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ORIGINAL PAPER

Towards a Sustainable Route for the Production of Squalene Using Cyanobacteria

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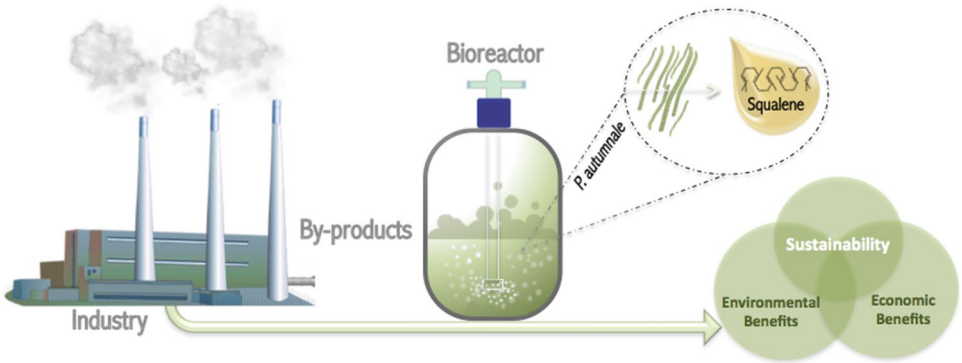
Abstract

Treatment of slaughterhouse wastewater is a huge industrial problem. The use of an algae biorefinery platform could be a sustainable technological alternative that produces value-added compounds instead of dumping the wastewater. For this reason, this research aimed to evaluate squalene production from the microalgae *Phormidium autumnale* cultivated using agroindustrial wastewater. A derivatization method was performed to determine the squalene and fatty acids content, evaluated by gas chromatography with flame ionization and mass spectrometry detectors. A total of 0.18 g/kg of squalene were found in the biomass, with a high content of unsaturated fatty acids (52%). Sensitivity analysis estimated production of 727–72,750 kg/year in industries with different capacities. In this sense, *P. autumnale* in agroindustrial wastewater could offer a potential alternative method of squalene production.

Graphical Abstract



Keywords Bioactive compound · Gas chromatography · Microalgae · *Phormidium autumnale* · Sensitivity analysis

Introduction

Squalene, a natural triterpene, is a putative head-to-head condensation product of farnesyl diphosphate, catalyzed by squalene synthase enzymes [1]. Squalene biosynthesis in prokaryotic microorganisms occurs until the building blocks isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP), a pathway that is also responsible for the formation of many terpenoids [2]. Squalene can be found in diverse types of cells, playing an important role

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as an intermediate in sterol biosynthesis, and also impacts human health [3]. Various positive effects of this triterpene on health have been reported, including for use in the treatment of cancer, in lipid-based anticancer prodrugs for chemotherapy, as an antiviral treatment against the hepatitis C virus, as a means of cardiovascular protection, and for its antioxidant activity [4–9].

Shark liver oil, traditionally, is the richest source of squalene, but use of this resource conflicts with marine wildlife preservation. In this sense, the biotechnological route of squalene production has been increasing, especially given the higher productivity and yield obtained from this process [10].

In this sense, microalgae biotechnology offers an alternative for squalene production, as shown by the microalgae *Aurantiochytrium mangrovei*, which presents high squalene content that ranges from 0.2 to 0.4 g/kg of cellular dry mass [11, 12]. The cyanobacteria *Synechocystis* sp. PCC 6803 was used for the same purpose, genetically engineered to optimize yield, with accumulation of 0.80 g/kg [13]. There are some differences among cell structures of microalgae. *Aurantiochytrium mangrovei* is a eukaryote algae, while *Synechocystis* sp. PCC 6803 is a prokaryote microorganism. The largest difference is the metabolic pathway involved in squalene production. In eukaryotic cells, two pathways are responsible for squalene production, the mevalonate pathway (MVA) and the methylerythritol pathway (MEP). For prokaryotic cells, by contrast, MEP is responsible for producing this secondary metabolite [14]. Cyanobacteria are today a potential source of many bioactive compounds, mostly secondary metabolites, and the production of these compounds is strongly related to environmental conditions [15]. In this sense, genetic engineering is not always necessary to produce squalene; it may be accomplished through environmental modification to activate a specific metabolic route.

