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CHEMICAL SCIENCES

Comparison of cell wall polysaccharides in *Schizophyllum commune* after changing phenotype by mutation

NICOLE DALONSO, CARMEN L.O. PETKOWICZ, LUIS G. LUGONES, MARCIA L.L. SILVEIRA & REGINA M.M. GERN

Abstract: The Agaricomycetes fungi produce various compounds with pharmaceutical, medicinal, cosmetic, environmental and biotechnological properties. In addition, some polysaccharides extracted from the fungal cell wall have antitumor and immunomodulatory actions. The aim of this study was to use genetic modification to transform *Schizophyllum commune* and identify if the phenotype observed (different from the wild type) resulted in changes of the cell wall polysaccharides. The plasmid pUCHYG-GPDGLS, which contains the *Pleurotus ostreatus* glucan synthase gene, was used in *S. commune* transformations. Polysaccharides from cell wall of wild (ScW) and mutants were compared in this study. Polysaccharides from the biomass and culture broth were extracted with hot water. One of the mutants (ScT4) was selected for further studies and, after hydrolysis/acetylation, the GLC analysis showed galactose as the major component in polysaccharide fraction from the mutant and glucose as the major monomer in the wild type. Differences were also found in the elution profiles from HPSEC and NMR analyses. From the monosaccharide composition it was proposed that mannogalactans are components of *S. commune* cell wall for both, wild and mutant, but in different proportions. To our knowledge, this is the first time that mannogalactans are isolated from *S. commune* liquid culture.

Key words: Genetic tools, fungus, mutant, cell wall, polysaccharides characterization.

INTRODUCTION

Glucans are one of the major polysaccharides found in the fungal cell wall. Depending on the species, culture medium and extraction conditions, these biomolecules may show structural differences. They can be linear or branched, with α, β or both configurations of glucose units and have different types of glycosidic bonds, like 1→3, 1→4 and/or 1→6 (Synytsya & Novak 2014).

Fungal glucans have been recognized for their antitumor and immunomodulatory activities (Dalonso et al. 2015, Wisbeck et al. 2017, Chakraborty et al. 2021). In addition, other polysaccharides isolated from mushrooms, such as galactans, fucans, xylans and mannans, have also shown significant biological activities. Some heterogalactans, such as mannogalactans, have shown anti-inflammatory (Silveira et al. 2015), immunomodulatory (Maity et al. 2014) and antitumor effects (Peng et al. 2005). Heterogalactans generally have a main chain composed of α-(1→6) galactose, such as the mannogalactans which may also have 3-*O*-methyl-galactose residues and are partially substituted at *O*-2 by β-D-mannose units (Rosado et al. 2003). The prevalence of one or other polysaccharide in the fungal cell wall is

subject to the developmental needs, adaptation, culture medium, growth conditions, among other factors and it is still not well understood (Dalonso et al. 2015).

Nuclear magnetic resonance (NMR) is an important tool for detecting responses to external agents that interfere in cell wall dynamics. The fungal cell wall is a result of highly complex and heterogeneous biosynthetic assembly. It performs versatile functions, providing rigidity and structure to the fungi (Kang et al. 2018, Zhao et al. 2020).

Cell wall stability or integrity is achieved through cross-links between glucan, mannoprotein, mannan, chitin, other polysaccharides and associated glycoproteins (Chatterjee et al. 2015). Mutations in the genes encoding glycosyltransferases, glycoproteins, or transcription factors may result in cell wall changes (Hu et al. 2020), affecting the assembly of various components, modifying hyphal growth, mating, fruiting bodies or pathogenicity (Wouw et al. 2009).

Under conditions of cell wall damage, repair mechanisms are activated and the assembly of wall components can be modified for adaptation to the stress condition (Gow et al. 2017). Different studies have demonstrated that mutations in fungi caused by ultraviolet light (Adeeyo et al. 2016), cosmic radiation (Zhao et al. 2016), phenolic compounds (Reverberi et al. 2004) or genetic manipulation (Ohm et al. 2010) can affect the biosynthesis of cell wall polysaccharides or exopolysaccharides (EPS), increasing their amount or changing the solubility.

Mutations in the *thn1* gene are often related to morphological changes in *S. commune*, resulting in a thin phenotype. Positively regulated genes that control stress-related protein function suggest a reorganization of the cell wall in this phenotype (Fowler & Mitton 2000, Erdmann et al. 2012).

In a previous work we determined the best way to fuse long DNA fragments for plasmid construction (Dalonso et al. 2017). Using an easy to transform fungus, *S. commune*, we constructed a plasmid (pUCHYG-GPDGLS) by Circular Polymerase Extension Cloning (CPEC). The plasmid used in the *S. commune* transformation was constructed to overexpress a glucan synthase (GLS) from *P. ostreatus*, which can induce an increase of glucan content or cause mutation in the cell wall dynamics, resulting in chemical changes of polysaccharides.

Spectroscopic analyses, such as infrared (FT-IR) and nuclear magnetic resonance (NMR), besides monosaccharide composition can contribute in understanding about the fungal cell wall (Synytsya & Novak 2014, Zhao et al. 2020) and phenotype changes. In the present study, this approach was used to identify the changes observed in the cell wall polysaccharides of *S. commune* mutant with a different phenotype.

