Different Approaches to Detect "Nanobacteria" in Patients with Kidney Stones: an Infectious Cause or a Subset of Life?

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Purpose: This research focused on the detection of nanobacteria in kidney stones of 30 Iranian patients without adding fetal bovine serum (FBS) to the culture media.

Materials and Methods: Nanobacteria were isolated from a nephro-ureterolithiasis extract of the urinary tract and kidney of patients and were cultured in the laboratory. The growth of nanobacteria was monitored using a spectrophotometer, and with inverted microscopy technique, their crystallization was analyzed after two days. The images from atomic force microscopy (AFM), transmission electron microscopy (TEM) and scanning electron microscopy (SEM) indicated the morphology and demonstrated the size of the cultured nanobacteria which is between 60 and 160 nm.
Fourier transform infrared spectroscopy (FTIR) and X-ray diffraction (XRD) were used to study the chemical composition, surface functional groups and crystal structure of the igloo-like nanobacteria shell. FTIR spectra in the region of 1000 to 1200 cm-1 and the XRD peaks provided evidence that the main components of the nanobacteria shell were apatite-based compounds.

Results: Nanobacteria infected all the 27 patients with apatite kidney stone, and none of the three patients who had uric acid kidney stone were infected as confirmed by the cultivation of the stones samples. The results showed that nanobacteria might play a fundamental role in the formation of apatite-based kidney stones.

Conclusion: The biomineralization ability of nanobacteria may lead to calcification of the soft tissues, which in turn may result in other diseases. It is also suggested that nanobacteria may be a factor in calcification-related diseases and disorders with poorly characterized etiologies. This research with its different approaches, clarified significant doubts that nanobacteria act as contaminant, warranting continued investigation of its role in other diseases.

Key words: Nanobacterium; biomineralization; calcium apatite; kidney stone; calcification; lithiasis.

INTRODUCTION

Nephrolithiasis is a condition with prevalence varying geographically, presenting with multiple etiologies, and showing increasing annual incidents, though it is still recognized as a relatively rare medical problem in the world. There are several known factors that can cause the disease, these include metabolic disorders, anatomical malformations, and environmental and dietary factors. Based on several studies, it has also been found that bacterial infections and metabolic factors can play a fundamental role in some urinary tract disorders such as biofilm formation on catheters[1], cancer[2-4] and lithiasis[5-9].
The term "nanoform" was described previously in both geology and biology[10], and the terms related to this novel category are classified into two. The first class comprised of living nanoforms and is referred to as nanobacteria[11-13], while the second class is composed of non-living particles, specifically, mineralo fetuin complexes[14] or the calcifying nanoparticles referred to this discovery.
Nanobacteria are the smallest cell-walled bacteria that have been discovered in human and cow blood, as well as in commercial cell culture serum. They can be grown under mammalian cell culture conditions for characterization and study. One of the most controversial issues about nanobacteria relates to its resistance to physical and chemical agents as well as its strong heat resistance. Nanobacteria produce mineralized shells made of carbonate apatite, and it is assumed that this apatite shell may be what protects the nanobacteria from various stresses[15].
Kajander[11] first reported nanobacteria as infectious agents in 1993. Although, based on the current criteria of living organisms, nanobacteria do not have living organism's features[16], but new evidence based on recent researches[17] and the findings of the current study support the idea that nanobacteria are living organisms.

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Table 1: Patients Demographic and Characteristic Table

Variables	Patient Statics
Age Group (Years).(Mean ± SD)	18-48. (34.77 ± 10.22)
Male to Female Ratio	20/10
BMI(kg/m^2). range (mean ± SD)	22.8-34.3. (28.05 ± 3.24)
Height	158-180. (168.97 ± 5.23)
Weight	31.2-97. (77.91 ± 12.86)
History of Previos stone sugeries	17
History of calcification diseases	20
- Gender ratio out of 20	11 (Male)/20
	9 (Female)/20
Positive samples out of 30	27 (18 male and 9 female)
Negative samples out of 30	3(2 male and 1 female)

Abbreviations: SD: Standard deviation, BMI: body mass index.