Cyanobacteria are photosynthetic, prokaryotic microorganisms and can produce numerous metabolites in diverse environments because they are robust and grow in different temperatures support high nutrient concentrations, and have metabolic versatility, belong to a group of diazotrophs, which allows some strains, to perform nitrogen fixation [16]. Therefore, some strains can also achieve respiration using an exogenous source of carbon [17]. These particularities allow wastewater to be used as a carbon source providing tertiary treatment, using substrates with higher concentrations of nitrogen and phosphorous. The *Phormidium autumnale* cyanobacteria is a filamentous cyanobacterium, that possesses these abilities [18]. Also, another study observed that this strain demonstrates high capacity for the removal of organic matter [19] alongside the simultaneous production of high-value, unsaponifiable compounds, such as carotenoids [15, 20, 21].

In fact, agroindustrial wastewater is a great alternative for use as a carbon source, offering new technological routes to produce valuable compounds from biomass [22]. In this context, another study reported using living organisms to produce fine chemicals, such as polyhydroxyalkanoates (PHAs), from slaughterhouse wastes, proving that these processes emerge as a "white biotechnology" process [23]. Also, use of industrial slaughterhouse waste reduces process costs while simultaneously minimizing hazardous environmental emissions [24].

For reasons of environmental policy, then, and in light of economic, sustainability, and practical perspectives, it is important to demonstrate the feasibility of such bio-products [25]. These bio-compounds could emerge from algae biorefinery platforms, thus reducing the environmental implications of their waste precursors [26]. In this regard, this work aims to evaluate a sustainable route to produce squalene from biomass of the cyanobacteria *Phormidium autumnale*.

Materials and Methods

Microorganisms and Culture Media

Stock axenic cultures of *P. autumnale*, originally isolated from the Cuatro Cienegas desert (26°59′N, 102°03′W, in Mexico), were propagated and maintained in solidified agar–agar (20 g/L) containing synthetic BG11 medium [27]. The incubation conditions used were 25 °C, a photon flux density of 15 µmol/(m s), and a photoperiod of 12/12 h light/dark.

Wastewater

Slaughterhouse wastewater was acquired from an industrial facility located in Santa Catarina, Brazil (27°14′02″S, 52°01′40″W). This wastewater, obtained from the discharge point of an equalization tank over a period of 1 year, was carefully studied for hydrogenionic potential (pH), chemical oxygen demand (COD), total phosphorus ($P–PO_4^{3-}$), Total Kjeldahl Nitrogen (N-TKN), volatile solids (VS), fixed solids (FS), total solids (TS), and suspended solids (SS), following the standard methods for the examination of water and wastewater [28]. The average composition of the wastewater was pH 5.9 ± 0.05, COD 4100 ± 874 mg/L, $P–PO_4^{3-}$ 2.84 ± 0.2 mg/L, N-TKN 128.5 ± 12.1 mg/L, VS 2.9 ± 1.4 mg/L, FS 0.9 ± 0.3 mg/L, TS 3.8 ± 2.7 mg/L, SS 1.9 ± 0.8 mg/L.

Production of Microalgal Biomass

Biomass was produced in heterotrophic conditions, using slaughterhouse wastewater as the culture medium.

Cultivations were performed in a bubble column bioreactor [29] operating under a batch regime and fed on 2.0 L of wastewater. The experimental conditions were as follows: initial concentration of inoculum 100 mg/L, temperature 26 °C, pH adjusted to 7.6, carbon-to-nitrogen ratio of 30 (adjusted when necessary with glucose), aeration of one unit volume of air per unit volume of wastewater per min, absence of light, and residence time of 168 h [17]. The biomass was separated from the wastewater by centrifugation and subsequently freeze-dried for 24 h at − 50 °C under − 175 mmHg. The cultivations were performed twice and in duplicate, so experimental data refer to the mean value of the four repetitions.