MATERIALS AND METHODS

Microorganism maintenance and growth

Mycelium from the *Schizophyllum commune* H4-8 and *Pleurotus ostreatus* Pc9 strains were obtained from the strain bank of the Microbiology Department of the Utrecht University.

S. commune H4-8 was kept on agar plates in minimal medium - MM (0.22% glucose, 0.15% asparagine, 0.05% $MgSO_4$.$7H_2O$, 1 ml of microelement solution for spore induction and 2.5 ml of phosphate buffer concentrate [184 g/L KH_2PO_4 and 400 g/L K_2HPO_4] pH 6.5) (Dons et al. 1979).

P. ostreatus Pc9 was kept on agar plates in complete medium for mushroom - MCM (0.2% yeast extract, 0.2% peptone, 2% glucose, 0.05% $MgSO_4$.$7H_2O$, 0.05% KH_2PO_4 and 0.1% K_2HPO_4) (Kim et al. 1999).

DH5α bacteria were grown overnight in Luria Bertani liquid medium (LB) (Bertani 1951) and then kept on agar plate at the same medium.

DNA extraction

Due to the similarities and conserved region of the glucan synthase gene from *P. ostreatus* and *S. commune*, this region was selected to construct a plasmid and to carry out a mutation in *S. commune*. Genomic DNA from H4-8 *S. commune* and Pc9 *P. ostreatus* were extracted according to literature (Ohm et al. 2010).

A small piece of each mycelium (H4-8 *S. commune* and Pc9 *P. ostreatus*) was scalped from the agar plate, placed in 2 mL Eppendorf tubes and frozen in liquid nitrogen. Two small metal balls were added in the tube to help with the homogenization process using a Retsch Tissue Lyser II for 1 min. After the removal of the metal balls, 1 mL of CTAB-buffer (2% CTAB, 0.1 mol/L Tris–HCl pH 8.0, 1.4 mol/L NaCl) was added and the tube heated at 65 ºC in a heat block for 20 min. Then, the tube was centrifuged (1 min at 20,000 x g), 500 µL of chloroform was added to the supernatant and mixed. The phase separation was induced by centrifugation (5 min, 20,000 x g) and the upper phase was transferred to a new tube. The DNA was precipitated by the addition of 640 µL of isopropanol, centrifuged (5 min, 20,000 x g), and the pellet was washed with 500 µL of 70 % ethanol. The residual ethanol was evaporated by heat (5 min, 60 ºC) and the DNA was dissolved in 50 µL of TE (Tris-EDTA) buffer.

The plasmid DNA from *Escherichia coli* (DH5α) was extracted using NucleoSpin Plasmid, according to the manufacturer instructions (MACHEREY-NAGEL).

PCR conditions and plasmid construction

For PCR, 1 µL of the DNA was mixed with a buffer, primers, Phusion High-Fidelity DNA Polymerase and 200 µM of each nucleotide, according to the manufacturer standard instructions (Thermo Scientific). The thermocycle conditions were set at denaturation (3 min, 98 ºC), annealing (56-62 ºC, 15 s) and the extension was adjusted according to the length of DNA fragment (30 s/kb). Table I shows the nucleotide sequence and TM of overlapping primers to obtain the plasmid pUCHYG-GPDGLS.

The hygromycin resistance gene linked to the GPD promoter (glyceraldehyde-3-phosphate dehydrogenase gene) of *S. commune* was extracted from the PHYM1.2 vector (Scholtmeijer et al. 2001) through digestion, with the assistance of the restriction enzymes *Xho*I and *Bam*HI. This fragment was then inserted into the *Sal*I and *Bam*HI sites of the pUC20 vector, using T4 ligase for overnight binding. DH5α cells were transformed through thermal shock with 2 µL of the binding product, at a ratio of 1:3 (vector:insert) and then selected through the blue and white colonies method in the presence of LB with ampicillin (50 µg/mL) (Sambrook & Russell 2001). After overnight growing, white colonies were checked, and the plasmid was extracted, digested for 16 h with *Bam*HI to promote its linearization and called pHYM20.

Figure S1 (See supplementary material figures S1-S4) shows step-by-step the process used for assembly of the plasmid by CPEC. The primers used to amplify the glucan synthase (GLSA/GLSB) and the GPD promoter of *P. ostreatus* were designed by the NEBuilder tool, available at the NEB website (http://nebuilder.neb.com/#). The linearized pHYM20 vector (0.016 pmol) was then mixed at the ratio of 1:3 with the other fragments (GLSA, GLSB and GPD) in an equimolar concentration (0.05 pmol each). The best way to connect long fragments targeting plasmid construction was previously established (Dalonso et al. 2017). Subsequently, 6 µL of this mixture was used for the Circular Polymerase Extension Cloning (CPEC) (Quan & Tian 2009)

Table I. Nucleotide sequence and TM (melting temperature) of overlapping primers to obtain the plasmid pUCHYG-GPDGLS.

