Screening and identification of a *Penicillium brevicompactum* strain isolated from the fruiting body of *Inonotus obliquus* and the fermentation production of mycophenolic acid

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Abstract

Purpose Mycophenolic acid (MPA) is a fungal metabolite with a variety of biological activities and widely applied in clinical practices. We herein aimed to isolate a new *Penicillium brevicompactum* strain from the fruiting body of *Inonotus obliquus* to improve the production of MPA.

Methods The fruiting body of *Inonotus obliquus* was used to isolate *P. brevicompactum* strains. Identification of the *P. brevicompactum* strain was performed by sequencing and phylogenetic tree analysis. HPLC assay was conducted to identify the production of MPA. Submerged liquid fermentation and bi-directional fermentation were applied to improve the yield of MPA.

Result The candidates of the *P. brevicompactum* strain were isolated and screened from the fruiting body of *Inonotus obliquus* collected from Changbai mountains in China. Based on sequencing and phylogenetic tree analysis of 18S rDNA-ITS, the strain MC-4 was finally identified as *P. brevicompactum*. And HPLC assay indicated that the isolated *P. brevicompactum* strain could produce MPA in metabolic products. The optimized conditions of submerged liquid fermentation were as follows: 100 g/L Chinese yam in a liquid PDB medium, pH 6, fermentation temperature of 24 °C, shaker speed of 130 r/min, and fermentation time of 6 days. The maximum value of MPA production was 1.415 g/L after submerged liquid fermentation. Furthermore, the yield of MPA could be significantly increased to 1.537 g/L after bi-directional fermentation with the extractive from *fructus Swietenia macrophylla* (FSM).

Conclusion We demonstrated that a *Penicillium brevicompactum* strain isolated from the fruiting body of *Inonotus obliquus* can be used to improve the production of MPA by submerged liquid fermentation and bi-directional fermentation. This would provide a novel approach for more efficient and safer production of MPA.

Keywords Mycophenolic acid, *Inonotus obliquus*, *P. brevicompactum*, *Fructus Swietenia macrophylla*, Bi-directional fermentation

Introduction

Mycophenolic acid (MPA) is a fungal metabolite that mainly exists in the *Penicillium* genus, including *P. brevicompactum*, *P. stoloniferum*, *P. viridicatum*, and

others (Regueira et al. 2011). MPA was firstly discovered in 1893 and has been identified as an important bioactive substance with immunosuppressive, antiviral, antitumor, antibacterial, antifungal, and various activities (Carter et al. 1969; Hood and Zarembski 1997; Vinci et al. 2018). Notably, its derivative, mycophenolate mofetil, is a key immunosuppressant that is widely applied in the clinical transplantation of the liver, kidney, and heart (Okour et al. 2018; Zhang et al. 2018).

Because of the excellent medicinal value of MPA, optimization for MPA production process has always been a research hotspot. Currently, MPA is mainly produced by fungal fermentation, including solid state and

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liquid state fermentation (Xu and Yang 2007). However, traditional fermentation has a great limitation in MPA production due to the low efficiency and productivity of fungal strain, the difference in fermentation approach, and the deficiency of technological parameter. Most importantly, there are often toxic and side effects in MPA produced by traditional fermentation, which restrict the use of MPA in clinical practice (Mudge et al. 2004). Hence, further exploring new fungal strain and fermentation method with higher efficiency is necessary for the application of MPA.

Penicillium brevicompactum, one of the main members in the *Penicillium* genus, is a kind of filamentous fungus with highly medicinal value. As the main fungal strain producing MPA, *P. brevicompactum* is widely used in fermentation production of MPA (Regueira et al. 2011; Xu et al. 2017). *P. brevicompactum* is distributed throughout the natural world and can be isolated from soil, rotting vegetables, food, grains, textiles, dyes, and various other sources (Xu et al. 2017). Nevertheless, different *P. brevicompactum* strains derived from diverse sources often have varying efficiency in fermentation production along with different yield of MPA.