Sampling and Data Analysis

The samples were collected aseptically every 24 h during the microorganism growth phase, and biomass data were used to calculate the biomass $[P_X = (X_i - X_i - 1)\cdot(t_i - t_{i-1})^{-1}$, mg/(L h)] and lipid $[P_L = P_X \cdot L$, mg/(L h)] productivities, in which X_i is the biomass concentration at time ti (mg/L), X_{i-1} is the biomass concentration at time ti − 1 (mg/L), t is the residence time (h), and L is the lipid content of the *Phormidium autumnale* biomass (%). Total concentrations of organic carbon were used to calculate the substrate consumption rate $r_S = dS/dt$, mg/(L h), and the biomass yield coefficient $Y_{X/S} = dX/dS$ (g cell/g) substrate, where S_0 is the initial substrate concentration (mg/L), S is the substrate concentration (mg/L), and t is the time (h).

Experiment

Reagents

The following analytical grade reagents were used: methanol and chloroform from Vetec (São Paulo, SP, Brazil); anhydrous sodium sulfate, sodium methoxide, methanolic solution (1 M), methyl acetate, and diethyl ether from Sigma-Aldrich (St. Louis, MO, USA); and oxalic acid from Synth (São Paulo, SP, Brazil). Hydrochloric acid and 0.05% butyl hydroxyl toluene (BHT) from Dinâmica (São Paulo, SP, Brazil) were used in the chloroform extractor. Hexane was also from Dinâmica (São Paulo, SP, Brazil). A squalene standard (98.9%) and a mixture of fatty acid methyl esters (FAME) Mix-37 (P/N 47885-U) were obtained from Sigma-Aldrich. The squalene stock solution, with 1 mg/mL concentration, was prepared by weighing 10 mg of the standard in a volumetric flask of 10 mL and completing with hexane.

Lipid Extraction

The total lipid fraction from the dry biomass was extracted by a modified version of the method of Bligh and Dyer [30]. In this method, around 0.5 g of cyanobacteria samples were pre-treated with 5 mL hydrochloric acid (2 M) solution to allows the cells rupture. Afterward, extraction was performed in the absence of light, and the lipid content was determined gravimetrically. After each extraction procedure, the chloroform–lipid extracts were evaporated at 50 °C under vacuum (− 760 mmHg) and submitted to the transesterification method.

Derivatization

Fatty acid methyl esters were obtained following Christie [31]. Around 50 mg of microalgal lipid extract was inserted into a flask tube, to which 2 mL of hexane plus 40 µL of methyl acetate were added. Homogenization by vortex followed for 30 s. Then, 60 µL of sodium methoxide methanolic solution (1 M; methylation solution) was added, with shaking for 2 min. A solution of oxalic acid in diethyl ether (0.4 M; termination solution) solubilized the polar lipids. Then, hexane was added, standing alone for 1 h at ambient temperature, and the extract was centrifuged at 1775×*g* for 5 min. The supernatant was transferred into a 1.5 mL vial for further chromatographic analysis.

Fatty Acid Profile

The methylated samples were analyzed using a gas chromatographic instrument equipped with a flame ionization detector (GC-FID), Varian 3400 (Palo Alto, CA, USA), and an autosampler, Varian 8200 (Palo Alto, CA, USA). An aliquot of 1 µL of the sample was injected into a split/splitless injector, operating in split mode, with a 50:1 ratio at 240 °C. The carrier gas was hydrogen under constant pressure of 20 psi. The FAME were separated using a capillary column, SP-2560 Supelco (Bellefonte, PA, USA, 100 m × 0.25 mm × 0.20 µm). The temperature of the oven was initially 80 °C (hold time of 5 min). Afterward, the temperature was increased to 175 °C at the rate of 15 °C/min, to 190 °C at the rate of 5 °C/min, and finally up to 240 °C at the rate of 8 °C/min, maintaining isothermal conditions for 15 min. The FID temperature was held steady at 280 °C.

The FAME were identified using the authentic standard, FAME Mix-37. The fatty acid methyl ester standard was evaluated under the same conditions; consequently, its retention times were used to identify the fatty acids, expressed as percentages of the total chromatographic area.

Squalene Determination

The same FAME extract was used for squalene analysis. The injection port of the GC-FID was operated in splitless mode (splitter valve off by 0.8 min; 30:1) at 280 °C. Hydrogen at constant pressure of 15 psi was used as the carrier gas. Separation was performed in a non-polar column, RTX-5MS Restek (Bellefonte, PA, USA, 30 m × 0.25 mm i.d. × 0.25 µm). The temperature was initially 200 °C, with an increase to 280 °C at a rate of 15 °C/min and then to 330 °C at a rate of 5 °C/min, maintaining isothermal conditions for 10 min. The temperature of the detector was maintained at 280 °C.