Being living or non-living, the fact or fiction of nanobacteria has been postponed for future research to show the final decision on this controversial scientific issue. Similar to other infectious agents, nanobacteria have the ability to spread to other organs and tissues through the bloodstream, and therefore can be found in the blood, urine and saliva of infected persons. The ability of nanobacteria to produce mineralized shells and their existence in the urinary tract of humans led to the hypothesis that they play a role in the production and progression of lithiasis. In some studies, it was shown that nanobacteria could induce cell apoptosis and calcification of soft tissues, and that the formation of kidney stones could be induced after intrarenal injection or infection with nanobacteria[18-20].

In this study, kidney stones from 30 Iranian patients were collected and analyzed for the presence of nanobacteria in nephro-ureteroliths. This study aimed to detect nanobacteria in the cultures of specimens taken after surgery for kidney stone disease and investigate the presence of these bacteria using inverted microscopy, SEM, TEM, AFM, XRD and FTIR.

MATERIALS AND METHODS

Cultivation of the nanobacteria

The samples used were obtained from thirty patients selected random, with kidney and upper urinary tract stones following nephrolithotomy surgery. The authors partnered with multiple hospital centers in Iran to secure patient's documented permission and provided the commitment to anonymity without any consideration of eligibility to participate in the research. The chemical composition of the isolated stones based on the initial urinary calculi analysis was calcium oxalate in more than 85% of the samples. After the collecting phase, a crucible grinder was placed in an autoclave (121°C, 20 min) before being used to powder the individual kidney stone completely. The powdered stones were demineralized by treatment with a 1 N solution of HCl for 3 min before neutralization by Tris buffer. The suspension was then centrifuged (14,000 ×g, 60 min) in a high-speed centrifuge from Sigma. The supernatant was discarded, and the precipitate was collected and filtered with DMEM prepared in the Pasteur Institute of Tehran through a syringe-type ultrafilter (0.1 µm) into flasks. Each flask contains 5 ml DMEM without fetal bovine serum (FBS) to avoid any possible contamination from FBS even if it was irradiated with gamma and in the presence of antibiotic (100 U/ml of penicillin and 100 µg/ml of streptomycin). The flasks were incubated under aseptic conditions in a cell incubator (CO_2: 5%, Air: 88%, 37°C).

Medium containing 5 ml DMEM in the presence of antibiotics lacking any powdered stone was used as a negative control. All the processes mentioned above were carried out under strict sterile conditions in a laminar flow hood. Nanobacteria (NB) growth was monitored

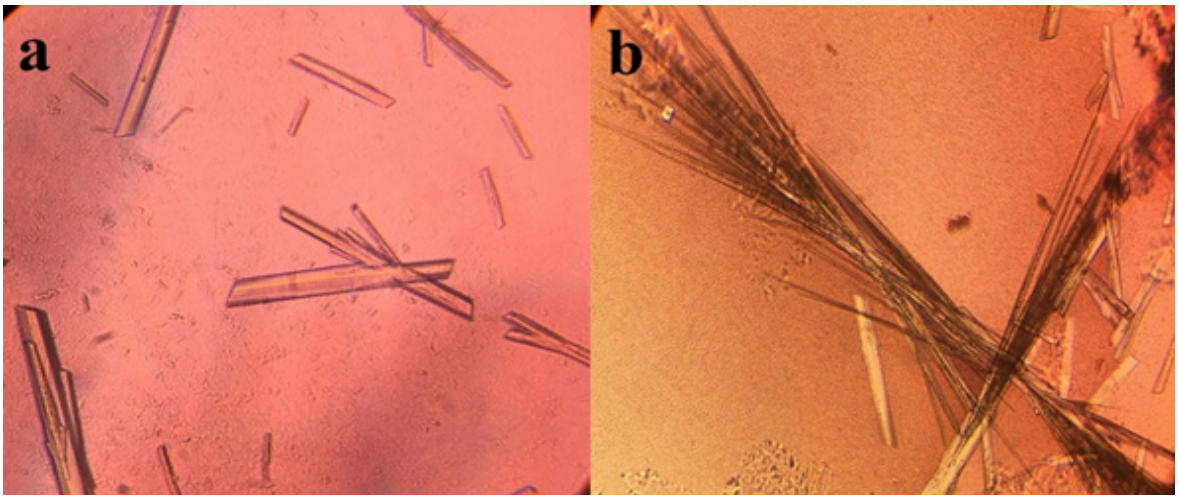

Figure 1: Crystal formation by nanobacteria in culture media using inverted microscopy (a) first day after cultivation and (b) two days after the culture (Magnification: 1000×).