Inonotus obliquus, also known as Chaga, is a parasitic fungus that has broad-spectrum health benefit in traditional medicine for various health problems (Cui et al. 2005; Nakajima et al. 2007). As a kind of medicinal and edible fungus, *Inonotus obliquus* is generally parasitic on the birch trees with many years old. In the long-term parasitic processes of *Inonotus obliquus*, there often are some other beneficial microorganisms that may also live symbiotically or parasitically with the fruiting body of *Inonotus obliquus*, to survive by using the nutrition of birch tree or *Inonotus obliquus* (Géry et al. 2018; Wold et al. 2018). In the present study, we isolated and identified a *P. brevicompactum* strain from the fruiting body of *Inonotus obliquus*. Furthermore, we applied the *P. brevicompactum* strain isolated from *Inonotus obliquus* to improve the production of MPA by submerged liquid fermentation and bi-directional fermentation with the extractive from *fructus Swietenia macrophylla* (FSM). Our results would provide a novel approach for more efficient and safer production of MPA.

Materials and methods

Materials

The wild fruiting body of *Inonotus obliquus* was collected from birch with many years old in Changbai

mountains in Jilin (China). FSM was purchased from Solomon Islands by online purchasing. Chinese yam was purchased from Wenxian (Henan, China) by online purchasing. The standard of MPA was purchased from Zhongkezhijian Biotechnology Co. Ltd (Beijing, China).

Screening of *P. brevicompactum* strain

Nine cubes (0.5 cm³) of the fruiting body of *Inonotus obliquus* were obtained using a knife from three different parts in the fruiting body of *Inonotus obliquus*. Each cube of the fruiting body of *Inonotus obliquus* was washed with 1 mL sterile water for 4–5 times. The washing fluid was collected in a sterile condition and planted on the PDA solid medium (200 g potato, 18 g agar, 20 g glucose/1 L medium, pH = 6.0) containing cephalosporin C (10 µg/mL) for 6–12 days at 28 °C. Then, the colony was collected and planted on the PDA solid medium containing cephalosporin C to culture at 28 °C. After 6–12 days, the above steps were repeated until a purified single colony was obtained.

Identification of *P. brevicompactum* strain

The purified single colony was cultured in the PDA liquid medium containing cephalosporin C for 48 h at 24 °C in a shaking table at 130 r/min. After standing for 24 h, pale yellow mycelium was collected by filtration and washing. The genomic DNA was extracted from mycelium strain through a benzyl chloride method (Yang et al. 2014). PCR assay was then performed to obtain the ITS sequences of fungi strain. The primers were used as follows: ITS1: 5'-TCCGTAGGTGAACC TGCGG-3', and ITS4: 5'-TCCTCCGCTTATTG ATATGC-3'. PCR fragments were then conducted with sequencing identification. After a manual correction with BioEdit v.7.0, sequence alignment (BLAST) was performed with homologous *P. brevicompactum* for strain identification in public databases (<http://www.ncbi.nlm.nih.gov/BLAST/>).

And phylogenetic tree was constructed by MEGA 6.0 using the N-J method according to BLAST results. The fungal colony was preserved in a refrigerator at 4 °C for short-term preservation and in liquid nitrogen for long-term storage.

HPLC analysis of MPA

Fermentation products were filtrated with gauze and centrifuged at 3×10^3 r/min for 5 min, followed by filtration by a 0.45-µm filter. Extracted solution was analyzed by

HPLC assay using Agilent 1260 Series LC system. Related parameters were as follows: Phenomenex® Luna 5u C18 column (250 nm × 4.6 mm), mobile phase (phosphoric acid/water 40:60), detecting wavelength 240 nm, flow rate 1 mL/min, and sample amount 20 µL. Sample was identified by comparing the retention time and spectra with MPA standard.

Submerged liquid fermentation of *P. brevicompactum*

The effects of Chinese yam content, speed of shaking table, initial pH, and temperature on the production of MPA were studied. Peeled and cleaned Chinese yam was sliced and homogenized, then different concentrations of Chinese yam were added into the medium, followed by high temperature and high pressure sterilization. For culture of *P. brevicompactum*, the collected *P. brevicompactum* strain was firstly seeded on the PDA solid medium containing cephalosporin C for 6 days at 28 °C. Then, *P. brevicompactum* was collected using inoculating loop (0.5 cm²) from *P. brevicompactum* colonies with the same thickness on medium. And *P. brevicompactum* from one inoculating loop was inoculated in a 100-mL medium with pH 6 in a conical flask, conducted with liquid fermentation at 24 °C in a shaking table at 130 r/min. Three repeats were conducted for each group. The production of MPA was analyzed by HPLC analysis at 2, 4, 6, 8, and 10 days after fermentation.

