**Spiribacter aquaticus** sp. nov., a novel member of the genus **Spiribacter** isolated from a saltern

María José León,1 Borja Aldeguer-Riquelme,2 Josefa Antón,2 Cristina Sánchez-Porro1 and Antonio Ventosa1,*

**Abstract**

A moderately halophilic bacterium, designated strain SP301, was isolated from a solar saltern located in Santa Pola, Alicante, on the East coast of Spain. It was a Gram-stain-negative, strictly aerobic bacterium, able to grow in 7.5–25% (w/v) NaCl and optimally in 12.5% (w/v) NaCl. Phylogenetic analyses, based on 16S rRNA gene sequences, showed that the novel isolate is a member of the genus **Spiribacter**, with the most closely related species being **Spiribacter roseus** SSL501 (99.9% sequence similarity) and **Spiribacter curvatus** UAH-SP711 (99.4% sequence similarity). The 16S rRNA gene sequence similarity with the type species **Spiribacter salinus** M19-401 was 96.6%. The DNA–DNA relatedness value between strain SP301 and **S. roseus** SSL501 and **S. curvatus** UAH-SP711 was 40 and 55%, respectively; these values are lower than the 70% threshold accepted for species delineation. The major fatty acids were C16:0, C18:1ω7c, C19:0 cyclo ω8c and C12:0. Similarly to other species of the genus **Spiribacter**, strain SP301 was observed as curved rods and spiral cells. Metabolic versatility was reduced to the utilization of a few organic compounds as the sole carbon and energy sources, as with other members of **Spiribacter**. However, it differed in terms of colony pigmentation (brownish-yellow instead of pink) and in having a higher growth rate. Based on these data and on the phenotypic, genotypic and chemotaxonomic characterization, we propose the classification of strain SP301 as a novel species within the genus **Spiribacter**, with the name **Spiribacter aquaticus** sp. nov. The type strain is SP301 (=CECT 92381=LMG 300051).

Hypersaline environments are widely distributed around the Earth. They are represented by aquatic and saline terrestrial habitats, as well as salted products, such as salted foods, and marine or rock salt [1–3]. Most microbiological studies carried out in these environments have focused on saline aquatic systems. However many of these studies have been based on culture-dependent techniques, allowing only the isolation of microorganisms that do not constitute the most abundant bacterial inhabitants in these natural environments for many years. This has, therefore, prevented an understanding of the relationships and primary functions of the microorganisms in these natural environments [3–5].

Solar salterns that are traditionally used for the commercial production of salt are excellent models for studying microbial diversity, offering a wide range of salinities from seawater to salt saturation. Santa Pola saltern, located on the East coast of Spain, is one of the best known solar salterns worldwide. Numerous studies have focused on this multi-pond saltern. Earlier studies focused on the isolation and characterization of microorganisms in pure cultures, and later ones on culture-independent techniques, and more recent research on metagenomics [6]. Metagenomic-based studies carried out in solar salterns located in Spain [4–7] permitted the isolation of a new Gammaproteobacterium that appeared to be a bacterial representative of the dominant microbial population at intermediate salinities in these environments. It was described taxonomically as a novel genus and species, **Spiribacter salinus** [8]. The genus **Spiribacter** belongs to the family **Ectothiorhodospiraceae**, within the order **Chromatiales**, class **Gammaproteobacteria** in the phylum **Proteobacteria**. At the time of writing, the genus **Spiribacter** comprises three species with validly published names: the above mentioned **Spiribacter salinus** [8] and **Spiribacter curvatus** [9], as well as the more recently described **Spiribacter roseus** [10]. The complete genomes of all these species have been sequenced [11], demonstrating the smallest genomes described for members of the family **Ectothiorhodospiraceae**. Recent studies have shown evidence of the presence of species of the genus **Spiribacter** worldwide [12–14], constituting a dominant group in the concentrator ponds of solar salterns with intermediate salinities (10–25% salts) [8]. The genus **Spiribacter** includes moderately halophilic, Gram-stain-negative, non-motile, curved rods—nearly closed rings to spiral-shaped cells at the stationary phase, which produce pink colonies [8].

