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Peer Review File

Topological transformation of microbial proteins into iron single-atom sites for selective hydrogen peroxide electrosynthesis

Corresponding Author: Professor Dingsheng Wang

This manuscript has been previously reviewed at another journal. This document only contains information relating to versions considered at Nature Communications.

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
I recommend the publication of this manuscript in Nature Communications. The authors present a new approach to synthesizing iron single-atom catalysts using iron-containing microbial waste as precursors through pyrolysis.This innovative method not only helps address the issue of waste management but also facilitates the development of high-performance catalytic materials.The synthesized catalysts exhibit good electrocatalytic 2e- oxygen reduction performance, achieving a current density of 200 mA cm-2 for H2O2 production. The identification of catalytic active sites through comprehensive experimental and theoretical analysis further underscores the robustness of this study. The authors have addressed all reviewer concerns, demonstrating the manuscript's readiness for publication in Nature Communications.

Reviewer #3

(Remarks to the Author)
The authors carefully addressed the questions from the last round, and I am pleased to see a significant improvement in the explanation, which enhances the overall soundness of the research. The electrochemical characterization, testing and results looks good now. And I believe this manuscript is suitable for Nature Communications.
Still, there are a few questions that need to be clarified for the design principle.
1. Will the performance be affected by using bacteria from different iterative cultures? Is this method suitable for mass production?
2. Why does the process need to reduce the Fe from the Bacillus pumilus?
3. Does the treatment process of Bacillus pumilus will damage its structure? Like cell wall? Does this cause other chemicals or ions to easily accumulate into the precious?
4. Even though the electrochemical performance looks good, it is still recommended to provide in-situ Ramen or XRD to analyze the catalytical principles.

The below sections are focused on the author's response to Reviewer 2's comments.

1. For the mechanism and structural design of single-atom sites by using bacterial comparing with the iron-included protein, I think I already provided similar comments during the first round review while the manuscript was submitted in Nature Synthesis. The author gives a full response and provides an XAS analysis of the bacteria and the relationship between the pyrolysis temperature and the coordinated environment of the formed single Fe atom sites. The results are fully provided, but the innovation should be lower than expected. For example, the advantages of using bacteria are low cost and ease of large batch production, not the unique properties of bacteria compared to the use of related proteins.
2. Although the use of (Fe—) bacteria to increase the selectivity of the obtained carbon materials seems to exhibit not too much obvious improvement in overpotential and Faradaic efficiency compared to some reported research.
3. For comment 3" it difficult to believe that the active site for H2O2 production is on the FeN5–xOx single atoms", even though the author give the trace of ORR sites, it still recommend the authors provide some kind of posioues expiration to

prove the active sites are from single-atom sites.

In summary, the whole work looks almost comprehensive, and the total innovation should match the requirements of Nature Communications. Hence, I recommend it be accepted after some minor data supplied.

Version 2:

Reviewer comments:

Reviewer #3

(Remarks to the Author)
The authors made good revisons which are recommended to accepted now.

Response to reviewers

Manuscript ID: NCOMMS-24-18154A

Title: Topological transformation of microbial proteins into iron single-atom catalysts for selective hydrogen peroxide electrosynthesis

Dear Editors and Reviewers,

We sincerely thank the editors and reviewers for their constructive suggestions and comments. According to the reviewers' comments and suggestions, we have made careful modifications in the revised manuscript (words highlighted), and we provide our point-by-point responses below.

Color code:

Blue: comments by the referees.

Black: answers to the referees.

Response to Reviewer #1:

Comment-1: I recommend the publication of this manuscript in *Nature Communications*. The authors present a new approach to synthesizing iron single-atom catalysts using iron-containing microbial waste as precursors through pyrolysis. This innovative method not only helps address the issue of waste management but also facilitates the development of high-performance catalytic materials. The synthesized catalysts exhibit good electrocatalytic $2e^-$ oxygen reduction performance, achieving a current density of 200 mA cm^{-2} for H_2O_2 production. The identification of catalytic active sites through comprehensive experimental and theoretical analysis further underscores the robustness of this study. The authors have addressed all reviewer concerns, demonstrating the manuscript's readiness for publication in Nature Communications.

Authors' Response: We sincerely appreciate your valuable suggestions during the initial review, which have greatly helped improve the quality of our manuscript.

Response to Reviewer #3:

The authors carefully addressed the questions from the last round, and I am pleased to see a significant improvement in the explanation, which enhances the overall soundness of the research. The electrochemical characterization, testing and results looks good now. And I believe this manuscript is suitable for *Nature Communications*.

