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Characterization of self-aggregation in *Bacillus licheniformis* strain RK14

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Abstract

Purpose Some *Bacillus licheniformis* strain isolated from the environment has been reported to form aggregates during the suspension culture under carbon limitation. The aim of this study is to characterize the aggregation process of *B. licheniformis* RK14 strain isolated from the soil.

Methods *B. licheniformis* RK14 was cultured in a glucose-free lysogeny broth (LB) to confirm the aggregate formation under glucose limitation conditions and compared to model *B. licheniformis* strains. To characterize the aggregation process of RK14, microscopic analyses and time-lapse observations were conducted. Thioflavin T (ThT) was used to assess the involvement of transient cell elongation in aggregate formation.

Results Although RK14 did not form aggregate when cultured in a glucose-rich γ-PGA production medium, it was found to self-aggregate when grown in a glucose-free LB. The optimal temperature for aggregation was approximately 40℃. Microscopic analysis showed that the aggregates were composed of viable cells with cell–cell cluster-like structures. Time-lapse observations clarified elongation of individual cells after 1 h of culture, followed by a return to rod-shape while maintaining the aggregation state. Addition of ThT at micromolar concentration inhibits aggregate formation, probably due to the suppression of cell elongation.

Conclusions The aggregation process of RK14 was similar to the pellicle and submerged biofilm formation process of *B. subtilis* in terms of morphological changes. It was concluded that transient cell elongation is critical for aggregate formation of RK14 strain.

Keywords *Bacillus licheniformis* RK14, Self-aggregation, Lysogeny broth, Cell elongation, Thioflavin T

Background

Flocculation is an aggregation phenomenon of bacterial cells in which they form flocs or clumps. Numerous microorganisms have been found to have floc-forming capabilities (Salehizadeh and Shojaosadati 2001) that can be applied to wastewater treatment. In an activated sludge, the components of flocs typically include polysaccharides, polynucleotides, and proteins (Tago and Aida 1977). However, cells may also aggregate to form cell–cell cluster-like structures without producing excessive extracellular matrix, as in the case of flocculating yeast (Soares 2011). In flocculating yeast, a specific lectin-like protein (Flo protein), only present in flocculating *Saccharomyces cerevisiae* cells, recognizes and interacts with the carbohydrate residues of α-mannans of neighboring cells. Calcium ions enable the lectins to adopt their active conformations (Soares 2011). There are several advantages of using flocculent yeast strains in alcoholic beverage production (wine, cachaça, and sparkling wine), renewable fuel production (bio-ethanol), biotechnology (production of heterologous proteins), and environmental applications (bioremediation of heavy metals).

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Some *Bacillus licheniformis* strain isolated from the environment have been reported to form aggregates during suspension culture under carbon limitation condition (da Silva et al. 2005). This is a phenomenon not seen in laboratory strains of *B. licheniformis* or *B. subtilis*. In that study, although culture engineering based on the substrate feeding condition have been well studied, the aggregate itself have not been characterized from a biological point of view, and the biomolecules that play an essential role in aggregate formation still need to be clarified. In general, *Bacillus* species have been reported to form biofilms on solid surfaces (Randrianjatovo-Gbalou et al. 2017; Arnaouteli and Bamford 2021). Biofilms are composed of closely associated cells encapsulated in an extracellular matrix mainly composed of polysaccharides, proteins, and DNA. The proteinaceous matrix of *Bacillus* species biofilms contains amyloid-like fibrils, which have been shown to play a determinant role in the structural integrity of the biofilm (Arnaouteli and Bamford 2021). Furthermore, most of *Bacillus* species form a pellicle biofilm that develops at the air–liquid interface under static culture conditions. This pellicle formation is a feature of obligate aerobes and a means of efficient oxygen acquisition. Although the mechanism of biofilm and pellicle formation in *Bacillus* species have been well reported, there is, to our knowledge, no report of characterization and mechanism of aggregate formation of *B. licheniformis* in suspension culture.

Meanwhile, we previously isolated an RK14 strain of *Bacillus licheniformis* with high γ-polyglutamic acid (γ-PGA) productivity from soil (Liu et al. 2017). γ-PGA from RK14 cells has a high molecular mass, and an enantiomeric composition that differs from those obtained from other *B. licheniformis* strains. RK14 strains produced 6.9 g/L γ-PGA without aggregate formation when cultured in semi-synthetic media, mainly containing fructose, glycerol, and L-glutamate at 37 °C.