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**Keywords:** halophilic bacteria; Extremophiles; **Spiribacter**; saltern; hypersaline habitats.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of SP301 is LT714150.
The optimized culture conditions already used for the cultivation of the previously described species of the genus *Spiribacter* facilitate the isolation of novel strains closely related to this genus. In this sense during the course of studies on the microbial diversity of salterns in the East coast of Spain a novel halophilic microorganism, designated as strain SP30\(^T\), phylogenetically related to the genus *Spiribacter* was isolated. Here, we describe the isolation and characterization, based on a polyphasic approach, of this bacterium and propose it as a novel species of the genus *Spiribacter*.

Strain SP30\(^T\) was isolated from a water sample with 34.2 % (w/v) total salts obtained in May 2015 from a pond of a marine saltern located in Santa Pola, Alicante, on the East coast of Spain. Samples were collected in sterile containers, transported to the laboratory and plated on a 25 % (w/v) seawater salts solution (SW) supplemented with 0.1 % yeast extract, solidified with 2 % agar, and incubated at 37 °C for one month. The 25 % SW solution (described in [15]) contained (g l\(^{-1}\)): NaCl, 195; MgCl\(_2\).6H\(_2\)O, 34.6; MgSO\(_4\).7H\(_2\)O, 49.5; CaCl\(_2\), 0.7; KCl, 5.0; NaHCO\(_3\), 0.17; NaBr, 0.65. The pH was adjusted to 7.5 with 1 M KOH. The strain was isolated by directly loop-streaking the water sample on this medium after incubation under aerobic conditions at 37 °C. The strain was routinely grown in SMM medium previously used by León *et al.* [10] at 37 °C. The composition of the SMM medium was (g l\(^{-1}\)): NaCl, 46.8; MgCl\(_2\).6H\(_2\)O, 19.5; MgSO\(_4\).7H\(_2\)O, 30.5; CaCl\(_2\), 0.5; KCl, 3.0; NaHCO\(_3\), 0.1; NaBr, 0.35; casein digest, 5.0 and sodium pyruvate, 1.1. The pH was adjusted to 7.5 with 1 M KOH. Cultures were maintained at −80 °C in SMM medium containing 30 % (v/v) glycerol. The type strains *S. salinus* M19-40\(^T\), *S. curvatus* UAH-SP71\(^T\) and *S. roseus* SSL50\(^T\) were used as reference strains for comparative purposes in our study. They were cultivated under the same conditions as strain SP30\(^T\).