Still, there are a few questions that need to be clarified for the design principle.

Comment-1: Will the performance be affected by using bacteria from different iterative cultures? Is this method suitable for mass production?

Authors' Response: Under the same cultivation conditions, the performance of microbe-derived carbon materials remains consistent across different microbial generations of microbial precursors. In preliminary experiments, we used consecutively passaged cultures as the inoculation source, followed by large-scale cultivation to obtain a significant amount of microbial biomass. The carbon materials derived from these microbes exhibited good reproducibility in catalytic performance.

By adjusting the culture conditions of microorganisms to control their elemental uptake, it is possible to influence their growth, including protein expression, and elemental composition. These changes can ultimately alter the microstructure of the resulting microbe-derived carbon materials and their catalytic properties. For instance, by limiting the supply of Fe nutrients in the microbial medium, the number of active Fe single-atom sites responsible for catalyzing ORR in *Bacillus pumilus*-derived carbon material is reduced, resulting in changes in its catalytic performance. In our text, we employed this method to demonstrate that the FeN_3O_2 sites significantly enhance the

ORR catalytic activity of *Bacillus pumilus*-derived carbon material.

Moreover, microorganisms are easily scalable in cultivation, a process that has already been widely applied in industrial production. The use of microbial precursors, combined with a simple pyrolysis treatment, ensures that microbe-derived carbon materials are suitable for large-scale production.

Comment-2: Why does the process need to reduce the Fe from the *Bacillus pumilus*?

Authors' Response: To assess the catalytic contribution of iron single-atom sites derived from iron-dependent proteins, we prepared two type catalysts: *Bacillus pumilus*-derived carbon material (with normal iron content) and *Bacillus pumilus*(Fe−)-derived carbon material (with low iron content). *Bacillus pumilus*(Fe−)-derived carbon material was synthesized using *Bacillus pumilus*(Fe−), a mutant strain cultured in an iron-deficient medium, which resulted in a 53.7% reduction in iron content. By comparing the catalytic performance of these two materials, we aimed to determine whether the iron-dependent proteins were topologically transformed into iron single-atom sites, enhancing the ORR catalytic activity of the microbe-derived carbon materials. Experimental results demonstrated that the *Bacillus pumilus*(Fe−)-derived carbon material exhibited inferior ORR activity and reduced selectivity for H_2O_2. This confirmed that the iron single-atom sites enhance the catalytic performance of the microbe-derived carbon material for H_2O_2 synthesis. To enhance clarity, we have revised the content related to the preparation of *Bacillus pumilus*(Fe−)-derived carbon

material in **Figure 2** of the main text, as reflected in the revised text attached.