In this study, *B. licheniformis* RK14 was cultured in glucose-free lysogeny broth (LB) to confirm the aggregate formation under glucose limitation conditions and compared to model *B. licheniformis* strains. To characterize the aggregation process of RK14, microscopic analyses and time-lapse observations were conducted. We finally examined the involvement of transient cell elongation in aggregate formation.

Results and discussion

Self-aggregation of *B. licheniformis* RK14

In our previous study, the RK14 strain was cultured in glucose-rich 3YD or YFEb medium for γ-PGA production (Liu et al. 2017). In this study, the RK14 strain was first cultured in glucose-free LB at 37 °C under shaking culture conditions. During suspension culture, we found that the RK14 strain forms aggregates. After 18 h, the precipitated aggregates were collected into microtubes and photographed as shown in Fig. 1a. The LB culture had significantly much more precipitated aggregates than the 3YD or YFEb cultures. Microscopic observation showed aggregation in LB culture of about several hundred μm^2 (Fig. 1b), and greater magnification clearly showed the cell–cell cluster-like structure as reported previously (da Silva et al. 2005). Evaluation of cell growth showed similar growth in LB ($OD_{600}=4.9$) and in 3YD medium ($OD_{600}=4.4$), while $OD_{600}=9.0$ and much higher in YFEb medium.

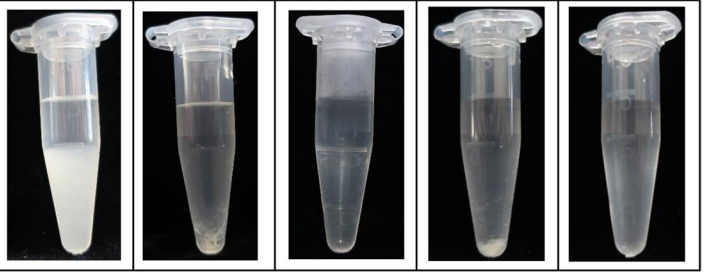
To evaluate whether this phenomenon is unique to strain RK14, we also cultured *B. licheniformis* ATCC14580 (Rey et al. 2004), which does not produce γ-PGA, and ATCC9945 (Mitsunaga et al. 2016), the typical γ-PGA producing strain, under the same conditions. The ATCC14580 strain grew well in LB and reached an $OD_{600}=7.5$, however, no precipitant was observed (Fig. 1a). The ATCC9945 strain also grew well ($OD_{600}=7.7$), and a very small amount of precipitate was observed. These results suggest that aggregate formation is unique to the RK14 strain cultured in LB and is not directly related to γ-PGA production.

To examine the effect of culture temperature on aggregate formation, the RK14 strain was cultured in LB at temperatures ranging from 30 to 50 °C. Culturing at 30 °C greatly decreased the amount of aggregate compared with that at 37 °C even though cell growth was similar. Aggregate formation at 42 °C was similar to that at 37 °C. A further temperature increase to 50 °C resulted in decreased aggregate formation despite promoting growth. The amount of aggregate was quantitatively determined by dry weight. As shown in Fig. 2b, the dry weight of aggregates at 37 °C and 42 °C were approximately 0.6 and 0.7 g/L, respectively, and significantly higher than those at both lower (30 °C) and higher (50 °C) temperatures. Thus, the optimal temperature for aggregate formation is approximately 40 °C.

Observation of cell aggregates of strain RK14

Aggregate formation is specific for the RK14 strain cultured in LB, and optimal aggregate formation occurs at approximately 40 °C. Furthermore, microscopic observation showed that aggregates have a cluster-structure. Considering that bacterial aggregate formation is usually induced when microbial cells are damaged or partial killed by physiological stress (Ojima et al. 2015, 2021), we evaluated cell viability of the RK14 strain aggregates with live/dead straining. In this assay, live cells were fluorescent green (Calcein-AM) and dead cells were fluorescent red (PI), and three-dimensional images were observed with the CLSM. As shown in Fig. 3, most cells were

(a)

