanding of hydrophobicity/hydrophilicity trends.
- Fig. 8 – were contact angle measurements on Cytochrome C substituted hydrogels preformed, as done with hydrogels in the previous studies?

Reviewer #3 (Remarks to the Author):

Bacterial growth on Agarose hydrogels as a model system in designing infection models are interesting, however, I have the following major comments on the study:

While the manuscript highlights the unique 3D porous structures of hydrogels in the introduction, the study focuses exclusively on the bacterial growth on the hydrogel surface. This is a significant limitation of the study, as the bulk phase of the hydrogels can influence bacterial growth behaviour quite differently compared to the surface.

Recommendations are techniques such as i) SEM or cryoSEM after fracture of hydrogels , ii) Confocal Laser Scanning Microscopy (CLSM) after staining for bacteria, optical sectioning will provide insight on the 3D bacterial distribution and its viability within the hydrogels.

Authors justification as electrostatic interaction for the selective promotion/inhibition of bacterial growth is not fully convincing. Authors should conduct zeta potential measurements for all bacteria used in the study. In general, Gram-negative bacteria typically exhibit more negative zeta potentials due to the presence of LPS in the outer membrane. However attachment and especially growth are not solely governed by electrostatic interactions.

This manuscript did not sufficiently consider the role of nutrients and its accessibility to bacteria in the growth, considering the architecture of hydrogels. When all bacteria are dead as shown As Figure S29 and S30, showed complete bacterial death, suggest possible nutrient limitation or bacterial mobility to access nutrients potentially due to hydrogel structure. Authors should investigate whether there is possible nutrient limitation or restricted mobility of bacteria in the hydrogels, both of which will impact bacterial growth.

CryoSEM images in Figure S10, specifically A and B shows a collapsed structure (could be due to over- etching), showing large pores with no significant differences. Authors in the results section claim they are from both US and S hydrogels contradicting the figure legend. The authors should systematically perform CryoSEM on both US and S hydrogels in the

absence and presence of bacterial growth to understand the the bacterial distribution in the hydrogel matrix.

Minor comments:

How thick are the hydrogels?

Figure 2, bacterial growth area scale, Is that the diameter of the bacterial growth measured? what units?

Figure S2-S9 says all microorganisms are Uropathogenic? This needs to be corrected. It is important to mention from where these microorganisms are isolated from?

Cryo-SEM - Was the imaging performed under low temperatures in SEM? This information was not clear in the methods.

There are few mismatch with figures in the text and from the legend with respect to numbers. For example: Frequency sweep figures in the results say Figures S3-S12, whereas the corresponding figures are from S11-S20.

Reviewer #4 (Remarks to the Author):

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Communications Materials initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

\*\* See Nature Research's author and referees' website at [www.nature.com/authors](http://www.nature.com/authors) for information about policies, services and author benefits

Version 1:

Decision Letter:

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Dear Dr Charmet,

Thank you once again for submitting your manuscript, "Dynamic Bacterial Growth Modulation in Structurally Distinct and Functionally Tuneable Agarose Hydrogels," to Communications Materials. It has now been seen again by the referees, whose comments are appended below. The concerns of our reviewers have now been addressed, but there are some amendments needed before we can accept your paper.

We ask that you edit your manuscript according to the attached table. **Please read this document carefully as we will be unable to further assess your revised paper until these important points are addressed.**

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**We hope to receive this updated version of your paper within 1 week, but please let us know if you find that you need more time.**

Best regards,

Steven Caliri, PhD  
Editorial Board Member  
Communications Materials  
[orcid.org/0000-0002-7506-3079](https://orcid.org/0000-0002-7506-3079)

**Reviewers' comments:**

Reviewer #1 (Remarks to the Author):

I have carefully reviewed the revised manuscript and the rebuttal letter, and I am satisfied that all previous comments have been fully and appropriately addressed. The authors have strengthened the mechanistic interpretation, clarified species-specific differences, integrated relevant quantitative analyses, and added supportive experiments that now robustly justify the conclusions. Revisions to the Introduction, Discussion, figures, and methodological details have improved clarity and coherence. I have no further substantive concerns, and the manuscript is acceptable for publication pending minor editorial polishing by the journal.