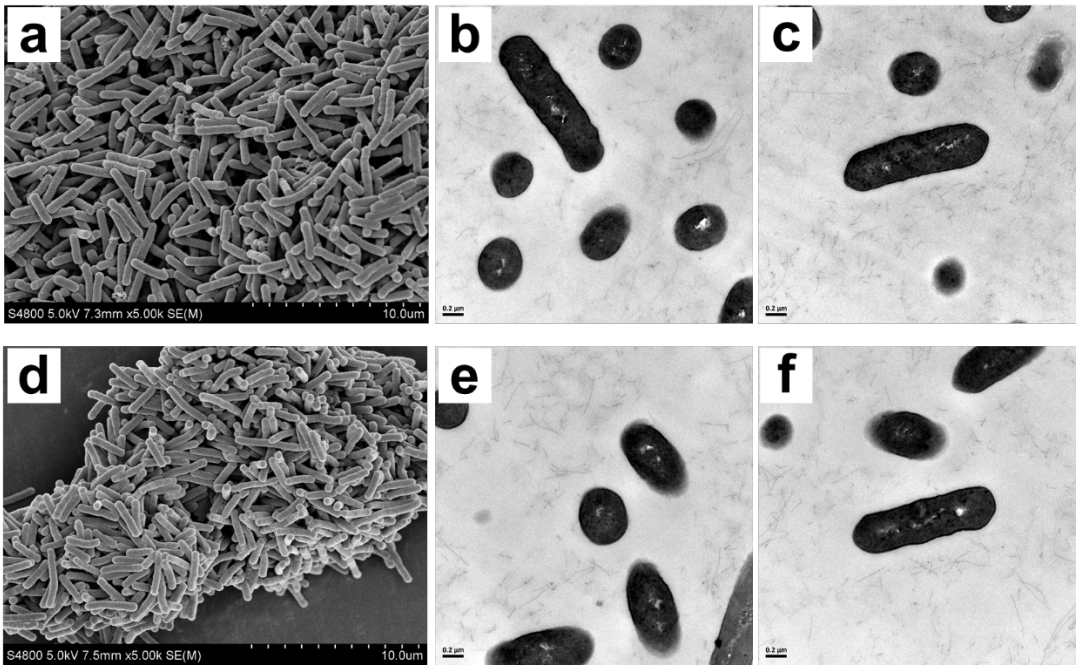
(Lines 160 – Lines 197) ‘To determine whether iron-dependent proteins are transformed into active Fe sites for H_2O_2 production, a vertical comparison of microbe-derived carbon materials was conducted. *Bacillus pumilus*, which was selected for its superior catalytic performance among microbe-derived carbon materials, underwent detailed investigation. This robust probiotic strain, commonly sourced from chassis cells utilized in industrial biochemical production[36], expresses a variety of iron-dependent proteins[37], including ferredoxin, cytochrome *c* oxidase, and iron-dependent superoxide dismutase. The expression of these proteins is closely linked to the microorganism's metabolic regulation, which enables the tailored cultivation of microbial strains with reduced iron-protein content for comparative studies. After four iterative cultures in customized iron-free medium (see Supplementary Table 1), *Bacillus pumilus* was transformed into a mutant strain, designated as *Bacillus pumilus*(Fe−), as shown schematically in Fig. 2a. The cell structures of *Bacillus pumilus* and *Bacillus pumilus*(Fe−) are essentially indistinguishable under electron microscopy, as shown in Supplementary Fig. 15. However, *Bacillus pumilus*(Fe−) exhibited a markedly reduced iron content, showing approximately a 72% decrease, as shown in Supplementary Fig. 16. During cultivation in the absence of iron sources, *Bacillus pumilus*(Fe−) compensates by assimilating other metal ions, which are used to express proteins that substitute for the deficient iron-dependent proteins[37, 38]. Consequently, the contents of Mg, Mn and Zn increased by 2.2, 6.6, and 0.1 times, respectively.

Bacillus pumilus(Fe−) cells were processed into *Bacillus pumilus*(Fe−)-derived carbon material, which functions as a comparative catalyst in contrast to the *Bacillus pumilus*-derived carbon material. Both catalysts underwent an identical pyrolysis process, leading to comparable levels of N and O doping and pore structure, as shown in Supplementary Figs. 17 and 18. However, they exhibited distinct metal contents, consistent with the differences in the contents of microbial precursors, as depicted in Fig. 2b and Supplementary Fig. 16. Compared to *Bacillus pumilus*-derived carbon material, the Fe in *Bacillus pumilus*(Fe−)-derived carbon material show a 53.7% decrease in Fe content and a 147% increase in Mn content. Additionally, the Mg content remained nearly unchanged, with only a marginal increase of 0.5%. This observation further corroborates that during pyrolysis, Mg tends to segregate and form MgO nanocrystallites, leading to its separation from the carbon matrix[39]. Assessing the corresponding difference in catalytic performance, *Bacillus pumilus*(Fe−)-derived carbon material exhibited a maximum decrease in H_2O_2 selectivity to 59.0% at 0 V *vs*. RHE and a negative shift in the half-wave potential to 0.55 V *vs*. RHE, as presented in Fig.2c, 2d and Supplementary Fig. 19. This drastic contrast illustrates that the presence of Mg does not contribute to the ORR catalytic performance. Despite the low Fe content of 0.05 wt%, the presence of active iron sites significantly enhances selectivity, resulting in an increase of at least 26%. This observed difference in catalytic behaviour underscores the crucial role of active Fe sites, which are pyrolytically transformed from Fe-dependent proteins, in catalysing the ORR for H_2O_2 production.’