For the determination of cellular morphology and motility, strain SP30\(^T\) was grown on SMM medium on a shaking incubator at 200 rpm and examined by light microscopy under a phase-contrast microscope (Olympus; CX41). In the same way, as with other species of the genus *Spiribacter*, cells of strain SP30\(^T\) formed curved rods-nearly closed rings and short spiral shapes, of size 0.4 µm (width) by 1.5 to 1.8 µm (length). In media with higher salt concentrations the morphology of the curved cell rods became straighter. Cells of the novel isolate, SP30\(^T\), were non-motile, Gram-stain-negative and strictly aerobic. The morphology of the colonies, their size and pigmentation were observed on SMM solid medium after 5 days of incubation at 37 °C. Colonies were circular, entire, yellow-brown and 0.5–1.0 mm in diameter. Optimal conditions for growth were determined by growing the strains in a modified SW liquid medium previously used by León *et al.* [8], supplemented with sodium pyruvate and casein digest and lacking yeast extract, at 0, 3, 5, 7.5, 10, 12.5, 15, 17.5, 20 and 25 % (w/v) NaCl. The pH range for the isolate was tested at the optimal salt concentration for the novel strain, adjusting the medium to pH 5.0, 6.0, 7.0, 7.5, 8.0, 9.0 or 10.0 with the addition of the appropriate buffers [15]. Growth rates were determined by monitoring the increase in optical density at 600 nm. The optimal temperature for growth and the range were determined by incubating strain SP30\(^T\) at 4, 15, 20, 28, 30, 37, 40 and 45 °C. Strain SP30\(^T\) is a moderately halophilic bacterium able to grow in media with 7.5–25 % (w/v) NaCl present, with optimal growth at 12.5 % (w/v) NaCl. It is not able to grow in the absence of NaCl. The pH range for growth was 7–9, with optimal growth at pH 8. Cells were able to grow from 20 to 40 °C, with optimal growth at 37 °C. All biochemical tests were carried out in SMM medium at 37 °C, unless otherwise stated. Growth under anaerobic conditions (with H\(_2/CO_2\)) was determined by incubation of strain SP30\(^T\) in SMM solid medium in anaerobic chamber using Anaerogen (Oxoid) to generate an anaerobic atmosphere and an anaerobic indicator (Oxoid). Catalase activity was determined by adding a 1 % (w/v) H\(_2\)O\(_2\) solution to colonies on SMM agar medium. Oxidase activity was examined with 1 % (v/v) tetramethyl-p-phenylenediamine [16]. Hydrolysis of aesculin, casein, gelatin, DNA, starch or Tween 80, Voges-Proskauer and methyl red tests, production of indole, phosphatase, urease, nitrate and nitrite reduction were determined as described by Cowan and Steel [17]. Citrate utilization was determined on Simmons’s citrate medium supplemented with SMM medium lacking pyruvate and casein digest. Acid production from carbohydrates was determined using a modified phenol red base medium supplemented with 1 % carbohydrate, as previously reported by Ventosa *et al.* [18], supplemented with SMM medium lacking pyruvate and supplemented with casein digest instead of yeast extract. The carbohydrates tested were: L-arabinose, D-fructose, D-galactose, D-glucose, maltose, D-mannose, melezitose, D-raffinose, D-ribose, trehalose and D-xylose. In order to determine the range of substrates used as carbon and energy sources or as carbon, nitrogen and energy sources, the classical medium of Koser [19], as modified by Ventosa *et al.* [18], was used. Substrates were added as filter-sterilized solutions to give a final concentration of 1 g l\(^{-1}\), except for carbohydrates, which were used at 2 g l\(^{-1}\). When the substrate was an amino acid the basal medium was prepared without KNO\(_3\) and (NH\(_4\))\(_2\)HPO\(_4\). The substrates tested as sole sources of carbon and energy were: D-arabinose, cellobiose, fructose, D-galactose, D-glucose, lactose, maltose, D-mannose, melezitose, raffinose, trehalose, D-mannitol, methanol, D-sorbitol, xylitol, benzoate, citrate, formate, hippurate, pyruvate, succinate and D-l-tartarate. The following substrates were tested as sole sources of carbon, nitrogen and energy: L-alanine, L-arginine, L-asparagine, L-cysteine, glutamine, L-isoleucine, L-lysine, L-methionine L-ornithine, L-phenylalanine, L-threonine, L-serine and L-valine. Strain SP30\(^T\) had catalase and oxidase activity. The novel isolate was phosphatase and urease positive. Gelatin, DNA, starch, Tween 80 and aesculin were not hydrolysed by strain SP30\(^T\). Nitrate was not reduced to nitrite. The Simmon’s citrate test was negative and indole was not produced. Acid was produced from D-ribose but was not produced from many other carbohydrates that were tested, for example: D-glucose, trehalose, D-fructose, melezitose or maltose.
As previously reported for the other species of the genus *Spiribacter* [8–11], based on the analysis of the complete genome as well as on the data obtained in the laboratory, strain SP30T has simplified metabolic versatility, showing a very reduced ability to utilize organic compounds. Of the compounds tested, only pyruvate in the presence of casein digest was used as a carbon and energy source. Differential characteristics between strain SP30T and other closely related species of the genus *Spiribacter* are listed in Table 1.