Reviewer #2 (Remarks to the Author):

The authors have provided clear and detailed responses to Reviewer #2 comments and have made the necessary revisions to significantly improve the manuscript. Overall, Reviewer #2 is satisfied with the revisions and recommends this manuscript for publication.

Reviewer #3 (Remarks to the Author):

The manuscript can be accepted in this form.

Reviewer #4 (Remarks to the Author):

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Communications Materials initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Version 2:

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Best regards,

Steven Caliri, PhD  
Editorial Board Member  
Communications Materials  
orcid.org/0000-0002-7506-3079

Version 3:

Decision Letter:

Dear Dr Charmet,

We are delighted to accept your manuscript titled "Dynamic Bacterial Growth Modulation in Structurally Distinct and Functionally Tuneable Agarose Hydrogels" for publication in Communications Materials. Thank you for choosing to publish your interesting work with us.

Please note that in advance of your paper being published we will host an early access version, known as an 'Article in Press,' on our journal website. For more information on this initiative please see our [author guidelines](https://support.springernature.com/en/support/solutions/articles/6000281821-what-is-an-article-in-press-).

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We look forward to publishing your paper.

Best regards,

Steven Caliri, PhD  
Editorial Board Member  
Communications Materials  
orcid.org/0000-0002-7506-3079

\*\*\*As a new journal, we would greatly appreciate any comments you have about your experience at Communications Materials. I hope that we have been able to meet your expectations and look forward to working with you again in the future.

We may promote your article on social media once it is published, so please feel free to send me the twitter handles of any authors or departments and we will be sure to tag them accordingly.\*\*\*

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We would like to thank the editor for giving us the opportunity to revise our manuscript, which has become stronger as a results of the insightful comments and suggestion of the reviewers. You will find our response to the reviewers, and corrections as per the guidelines in the documents provided herein.

## Reviewers' comments:

### Reviewer #1 (Remarks to the Author):

#### Comments

#### 1. Interpretive Hierarchy of Findings

The conclusion that “bacterial growth is primarily shaped by stiffness and water loss” is well supported in general, but Gram-positive inhibition in unsubstituted hydrogels constitutes an important exception. The Discussion section should more clearly stratify conclusions by bacterial species group to avoid oversimplification. Currently, this major finding is addressed in only a single paragraph of approximately five lines (lane 333-338), which is insufficient to match the significance of the discovery. Expanding this section with dedicated discussion for each species would strengthen the interpretation and impact.

We thank the reviewer for this valuable suggestion. In response, we have added a concise paragraph in the Discussion to clearly highlight species-specific responses to hydrogel properties:

*“Our results reveal species-specific responses to hydrogel properties. Gram-negative bacteria (*E. coli*, *P. fluorescens*) grew best in softer, hydrated hydrogels, with *E. coli* showing elongation in the softest gels. Gram-positive bacteria (*S. aureus*, *B. subtilis*) were more sensitive to polymer concentration, with growth restricted in stiffer, dehydrated matrices. These findings indicate that while stiffness and hydration broadly influence growth, species-specific traits produce important exceptions, emphasizing the need to consider both bacterial and matrix properties. Notably, we identify electrostatic interactions as a species-selective regulatory mechanism. Growth inhibition of Gram-positive bacteria in negatively charged unsubstituted agarose is attributable to surface charge repulsion, which was reversed by modifying the hydrogel charge via cytochrome C encapsulation. This observation highlights an underexplored dimension of hydrogel-bacteria interactions, in which physicochemical mismatch between hydrogel charge and bacterial envelope can serve as a selective growth-modulating mechanism.”*

#### 2. Statistical Synthesis and Effect Size Reporting

While a four-way ANOVA is performed, the link between stiffness/water retention and bacterial growth is shown in supplementary Figure S21-22. Bringing key quantitative effect sizes or correlation coefficients into the main text would make the mechanistic argument more robust.

We thank the reviewer for this suggestion. While we had already reported the negative associations between bacterial growth, hydrogel stiffness, and water loss using Spearman's correlations in the supplementary tables (S25-S26), we have now included the range of Spearman correlation coefficients in the main text under the results section (Combined effects of stiffness and water loss on bacterial growth) ( $P = -0.56$  to  $-0.80$  across species) to highlight the quantitative relationship between hydrogel properties and bacterial growth.