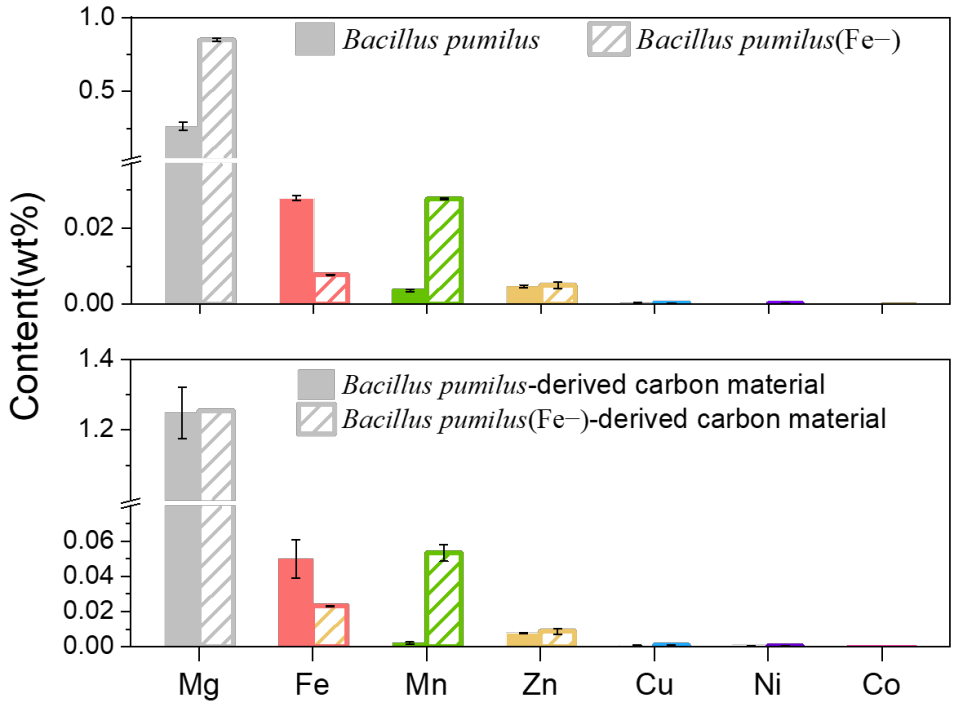
Comment-3: Does the treatment process of *Bacillus pumilus* will damage its structure? Like cell wall? Does this cause other chemicals or ions to easily accumulate into the precious?

Authors' Response: During the iron-deficient cultivation process, the cellular structure of *Bacillus pumilus* showed no significant changes under electron microscopy. However, ICP-MS analysis revealed that the cells accumulated more Mg, Mn, and Zn. Detailed information on the structural and elemental composition changes in the *Bacillus pumilus*(Fe−) strain and its derived carbon materials is presented in Supplementary Figures 15–16, as reflected in the revised figure attached. Under electron microscopy, *Bacillus pumilus*(Fe−) exhibited no observable differences in cell structure compared to *Bacillus pumilus*. However, iron-containing proteins play a crucial role in the metabolism of *Bacillus pumilus*. To compensate for the absence of iron-containing proteins, *Bacillus pumilus*(Fe−) intake other metal ions to express proteins with similar functions, leading to increases in Mg, Mn, and Zn content by 2.2, 6.6, and 0.1 times, respectively. During pyrolysis, the relatively low Zn content resulted in a limited number of active sites, while the higher concentrations of Mg and Mn tended to aggregate, making it difficult for them to form highly active ORR catalytic sites. Therefore, apart from Fe, the catalytic contribution of active sites derived from other metal elements on the catalytic performance of *Bacillus pumilus*(Fe−)-derived carbon material is minimal. In addition, we have supplemented the discussion of this part in the main text (**Lines 160 – Lines 197**), as referenced in **Comment-2**.

(Supplementary Information, Page 19 – Page 20) ‘



Supplementary Figure 15 | The morphology of *Bacillus pumilus* and *Bacillus pumilus*(Fe−). *Bacillus pumilus* is often used as an industrial fermentative bacterium, being easy to culture with a short growth period. *Bacillus pumilus* will express a variety of iron-containing structures, for example, Siderophores, Fe-superoxide dismutase, Heme-containing enzymes/proteins. **a,** SEM images of vegetative *Bacillus pumilus* cells. **b-c**, TEM images of the biology slice taken from biopsy samples of osmium acid and lead acetate pre-treated *Bacillus pumilus*. **d**, SEM images of vegetative *Bacillus pumilus*(Fe−) cells. **e-f**, TEM images of the biology slice taken from biopsy samples of osmium acid and lead acetate pre-treated *Bacillus pumilus*(Fe−) cells.



Supplementary Figure 16 | The content of metal elements in *Bacillus pumilus*, *Bacillus pumilus*(Fe−), *Bacillus pumilus*-derived carbon material and *Bacillus pumilus*(Fe−)-derived carbon material.'

Comment-4: Even though the electrochemical performance looks good, it is still recommended to provide in-situ Ramen or XRD to analyze the catalytical principles.