Genomic DNA from strain SP30T was extracted and purified using the method described by Marmur [20]. The 16S rRNA gene was amplified by PCR with the forward primer 16F27 and the reverse primer 16R1488 [21]. Direct determination of the sequence of the PCR-amplified DNA was carried out using an automated DNA sequencer (model ABI 3130XL; Applied Biosystems). The almost-complete 16S rRNA gene sequence of strain SP30T (1411 bp) was deposited in GenBank and used for initial blast searches in GenBank and for phylogenetic analysis. The identification of phylogenetic neighbours and the calculation of pairwise 16S rRNA gene sequence similarities were achieved using the EzBioCloud tool [22]. 16S rRNA gene sequence analysis showed that strain SP30T was a member of the genus *Spiribacter*. The closest relatives were *S. roseus* SSL50T (99.9% sequence similarity) and *S. curvatus* UAH-SP71T (99.4% sequence similarity). The 16S rRNA gene sequence similarity with the type species of the genus *S. salinus* M19-40T was 96.6%. Other related species *Halopeptonella vilamensis* SV525T, *Alkalilimnicola ehrlichii* MLHE-1T, *Arhodomonas aquaeolei* ATCC 49307T and *Arhodomonas rectens* RS91T had 98.5, 95.4, 94.9 and 94.9% similarities to the novel isolate, respectively. The 16S rRNA gene sequence analysis was performed with the arb software package [23]. The 16S rRNA gene sequence was aligned with the published sequences from closely related bacteria and the alignment was confirmed and checked against both primary and secondary structures of the 16S rRNA molecule using the alignment tool of the arb software package. Phylogenetic trees were reconstructed using three different treeing methods: the maximum-parsimony [24], neighbour-joining [25] and maximum-likelihood [26] algorithms integrated in the arb software for phylogenetic inference. Bootstrap analysis was based on 1000 resamplings [27]. The 16S rRNA gene sequences used for phylogenetic comparisons were obtained from the GenBank database and their strain designations and accession numbers are shown in Fig. 1. The 16S rRNA-based phylogenetic trees clustered strain SP30T into a branch that was clearly related to species of the genus *Spiribacter* and it is further related to species of *Alkalilimnicola* and *Arhodomonas*. Strain SP30T was most closely related with the species *S. roseus* and *S. curvatus*. The high similarity value with respect to *S. roseus* SSL50T might indicate that the novel strain could constitute a member of this species. However, significant differences in cultures of strain SP30T with respect to the species *S. roseus*, and also *S. curvatus* were observed; strain SP30T was brownish-yellow instead of pink as are the other species of *Spiribacter*. In addition, the incubation time shown by this novel strain was also shorter, reaching higher optical densities than species of *Spiribacter* with validly published names. Thus, we decided to investigate the novel strain in more detail. Our phylogenetic analyses also show that the species *Halopeptonella vilamensis* is phylogenetically related to species of *Spiribacter* and it would be necessary to carry out a comparative study in order to determine its exact taxonomic position.

The G+C content of the genomic DNA was determined from the midpoint value (Tm) of the thermal denaturation profile [28] by using the equation of Owen and Hill [29],

### Table 1. Differential characteristics of strain SP30T and the type strain of the most closely related species of the genus *Spiribacter*

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell morphology</td>
<td>Curved rods-nearly closed rings, short spiral cells Brownish yellow</td>
<td>Curved rods-nearly closed rings, short spiral cells Pink</td>
<td>Thin curved rods, some spiral cells Dark pink</td>
<td>Thin curved rods, long spiral cells Pink</td>
</tr>
<tr>
<td>Colony pigmentation</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cell size (µm)</td>
<td>0.4×1.5–1.8</td>
<td>0.2–0.3×1.6–1.8</td>
<td>0.5–1.5</td>
<td>0.3×0.8–1.8</td>
</tr>
<tr>
<td>NaCl concn range</td>
<td>7.5–25</td>
<td>7.5–20</td>
<td>5–20</td>
<td>10–25</td>
</tr>
<tr>
<td>NaCl optimum</td>
<td>12.5</td>
<td>15.0</td>
<td>10.0</td>
<td>15.0</td>
</tr>
<tr>
<td>Temperature range for growth (°C)</td>
<td>20–40</td>
<td>15–40</td>
<td>5–40</td>
<td>15–40</td>
</tr>
<tr>
<td>pH range for growth</td>
<td>7.0–9.0</td>
<td>7.0–9.0</td>
<td>5.0–10.0</td>
<td>6.0–9.0</td>
</tr>
<tr>
<td>pH optimum</td>
<td>8.0</td>
<td>7.5–8.0</td>
<td>8.0</td>
<td>7.5–8.0</td>
</tr>
<tr>
<td>Urease</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Phosphatase</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>DNA G+C content (mol%)</td>
<td>65.4</td>
<td>64.2 (66.0*)</td>
<td>60.4 (63.9*)</td>
<td>60.0 (62.7*)</td>
</tr>
</tbody>
</table>