Strain	RK14	RK14	RK14	ATCC 9945	ATCC 14580
Medium	LB	3YD	YFEb	LB	LB
OD_{600}	4.9	4.4	9.0	7.7	7.5
Photo					

(b)

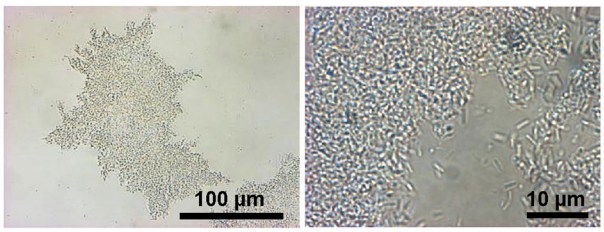
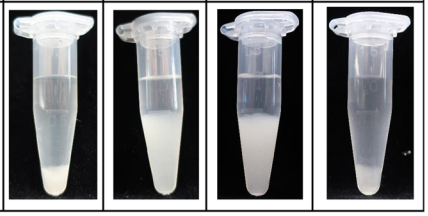


Fig. 1 Aggregate formation and cell growth of *Bacillus licheniformis* strains RK14, ATCC9945, and ATCC14580 at 37 °C. **a** OD_{600} and photos of aggregates in microtubes at 18 h with different culture media. OD_{600} was measured after removing aggregates through precipitation. **b** Microscopic images of aggregates formed by strain RK14 after 18 h of culture in LB

(a)

Temperature	30	37	42	50
OD_{600}	4.2	4.9	5.6	9.2
Photo				

(b)

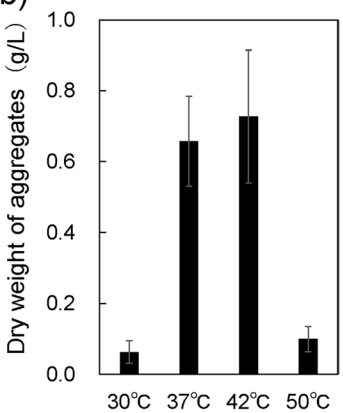


Fig. 2 Aggregate formation and cell growth of the RK14 strain cultured under different temperature conditions in LB. **a** OD_{600} and photos of aggregates in microtubes at 18 h. **b** Dry weight of aggregates formed by strain RK14 after 18 h. Data were obtained from 3 independent experiments. Vertical bars indicate standard deviations

fluorescent green and only a small number of cells were red, suggesting that the aggregates are mainly composed of viable cells. The merged image showed that dead cells were dispersed within the aggregate structure, indicating that aggregates do not originate from dead cell clumps.

Next, we performed time-lapse microscopic observations with photographs taken every hour. Figure 4 shows photographs of morphological changes at 1, 2, 4, 6, 8, and 10 h. After 1 h of culture, aggregates had already started to form, and microscopic images showed that most cells are several times longer than planktonic cells (Fig. 4a). The higher magnification image showed the presence of very long cells, especially at the edges of aggregates. At 2 h, the aggregates

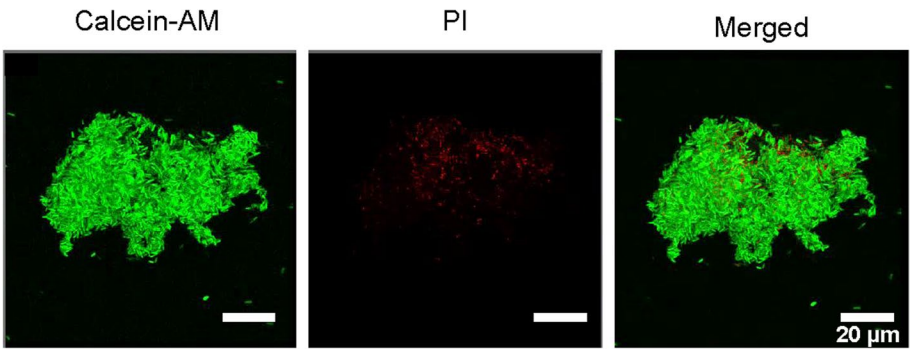


Fig. 3 Confocal scanning laser microscopy images of strain RK14 aggregates with live/dead staining. Green fluorescence shows the living cells stained with calcein-AM; red fluorescence shows the dead cells stained with PI