Authors' Response: Following your suggestion, we have attempted to use surface-enhanced Raman spectroscopy (SERS) to detect the ORR catalytic process. The experiment utilized 55 nm gold nanoparticles to enhance the Raman signal (*Nature* 464, 392–395, 2010), and the results are shown in the Fig. 1 below.

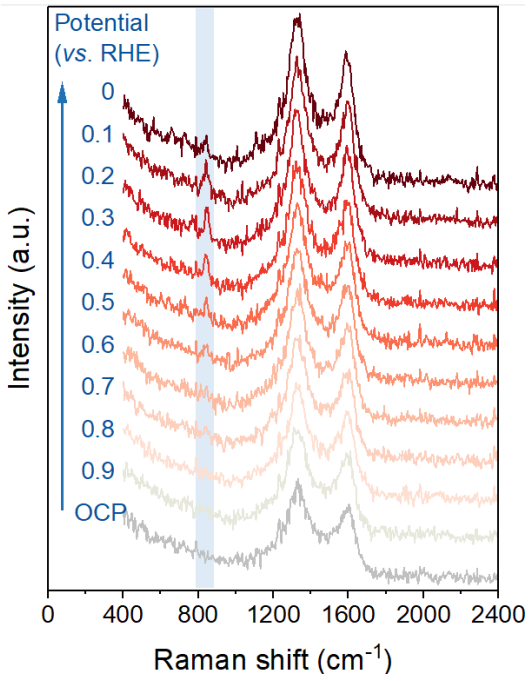


Fig. 1 *In situ* surface-enhanced Raman spectra of oxygenated species adsorbed on the surfaces of FeN_3O_2 catalyst. at different potentials during the ORR test in O_2-saturated 0.1 M KOH solutions.

Unfortunately, we only detected a characteristic peak at 840 cm^{-1}, which corresponds to the adsorption of H_2O_2 on the surface of the gold nanoparticles (*J. Am. Chem. Soc.* 2003, 125, 7086-7099.). Potential signals from various oxygen species (*Nat. Energy*. 2019, 4, 60–67; *J. Am. Chem. Soc*. 2021, 143, 1318−1322.), typically observed between 600 and 2000 cm^{-1}, were not detected. This absence may be attributed to several factors: 1. The signals from intermediate products may have been too weak. Although gold nanoparticles are known to enhance Raman signals, the catalytic sites may not have been within the effective enhancement range of the nanoparticles, leading to an insufficient boost of the oxygen species signals. Additionally, Raman spectroscopy collects signals from a very small area, and even after multiple tests, it is possible that

the signals from intermediates were missed.

2. The microbial-derived carbon material we prepared has an amorphous carbon structure. The characteristic D and G bands of the carbon structure are relatively broad and indistinct, making it more difficult to resolve potential oxygen species signals in the 1200–1700 cm^{-1} region.

To date, there have been a few reports of successful SERS detection of intermediates in the ORR process catalyzed by non-metallic doped sites or trace metal single-atom sites. But the field is essential and has arisen much attention. We will investigate the catalytic mechanisms of the ORR process in future experiments.

The below sections are focused on the author's response to Reviewer 2's comments.

Comment-1: For the mechanism and structural design of single-atom sites by using bacterial comparing with the iron-included protein, I think I already provided similar comments during the first round review while the manuscript was submitted in Nature Synthesis. The author gives a full response and provides an XAS analysis of the bacteria and the relationship between the pyrolysis temperature and the coordinated environment of the formed single Fe atom sites. The results are fully provided, but the innovation should be lower than expected. For example, the advantages of using bacteria are low cost and ease of large batch production, not the unique properties of bacteria compared to the use of related proteins.

Authors' Response: Thank you for the comments. The development of catalyst

research remains a large gap between laboratory-scale research and industrial applications. Many catalysts have shown promising lab results, but high costs and difficulties in large-scale production hinder their commercialization. This gap has garnered significant attention and needs to be addressed. In our study, microorganisms are employed as low-cost, scalable precursors, which significantly reduce catalyst costs and enhance feasibility for practical applications.