Quantification was acquired using a five-point analytical curve (10–50 mg/L), and some validation parameters were studied, such as linearity. For this purpose, a linear regression equation was used to determine the linear correlation coefficient (R^2) of the calibration curve, with precision expressed as relative standard deviation (RSD). The limit of detection (LOD) was estimated according to the concentration of the compound at a signal-to-noise ratio of three. The limit of quantification (LOQ) was achieved by injecting sequential dilutions of the standards, with the LOQ calculated as the concentration that results in a signal-to-noise ratio greater than or equal to 10. Accuracy was determined by a recovery assay from samples spiked with a known (20 mg/L) amount of the standard, with accuracy expressed as percentage recovered of the standard.

Positive identification of squalene in the samples was performed by comparing the retention time and mass spectra obtained experimentally from an authentic standard solution. Identification was performed using gas chromatography coupled to a mass spectrometer (GC/MS), Shimadzu QP-2010 Plus (Tokyo, Japan), under the same chromatographic conditions as described for GC-FID, except with helium as the carrier gas. The GC/MS interface and ion source (+ 70 eV) were held at 280 °C, and the single, quadrupole mass analyzer was operated in scan mode (35–350 m/z).

Sensitivity Analysis: Estimate of Squalene Production

The annual production of squalene by the biomass was estimated for industries with different capacities (100; 1000; and 10,000 m^3/d) operating 24 h per day, 336 days per year. Biomass and squalene concentration data were used to calculate squalene productivity [$P_S = P_X \cdot S$, mg/(L h)], where P_X is the biomass productivity and S is the squalene concentration in the biomass (mg/g).

Results and Discussion

Squalene Determination in Cyanobacterial Biomass

Squalene was positively identified in the biomass lipid extract, and suitable chromatographic selectivity (Fig. 1). In the evaluated linear range (10–50 mg/L), a correlation coefficient of 0.998 was observed, indicating satisfactory linearity under this method. The calibration curve was constructed by plotting the peak area versus the squalene concentration, with parameters of 3822.1 for the slope and 14,216 for the intercept. This method shows a LOD and LOQ of 0.3 and 1.0 ng/L, respectively. The precision of this extraction method, expressed as relative standard deviation (RSD), was 12.0%. Accuracy was acquired in triplicate, with a spike of 20 mg/L standard recovered and an average result of 101%. to European Commission [32], acceptable values of recovery range from 70 to 120%; hence, the result in this instance can be considered acceptable.

Estimate of Squalene Production in Heterotrophic Cultivation

Squalene production by the cyanobacteria *Phormidium autumnale* was observed, with the values shown in Table 1. Squalene was quantitatively evaluated in dry biomass to have a concentration of 0.18 g/kg. The total lipid fraction

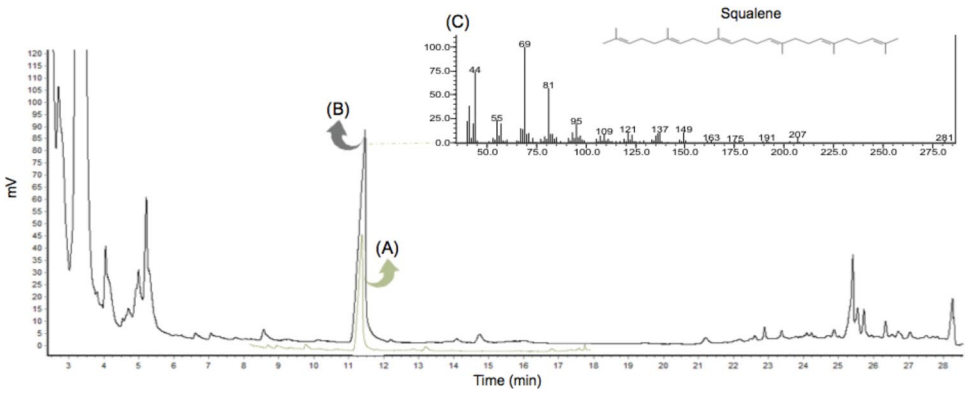

Fig. 1 GC-FID chromatograms of squalene from (*A*) authentic standard and (*B*) extracted from *Phormidium autumnale* wastewater biomass and (*C*) squalene mass spectrum and chemical structure