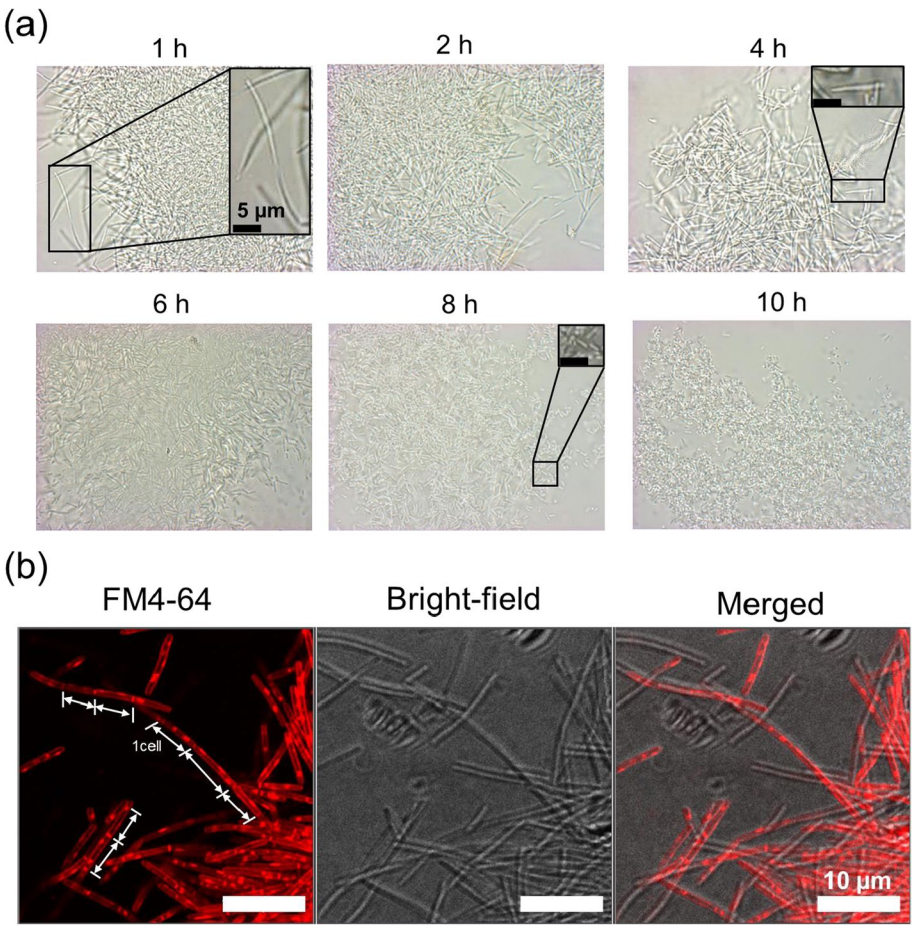


Fig. 4 Morphological changes in RK14 cells during aggregate formation. The RK14 strain was grown under shaking culture conditions in LB at 37 °C. **a** Time-lapse observation of aggregates at 1, 2, 4, 6, 8, and 10 h. **b** Aggregates at 2 h are stained with FM4-64, a membrane-specific dye

still included long cells. To confirm whether these long cells were elongated single cells or a chain of several cells, the aggregate at 2 h was stained with FM4-64, a plasma-membrane dye. Visualization of membranes with FM4-64 revealed that these long cells had a clear division plane for each cell unit, suggesting that the long cells were cell chains (Fig. 4b). It seems that these chains were tightly connected to form clusters (Fig. 4a)