It should be noted that the structure and elemental composition of microbial cells are much complex compared to pure proteins. One of the major innovations of our text lies in uncovering the origins of the superior performance of catalysts derived from microbial precursors. In our work, we focus on the formation of iron single-atom sites through the topological transformation of iron-dependent proteins during the preparation process, and on how these sites influence the ORR catalytic performance of microbe-derived carbon materials. To clarify this point, we have further revised the second paragraph of the **Introduction** as follows:

(**Lines 48 – Lines 72**) 'Single-atom catalysts (SACs) are cutting-edge electrocatalysts that enable cost-effective electrochemical production of H_2O_2, demonstrating superior catalytic activity and target product selectivity in the $2e^-$ ORR[7-10]. Despite their ultralow active site loadings, SACs exhibit exceptional performance[11]. These trace single-atom sites, often found in heteroatom-doped carbon materials, form spontaneously during pyrolysis and work synergistically with non-metallic doping sites[12, 13]. Utilizing microbes as precursors facilitates the scalable gram-level synthesis of heteroatom-doped carbon materials, which bridges the gap between lab-scale advancements and

industrial applications[14]. In microbial precursors, trace amounts of Fe act as metalloprotein centers, confined by protein ligands with well-defined stereo-configurations, providing an ideal framework for forming Fe single-atom sites. While these trace Fe single-atom sites significantly contribute to ORR performance, their optimal local structure for achieving the best catalytic performance, as well as the structure–activity relationship, remain unclear[15, 16]. This is particularly evident when discussing the active site that catalyzes H_2O_2 production, as Fe SACs are traditionally viewed as less effective for catalyzing the $2e^-$ ORR due to their tendency, via heme sites (FeN_4 moiety), to catalyse the reduction of O_2 to H_2O. However, Fe SACs possess a moderate O_2 binding affinity, allowing them to readily convert between various Fe–O intermediates[17-20]. By fine-tuning the coordination environment of Fe SACs, selective conversion of different ORR products can be achieved[21-23]. When Fe SACs mimic the distinct metal environment of superoxide dismutase and superoxide reductase, they are endowed with the ability to undergo oxidative metabolism and protection against reactive oxygen species[24-27]. Their nonheme configurations, characterized by distorted square pyramidal geometries and electronic asymmetry, provide the structural basis for the selective reduction of Fe–O intermediates to H_2O_2[28, 29]. Mimicking these natural nonheme iron-dependent enzymes or inheriting their characteristics at the atomic level[30] is essential for unlocking their full potential for sustainable H_2O_2 production.'

Comment-2: Although the use of (Fe−) bacteria to increase the selectivity of the obtained carbon materials seems to exhibit not too much obvious improvement in overpotential and Faradaic efficiency compared to some reported research.

Authors' Response: There may be a misunderstanding regarding this comment. To assess the catalytic contribution of iron single-atom sites derived from iron-dependent proteins, we prepared two type catalysts: *Bacillus pumilus*-derived carbon material (with normal iron content) and *Bacillus pumilus*(Fe−)-derived carbon material (with low iron content). *Bacillus pumilus*(Fe−)-derived carbon material was synthesized using *Bacillus pumilus*(Fe−), a mutant strain cultured in an iron-deficient medium, which resulted in a 53.7% reduction in iron content. By comparing the catalytic performance of the two materials, we aimed to determine whether the iron-dependent proteins were topologically transformed into iron single-atom sites, enhancing the ORR catalytic activity of the microbe-derived carbon materials. Experimental results demonstrated that the *Bacillus pumilus*(Fe−)-derived carbon material exhibited inferior ORR activity and reduced selectivity for H_2O_2. This confirmed that the iron single-atom sites in the microbe-derived carbon material enhance its ORR catalytic performance. Further discussion clarified that the O adjacent-C atoms in FeN_3O_2 sites actively catalyze H_2O_2 production. To enhance clarity, we have revised the content related to the preparation of *Bacillus pumilus*(Fe−)-derived carbon material in **Figure 2** of the main text, as reflected in the revised text attached.

We agree with the reviewer' comments, *Bacillus pumilus*-derived carbon material exhibits the characteristics of amorphous carbon, with lower surface charge transfer capabilities compared to the sophisticated carbon materials described in the literature. Improving the overpotential and Faradaic efficiency is the direction for further

enhancing the performance of microbe-derived carbon materials.