Bi-directional fermentation for MPA production

Bi-directional fermentation for MPA production was performed by incubating with the extractive from FSM. In brief, FSM extractive was prepared by an ultrasonic method as the following parameters: methanol 85%, extraction ratio 1:15, time 40 min, and temperature 70 °C. Then, different concentrations (0, 0.1, 0.2, 0.3, 0.4, 0.5 g/L) of FSM extractive were added into the 100-mL liquid PDB medium (200 g potato, 20 g glucose/1 L medium, pH=6.0) supplemented with 100 g/L Chinese yam. The *P. brevicompactum* strain was seeded in the above medium and incubated for 6 days at 24 °C in a shaking table at 130 r/min. The production of MPA was analyzed by HPLC analysis.

Statistical analysis

Data were expressed as mean ± SEM. Differences between groups were analyzed by one-way ANOVA

using GraphPad Prism 6 (La Jolla, CA, USA). All experiments were performed independently at least three times. Statistical significance was defined by $P < 0.05$.

Results

Screening and characteristics of *P. brevicompactum* strain

To improve the production of MPA, we firstly isolated a *P. brevicompactum* strain from the fruiting body of *Inonotus obliquus*. As shown in Fig. 1, we screened potential *P. brevicompactum* strains by single colony and antibiotic screening method. Five possible strains were isolated from the fruiting body of *Inonotus obliquus* collected from Changbai mountains in China. The colonial morphology was seen in Fig. 2. Among all strains, MC-4 gave the characteristics of the *P. brevicompactum* strain that colony grew densely with villous texture, spore-producing structure was formed in large quantities, it was livid on the spore surface and light brown on the underside of colony, and there was no water-soluble pigment. Therefore, MC-4 was primary selected as the candidate of the *P. brevicompactum* strain.

Sequencing identification of *P. brevicompactum* strain

We further performed the sequencing analysis by 18S rDNA-ITS assay to identify the *P. brevicompactum* strain from five possible strains. The genomic DNA extracted from five strains were shown in Fig. 3a. Then, PCR assay was conducted with the primers of ITS1/ITS4. There were amplification products with 600 bp in all five strains. And sequence similarities approached 99% with the candidate sequences at GenBank in all cases, as seen in Table 1. According to the BLAST, strain MC-1 was identified as *Penicillium rudallense*, MC-2 and MC-3 were identified as *Alternariaster helianth*, MC-4 was identified as *Penicillium brevicompactum*, and MC-5 was *Cladosporium* sp. HNC12-38. Then, we constructed the phylogenetic tree of strain MC-4 based on ITS sequences. The strain MC-4 had the highest homology with *P. brevicompactum* (Fig. 3b, c). Taken together, these results suggest that the isolated strain MC-4 is *P. brevicompactum* and was selected for further study.

Fig. 1 Screening of the *P. brevicompactum* strain. The cubes of the fruiting body of *Inonotus obliquus* were collected and cultured on the PDA solid medium containing cephalosporin C (10 µg/mL) for 6–12 days at 28 °C. Then, the colony was collected and planted on the

PDA solid medium at same condition. The above steps were repeated until a purified single colony was obtained. **a** The primary fruiting body of *Inonotus obliquus*. **b** The screening of strains. **c** Cloning and purification of single colony

HPLC identification for the fermentation product of *P. brevicompactum*

To further determine the feasibility of isolated *P. brevicompactum* as the candidate strain for production of MPA, we analyzed the fermentation products of strain MC-4 by HPLC assay. Firstly, we confirmed that the parameters of HPLC assay for MPA standard were as follows: C18 column (250 nm × 4.6 mm, 5 µm), mobile phase (0.1% phosphoric acid/water 40:60), detecting wavelength 240 nm, flow rate 1 mL/min, temperature 40 °C, and sample amount 20 µL (Fig. 4a). Compared with the MPA standard, there was a

spectrum peak in the HPLC result of liquid fermentation broth from strain MC-4 at the same retention time 4.763 min with MPA standard (Fig. 4b). And the concentration of MPA was 65.40% in the sample of fermentation broth. This data indicate that the isolated *P. brevicompactum* strain can produce MPA in metabolic products.