and many chains are still observed in the aggregate structure. At 6 h, longer chains were observed at the center of aggregates, whereas shorter chains increased at the edges of aggregates, indicating that cell separation had occurred. At 8 h, most cells had returned to rod-shape and at 10 h most chains had disappeared, although the cells maintained the cluster structure. This temporal change in cell morphology was previously reported for *B. subtilis* pellicle formation (Kobayashi 2007), during which cell chains are formed, followed by clustering and degradation of cells chains. While pellicles are floating aggregates that form at the liquid–air interface, the aggregate formation in this study occurs submerged during shaking test-tube culture. From this point of view, the mechanism of submerged biofilm formation by *B. subtilis* was recently reported (Sanchez-Vizuete et al. 2022). In that report, cells adhering to the surface firstly form elongated chains before being suddenly fragmented and released as free motile cells in the medium. That switching coincided with an oxygen depletion in the well which preceded the formation of the pellicle at the liquid–air interface. Residual bacteria still associated with the solid surface at the bottom of the well started to express matrix genes under anaerobic metabolism to build the typical biofilm protruding structures. It was concluded that a floating pellicle and a submerged biofilm can form consecutively in the same system. In this study, oxygen supply seems to be higher than when cultured in static wells because the aggregate formation occurs during shaking test-tube culture. It is possible that the higher oxygen supply inhibits the release of free motile cells and leads to the maintenance of aggregates. Thus, the mechanism of aggregate formation in this study may be related to that of floating pellicle or submerged biofilm formation reported previously.

Effect of inhibition of cell elongation on aggregate formation

It was confirmed that transient cell elongation is important for aggregate formation of RK14 strain. From the aspect of cell elongation, recent single cell measurements revealed that Thioflavin T (ThT) decreased the elongation rate of *B. subtilis* at micromolar concentration (Han and Payne 2022). Therefore, we hypothesized that ThT might act as an inhibitor against aggregate formation due to the suppression of cell elongation. Aggregate formation of RK14 strain was examined in the presence of ThT. According to the previous report (Han and Payne 2022), ThT was added in LB at the range from 10 to 100 μM, and cell growth and aggregate formation were determined at 18 h. As results, the OD_{600} slightly decreased with 10, 20, and 50 μM ThT compared to without addition (Fig. 5). These results are corresponding to those of *B. subtilis* in the previous paper (Han and Payne 2022). At 100 μM, OD_{600} value was 0.1, indicating that higher concentration of ThT has an anti-proliferative effect on RK14 strain. The aggregate formation slightly decreased at 10 μM and dramatically decreased at 20 μM. At 50 μM, no aggregates were observed. Considering that the OD_{600} values at 10 and 20 μM are comparable, ThT seems to act as an inhibitor of aggregation rather than suppressing proliferation. In the previous paper, single cell analysis revealed that the elongation rate decreased by half even at 10μM ThT, suggesting that ThT inhibits aggregate formation by suppressing cell elongation. These results support the conclusion that transient cell elongation is key factor for aggregate formation of RK14 strain.

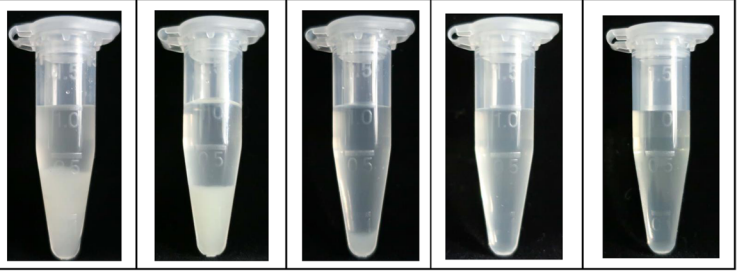
Thioflavin T [μM]	0	10	20	50	100
OD_{600}	5.1	4.4	4.2	3.7	0.1
Photo					

Fig. 5 Aggregate formation and cell growth of the RK14 strain cultured at 37 °C in LB in the presence of thioflavin T. OD_{600} values and photos of aggregates were obtained at 18 h

Table 1 Bacterial strains used in this study

Strains	Note	References
B. licheniformis RK14	High γ-PGA production strain isolated from the soil	Liu et al. 2017
B. licheniformis ATCC14580	Laboratory strain, no γ-PGA production	Rey et al. 2004
B. licheniformis ATCC9945	Model high γ-PGA production strain	Mitsunaga et al. 2016

Conclusions

In this study, self-aggregation of *B. licheniformis* RK14 was observed when cultured with LB at 37 to 42 °C with shaking. This phenomenon is not seen in laboratory strains of *B. licheniformis* or *B. subtilis*. The aggregates were composed of viable cells with cell–cell cluster-like structures. Time-lapse observation revealed that the individual cells grew longer after 1 h of culture and then returned to rod-shape over time, which is similar to previously reported morphological changes during *B. subtilis* pellicle formation. Aggregate formation was inhibited by ThT due to the suppression of cell elongation. It was concluded that transient cell elongation is critical during aggregation process of RK14 strain.