### 3. Mechanistic Depth for Gram-Positive Inhibition

The electrostatic repulsion hypothesis is convincingly demonstrated via cytochrome C rescue. However, potential contributions from cell wall structure, biofilm initiation capacity, or hydrophobicity differences should be more thoroughly discussed, even if results are negative.

We thank the reviewer for this suggestion. We have added a sentence in the results section (Unsubstituted agarose hydrogels selectively promote/inhibit bacterial growth), acknowledging additional factors such as cell wall structure, biofilm initiation capacity, and surface hydrophobicity, and note that our measurements of hydrophobicity, membrane rigidity, and polarization showed no consistent correlation with growth patterns. This reinforces that electrostatic interactions are the dominant mechanism under the conditions tested.

### 4. Nutrient-Hydrogel Interaction Mechanisms

The observation that certain media (e.g., M2) soften gels and improve water retention is intriguing. Please expand the discussion on possible physicochemical mechanisms, e.g., ionic strength effects, protein-polysaccharide binding, that could underpin these media-induced changes.

We thank the reviewer for highlighting this point. We have added a sentence in the results section (Encapsulated nutrient media impacts bacterial growth by altering hydrogel stiffness and water loss) acknowledging that media such as TSB, M1, and M2 soften hydrogels and improve water retention, and we note that the precise physiochemical mechanisms such as ionic strength, protein-polysaccharide interactions were not investigated in this study and remain an interesting topic for future work.

### 5. Experimental Reproducibility of Inoculation Method

The inoculation approach may introduce variability in bacterial penetration depth and distribution. Clarify whether depth and force were standardized (e.g., fixed-depth needles) and whether this was validated for reproducibility.

We thank the reviewer for this suggestion. Bacterial inoculations were performed by pricking the hydrogel surface with a pipette tip. Although penetration depth was not mechanically fixed, experiments were repeated at multiple time points to confirm that growth patterns were reproducible.

### 6. Presentation Suggestions

At the end of the Introduction, authors typically state the purpose of the study, the critical problem it addresses, and provide a brief overview of the approach and main findings. In the current manuscript, this section reads more like a conclusion rather than a concise statement of study objectives. I recommend revising it to clearly articulate the research aim, the specific gap this work seeks to fill, and a succinct summary of the key results.

We thank the reviewer for this suggestion. We have revised the Introduction to clearly state the study aim, the knowledge gap, and a brief overview of the approach, while retaining a concise summary of the main findings.

In the Results section, most of the text is written in the past tense, which is appropriate for describing experimental findings. However, in the second paragraph beginning on Page 5,

line 158, the sentences shift to the present tense. For consistency and clarity, please revise this section to maintain the past tense throughout.

We thank the reviewer for noting this. We have revised the relevant section of the Results to maintain past tense consistently when describing experimental findings, ensuring clarity and uniformity throughout.

Figure 7 is information-dense. Consider annotating key trends directly on the figure or using arrows to guide interpretation. Simplifying colors or breaking complex panels into multiple sub-figures could improve reader comprehension.

We thank the reviewer for this suggestion. We have considered simplifying or annotating Figure 7; however, we decided to retain the current format to preserve the integrity of the full dataset and maintain consistency with the other figures. We have ensured that the figure legend clearly describes the key trends and provides guidance for interpretation to facilitate reader comprehension.

## **Reviewer #2 (Remarks to the Author):**

This manuscript by Dsouza et al. provides a multi-parametric study that explores the effects of different physicochemical parameters of agarose hydrogels on bacteria growth using four different bacterial species. Observations are made on how hydrogel stiffness, water loss, electrostatic repulsion, and hydrogel stiffening from media impact bacteria growth.

The paper gives a fresh perspective on hydrogel properties that can impact bacterial growth, as most researchers only consider growth media/cellular metabolism for plate cultures. The study is systematic, experiments are well-organized and logical, reproducible, the statistical analysis appears to be well done, and conclusions are generally supported by the data. The materials and cells used are not novel – but since agarose is a very common and widely used material, the findings could be broadly useful to other researchers.

