Response of \text{N}_2\text{O} emission and denitrification genes to different inorganic and organic amendments

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Denitrification is a key biochemical process in nitrogen cycling and nitrous oxide (\text{N}_2\text{O}) production. In this study, the impacts of different inorganic and organic amendments (OAs) on the abundance of denitrifying genes (\textit{nirS}, \textit{nirK} and \textit{nosZ}) and the level of \text{N}_2\text{O} emission were examined with incubation experiments. Six treatments included the indicated applications: (i) no fertilization (CK); (ii) urea application alone (U); (iii) wheat straw plus urea (U + WS); (iv) pig manure plus urea (U + PM); (v) compost product plus urea (U + CP); and (vi) improved compost product plus urea (U + IC). The results indicated that all fertilization treatments increased accumulative \text{N}_2\text{O} emissions compared with the CK treatment. The U + WS, U + PM and U + CP treatments increased \text{N}_2\text{O} emissions by 2.12–141.3%, and the U + IC treatment decreased \text{N}_2\text{O} emissions by 23.24% relative to the U treatment. \textit{nirK} was the dominant denitrification gene rather than \textit{nirS} and \textit{nosZ} found in soil. Additionally, the highest abundance of \textit{nirK} gene was that with the U + PM treatment, and the lowest was that with the U + IC treatment. Additionally, changes in the \textit{nirK} gene were highly correlated with levels of dissolved organic carbon (DOC), dissolved organic nitrogen (DON) and nitrate nitrogen (\text{NO}_3^-\text{N}). Automatic linear modeling revealed that \text{N}_2\text{O} emission was closely related to the \textit{nirK} gene, DOC and \text{NO}_3^-\text{N}.

Overall, the use of urea and improved compost as co-amendments retarded \text{N}_2\text{O} emission to a considerable degree compared with other OA additions.

Nitrous oxide (\text{N}_2\text{O}) is one of the most prevalent greenhouse gases, and its warming potential is approximately 265 times that of \text{CO}_2 on the 100-year scale\textsuperscript{1}. In addition, \text{N}_2\text{O} is an important substance that destroys the ozone layer\textsuperscript{2}. Annual average \text{N}_2\text{O} emissions from global cultivated fields total 6.4 × 10\textsuperscript{12} g [calculated as nitrogen (N)], which accounts for 25% of total global \text{N}_2\text{O} emissions\textsuperscript{3}. Agricultural fertilization management is the main source of \text{N}_2\text{O} emissions. Application of nitrogen fertilizer stimulates \text{N}_2\text{O} emission from cultivated soils\textsuperscript{4,5}. Therefore, it is urgent to find an effective strategy for decreasing \text{N}_2\text{O} emissions.

\text{N}_2\text{O} is produced mainly through biological and abiotic processes. Denitrification is one of the main ways \text{N}_2\text{O} is produced under microbe-driven conditions in soil\textsuperscript{6}. Denitrification is a process in which nitrate (\text{NO}_3^-\text{N}) is sequentially reduced to nitrite (\text{NO}_2^-\text{N}), nitric oxide (NO), nitrous oxide (\text{N}_2\text{O}), and finally dinitrogen gas (N\textsubscript{2}). It is essentially the inverse process of nitrification. Nitrite-reducing bacteria are encoded by nitrite reductase genes (\textit{nirK} and \textit{nirS}). In addition, reduction of \text{N}_2\text{O} to N\textsubscript{2} is one of the key steps in the whole process, and is encoded by the \textit{nosZ} gene\textsuperscript{8,9}. Nos-catalyzed \text{N}_2\text{O} reduction plays a significant role in \text{N}_2\text{O} emissions. Therefore, the abundance of \textit{nirS}, \textit{nirK} and \textit{nosZ} denitrifying genes was used to study soil nitrogen cycling and \text{N}_2\text{O} emissions\textsuperscript{10,11}. At present, nitrogen addition affecting \text{N}_2\text{O} emissions has attracted wide concern\textsuperscript{12,23}. Wang et al.\textsuperscript{14} reported that nitrogen addition at 250 kg ha\textsuperscript{-1} promoted denitrification, thus contributing to comparable stimulation of \text{N}_2\text{O} emissions. Albanito et al.\textsuperscript{15} proposed a linear relationship between \text{N}_2\text{O} emissions and amounts of nitrogen fertilizer applied in tropical and subtropical regions. To reduce \text{N}_2\text{O} emissions, combined application of organic materials and inorganic fertilizer has been endorsed by some scholars. The addition of organic material altered the abundance of denitrifiers and thus influenced the denitrification process\textsuperscript{16,17}. Wang et al.\textsuperscript{18} found that organic materials stimulated the activity of the \textit{nosZ} gene and decreased \text{N}_2\text{O} emissions. However, some studies have suggested that organic materials (i.e., wastewater sludge, manure and vermicompost) provide carbon sources for denitrifiers and accelerate denitrification, thus increasing \text{N}_2\text{O} emissions\textsuperscript{11,19}. Although many studies have