(Lines 160 – Lines 197) ‘To determine whether iron-dependent proteins are transformed into active Fe sites for H_2O_2 production, a vertical comparison of microbe-derived carbon materials was conducted. *Bacillus pumilus*, which was selected for its superior catalytic performance among microbe-derived carbon materials, underwent detailed investigation. This robust probiotic strain, commonly sourced from chassis cells utilized in industrial biochemical production[36], expresses a variety of iron-dependent proteins[37], including ferredoxin, cytochrome *c* oxidase, and iron-dependent superoxide dismutase. The expression of these proteins is closely linked to the microorganism's metabolic regulation, which enables the tailored cultivation of microbial strains with reduced iron-protein content for comparative studies. After four iterative cultures in customized iron-free medium (see Supplementary Table 1), *Bacillus pumilus* was transformed into a mutant strain, designated as *Bacillus pumilus*(Fe−), as shown schematically in Fig. 2a. The cell structures of *Bacillus pumilus* and *Bacillus pumilus*(Fe−) are essentially indistinguishable under electron microscopy, as shown in Supplementary Fig. 15. However, *Bacillus pumilus*(Fe−) exhibited a markedly reduced iron content, showing approximately a 72% decrease, as shown in Supplementary Fig. 16. During cultivation in the absence of iron sources, *Bacillus pumilus*(Fe−) compensates by assimilating other metal ions, which are used to express proteins that substitute for the deficient iron-dependent proteins[37, 38]. Consequently, the contents of Mg, Mn and Zn increased by 2.2, 6.6, and 0.1 times, respectively.

Bacillus pumilus(Fe−) cells were processed into *Bacillus pumilus*(Fe−)-derived carbon material, which functions as a comparative catalyst in contrast to the *Bacillus pumilus*-derived carbon material. Both catalysts underwent an identical pyrolysis process, leading to comparable levels of N and O doping and pore structure, as shown in Supplementary Figs. 17 and 18. However, they exhibited distinct metal contents, consistent with the differences in the contents of microbial precursors, as depicted in Fig. 2b and Supplementary Fig. 16. Compared to *Bacillus pumilus*-derived carbon material, the Fe in *Bacillus pumilus*(Fe−)-derived carbon material show a 53.7% decrease in Fe content and a 147% increase in Mn content. Additionally, the Mg content remained nearly unchanged, with only a marginal increase of 0.5%. This observation further corroborates that during pyrolysis, Mg tends to segregate and form MgO nanocrystallites, leading to its separation from the carbon matrix[39]. Assessing the corresponding difference in catalytic performance, *Bacillus pumilus*(Fe−)-derived carbon material exhibited a maximum decrease in H_2O_2 selectivity to 59.0% at 0 V *vs*. RHE and a negative shift in the half-wave potential to 0.55 V *vs*. RHE, as presented in Fig.2c, 2d and Supplementary Fig. 19. This drastic contrast illustrates that the presence of Mg does not contribute to the ORR catalytic performance. Despite the low Fe content of 0.05 wt%, the presence of active iron sites significantly enhances selectivity, resulting in an increase of at least 26%. This observed difference in catalytic behaviour underscores the crucial role of active Fe sites, which are pyrolytically transformed from Fe-dependent proteins, in catalysing the ORR for H_2O_2 production.’

Comment-3: For comment 3 “it difficult to believe that the active site for H_2O_2 production is on the $FeN_{5-x}O_x$ single atoms”, even though the author give the trace of ORR sites, it still recommend the authors provide some kind of posioues expiration to prove the active sites are from single-atom sites.

Authors’ Response: Thanks for the comments. In *Bacillus pumilus*-derived carbon material, iron single-atom sites contribute 26% to the H_2O_2 selectivity, the other contribution coming from various non-metal doped sites, e.g., oxygen-doped carbon and nitrogen-doped carbon. The catalytic contribution of the iron single-atom sites can be concluded by the following three reasons:

First, highly active metal single-atom sites can exhibit excellent catalytic performance even at very low loadings. Similar phenomena have been reported for heteroatom-doped carbon materials and graphene materials (*Nat. Commun.* 2023, 14, 6666; *Carbon* 2023, 203, 237-260; *Adv. Mater.* 2018, 30, 1703691). The FeN_3O_2 sites resemble the configuration of the metal center in iron superoxide dismutase, an enzyme with exceptionally high catalytic activity (*Chem. Rev*. 2014, 114, 3854-3918). Therefore, trace amounts of FeN_3O_2 sites can efficiently catalyze H_2O_2 production. In our work, iron single-atom sites contribute 26% to the H_2O_2 selectivity.

Second, we have prepared a compared catalyst e.g. *Bacillus pumilus*(Fe−)-derived carbon material, which contains fewer iron single-atom sites. By comparing with the catalytic performance of *Bacillus pumilus*-derived carbon material and *Bacillus pumilus*(Fe−)-derived carbon material, we confirmed that the reduction in FeN_3O_2 site

numbers leads to a decline in H_2O_2 electrosynthesis performance.