*Data from the genome sequence.*
Spiribacter aquaticus sp. nov. was isolated from a sample of water collected from a lake in Spain. The G+C content of the DNA for strain SP30\textsuperscript{T} was 65.4 mol\%. This value is within the range reported for species of the genus *Spiribacter*. Fatty acid analysis was performed using the MIDI Microbial Identification System [35]. Cells of strain SP30\textsuperscript{T} were cultured on SM15 agar medium at 37 °C [8] up to the late exponential phase, following the same methods described previously for species of the genus *Spiribacter* by León et al. [8–10]. The extraction and analysis of fatty acids were performed according to the recommendations of the MIDI system. This analysis was carried out by the CECT (Spanish Collection of Type Cultures, Valencia, Spain) using gas chromatography (Agilent 6850) and a standardized protocol, according to the MIDI Sherlock system [36].

The cellular fatty acid profile of strain SP30\textsuperscript{T} was characterized by the fatty acids C\textsubscript{16:0} (31.9 %), C\textsubscript{18:1\delta9c} (31.1 %), C\textsubscript{19:0 cyclo \omega8c} (9.9 %) and C\textsubscript{12:0} (8.5 %) as the major fatty acids (Table 2). The fatty acid composition of strain SP30\textsuperscript{T} was found to be similar to those of other species of the genus *Spiribacter*. Nevertheless, there were some differences in the proportions of some of the major fatty acids present in strain SP30\textsuperscript{T} compared to *S. roseus* and *S. curvatus*. For example, C\textsubscript{19:0 cyclo \omega8c}, which is present at a higher percentage in the novel isolate than in the other species of *Spiribacter*.

On the basis of evidence obtained from the polyphasic taxonomic analysis it is proposed that strain SP30\textsuperscript{T} should be considered to be a novel species within the genus *Spiribacter*, for which the name *Spiribacter aquaticus* sp. nov. is proposed.

**DESCRIPTION OF SPIRIBACTER AQUATICUS SP. NOV.**

*Spiribacter aquaticus* (a.qua‘ti.cus L. masc. adj. aquaticus living or found in the water, aquatic).
the presence of casein digest, is used as a carbon and energy source. Pyruvate, in xylene. Possess a very reduced ability to utilize organic compounds at 37°C. Moderately halophilic, able to grow in media with 7.5–12.5% (w/v) NaCl. No growth occurs in the absence of NaCl. The type strain is SP30T (=CECT 9238T=LMG 30005T), isolated from the water of a saltern pond located in Santa Pola, Alicante, Spain. The DNA G+C content of the type strain is 65.4 mol%.

Cells are Gram-stain-negative, non-motile, aerobic, curved rods-nearly closed rings and short spiral shapes, 0.4×1.5–1.8µm. Colonies are circular, entire, yellow-brown and 0.5–1.0 mm in diameter on SMM medium after 5 days of incubation at 37°C. Moderately halophilic, able to grow in media with 7.5–25% (w/v) NaCl, with optimal growth at 12.5% (w/v) NaCl. No growth occurs in the absence of NaCl. Able to grow in the pH range 7–9 and able to grow from 20 to 40°C, with optimal growth at pH 8 and at 37°C. Catalase- and oxidase-positive. Gelatin, DNA, starch, Tween 80 and asculin are not hydrolysed. Nitrate is not reduced to nitrite. Phosphatase- and urease-positive. Simmons’ citrate, methyl red and Voges-Proskauer tests are negative. Indole is not produced. Acid is produced from D-ribose, D-fructose, D-galactose, D-glucose, maltose, D-mannose, melezitose, raffinose, trehalose and D-xylene. Possess a very reduced ability to utilize organic compounds as the sole carbon and energy source. Pyruvate, in the presence of casein digest, is used as a carbon and energy source. The following compounds are not utilized as the sole carbon, nitrogen and energy source: L-alanine, L-arginine, L-asparagine, L-cysteine, glutamine, L-isoleucine, L-lysine, L-methionine L-ornithine, L-phenylalanine, L-threonine, L-serine and L-valine. The major cellular fatty acids are C₁₆₀, C₁₈₀:1ω7c, C₁₉₀:0 cyclo ω8c and C₁₂:0.