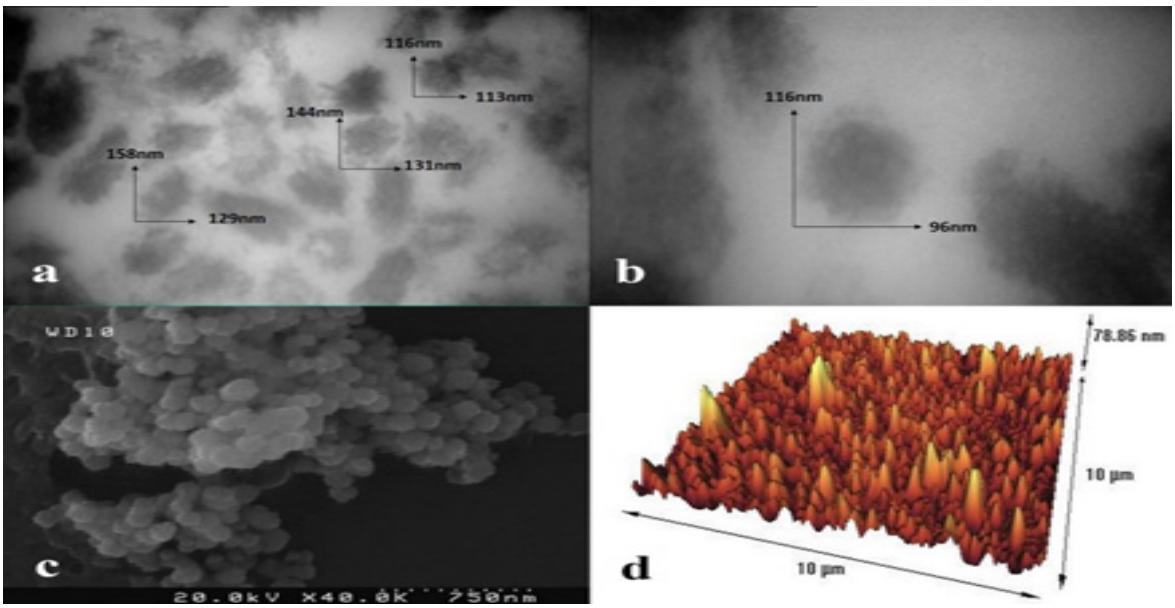

Figure 2. a) SEM images of nanobacteria isolated from cell cultures of kidney stone specimens showing nanobacteria as a cluster with spherical coccoid shape, and similar morphology and a size distribution ranging from 60 to 160 nm. **b & c)** TEM images of the cultured samples revealed that nanobacteria had hairy or needle-like structures within variable sizes of 68 x 64 to 158 x 129 nm. **d)** AFM 2D and 3D images illustrate the structure of the nanobacteria and their spherical shape. The scale bar represents 78 nm and also corresponds to the maximum size range of the analyzed nanobacterial specimens.

for up to five days by inverted microscopy. Crystallization was initiation by nucleation of the NB, and turbidity of the culture media was considered as the NB growth index.

TEM examination

Morphological characteristics of the cultured NB were examined by high-resolution transmission electron microscopy (TEM). The cultivated NB were incubated in a solution of 2.5% glutaraldehyde in 0.1 molar cacodylate buffer at 4°C for 1 h, then washed twice with sodium cacodylate buffer and incubated in a solution of 1% osmium tetroxide at room temperature for 1 h. Afterward, all the samples were rinsed with buffer and dehydrated with different concentrations of ethanol (25, 50, 70, 90 and 100%). The samples were then soaked in propylene oxide (15 min) and then molded by resin before being placed in an oven (60°C) for two days. The blocks were then cut into 70 – 120 nm thicknesses by ultra-microtome.

Uranyl acetate 3% (30 min) and lead citrate 2% (7 min) were used to stain the ultra-thin cut blocks which were then viewed under a Carl Zeiss EM10C transmission electron microscope (Carl Zeiss, Jena, Germany) operating at 80 KV.