Table 1 Kinetic of growth and squalene productivity of *Phormidium autumnale*

Squalene (g/kg CDM)	0.18
Total Lipids (g/kg)	103.0 ± 0.01
P_{sq} [mg/(L h)]	$5.5 \times 5.6 \times 10^{-3} \pm 0.24$
P_x [mg/(L h)]	15.0 ± 6.40
P_L [mg/(L h)]	1.5 ± 1.10
$Y_{x/s}$ (mg biomass/g carbon)	320.0 ± 0.0
$Y_{pl/s}$ (mg squalene/g carbon)	$5.8 \times 10^{-2} \pm 0.0$
$Y_{ps/s}$ (mg lipids/g carbon)	33.0 ± 0.0
tg (h)	48 ± 0.0

CDM Cellular dry mass, *Px* biomass productivity, *Psq* squalene productivity, P_L lipid productivity, $Y_{x/s}$ biomass yield coefficient, $Y_{p/s}$ squalene yield coefficient, $Y_{p/s}$ lipid yield coefficient, *tg* generation time

extracted was 103.0 g/kg. Regarding growth kinetics, this study showed high biomass productivity of 15 mg/(L h). Also, considering that this bioactive compound is lipophilic and intracellular, squalene productivity is expressed as squalene content multiplied by biomass productivity, giving squalene productivity of 5.6×10^{-3} mg/(L h) in parallel with 1.5 mg/(L h) of lipid productivity. Additionally, the cultivation system is related to conversion of organic carbon alongside the production of compounds with metabolic activity. Observed carbon conversion yields amounted to 5.8×10^{-2} ($mg_{squalene}/g_{carbon}$), lipid conversion yields were 33.0 (mg_{lipids}/g_{carbon}), and biomass yields were 320 ($mg_{biomass}/g_{carbon}$) with the cultivation system used.

In this sense, considering that today's global market has increased demand for bioactive compounds, exploration of cyanobacterial biomass for industrial-scale production, has a great future, because such biomass is established as a commercial source of high-value chemicals and commercial products, such as β-carotene, docosahexaenoic, eicosapentenoic acid, and phycobilin pigments [33–36].

Distinct studies have not detected squalene in wild types microalgae cell of the species *Chlamydomonas reinhardtii* and *Phaeodactylum tricornutum* [37, 38]. Therefore, some strains are squalene accumulators, such as *Schizochytrium mangrovei* and *Botryococcus braunnii*, for which studies found concentrations of 0.16 and 1.80 g/kg, respectively [39, 40].

Likewise, distinct biotechnological routes for the microbial production of squalene have been explored, including using the *Saccharomyces cerevisiae* strain, which presents a concentration of 0.04 g/kg, lower than the result acquired from the present work. On the other hand, in one study, *Saccharomyces cerevisiae* presented a concentration of 1.30 g/kg at the end of the fermentation process [41]. Also, the yeast *Torulaspora delbrueckii* showed elevated production of 0.43 g/kg [42].

Unfortunately, deep-sea shark liver oil is the most common form of squalene isolated for supplemental use by the pharmaceutical industry. Squalene is commonly found in deep-sea sharks due to its role in neutral buoyancy. The squalene concentration is species-dependent. For example the *Portuguese dogfish* presents with 37.37%, *Leafscale gueper* with 49.89% and *Black dogfish* with 35.38% of the liver oil [43], while *Centrophorus scalpratus*, at 82.00%, presents substantially higher [44].

However the cyanobacteria can stand out as squalene producer, because demonstrate higher production rates during entire year and do not require a long period of time to acquire the compound, representing a renewable source. In addition, squalene's several demonstrated applications in the food industry [45]. Also, considering that, the *Phormidium autumnale* possess ability to grow in diverse environments, as shown by the high biomass conversion efficiency of manipueira (cassava wastewater) under heterotrophic cultivation, which results in biofuel production [46]. Likewise, this cyanobacteria have been reported as potential producer of carotenoids; when biomass is cultivated in agroindustrial wastewater, pronounced production of 107,902 kg/year was obtained [21]. Therefore, a techno-economic analysis using the *Phormidium autumnale* strain showed that, using agroindustrial wastewater and estimating commercial biomass value at USD 480/ton, a profit margin of 94% is possible to obtain [47].