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been focused on combined application of organic and inorganic fertilizers and its effect on N\textsubscript{2}O emissions in agricultural soils, the research conclusions are not yet consistent. Therefore, it is necessary to carry out research to determine the drivers of N\textsubscript{2}O emissions and propose reasonable fertilization strategies.

In addition, the identification of major denitrification-driving genes plays an important role in predicting N\textsubscript{2}O emissions. Yin et al.\textsuperscript{20} indicated that denitrifiers were more sensitive to inorganic fertilization than to organic fertilization. Harter et al.\textsuperscript{21} revealed that the nosZ gene is the decisive factor for N\textsubscript{2}O emissions. Hai et al.\textsuperscript{22} found that organic fertilizers increased the abundance of nirS and nirK genes and enhanced N\textsubscript{2}O emissions. Huang et al.\textsuperscript{23} reported that co-addition of urea and cattle manure decreased the abundance of nirS and nirK but increased N\textsubscript{2}O emissions. In addition, Xu et al.\textsuperscript{24} suggested that nirS-type denitrifying genes showed strong correlations with significant increases in N\textsubscript{2}O emissions from soils undergoing organic fertilization. Henderson et al.\textsuperscript{25} questioned whether the relationship between N\textsubscript{2}O emissions and the abundance of nirS and nosZ genes was significant. Other studies have focused on the impacts of co-amendment with inorganic and organic fertilizers on the abundance of denitrification bacterial communities and changes in soil properties\textsuperscript{21,26–28}. However, there was still a knowledge gap regarding the dominant drivers of N\textsubscript{2}O emissions, including denitrifiers and environmental factors.

Therefore, this study was focused on the impacts of co-additions of urea and four organic additions (OAs), including wheat straw, pig manure, compost products and improved compost products, on N\textsubscript{2}O emissions. Additionally, fluorescent quantitative PCR and 16S rDNA sequencing were used to study changes in the abundance of denitrifying genes in soil caused by OAs and clarify the mechanism for the response of denitrifying gene abundance to added OAs. Particularly, the purposes of this research were (i) to compare the impacts of different OAs with urea on changes in N\textsubscript{2}O emissions; (ii) to understand the abundance of denitrifiers affected by different OAs; and (iii) to identify the relationships among N\textsubscript{2}O emissions, soil properties and the abundance of denitrifiers.

Materials and methods

Experimental soil collection. The tested soil was collected from the experimental station around Northwest A&F University in Shaanxi Province, China. The tested plot lies in a typical semiarid region with average annual precipitation of 533–631 mm. The basic soil properties before the experiment were as follows: pH = 7.53, soil organic carbon (SOC) = 0.79%, total nitrogen (TN) = 0.075%.

Experimental materials collection. The four OAs included compost product (CP), wheat straw (WS), pig manure (PM) and improved-compost product (IC). PM and WS were collected from the farm and field of the experimental station. CP and IC products were produced in the greenhouse on the campus. CP was manufactured with PM and WS. IC was produced by the addition of bean dregs and biochar to the compost product. Previous studies confirmed that IC products increased SOC and TN contents more than compost products\textsuperscript{29,30}. All OA materials were dried at 55 °C and then sieved through a 2 mm mesh. Basic physicochemical properties for all OAs are shown in the Supplementary Material.