However, there is (1) a poor connection to applications enabled from the findings and (2) a general lack of discussion as to why specific trends are observed. These are weaknesses that dampened this reviewer's enthusiasm for the paper. More specifics are described below.

### **Major weaknesses:**

1. The goals / main findings of the manuscript are disconnected from the stated motivation to better understand infection, persistence, and antibiotic resistance. It is unclear how the results in this paper advance the understanding of bacteria behavior in any of these scenarios.

We thank the reviewer for this thoughtful and important comment. We agree that clarifying the connection between our experimental findings and infection-relevant bacterial behavior strengthens the manuscript. To address this, we have revised both the Introduction and Discussion sections to more explicitly link our results to infection persistence, bacterial colonization, and antibiotic tolerance. Specifically, the

Introduction now highlights that bacteria in infection sites (e.g., wounds, tissues, and implant surfaces) experience hydrated, polymer-rich environments resembling hydrogels, and that understanding how hydrogel properties affect bacterial proliferation provides mechanistic insight into infection persistence and material-associated colonization:

*“Despite growing interest in using hydrogels to understand and control bacterial behaviour, the fundamental properties that govern bacterial growth within these matrices remain poorly understood. This gap is particularly important because bacteria in real infection settings such as wound tissues, abscesses encounter soft and hydrated environments that closely resemble hydrogels. In such environments, bacterial persistence is often linked to altered growth, physiology, and reduced antibiotic efficacy, all of which can be influenced by the nature of surrounding matrix. However, it remains unclear how specific hydrogel properties including stiffness, water availability, and surface charge, contribute to bacterial colonization and expansion in these environments. “*

*“To address this knowledge gap, our study focuses specifically on agarose hydrogels as a controllable platform to decouple and systematically investigate the physicochemical parameters that regulate bacterial growth. By determining how mechanical stiffness, hydration, and matrix charge influence species-specific proliferation, we aim to generate mechanistic insights that are relevant to bacterial colonization of soft tissue-like environments, infection persistence, and the design of antibacterial biomaterials.”*

Additionally, the Discussion now explicitly interprets our findings in the context of infection-relevant environments, explaining how hydrogel stiffness, hydration, and charge can influence bacterial proliferation, persistence, and antibiotic tolerance:

*“Our findings have direct implications for understanding bacterial behaviour in infection-relevant environments. Soft, hydrated matrices such as those found in wound beds, damaged tissue, or biomaterial coatings may promote bacterial proliferation by reducing mechanical resistance preventing desiccation. Conversely, stiffer or dehydrated matrices may hinder bacterial expansion and nutrient access, helping to restrict colonization. Furthermore, the observed role of matrix charge in selectively inhibiting certain species suggests a material-dependent mechanism that could be exploited to prevent surface colonization on medical devices or wound dressings. Since bacterial persistence and antibiotic tolerance are strongly influenced by local microenvironments, these results provide a mechanistic foundation for designing more realistic in vitro infection models and for engineering materials that either prevent or sustain bacterial colonization in a controlled manner. “*

2. The advantages of using (un)substituted agarose hydrogels, and agarose hydrogels as a whole, are not discussed in the introduction.

We thank the reviewer for this constructive suggestion. We have revised the Introduction to explicitly discuss the advantages of using agarose hydrogels, both substituted and unsubstituted, and to explain why they serve as a suitable platform for study material-bacteria interactions. The revised text now emphasizes agarose's chemical inertness, tunable physicochemical properties, and lack of inherent

antimicrobial effects, which make it ideal for decoupling material-driven influences on bacterial behaviour:

*“However, agarose offers more than routine use as a culture medium. Its chemically defined, inert backbone enables controlled tuning of stiffness, porosity, water retention, and surface charge without introducing antimicrobial effects, making it ideal for isolating material-driven influence on bacterial behaviour. Chemical substitutions such as hydroxyethylation modify these physical properties while preserving the same polysaccharide structure. Thus, comparing unsubstituted and substituted agarose provides a reductionist platform to decouple material chemistry from bacterial response. Yet, despite agarose’s widespread use, the advantages and limitations of its variants as bacterial growth matrices have not been systematically assessed.”*

3. Data presented in Figures 3-5 feel repetitive with minimal discussion as to why increased hydrogel stiffness causes higher water loss and why hydrogel stiffness and water loss enhance bacteria growth.