Given this possibility, a sensitivity analysis was performed to express the production of squalene from cyanobacterial biomass. The calculations assumed different industrial capacities. Given a small industry with 100 m^3/days of capacity it is possible to produce 727 kg/year of squalene. In turn, a midsize industry of 1000 m^3/days, can be produced 7275 kg/year of squalene, and a large industry of 10,000 m^3/days, can produce 72,750 kg/year of squalene.

Further, in comparison with deep-sea sharks. For an adult *Echinorhinus brucus* shark species, the average squalene concentration in the liver oil on average is 38.5% [48], the liver from male sharks weights about 4.25 kg [49], and it is well known that the oil content can range from 10 to 70% in shark livers [50]. Given 10% squalene concentration in shark liver oil, one animal on average reaches 0.16 kg of squalene, while 70% squalene concentration in shark liver oil would reach 1.15 kg of squalene per animal. These concentrations promote intense shark hunting. From this traditional source, in order to obtain an amount similar to that which could be acquired from a small industry, it is necessary to slaughter at least 635 of sharks containing 70% of liver oil and 4446 animals with livers containing 10% of the oil. Squalene extraction from cyanobacteria by using food wastewater as a substrate proved to be an alternative to shark slaughter. For this reason, extracting squalene from cyanobacteria using

food wastewater as a substrate proved to be an alternative to shark slaughter.

Finally, the squalene content of a microorganism is located in the cells, more specifically, the configuration of squalene determines its location, for example, if the structure of squalene is similar to a sterol configuration, it probably stays into the cellular membrane [2]. Thus, the squalene in cyanobacteria should be considered an intracellular component, situated in the lipid fraction, the extraction of which occurs simultaneously with the extraction of lipid content from the biomass.

Generally, the oil composition of single cell lipid presents metabolites with high nutritional value beyond squalene, such as fatty acids, particularly those with an unsaturated profile. Ten fatty acids were identified in the cyanobacterial biomass, as shown in Table 2. Altogether, 48% were saturated (SFA) and 52% were unsaturated fatty acids; 26.4% were monounsaturated (MUFA) and 25.5% were polyunsaturated fatty acids (PUFA). Also, a representative amount of palmitic acid (C16:0), oleic acid (18:1n9), and stearic acid (C18:0) were found in this biomass. Similarly, in the same strain, although in another study, shared C18:0, C16:0 and C18:1n9 fatty acids were demonstrated as major compounds [51]. Besides, cyanobacterial oil contains a composition of all lipid, including a fatty acid profile similar to that of shark liver oil [52].

Conclusion

This study investigated cyanobacteria as a powerful, sustainable, novel route to obtain squalene and compares this method with non-renewable sources. Also, the results demonstrated effective kinetic parameters. Indeed, the sensitivity analysis demonstrates the production of bioactive compounds in the amounts of kilograms per year given diverse industrial capacities and quantities of slaughterhouse wastewater emissions.

Table 2 Fatty acids profile of cyanobacteria *Phormidium autumnale* oil

Fatty acids	(%)
16:0	31.41 ± 1.48
16:1	4.53 ± 0.50
17:0	2.33 ± 0.40
18:0	10.35 ± 2.57
18:1n9c	21.96 ± 4.72
18:2n6c	16.90 ± 1.85
18:3n6	4.17 ± 0.01
18:3n3	2.70 ± 0.59
20:4n6	0.45 ± 0.04
22:2	1.27 ± 0.26
SFA ∑	48.02 ± 1.56
MUFA ∑	26.49 ± 2.61
PUFA ∑	25.49 ± 0.55

SFA Saturated fatty acids, *MUFA* monounsaturated fatty acids, *PUFA* polyunsaturated fatty acids

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Compliance with Ethical Standards

Conflict of interest Mariane Bittencourt Fagundes declares that she has no conflict of interest and all the other authors: Raquel Guidetti Vendruscolo, Mariana Manzone Maroneze, Cristiano Ragagnin Menezes, Leila Queiroz Zepka, Juliano Smanioto Barin, Eduardo Jacob-Lopes and Roger Wagner also declares that they have no conflict of interest.

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