Experimental design. Six incubation experiments, including no fertilization (CK), urea N alone (U), 70% urea N plus 30% N from PM (U + PM), 70% urea N plus 30% N from WS (U + WS), 70% urea N plus 30% N from CP (U + CP) and 70% urea N plus 30% N from IC (U + IC), were conducted with three replicates to understand the responses of N\textsubscript{2}O emissions and denitrification genes to different fertilization practices. Fresh soil samples were first weighed (700 g for dry weight basis) and placed into plastic bottles. A total of 0.5 mg N g\textsuperscript{-1} of dry soil was added to each treatment. The amounts of urea and OAs were calculated by their total nitrogen content. The content of soil moisture was controlled at 60% of the water-holding capacity. Incubation was performed in a 25 °C environment for 77 days. Each plastic bottle was weighed, and distilled water was added to maintain constant moisture in the soil. All soil samples were divided into three parts after incubation: one was used for determination of soil properties after drying, one was used for measurements of NO\textsubscript{3}·N and NH\textsubscript{4}·-N at 4 °C, and the other was used for DNA determination at ~80 °C.

Properties determination. Soil NO\textsubscript{3}·N and NH\textsubscript{4}·-N were determined with an AA3 flow analyzer by extraction with 2 mol L\textsuperscript{-1} KCl. Soil organic carbon (SOC) was measured through K\textsubscript{2}Cr\textsubscript{2}O\textsubscript{7} digestion. Total nitrogen (TN) was measured by a Kjeldahl nitrogen analyzer\textsuperscript{31}. In addition, soil moisture content was determined by weighing after drying. In addition, the soil microbial biomass carbon (MBC) and microbial biomass nitrogen (MBN) contents were determined using the chloroform fumigation-extraction method\textsuperscript{32,33}. Briefly, triplicate soil samples were weighed and fumigated with ethanol-free chloroform for 24 h at 25 °C. The three fumigated and unfumigated samples from each treatment were extracted with 100 mL of 0.5 mol L\textsuperscript{-1} K\textsubscript{2}SO\textsubscript{4} on a shaker at 220 rpm for 30 min and then filtered and determined with a TOC/TN analyzer. Dissolved organic carbon (DOC) was determined by a TOC-analyzer after extraction and filtering\textsuperscript{34}. Dissolved organic nitrogen (DON) was measured using a flow analyzer after extraction with H\textsubscript{2}O\textsubscript{2}\textsuperscript{35}. N\textsubscript{2}O samples were collected on days 0, 3, 7, 14, 21, 28, 35, 49, 63 and 77 during incubation. All bottles were sealed for 10 min before sampling to ensure concentration of N\textsubscript{2}O emissions. N\textsubscript{2}O samples were determined by gas chromatography. The N\textsubscript{2}O fluxes and cumulative N\textsubscript{2}O emissions were calculated as described by Huang et al.\textsuperscript{36}. The specific calculation formula is as follows:

\[
F = \frac{\Delta C}{\Delta t} \cdot \frac{V}{m} \cdot \frac{M}{22.4} \cdot \frac{273}{273 + T}
\]

\(F\) is the N\textsubscript{2}O fluxes (μg/kg/day), \(\Delta C/\Delta t\) is the variation of concentration per unit time (μg/kg/day), \(V\) is the volume of the device (L), \(m\) is the dry soil weight (g), \(M/22.4\) is the mass density of standard gases (g/L), and \(T\) is the incubation temperature (°C).
Cumulative N₂O emission is the accumulation of the N₂O fluxes during the incubation period (μg/kg).

Microbial analyses. Soil DNA was extracted from 0.5 g of fresh soil using a DNA kit (Omega, GA, USA). The DNA solution extracted from each sample was studied with 1% agarose gel electrophoresis. The nirK, nirS and nosZ denitrifying genes were amplified by PCR using primers (Supplementary Materials). The abundances of denitrifiers were quantified by quantitative real-time PCR (qPCR) on an Illumina MiSeq platform in Beijing.

Data analyses. Figures were prepared with Origin 2018. Significant differences among the treatments were analyzed by Duncan’s multiple range test using IBM SPSS Statistics (version 20.0). Automatic linear modeling was conducted using IBM SPSS Statistics (version 20.0). Redundancy analyses (RDA) and principal component analyses (PCA) were conducted with Canoco (version 4.5).