We thank the reviewer for this valuable comment. We have revised the Results and Discussion section to provide a more detailed mechanistic explanation linking hydrogel stiffness, water loss, and bacterial growth behaviour. Specifically, we now discuss how increased crosslinking density in stiffer agarose networks reduces water-holding capacity, leading to enhanced capillary-driven dehydration and restricted nutrient diffusion. These physical changes impose mechanical and osmotic stresses on bacteria, thereby modulating their proliferation in a stiffness- and species-dependent manner:

*“Importantly, these trends can be explained by higher crosslinking density of stiffer agarose networks, which reduces water-holding capacity and increases water loss. As a result, bacteria in stiffer, dehydrated hydrogels experience limited nutrient diffusion, greater mechanical confinement, and desiccation stress, whereas softer, more hydrated networks provide a more permissive environment for expansion and metabolic activity. Overall, these results demonstrate that lower hydrogel stiffness and reduced water loss promote bacterial growth, although the magnitude and nature of this effect remain species dependent.”*

4. The dramatic increase in Gram-positive bacteria growth area across all growth media in unsubstituted hydrogels (0.2%, Figure 2A) should be discussed further.

We thank the reviewer for this insightful comment. We have added a detailed discussion of the observed growth of Gram-positive bacteria in low-concentration unsubstituted hydrogels (0.2%). The revised text now explains how the combination of softer hydrogel mechanics, higher water retention, and bacterial envelope properties facilitate proliferation of *S. aureus* and *B. subtilis* under these conditions:

*“The pronounced growth of Gram-positive bacteria (*S. aureus* and *B. subtilis*) in 0.2% US hydrogels reflects the interplay of hydrogel mechanics, hydration, and bacterial envelope properties. At this low polymer concentration, the network is softer and retains more water, reducing mechanical confinement and desiccation stress, which facilitates proliferation. The low crosslinking density also likely diminishes electrostatic inhibition, allowing Gram-positive bacteria with thick peptidoglycan walls to grow more*

effectively than in stiffer matrices. These results highlight that bacterial responses are species-specific and shaped by a combination of stiffness, hydration, and surface interactions, rather than bulk hydrogel properties alone.”

5. Literature values of similar systems with hydrogel mechanical properties/bacteria growth should be given where possible. Contact angle and storage modulus data should be available given the common use of agarose hydrogels.

We thank the reviewer for this valuable suggestion. Guégan et al. (2014), reported water contact angles of approximately 37-43° for hydroxyethyl-substituted agarose, consistent with its hydrophilic nature. In contrast, our substituted agarose exhibited higher contact angles 58-77° but became more hydrophilic upon nutrient media encapsulation. We attribute the differences in contact angles to differences in agarose type, substitution degree, and encapsulation method (bacterial nutrient media versus water or organic solvents). Notably, nutrient media encapsulation reduced the contact angles in our case, increasing hydrophilicity, highlighting the effect of media on surface wettability.

We have now also included the literature context for rheological measurements in the results section (Hydrogel stiffness scales with polymer concentration). Specifically, our rheology data show that storage modulus ( $G'$ ) decreases with decreasing agarose concentration, from  $3825 \pm 630$  Pa (US, 1%) and  $1479 \pm 270$  Pa (S, 1%) to  $240 \pm 24$  Pa (US, 0.2%) and  $156 \pm 46$  Pa (S, 0.2%), consistent with the observed increase in bacterial growth at lower concentrations. For comparison, Guégan et al. (2014) reported higher  $G'$  values ( $\sim 6.6 \times 10^3$  Pa at 0.75% and  $1.1 \times 10^5$  Pa at 3%) for hydroxyethyl-substituted agarose, reflecting differences in polymer concentration, substitution chemistry, and the absence of varying nutrient media.