SEM examination

The morphology of the cultured NB was analyzed using scanning electron microscopy (SEM). One drop of the freshly prepared solution of the ultra-centrifuged cul-

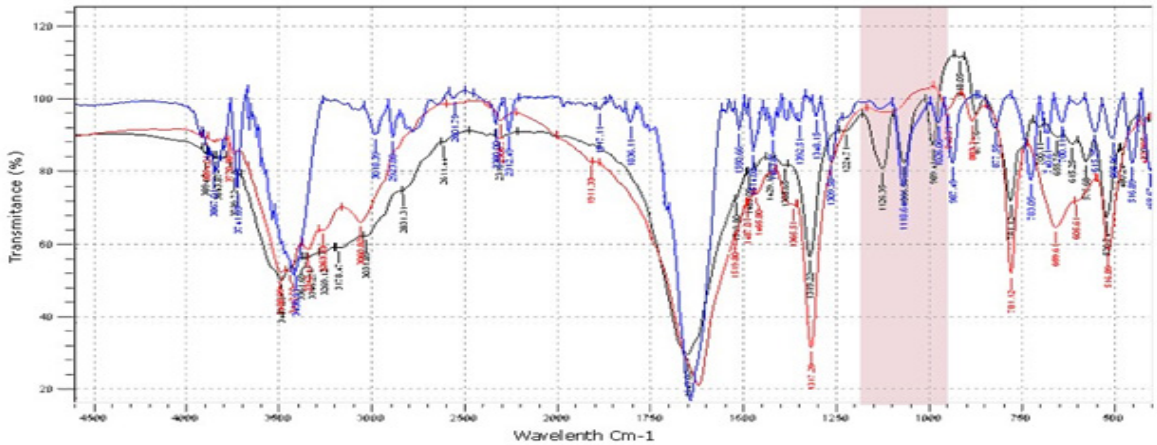

Figure 3. FTIR of the cultivated nanobacteria from positive samples (blue line) and the apatite kidney stone (black line). The peaks in 1000 to 1200 cm^{-1} range belong to phosphate absorption and the stretching bond of phosphate in the structure of the mineralized shell. FTIR spectra of the negative sample are shown with the red line. The peaks in 1000 to 1200 cm^{-1} range are absent (highlighted zone).

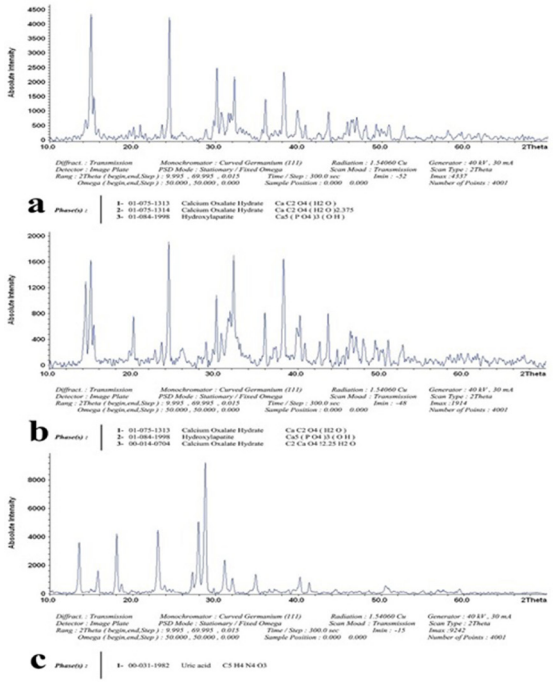

Figure 4. The XRD results showing that the main components of the shell of the cultivated nanobacteria are hydroxyapatite and calcium oxalate hydrate. **a)** An apatite kidney stone used for cultivation,
b) hydroxyapatite and calcium oxalate hydrate, and **c)** the negative sample consisting of mostly uric acid.

ture media was placed on carbon stickers, dried under air and coated with gold. A low voltage (10 KV) was set to observe NB particles with a Hitachi S-4160 scanning electron microscope (Japan).

AFM examination

For atomic force microscopy (AFM) examination, one drop of the ultra-centrifuged culture media was placed on sterile mica, then air dried under sterile conditions. The prepared mica was examined in an ARA-AFM device at the pharmaceutical research complex, Azad University Pharmaceutical Science Branch, Tehran, Iran.
FTIR examination
The IR spectrum of the cultured NB was recorded on an FTIR-430 spectrometer at room temperature. The instrument was operated at a frequency range of 500 – 4000 cm-1. For this spectroscopic analysis, 2 mg of the cultured NB was mixed with 100 mg of KBr and made into a pellet. Finally, the FTIR spectra were obtained using an 8400S (Shimadzu Co., Japan).