Submerged liquid fermentation to improve the yield of MPA

As submerged liquid fermentation is an efficient method for obtaining fermentation products, we herein conducted and

Fig. 2 Characteristics of *P. brevicompactum* strain candidates. Five single colonies were selected as the candidates of *P. brevicompactum* strain. The characteristics of MC-1, MC-2, MC-3, MC-4, and MC-5 candidate strains were showed

Fig. 3 Sequencing identification of the *P. brevicompactum* strain. **a** The genomic DNA was extracted from five candidate strains and detected by electrophoresis assay. Then, PCR assay was conducted with the primers

of ITS1/ITS4. **b** The sequence of candidate strain MC-4 was showed. **c** The phylogenetic tree of strain MC-4 was constructed based on the ITS sequences by the N-J method

optimized the submerged liquid fermentation of the isolated *P. brevicompactum* strain to improve the yield of MPA. Firstly, the effects of Chinese yam content, initial pH, temperature, and speed of shaking on the production of MPA were investigated (Fig. 5A–D). We finally performed the submerged liquid fermentation of strain MC-4 in the liquid PDB medium supplemented with 100 g/L Chinese yam as source of nutrients at 24 °C and pH 6 condition in a shaking table at 130 r/min. Besides, we determined the effect of fermentation time on the production of MPA. Results showed that the yield of MPA was increased over time and reached the peak at 6 days after fermentation (Fig. 5E).

Furthermore, there was a significant change in the morphological feature of mycelium pellets of strain MC-4. The mycelium pellets have comparatively smaller volume with irregular spherical shape in the primary fermentation, whereas the volume of mycelium pellets were obviously enhanced and the shape were changed to regular sphere after submerged liquid fermentation (Fig. 5F). The maximum value of MPA production was 1.415 g/L after submerged liquid fermentation, which was significantly higher than 0.856 g/L in primary fermentation. These results indicate that the production of

MPA from the isolated *P. brevicompactum* strain MC-4 could be effectively improved by submerged liquid fermentation.

Bi-directional fermentation to improve the properties of MPA

Bi-directional fermentation is a new Chinese herbal fermentation technology that can effectively reduce the toxicity and improve the production of fermentation products (Xia and Pan 2010). In the present study, we performed the bi-directional fermentation of isolated *P. brevicompactum* strain with the extractive from FSM to further improve fermentation production of MPA. Firstly, FSM extractive was prepared by an ultrasonic method with the yield of 2.189 mg alcohol soluble substance in 1 g FSM. Then, the FSM extractive was added into the fermentation broth of *P. brevicompactum*, followed by submerged liquid fermentation. As seen in Fig. 6 a and b, yield of MPA was gradually enhanced with the increase of FSM extractive concentration and reached the peak (1.537 g/L) when the concentration of FSM extractive was 3 g/L. And there were also changes in the phenotypic characteristic of fermentation products. Taken together, these

Table 1 The identification of five *Inonotus obliquus*-derived candidate strains through sequencing of ITS1/ITS4 gene

Strains	Closest relative species	Accession number	Total scores	Identity percentage
MC-1	<i>Penicillium rudallense</i>	LT558912.1	1066	99
MC-2	<i>Alternariaster helianth</i>	AY154713.1	1026	99
MC-3	<i>Alternariaster helianth</i>	AY154713.1	1038	99
MC-4	<i>Penicillium brevicompactum</i>	LT558911.1	1075	99
MC-5	<i>Cladosporium</i> sp. HNC12-38	KT989383.1	941	99

results suggest that the *Penicillium brevicompactum* strain isolated from the fruiting body of *Inonotus obliquus* can be used to further improve the production of MPA by bi-directional fermentation with FSM extractive on the basis of submerged liquid fermentation.

Discussion

Mycophenolic acid (MPA) is a crucial fungal metabolite that has immunosuppressive, antiviral, antitumor, antibacterial, antifungal, and various bioactivities, and widely applied in clinical practice (Juki et al. 2006; Domhan et al. 2008; Morales et al. 2008; Heischmann et al. 2017). Therefore, it is always the research hotspot that finding more efficient strain producing MPA and optimizing the production process of MPA. In the present study, a multiphasic approach was applied to obtain the excellent *P. brevicompactum* strain and to improve the fermentation production of MPA.