6. Controlling growth with varying polymer concentration is not specifically discussed in much detail.

We thank the reviewer for this important comment. We have revised the Discussion section to explicitly describe how varying hydrogel polymer concentration controls bacterial growth. The revised text explains that higher polymer concentrations create stiffer, less hydrated networks that restrict expansion and nutrient transport, whereas lower concentrations produce softer, hydrated matrices that promote proliferation:

*“Varying hydrogel polymer concentration modulates bacterial growth. Higher concentrations produce stiffer, less hydrated networks that restrict expansion and nutrient diffusion, while lower concentrations create softer, hydrated matrices that promote proliferation. Heatmaps (Figures 5 and S23, S24) show growth increasing from 1% to 0.2% for most species, with Gram-positive bacteria also affected by electrostatic interactions in unsubstituted hydrogels. These results demonstrate that polymer concentration controls growth through mechanical, hydration, and species-specific factors.”*

7. With inoculating bacteria within the hydrogel as a stab, pore size will play a large role in growth across different bacterial species. It was unclear whether the observed growth occurs on the hydrogel exterior, within the hydrogel, or both a combination of

the two. The paper does not distinguish between the two environments. Bacteria growth will behave differently when fully confined in the hydrogel versus when bacteria are at the hydrogel exterior, and perhaps there are differences in bacteria penetration into the hydrogel at larger pore sizes / lower agarose concentration.

We thank the reviewer for highlighting the importance of bacterial distribution within the hydrogel network. To address this, we performed fluorescence Z-stack imaging of *E. coli* in 1% and 0.2% US hydrogels. The analysis shows that bacteria are present both at the hydrogel surface and within the bulk, with deeper penetration and more uniform distribution observed in the softer 0.2% gels (Figure S11-S12). These results indicate that bacterial growth occurs throughout the 3D network and support our conclusion that hydrogel physicochemical properties, including stiffness and hydration, influence colonization beyond the surface. We have included a description of this imaging in the Methods and added the results in the main text with references to the supplementary figures.

8. The authors use Fig. S10 to discuss US hydrogels, but Fig. S10 only appears to show S hydrogels, according to the caption. This makes the discussion in lines 165-171 rather confusing.

We thank the reviewer for pointing out this discrepancy. The original Supplementary Figure S10 indeed only displayed S hydrogels. We have now added the corresponding data for US hydrogels to the figure and updated the caption accordingly.

#### Minor weaknesses:

1. Fig. 1 – Flagella and motility – not really addressed in paper but listed in Fig. 1.

We thank the reviewer for this comment. We have added clarification in the Discussion regarding flagella and motility:

*“Some species in our study are flagellated (*E. coli*, *P. fluorescens*, *B. subtilis*) while *S. aureus* is non-flagellated. Although motility phenotypes were confirmed in standard soft-agar assays, active motility was not tested directly in hydrogels. Nonetheless, observed growth patterns across species suggest that hydrogel physicochemical properties, rather than motility, are the primary determinants of proliferation. Together, these findings indicate that the physical and chemical properties of the hydrogel matrix are dominant regulators of bacterial growth, highlighting the importance of considering matrix stiffness, hydration, and electrostatic interactions when designing hydrogel-based bacterial models or antimicrobial materials.”*

2. Line 179 – Incorrect reference to supplementary figures.

We thank the reviewer for pointing this out. We have now provided the correct reference to supplementary figures.



3. Why was 37 °C used for all growth experiments? *E. coli* grows optimally at 37 °C, but other bacteria such as *P. fluorescens*, grow optimally at lower temperatures.

We thank the reviewer for raising the point. All bacterial growth experiments were conducted at 37 °C to provide a consistent, physiologically relevant temperature for infection-related conditions. We have now explained this in the discussion:

*“Bacterial growth experiments were performed at 37 °C to provide a consistent, physiologically relevant temperature for infection-related conditions. While *E. coli* and *S. aureus* are naturally adapted to this temperature, *P. fluorescens* and *B. subtilis* may have slightly lower optimal growth temperatures. Pilot experiments confirmed that all species grew robustly at 37 °C, allowing direct comparisons across hydrogel types and concentrations without introducing temperature-dependent variability.”*

4. Lines 247-253. A figure that provides more detailed chemical structures in S and US hydrogels would make for easy understanding of hydrophobicity/hydrophilicity trends.