Results
Changes in N₂O emission. N₂O emissions influenced significantly by different OAs are shown in Fig. 1a. A similar trend for N₂O emissions among all treatments was observed. N₂O emissions increased within the first 7 days and declined thereafter. N₂O emissions peaked on day 3 for the CK, U, U + WS and U + PM treatments and peaked on day 4 for the U + IC and U + CP treatments. The OAs increased N₂O emissions during the whole incubation process compared with the CK treatment. The maximum peak value for N₂O emissions among all OAs was that seen with the U + PM treatment (76.04 mg/kg/day), and the minimum value of N₂O emissions was that seen with the U + IC treatment (21.44 mg/kg/day). Compared with other OA treatments, compost amendments (U + IC, U + CP) decreased the peak values of N₂O emissions. Furthermore, the highest cumulative N₂O emissions were seen with the U + IC treatment (21.44 mg/kg/day), which were 241.8% higher than the levels seen with the CK sample; this was followed by the U + WS (111.1%), U + CP (44.63%), U + IC (41.61%) and U + CP (8.70%) treatments (Fig. 1b). In addition, cumulative N₂O emissions for the U + WS, U + PM and U + CP treatments were 49.05%, 141.3% and 2.12% higher relative to the U treatment, but the cumulative N₂O emission for the U + IC treatment was 23.24% lower compared with that of the U treatment. These results indicated that relative to application of urea alone, mixing urea with wheat straw, pig manure and compost products promoted N₂O emissions during incubation. In addition, IC retarded N₂O emissions to a greater degree compared with other OA additions.

Changes in soil properties. The basic properties of soils are shown in Table 1. The highest SOC content was observed for the U + WS treatment, followed by the U + IC and U + CP treatments, and these levels were significantly higher than that of the CK treatment (P < 0.05). However, there was no significant difference in SOC contents for the U + PM and CK treatments (P > 0.05). Relative to the CK treatment, OAs significantly increased the levels of DON, NH₄⁺-N and NO₃⁻-N. The highest NO₃⁻-N, NH₄⁺-N and DON contents were found for the U + PM treatment, and these were higher than those seen for other OA treatments (P < 0.05). However, the DOC contents with all OA treatments except for U + IC differed significantly from that of the CK treatment (P < 0.05). The maximum DOC concentration was observed for the U + WS treatment. In addition, only the U + PM treatment, among all OA treatments, failed to increase the MBN content significantly (P > 0.05). The U treatment did
Abundances of nirK, nirS and nosZ denitrifying genes. The relative abundances of denitrification genes (nirK, nirS and nosZ) were determined during incubation for all treatments (Fig. 2). The copy numbers for nirK, nirS and nosZ varied within the ranges 5.26 × 10^3–4.47 × 10^4, 1.26 × 10^3–9.36 × 10^3 and 1.04 × 10^3–2.51 × 10^3, respectively. The nirK-type denitrification genes exhibited the highest abundance after incubation. Compared with the CK treatment, all OA treatments (U + CP, U + IC, U + PM and U + WS) significantly increased the abundance of the nirK gene. The highest copy number of the nirK gene was seen for the U + PM treatment, followed by the U + CP, U + IC and U + WS treatments. In addition, there were no significant differences between the U + IC and U + CP treatments. However, the nosZ gene had the lowest abundance after incubation. Compared with the CK treatment, all OA treatments except the U + WS treatment significantly increased the copy number not significantly increase any soil properties during incubation relative to the CK treatment except for NO$_3^-$N (P > 0.05).

Table 1. Soil properties in different treatments with OAs. CK no urea and OAs, U urea, U + WS urea plus wheat straw, U + PM urea plus pig manure, U + CP urea plus compost, U + IC urea plus improved compost. Values are mean ± standard deviation (n = 3). The different letters indicate significant differences at the 0.05 probability level.