XRD examination

A small volume of the powdered kidney stones was reserved in the preparation stages for XRD. It was moved into sterile autoclaved microtubes and transferred to the XRD laboratories of Azad University of Tehran, North Branch. The technicians in the XRD laboratory shaped the samples into tablets and performed the X-ray diffraction analysis according to their standard protocols.

RESULTS

Inverted Microscopy Images
Three days after the cultivation of the kidney stones, nanobacteria growth was monitored by a turbidity assay of the culture media using a spectrophotometer set at 650 nm. The results from the inverted microscope (**Fig 1**) showed different stages of crystallization in the shell of the nanobacteria.

Electron and Atomic Force Microscopy Images

SEM, TEM and AFM images show that the size of the nanobacteria ranged from 60 to 160 nm. The morphology of the nanobacteria was spherical, and all the imaged nanobacteria had similar appearance.
The SEM micrograph (**Figure 2**) showed spherical igloo-like structures with variable sizes mostly less than 100 nm, and in some scaled cases, the size was less than 50 nm or even 20 nm, as observed in different samples. The TEM observations also indicate various sizes of nanobacteria. These images were taken by gold labeling of the samples with a hairy appearance, which is a discriminating feature of the nanobacteria. It is a controversial issue for researchers, as some of them believe that the edges are curled because of the dehydration of the samples during preparation while others, including the authors of this paper, think that this hairy appearance is due to the coat of the bacteria. In fact, the TEM images revealed that the nanobacteria are surrounded by needle-like deposits that coat the bacteria in needle-like apatite crystals, causing the hairy appearance. AFM analyses of the nanobacteria, including both 2D and 3D images, provided details of the morphological features and sizes of the nanobacteria, and also accentuate that the spherical bacteria are less than 100 nm in size, to be more exact, about 60 nm.
(**Figure 2:a**) SEM images of nanobacteria isolated from cell cultures of kidney stone specimens showing nanobacteria as a cluster with spherical coccoid shape, and similar morphology and a size distribution ranging from 60 to 160 nm. b & c) TEM images of the cultured samples revealed that nanobacteria had hairy or needle-like structures within variable sizes of 68 x 64 to 158 x 129 nm. d) AFM 2D and 3D images illustrate the structure of the nanobacteria and their spherical shape. The scale bar represents 78 nm and also corresponds to the maximum size range of the analyzed nanobacterial specimens.

FTIR

FTIR spectrum analysis showed an abnormal peak of between 1000 and 1200 cm^{-1} which belonged to phosphate absorption, and a peak less than 900 cm-1 belonging to the stretching bond of phosphate in the hydroxyapatite structure of the nanobacteria's mineralized shell.

XRD

The X-ray diffraction results showed that hydroxyapatite, $Ca_5(PO4)_3(OH)$, and calcium hydrate, $CaC2O4(H_2O)$ were the main components of the mineralized shell of the nanobacteria in positive samples, while in the negative samples, uric acid was the main crystal.

DISCUSSION

In the present study, the kidney stones of 30 Iranian patients were examined for infection by nanobacteria through culturing of the samples. Because nanobacteria produce a mineralized shell, it was possible to follow the growth of their cultures by measuring the turbidity of the media by a spectrophotometer. The combined

results from the turbidity assay, SEM, TEM, AFM, FTIR and XRD showed that 27 of these samples were infected by nanobacteria, while only three patients were free of the infection. The morphology and size of the cultivated nanobacteria were characterized using SEM, TEM and AFM. The average size of the nanobacteria was measured to be between 60 and 160 nm as estimated by the SEM and TEM images. FTIR and XRD techniques were used to analyze the composition of the mineralized shell. These results revealed that the main components of the shell were hydroxyapatite and calcium apatite. These apatite complexes did not exist in the kidney stones of the patients that were not infected with the nanobacteria. Based on the results of this study, it seems that the presence of nanobacteria in the kidney is one of the main factors contributing to kidney stone formation. Since first identified, the tentatively named nanobacteria were considered to be novel biofilm-producing or autonomously replicating bacteria that had been characterized by scanning electron microscopy to determine their morphology[11], which is one of the first steps required to start screening for their association with other diseases. Labelling nanobacteria as controversial or incomprehensible would be correct since recent papers widely varied in its classification. For example, the author of one paper claims that nanobacteria are nonliving microorganisms that only mimic living organisms[20], while other authors argue that this ultra-small bacteria is an example of a subset of the microbial life on earth that we know almost nothing about[17]. The distribution of these bacteria to different tissues in the body, evaluated by their injection into rabbits, facilitated the measurement of their in vivo effects and activities in the blood, plasma, liver, bone, kidney and spleen[18].