Previous reports have demonstrated that screening on strain sources is necessary to obtain the suitable culture strains for fermentation production of MPA because of the greatly intraspecific differences (Vinokurova et al. 2005; Xu and Yang 2007). The main sources of MPA are the *Penicillium* genus, such as *P. brevicompactum* and *P. viridicatum*, which are widely distributed in food and other resources (Shaligram et al. 2008; Dong et al. 2015; Xu et al. 2017). *Inonotus obliquus*, an edible and medicinal fungus, is applied in food and health products in traditional medicine (Cui et al. 2005; Frisvad et al. 2013; Géry et al. 2018). And *Inonotus obliquus* has also been used to produce polysaccharides, phenols, and other secondary metabolites by specific liquid fermentation (Baek et al. 2018). However, it has not been used as the source of strain producing MPA in previous studies. We herein firstly isolated and identified a *P. brevicompactum* strain MC-4 producing MPA from the fruiting body of *Inonotus obliquus* by single colony screening and sequencing identification.

Notably, we also screened other four fungal strains from the fruiting body of *Inonotus obliquus* that belongs to the *Penicillium* genus and other imperfect fungi. A possible reason for this might be associated with the unknown complex composition of *Inonotus obliquus* as a parasitic fungus. *Inonotus obliquus* is generally parasitic on the birch trees with many years old and takes a long time to grow, in which there are some other microorganisms that may also live symbiotically or parasitically with the fruiting body of *Inonotus obliquus* (Géry et al. 2018; Wold et al. 2018).

Compared to traditional fermentation, submerged liquid fermentation has many advantages of shorter fermentation cycle, larger fermentation scale, and higher fermentation yield.

Fig. 4 HPLC identification for the fermentation product of the *P. brevicompactum* strain MC-4. **a** The HPLC analysis of MPA standard. **b** The HPLC result of liquid fermentation broth from the *P. brevicompactum* strain MC-4

Fig. 5 Submerged liquid fermentation of the *P. brevicompactum* strain MC-4. The collected *P. brevicompactum* strain MC-4 was seeded in a 100-mL medium with different Chinese yam content and pH conditions in a conical flask, conducted with liquid fermentation at different temperatures in a shaking under different speed, respectively. Then, **A–D** effects of Chinese yam content, pH, fermentation temperature, and

shaking speed on the production of MPA were tested. **E** The production of MPA was analyzed at 2, 4, 6, 8, and 10 days after fermentation. **F** The changes in the morphological feature of mycelium pellets of the *P. brevicompactum* strain MC-4 were analyzed in traditional (a) and (b) submerged liquid fermentation (* $P < 0.05$ vs. 60, 5.0, 20, 100, 2)

Additionally, it can increase the quantity of mycelium from fungal strain and produce more polysaccharides, terpenoids, vitamins, and other active ingredients, contributing to improve the efficacy, security, and quality control of fermentation products as potential drugs (Fazenda et al. 2008; Chi et al. 2018). In this study, we performed the submerged liquid fermentation with the isolated *Inonotus obliquus*-derived *P. brevicompactum* strain MC-4 to improve the production of MPA. The maximum value of MPA product was significantly increased to 1.415 g/L after submerged liquid fermentation, which was significantly higher than those in primary fermentation.

There are various factors that can influence the production of submerged liquid fermentation, including sugar content, speed of shaking table, initial pH, temperature, and fermentation time (Huang et al. 2009; Meng et al. 2016). We employed

the Chinese yam that contains abundant sugar, protein, vitamin, free amino acids, and variety of trace elements, to provide polysaccharides and nutrients for submerged liquid fermentation of the *P. brevicompactum* strain (Kong et al. 2009). Basing on the metabolism and life activities of *P. brevicompactum*, active components in Chinese yam can be bio-transformed, furthering to improve the metabolites and production of *P. brevicompactum* in submerged liquid fermentation (Dong et al. 2015; Xu et al. 2017). Herein, we observed significant increase of MPA production in submerged liquid fermentation with Chinese yam compared to those in traditional fermentation, that is consistent with previous reports in which Chinese yam was also used in fermentation to improve the properties of production (Kong et al. 2009; Shin et al. 2006).