| Treatments | SOC (g/kg) | NH$_4^+$-N (mg/kg) | NO$_3^-$N (mg/kg) | MBN (mg/kg) | DON (mg/kg) | DOC (mg/kg) |
|------------|-----------|-------------------|-------------------|------------|-------------|-------------|
| CK         | 6.53 ± 0.85a | 0.58 ± 0.01a      | 49.35 ± 10.6a     | 0.48 ± 0.24a | 7.14 ± 3.06a | 150 ± 48a   |
| U          | 6.59 ± 0.80a | 0.63 ± 0.01a      | 80.60 ± 11.2b     | 0.53 ± 0.18a | 7.21 ± 3.01a | 155 ± 44a   |
| U + PM     | 6.91 ± 0.58a | 0.37 ± 0.02b      | 219.4 ± 16.94c    | 0.85 ± 0.15a | 60.53 ± 4.19b | 290 ± 22b |
| U + WS     | 8.88 ± 0.32b | 0.13 ± 0.07c      | 147.1 ± 10.05d    | 1.49 ± 0.26b | 54.22 ± 3.08c | 330 ± 32b |
| U + CP     | 8.13 ± 0.65b | 0.18 ± 0.03kc     | 182.6 ± 16.25e    | 0.97 ± 0.15ac | 54.79 ± 1.58bc | 210 ± 18c |
| U + IC     | 8.63 ± 0.55b | 0.25 ± 0.02d      | 178.1 ± 10.45e    | 0.94 ± 0.25c | 48.14 ± 4.22d | 180 ± 9.1ac |

Figure 2. Relative abundance of denitrification genes during the incubation affected by different OAs. Error bars indicate standard deviations (n = 3). The different letters indicate significant differences (P < 0.05) (nirS (a); nirK (b); nosZ (c); CK no urea and OAs, U urea, U + WS urea plus wheat straw, U + PM urea plus pig manure, U + CP urea plus compost, U + IC urea plus improved compost).
of the nosZ gene. The maximum abundance of the nosZ gene was observed for the U + PM treatment. There was no significant difference between the CK and U treatments. For abundance of the nirS gene, the U and OA treatments all significantly increased the abundance relative to the CK treatment. The highest nirS gene abundance was seen for the U + PM treatment, followed by the U + WS, U + CP and U + IC treatments. However, there was no significant difference between the U and U + CP treatments. Among all denitrification genes, the U treatment significantly increased only the abundance of nirS genes. These results indicated that co-additions of urea and OAs increased the abundance of nirK genes more, while urea alone increased the abundance of nirS genes more.

**Correlations of denitrifying gene abundance and soil properties.** PCA was used to analyze the abundances of denitrifiers among all treatments (Fig. 3a). The results showed that the explanations of Axis 1 and Axis 2 contributed 73.66% and 10.56%, respectively, to changes in denitrifying genes (nirS, nirK and nosZ).
The OA treatments were largely separated from the CK and U treatments along PCA1. Additionally, the U + PM and U + WS treatments were segregated from the U + CP and U + IC treatments along PCA2. The results suggested that OA application significantly influenced the abundance of the three types of denitrifiers, and OAs had stronger effects on the abundance of denitrifiers than urea application alone. Correlations among soil properties, cumulative N₂O emissions and denitrifying gene abundance showed that the abundance of the nirS gene was significantly associated with cumulative N₂O emissions (r = 0.876, P < 0.05) and DOC (r = 0.859, P < 0.05). The abundance of the nirK gene was correlated with DON (r = 0.977, P < 0.1), NO₃⁻N (r = 0.880, P < 0.05), DOC (r = 0.865, P < 0.05), MBN (r = 0.852, P < 0.05) and NH₄⁺-N (r = -0.880, P < 0.05). In addition, the abundance of the nosZ gene was mainly related to SOC (r = 0.965, P < 0.1), MBN (r = 0.836, P < 0.05) and NH₄⁺-N (r = -0.931, P < 0.1). In addition, the results of automatic linear modeling showed that nirK abundance, DOC, NO₃⁻N, MBN and DON were the main factors influencing N₂O emissions (Fig. 3b).