There are different hypotheses on the possible pathogenicity of these bacteria, which include malacoplakia, a rare inflammatory disease with an unknown cause that is characterized by the presence of histocytes containing both intra and extracellular calcospherules called Michaelis-Gutmann bodies. Researchers believe and propose that nanobacteria may cause this disease due to the structural resemblance of these spherules with nanobacteria[21]. Other researchers proposed a more astounding potential connection between HIV and nanobacteria, reporting in the first clinical study on this issue that 85% of HIV positive mothers had antibodies signifying exposure to nanobacteria[22]. The presence of unique nanobacteria correlate with other serious health disorders such as prostate cancer[23], chronic prostatitis [24], Alzheimer's disease[25], gall stone inflammation [26], aortic valve and vascular calcification[27,28], dental pulp stones[29] and kidney stones[13,19,30,31].

Since nanobacteria produce a mineralized shell, it was possible to follow the growth of their cultures by measuring the turbidity of the media with a spectrophotometer. The combined results from the turbidity assay, SEM, TEM, AFM, FTIR and XRD showed that 27 of these samples were infected by nanobacteria, while only three were free of the infection. The morphology and size of the cultivated nanobacteria were characterized via SEM, TEM, and AFM. The average size of the nanobacteria was measured to be between 60 –160 nm as estimated by the SEM and TEM images. FTIR and XRD techniques were used to analyze the composition of the mineralized shell. These results revealed that the main components of the shell were hydroxyapatite and calcium apatite. These apatite complexes did not exist in the kidney stones of the patients that were not infected by the nanobacteria. The following interpretation is definable by the nanobacteria major function which it is biomineralization; nanobacteria by biomineralization activity, involves apatite minerals to form crystal. For this reason, nanobacteria have thick hard apatite shell covers which are impenetrable to many inhibitory materials and may be the main reason why they grow in exposure to broad-spectrum antibiotics such as penicillin and streptomycin. Based on the results of this study, it seems that the presence of nanobacteria in the kidney is one of the main factors contributing to kidney stone formation.

With regards to treatment of nanobacteria, a recent interesting paper claims that according to the surveys on patients suffering from prostatitis caused by nanobacteria, antibiotic therapy showed improvement in some patients, while other therapies were also suggested[32]. All the new nanobacterial treatments need blind studies because much is not known about nanobacteria, and many aspects of its growth and development have not been investigated yet. Additional studies are needed to explore the molecular mechanisms that lead to nanobacterial biofilm formation[33], as well as to discern the role of nanobacteria in infections, cancers, lithiasis, and in the current case, biomineralization mechanisms. The clinical reality of microbial infections as a major menace to health care is an obvious fact, and the selection of a suitable antibiotic therapy will be crucial to the treatment of urinary tract infections, and also the treatment of nanobacteria as pointed out by the authors.

CONCLUSIONS

It is proposed that after detecting nanobacteria in the soft tissues of the body, they can act as nidi for nucleation, initiating biomineralization and stone formation. They might also play a role in tumor-inducing processes in the soft tissues. According to the current findings, stone formation is a complex process with different influences, and nanobacteria play the role of an initiator by favoring nucleation and crystal formation. In fact, because of the nanometer scale of these organisms and the fact that they can translocate via the bloodstream to any organ of the body, it is evident that there is no barrier for nanobacteria.

The most important issue now is to find out more about the functions of these nanobacteria, from adherence, internalization and cytotoxicity to other biomineralization functions. This full characterization will occur only by sequencing nanobacteria DNA and performing genetic scans to characterize its possible related roles and mechanisms, which is an ongoing study that is being conducted by the authors.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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