Fig. 6 Bi-directional fermentation of the *P. brevicompactum* strain MC-4. The FSM extractive was prepared from *fructus Swietenia macrophylla* by the ultrasonic method and was added into the fermentation broth of *P. brevicompactum*, followed by submerged liquid fermentation. **a** The

changes in the phenotypic characteristic of fermentation products were determined after bi-directional fermentation. **b** Effects of FSM extractive on the production of MPA were analyzed (* $P < 0.05$ vs. 1)

Besides, temperature and/or pH could affect the activity of enzymes in fungal strains, to influence the production of fermentation substances. And the shaking speed can change fermentation quality by affecting the formation of mycelium pellets (Shih et al. 2009; Jagadeeshbabu and Viswanathan 2010). We determined the optimal condition of submerged liquid fermentation as temperature 24 °C, pH 6, and shaking speed at 130 r/min. Another factor affecting the yield of fermentation products is fermentation time (Wang et al. 2016). We observed that yield of MPA products reached the peak at 6 days after submerged liquid fermentation. This might be associated with the changes of mycelium pellets and biomass accompanied with the change of fermentation time. In submerged liquid fermentation, the *P. brevicompactum* strain goes into adjustment period at 0–2 days, logarithmic growth period at 2–6 days, stable growth period at 6–8 days, and decline period at 8–12 days.

Bi-directional fermentation is a new modern fermentation technology by constituting the mixed fermentation with medical fungi and traditional Chinese medicines (Yi et al. 2007). Through bi-directional fermentation, medical fungi can obviously improve the production and properties of fermentation products compared to those in traditional fermentation. And the toxic components of Chinese medicines, including alkaloid, lactone substances, glycoside compounds, toxic protein, heavy metals, and aristolochic acids anthraquinone and tannin, can be significantly reduced (Xia and Pan 2010; Liu et al. 2018). In previous literature reports, bi-directional fermentation technology has been widely used in fermentation applications related to food, medicine, cosmetics, and other industries and achieved very good application effects (Liang 2017; Ming et al. 2010; Chong et al. 2018).

Fructus Swietenia macrophylla (FSM), also known as *Swietenia macrophylla* King, is the fruit of a Meliaceae plant growing in Solomon Islands. There are abundant compounds

in the fruit of FSM, such as saponins, flavonoids, proteins, vitamins, and minerals, which contribute to the clinical application of FSM in treating diabetes, hyperlipidemia, hypertension, and other chronic non-communicable diseases (Lowe et al. 2003; Dewanjee et al. 2009). However, the negative factors, including bitter and side effect toxicity, limit the medicinal value of FSM. And there is cytotoxicity in the *P. brevicompactum* strain, which affect the production and application of MPA and other metabolites (Frisvad et al. 2013; Géry et al. 2018). Hence, based on the characteristics of *P. brevicompactum* and FSM, we performed the bi-directional fermentation of the isolated *P. brevicompactum* strain with FSM extractive for the first time. The yield of MPA was remarkably increased to 1.537 g/L compared to those in submerged liquid fermentation, with changed phenotypic characteristics of MPA products. This is mainly associated with the advantages of bi-directional fermentation technology (Ming et al. 2010; Chong et al. 2018; Sun et al. 2018). In the specific bi-directional fermentation, medicinal fungus *P. brevicompactum* was used as the fermentation strain, to form a fermentation combination with the medicinal matrix from FSM extractive. On the one hand, the active ingredients from FSM extractive can neutralize and improve the cytotoxicity of *P. brevicompactum*, thus increasing the production of MPA (Djalil et al. 2018; Dharmalingam et al. 2012). On the other hand, metabolites and fermentation of *P. brevicompactum* can also neutralize the side effect toxicity of FSM, achieving mutual benefits (Shaligram et al. 2008).

However, our study is not without its limitation. Whether and what changes are there in the properties of MPA products after bi-directional fermentation with FSM extractive should be determined via further pharmacological experiments in the future study.

Conclusion

We demonstrated that a *Penicillium brevicompactum* strain isolated from the fruiting body of *Inonotus obliquus* can be used to improve the production of MPA by bi-directional fermentation with FSM extractive on the basis of submerged liquid fermentation. These findings would provide a novel approach for more efficient and safer production of MPA.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Research involving human participants and/or animals N/A

Informed consent N/A

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