We thank the reviewer for the suggestion. To improve clarity, we have added a schematic showing the chemical structures of US and S agarose hydrogels, highlighting the hydroxyl (-OH) and hydroxyethyl (-C<sub>2</sub>H<sub>5</sub>OH) groups. This figure illustrates the chemical basis for the observed differences in hydrophilicity and hydrophobicity (Figure S27). We have also mentioned the structural difference in figure caption of figure 5 in the main manuscript.

5. Fig. 8 – were contact angle measurements on Cytochrome C substituted hydrogels performed, as done with hydrogels in the previous studies?

We thank the reviewer for the comment. Contact angle measurements were not performed on cytochrome C-modified hydrogels because our data indicate that hydrophilicity alone does not explain the observed growth inhibition of Gram-positive bacteria. Specifically, the same hydrogels supported growth of Gram-negative species, suggesting that the selective inhibition is due to electrostatic interactions between the negatively charged hydrogel and the bacterial envelope, rather than a change in surface hydrophilicity. Therefore, while contact angle could be measured, it was not expected to provide additional mechanistic insight in this context.

### **Reviewer #3 (Remarks to the Author):**

Bacterial growth on Agarose hydrogels as a model system in designing infection models are interesting, however, I have the following major comments on the study:

1. While the manuscript highlights the unique 3D porous structures of hydrogels in the introduction, the study focuses exclusively on the bacterial growth on the hydrogel surface. This is a significant limitation of the study, as the bulk phase of the hydrogels can influence bacterial growth behaviour quite differently compared to the surface.

Recommendations are techniques such as i) SEM or cryoSEM after fracture of hydrogels , ii) Confocal Laser Scanning Microscopy (CLSM) after staining for bacteria, optical sectioning will provide insight on the 3D bacterial distribution and its viability within the hydrogels.

We thank the reviewer for highlighting the importance of bacterial distribution within the hydrogel network. Due to time and instrument access constraints, we could not perform SEM, Cryo-SEM, or confocal/optical sectioning. However, we performed fluorescence Z-stack imaging of *E. coli* in 1% and 0.2% US hydrogels. The analysis shows that bacteria are present both at the hydrogel surface and within the bulk, with deeper penetration and more uniform distribution observed in the softer 0.2% gels (Figure S11-S12). These results indicate that bacterial growth occurs throughout the 3D network and support our conclusion that hydrogel physicochemical properties, including stiffness and hydration, influence colonization beyond the surface. We have included a description of this imaging in the Methods and added the results in the main text with references to the supplementary figures.

2. Authors justification as electrostatic interaction for the selective promotion/inhibition of bacterial growth is not fully convincing. Authors should conduct zeta potential measurements for all bacteria used in the study. In general, Gram-negative bacteria typically exhibit more negative zeta potentials due to the presence of LPS in the outer membrane. However attachment and especially growth are not solely governed by electrostatic interactions.

We thank the reviewer for this insightful comment. We agree that overall electrostatic charge alone cannot fully explain the selective bacterial growth observed on unsubstituted (US) hydrogels. To clarify this mechanism, we performed Zeta potential measurements for all four bacterial species. Gram-negative bacteria (*E. coli*, *P. fluorescens*) exhibited more negative overall charges (−37 mV and −33 mV) compared to Gram-positive bacteria (*S. aureus*, *B. subtilis*, −18 mV and −25 mV). However, cytochrome C binding assays, which probe exposed surface charges, revealed that Gram-positive bacteria have more accessible negative charges (*S. aureus* 56%, *B. subtilis* 62.5%) than Gram-negative bacteria (*E. coli* 43%, *P. fluorescens* 32%). In Gram-negative bacteria, the negatively charged LPS is largely shielded by the outer membrane, whereas Gram-positive bacteria have exposed teichoic acids that readily interact with positively charged Cytochrome C. Embedding positively charged cytochrome C into 1% US hydrogels restored growth of Gram-positive bacteria, confirming that exposed surface charges, rather than overall zeta potential, govern selective bacterial compatibility.