Materials and methods

Bacterial strains and media

Bacterial strains used in this study are listed in Table 1. The *Bacillus licheniformis* RK14 strain, which produces γ-PGA, was the same as that used in previous studies (Liu et al. 2017). *B. licheniformis* RK14 was originally isolated from the soil. A laboratory *B. licheniformis* strain ATCC14580, which does not produce γ-PGA, and the typical γ-PGA producing strain ATCC9945 were also used. Preculture of the *B. licheniformis* strains was performed in lysogeny broth (LB; 10 g/L Bacto^{TM} Tryptone, 5 g/L yeast extract, and 10 g/L NaCl). Aggregation test cultures of the *B. licheniformis* strains were performed in LB, typical culture medium 3YD (30 g/L yeast extract and 20 g/L D-glucose), and γ-PGA production medium YFEb (30 g/L yeast extract, 20 g/L D-fructose, 60 g/L sodium L-glutamate·H_2O, 20 g/L glycerol, and 30 g/L NaCl). All media were initially adjusted to pH 7.0 using NaOH solution.

Aggregation test culture of each *B. licheniformis* strain

For aggregation test culture, *B. licheniformis* was precultured in LB for 18 h at 37 °C and subsequently inoculated into test tubes containing 4 mL fresh LB, 3YD, or YFEb to an optical density (OD) at 600 nm = 0.01 and cultured at 37 °C with shaking at 140 strokes/min for 18 h. Thioflavin T (0 ~ 100 μM) was added in LB as an inhibitor for cell elongation (Han and Payne 2022). The effect of temperature was evaluated by culturing at 30, 42, and 50 °C, in addition to 37 °C. Cell growth was measured by OD_{600}. After 18 h of culture, the test tubes containing the aggregates and cell suspension were left to stand for 15 min. After 15 min, aggregates precipitated, whereas suspension cells remained in the supernatant. The OD_{600} of the supernatant was measured as an indicator of cell growth. Precipitates were resuspended in a 0.9% (w/v) NaCl solution and left to stand for 15 min. This step was repeated twice to remove cells loosely adsorbed onto the aggregates. The aggregates were transferred to microtubes and photographed using a digital camera. For quantitative assays, the aggregates were collected from the culture broth in ten test tubes and dried at 80 °C and the constant dry weight of aggregates determined.

Live/dead staining and FM4-64 staining

For live/dead staining, the aggregates were resuspended in phosphate-buffered saline (PBS) and then stained with 0.7μM Calcein-AM and 1.3μM propidium iodide (PI) in PBS for 15 min. The excess dye was washed with PBS. The aggregates were observed using a confocal laser scanning microscope (CLSM) (model DM6000B) with TCS SP8 software (Leica, Germany), with excitation at 488 nm and emission at 515 nm for Calcein-AM, and excitation at 538 nm and emission at 617 nm for PI.

For FM4-64 staining, the aggregates were fixed with 4% paraformaldehyde in phosphate buffer (PB) for 10 min. After washing with PBS, the aggregates were stained with 2 μg/mL FM4-64 (Kobayashi 2007) and then observed with the CLSM with excitation at 505 nm and emission at 725 nm.

Time-lapse observation of aggregation

For time-lapse observation of aggregate formation, strain RK14 was inoculated into test tubes containing 4 mL fresh LB to an OD_{600} = 0.01 and cultured at 37 °C with shaking at 140 strokes/min. The aggregate samples were removed from the test tubes and observed under a microscope (BX50, Olympus) every hour.

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Authors' contributions

YO and MA proposed the research concept and provided the necessary tools for the experiments and experimental instructions. YO wrote the manuscript.

SM designed and conducted the experiments and analyzed the data. The authors read and approved the manuscript.

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Availability of data and materials
The original contributions presented in the study are included in the article/supplementary material; further inquiries can be directed to the corresponding author.

Declarations

Ethics approval and consent to participate.
This article does not contain any studies with human participants or animals performed by any of the authors.

Consent for publication
N/A. This article does not involve human participants.

Competing interests
The authors declare that they have no competing interests.

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