Discussion
Different fertilization practices influenced N₂O emissions from soil. Soil N₂O emissions peaked on day 3 for the CK, U, U + WS and U + PM treatments, while those for the U + CP and U + IC treatments peaked on day 7. This may be attributed to the fact that WS and PM provided more rapidly available N than CP and IC37. In addition, compared with urea application alone, the U + CP, U + WS and U + PM treatments increased cumulative N₂O emissions by 2.13–49.06%, which was within the increase range of 27–74% seen with pig slurry and compost amendments38. However, the U + IC treatment decreased cumulative N₂O emissions by 23.24% relative to urea alone. Therefore, the combined application of IC and urea reduced N₂O emissions in soil because IC was more stable than CP, PM and WS. Additionally, OA treatment increased cumulative N₂O emissions during incubation relative to the CK treatment. This might be because OAs provided more substrates for denitrification through mineralization. Denitrification could result in a rapid reduction in NO₃⁻N content and promote the emission of N₂O. Previous studies showed that the NO₃⁻N concentration was an important factor affecting the denitrification rate and N₂O release40–43. Increasing the concentration of NO₃⁻N could significantly increase N₂O release. Compared with urea application alone, the OA additions increased the content of NO₃⁻N in soil; furthermore, PM application provided more NO₃⁻N than WS, CP and IC. Therefore, PM application promoted changes in N₂O emissions, which was consistent with the variations in NO₃⁻N. In addition, SOC increased by addition of OAs to the soil. The importance of SOC as a factor affecting denitrification and N₂O emissions has been reported by Chen et al.44. However, in this study, the effect of SOC on denitrifiers and N₂O emissions was lower than that of DOC due to the narrow range of SOC changes occurring in a short-term incubation experiment. Carbon availability was the key controlling factor for denitrification in soil45. Compared with the U + PM treatment, the U + WS treatment produced a higher DOC content but lower N₂O emissions, which may be due to the higher C/N ratio in WS. Microbial fixation of carbon in the straw treatment limited carbon effectiveness. In addition, all treatments involving addition of organic material, the modified compost addition treatment produced the lowest DOC content due to its higher degree of humification, which reduced the abundance of nirK genes and therefore reduced N₂O emissions from the soil46.

N₂O emissions were highly related to soil physiochemical properties and the abundance of denitrifiers, which was consistent with the findings of Sun et al.46. Denitrifying bacteria are active in soil biological denitrification45,47. Many studies showed that fertilization increased the number of denitrifying microorganisms in the soil by providing substrates and energy for denitrifying bacteria and promoting their growth and reproduction37,48. In this study, the OA addition increased the abundance of denitrifying genes in soil. The contributions of nirK to N₂O emissions were higher than those of nirS and nosZ. In addition, application of pig manure significantly increased nirK gene abundance and thus promoted N₂O emissions more than other OAs. Yoshida et al.49 also found that application of organic manure increased the abundance of nirS genes in rice paddy soil more than that of nirS genes. However, there are some studies showing the opposite results. For example, Yin et al.50 found that organic manure changed the abundance of the nirS gene community in black soil during long-term treatment but not that of nirK. Barrett et al.51 confirmed that a higher abundance of nirS-type genes was observed in carbon-amended soil relative to other genes. This may be related to the different sources and nature of available carbon in amended soil. Environmental factors significantly influenced the abundance of denitrifiers in this study52,53. Positive correlations were observed between NO₃⁻N, DON, DOC and the abundance of the nirK gene. These findings suggested that NO₃⁻N, DON and DOC levels were important factors affecting the abundance of the soil denitrifying bacterial community, thus affecting denitrification with different OA applications. This may be due to differences in the availability of C and N in OA-amended soil.

Conclusion
Fertilization treatments increase denitrification and N₂O emissions relative to the CK treatment. In addition, compared with urea alone, combined application of pig manure and urea provided more available N (DON and NO₃⁻N) and increased the abundance of nirK genes, thus increasing cumulative N₂O emissions from the soil. However, combined applications of improved compost products and urea reduced accumulated N₂O emissions by decreasing the abundance of nirK genes. Overall, the different fertilization practices affected the abundance of denitrifiers, denitrification and soil properties. From the perspective of soil N₂O emission reduction, we recommend the application of improved compost products and urea to the soil.

Data availability
All data generated or analysed during this study are included in this published article (and its Supplementary Information files).
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Acknowledgements
This study was supported by National natural science foundation of China (Project No. 42077135), Integration and demonstration of remediation of heavy metal pollution in typical farmland of Shaanxi Province in China (Project No. NYK-2020-YL-21) and Talent Special Fund Grant from Northwest A&F university.

Author contributions
Y.Y. wrote the main manuscript text and H.L. prepared Figs. 1, 2 and 3. All authors reviewed the manuscript.

Competing interests
The authors declare no competing interests.

Additional information
Supplementary Information The online version contains supplementary material available at https://doi.org/10.1038/s41598-022-07753-9.

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