Third, our theoretical calculations also validate the catalytic role of $FeN_{5-x}O_x$ sites in H_2O_2 production. Notably, the catalytic center of the $FeN_{5-x}O_x$ local structure involves the O adjacent-C atom (*Nat. Commun.* 2019, 10, 3997). The O atom in the FeN_3O_2 local structure act as charge-transfer bridges, synergistically enhancing the O adjacent-C atom's ORR catalytic activity. Based on the analysis of Bader charge and catalytic energy barrier, the O adjacent-C atom can effectively bind *OOH and catalyze *OOH desorption with a lower energy barrier.

In addition, several methods reported in the literature can be used to verify the catalytic role of metal single-atom sites, such as Mössbauer spectroscopy, acid leaching treatment for different types of active sites and *in situ* surface-enhanced Raman spectroscopy. However, Mössbauer spectroscopy is not applicable for detecting trace amounts of iron in microbe-derived carbon materials, as the iron content of 0.05 wt% is below the detection limit of Mössbauer spectroscopy. Furthermore, acid leaching treatment of microbe-derived carbon materials may lead to the destruction of certain non-metal-doped sites, resulting in a decline in catalytic performance. We supplemented the data with the catalytic performance changes of *Bacillus pumilus*-derived carbon material before and after acid leaching is shown as following Fig.2. After acid leaching, the ORR catalytic activity of the *Bacillus pumilus*-derived carbon material showed a H_2O_2 selectivity dropped by 12.2% at 0.6 V *vs*. RHE. This is attributed to the number of non-metal-doped sites in microbe-derived carbon materials

far exceeds that of iron single-atom sites, it is more possible damaged some of these non-metallic sites.

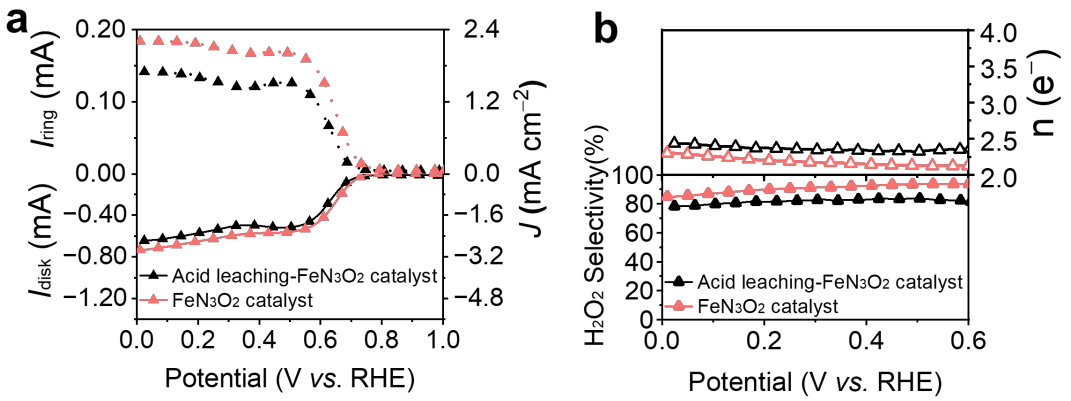


Fig. 2 ORR electrochemical performance of *Bacillus pumilus*-derived carbon material (FeN_3O_2 catalyst) and acid leaching *Bacillus pumilus*(Fe−)-derived carbon material (Acid leaching-FeN_3O_2 catalyst). a, ORR polarization curve. b, Calculated ORR electron transfer number and H_2O_2 selectivity.

Additionally, we have attempted to use surface-enhanced Raman spectroscopy (SERS) to detect the ORR catalytic process. The experiment employed 55 nm gold nanoparticles to enhance the Raman signal, and the results are shown in Fig. 1 of **Comment-4**. Unfortunately, only a characteristic peak at 840 cm^{-1}, corresponding to H_2O_2 adsorption on the surface of the gold nanoparticles (*J. Am. Chem. Soc.* 2003, 125, 7086-7099.), was detected. Characteristic signals for various oxygen species reported in the literature (*Nat. Energy*. 2019, 4, 60–67; *J. Am. Chem. Soc*. 2021, 143, 1318−1322.), located between 600 and 2000 cm^{-1}, were not detected. This might be due to the weak signal of intermediate products and the broad and blunt D and G peaks of the microbe-derived carbon material, making the oxygen species' signals in the 1200–

1700 cm^{-1} range harder to distinguish.

In summary, the whole work looks almost comprehensive, and the total innovation should match the requirements of *Nature Communications*. Hence, I recommend it be accepted after some minor data supplied.

Authors' Response: We sincerely appreciate your valuable suggestions, which have significantly enhanced the quality of our manuscript.