### Table 2. Cellular fatty acid composition of strain SSL50T and the most closely related species of the genus Spiribacter

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
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<tbody>
<tr>
<td>C₀₉₀</td>
<td>–</td>
<td>–</td>
<td>0.6</td>
<td>–</td>
</tr>
<tr>
<td>C₁₀₀</td>
<td>1.4</td>
<td>0.7</td>
<td>0.6</td>
<td>1.2</td>
</tr>
<tr>
<td>C₁₀₀:3-OH</td>
<td>3.3</td>
<td>3.3</td>
<td>4.0</td>
<td>6.4</td>
</tr>
<tr>
<td>C₁₁₀</td>
<td>8.5</td>
<td>6.7</td>
<td>3.5</td>
<td>5.7</td>
</tr>
<tr>
<td>C₁₂₀:3-OH</td>
<td>3.2</td>
<td>3.1</td>
<td>1.3</td>
<td>1.2</td>
</tr>
<tr>
<td>C₁₄₀:ω5c</td>
<td>–</td>
<td>–</td>
<td>0.8</td>
<td>–</td>
</tr>
<tr>
<td>Summed feature 2*</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>2.0</td>
</tr>
<tr>
<td>Summed feature 3*</td>
<td>1.9</td>
<td>2.5</td>
<td>8.6</td>
<td>3.6</td>
</tr>
<tr>
<td>C₁₄₀</td>
<td>1.8</td>
<td>0.6</td>
<td>0.8</td>
<td>0.8</td>
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<tr>
<td>C₁₆₀</td>
<td>31.9</td>
<td>28.3</td>
<td>9.3</td>
<td>13.4</td>
</tr>
<tr>
<td>Summed feature 8*</td>
<td>31.1†</td>
<td>50.0†</td>
<td>60.6†</td>
<td>60.6†</td>
</tr>
<tr>
<td>C₁₇₀</td>
<td>–</td>
<td>–</td>
<td>1.8</td>
<td>–</td>
</tr>
<tr>
<td>C₁₇₀:ω8c</td>
<td>–</td>
<td>–</td>
<td>2.1</td>
<td>–</td>
</tr>
<tr>
<td>C₁₇₀:ω6c</td>
<td>–</td>
<td>–</td>
<td>1.0</td>
<td>–</td>
</tr>
<tr>
<td>C₁₈₀</td>
<td>4.6</td>
<td>2.1</td>
<td>1.2</td>
<td>1.6</td>
</tr>
<tr>
<td>C₁₈₀:ω9c</td>
<td>–</td>
<td>0.8</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Summed feature 7*</td>
<td>2.3</td>
<td>3.0</td>
<td>1.9</td>
<td>0.6</td>
</tr>
<tr>
<td>C₁₉₀:ω6c</td>
<td>9.9</td>
<td>3.9</td>
<td>1.0</td>
<td>2.2</td>
</tr>
</tbody>
</table>

*Summed features are groups of two or three fatty acids that could not be separated by GC with the MIDI system. Summed feature 3 comprised C₁₆₀:ω5c and/or C₁₈₀:ω7c, summed feature 7 comprised un 18,846/C₁₉₀:ω6c and summed feature 8 comprised C₁₈₀:ω6c and/or C₁₈₀:ω7c.

†This percentage corresponds to C₁₈₀:ω7c.
‡This percentage corresponds to a mixture of C₁₈₀:ω7c and/or C₁₈₀:ω6c.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

Ethical statement
No experimental work with animals or human has been carried out in this study.

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