These results reconcile the apparent discrepancy between bulk charge and growth patterns and support our conclusion that electrostatic interactions at the cell surface are the dominant factor controlling selective bacterial growth on US hydrogels. We have described the results in the main text and have included the methodology in the methods section.

3. This manuscript did not sufficiently consider the role of nutrients and its accessibility to bacteria in the growth, considering the architecture of hydrogels. When all bacteria are dead as shown As Figure S29 and S30, showed complete bacterial death, suggest possible nutrient limitation or bacterial mobility to access nutrients potentially due to



hydrogel structure. Authors should investigate whether there is possible nutrient limitation or restricted mobility of bacteria in the hydrogels, both of which will impact bacterial growth.

We thank the reviewer for this comment. To address the possibility of nutrient limitation or restricted mobility, we note that the same US hydrogels (0.5% and 1%) promoted growth of Gram-negative bacteria (*E. coli* and *P. fluorescens*) across all nutrient media tested (NB, LB, TSB, M1 and M2). This indicates that nutrients are accessible within the hydrogel matrix and are not a limiting factor. Propidium iodide staining confirmed that Gram-positive bacteria were dead on these hydrogels, despite the presence of nutrients. Using *B. subtilis* and by embedding positively charged cytochrome C into 1% US hydrogels restored growth, confirming that electrostatic repulsion, rather than nutrient limitation or restricted mobility, is the primary mechanism governing selective bacterial growth. These results indicate that while nutrient availability and hydrogel architecture can influence bacterial growth in principle, the selective inhibition of Gram-positive bacteria on negatively charged US hydrogels is predominantly driven by electrostatic interactions under the tested conditions.

4. CryoSEM images in Figure S10, specifically A and B shows a collapsed structure (could be due to over-etching), showing large pores with no significant differences. Authors in the results section claim they are from both US and S hydrogels contradicting the figure legend. The authors should systematically perform CryoSEM on both US and S hydrogels in the absence and presence of bacterial growth to understand the the bacterial distribution in the hydrogel matrix.

We thank the reviewer for this suggestion. We have corrected Figure S10 to include Cryo-SEM images of both US and S hydrogels, resolving discrepancy with the figure legend. We agree that imaging bacterial distribution within the hydrogel matrix using Cryo-SEM would provide additional insights. However, due to limited access, time, and funding, performing Cryo-SEM with bacteria embedded in the hydrogels is not feasible in the current study. To address bacterial distribution, we instead performed fluorescence Z-stack imaging of bacteria within 1% and 0.2% US hydrogels, which allows visualization of bacterial localization in 3D and is presented in the Supplementary Materials (Figures S11-12). While not at Cryo-SEM resolution, these data provide a reliable assessment of bacterial distribution within the hydrogel network.

#### Minor comments:

1. How thick are the hydrogels?

We thank the reviewer for this helpful comment. The gels used in this study had an average thickness of approximately 2.1mm. This information has been added to the materials section of the manuscript.

2. Figure 2, bacterial growth area scale, Is that the diameter of the bacterial growth measured? what units?

We thank the reviewer for pointing out the need for clarification. The bacterial growth area refers to the 2-dimensional area covered by visible bacterial growth, not the diameter. This has now been clarified in the Materials section as follows:

*“The bacterial growth area was quantified from the binary images using the measure function in Fiji, representing the two-dimensional projected area of bacterial spread”.*

3. Figure S2-S9 says all microorganisms are Uropathogenic? This needs to be corrected. It is important to mention from where these microorganisms are isolated from?

*We thank the reviewer for this important comment. The strain information has been corrected in the Figures S2-S9. Only *E. coli* CFT073 is a uropathogenic strain.*

4. Cryo-SEM - Was the imaging performed under low temperatures in SEM? This information was not clear in the methods.

*We thank the reviewer for this observation. The Cryo-SEM imaging was indeed performed under low-temperature conditions of approximately -150°C.*

5. There are few mismatch with figures in the text and from the legend with respect to numbers. For example: Frequency sweep figures in the results say Figures S3-S12, whereas the corresponding figures are from S11-S20.

*We thank the reviewer for pointing this out. We have now corrected the figure numbers in the text, legend and supplementary material.*

## **Reviewer #4 (Remarks to the Author):**

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Communications Materials initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.