Primers	Sequences	Tm (°C)
GLSB-F[a]	cccagcactcgtccgagggcaaaggaatagGGAAATAAATTAAATGTATTCACTGCTTTGC	66,4
GLSB-R[b]	TGAACTACTCGAAGGCAATCAAGCTCCTTTACCGTGTCGA	70,6
GLSA-F[b]	AAAGGAGCTTGATTGCCTTCGAGTAGTTCATCATCCCGGA	71,6
GLSA-R[b]	CCACCCATCCTTGTGATGTCCGCAGAAGAGATAGAGGATATTTTC	68,4
GPD-F[b]	CTCTTCTGCGGACATCACAAGGATGGGTGGTTGGGGATGG	77,7
GPD-R[a]	cagctatgaccatgattacgaattcccgggGTTGCCCTCAAGGGTCTTCGAGCCTTC	76,7

[a] **The lower case letters correspond to the overlapping regions (30 bp) of the primers with the pUC20 vector.**
[b] **The underlined portions correspond to the overlapping regions of the reverse (R) and forward (F) primers in subsequent fragments.**

for 25 cycles in a reaction volume of 25 µL. A sequence of steps composed of the initial denaturation at 98 °C for 30 s and 25 cycles at 98 °C for 10 s, 55 °C for 30 s, 72 °C for 160 s, followed by a final extension of 10 min at 72 °C was used. The reaction components were the same for a standard reaction with the Phusion polymerase, although no primer was added.

Chemically competent cells (25 µL) (Stellar, TaKaRa) were then heat shock-transformed with 2.5 µL of the CPEC reaction product. After the selection in LB with ampicillin 50 µg/mL, transformant colonies were subjected to the extraction of plasmid DNA (NucleoSpin Plasmid) and digested with the *Sal*I restriction enzyme. Once the expected digestion profile was confirmed, according to the prediction in J5 (https://j5.jbei.org/), other restriction enzymes (*Hin*dIII, *Sma*I, *Sal*I and *Bam*HI) were used to confirm the plasmid construction. Plasmid pUCHYG-GPDGLS contains the glucan synthase gene from *P. ostreatus* (Agaricomycete) driven by glyceraldehyde-3-phosphate promoter, linked to hygromycin marker. Glucan synthase is the UDP-glucose 1,3-β-D-glucan 3-β-D-glucosyltransferase (EC 2.4.1.34) composed of a catalytic subunit FKS and a regulatory subunit RHO, responsible for polymerizing glucans, and it is involved in the building of cell wall in fungi.

Transformation of *S. commune*

The protoplasts (200 µL – 10^7 protoplasts/mL) were transformed with 10 µg of the purified plasmid pUCHYG-GPDGLS for 15 min on ice. Negative control (without plasmid) was included to check backgrounds. Then, one volume of PEG 4000 (40 % PEG 4000, 10 mM Tris-HCl) was added and the mixture was incubated at room temperature for 5 min. The regeneration medium (0.5 mol/L $MgSO_4.7H_2O$, 0.04 mol/L phosphate buffer, 10 µg/mL Zeocin, 50 µg/mL Ampicillin) was added up to the mark of 3 ml and then the protoplasts were incubated overnight (Van Peer et al. 2009). The regenerated protoplasts were placed on agar plates (Minimal medium, 0.04 M phosphate buffer, 1 % agar) with hygromycin (15 µg/mL). The amount of hygromycin that was enough to inhibit growth was tested. The viability of the protoplast after transformation was also

checked in a medium without hygromycin. After 12 days, five transformants were randomly selected for further studies.

Confirmation of plasmid integration

Hygromycin resistant transformants were confirmed through resistance maintenance once they were replated and used to check for plasmid integration into the fungal DNA. After the fungal DNA extraction, the hygromycin resistance gene was amplified by PCR using the primers HYG-F (5´-CCATGGCTGAACTCACCG-3´) and HYG-R (5´-CTATTCCTTTGCCCTCGG- 3´). The PCR product (5 µL) was applied on agarose gel (1 %), prepared in a TBE buffer (Tris 89 mmol/L, 89 mmol/L boric acid and 2 mmol/L EDTA) containing 0.5 µg/mL ethidium bromide and submitted to electrophoresis at 80 V for 1 h. The DNA was checked in a UV (λ 302 nm) transilluminator (Bio-Image Systems). The standard molecular weight (1 kb) was used to compare the expected DNA bands (Fermentas, Burlington, Canada).

In order to verify the influence of the hygromycin resistance gene and observed phenotype (less thick hyphae), one transformant was randomly selected for crossing with the compatible B strain of *S. commune*. After 10 days of growth in inverted Petri dishes, the basidia spores were collected from the lid's surface and inoculated into a culture medium containing 20 µg/mL hygromycin. To identify whether the hygromycin resistance gene is present in a single or multiple integration, the phenotype distribution, hypha morphology, and the ability to grow in the selective medium were analyzed in sibling cells.

Polysaccharide production and extraction

Polysaccharide production was performed in MCM medium without yeast extract because it may contain water soluble polysaccharides that would interfere in the final analysis. The MCM medium was chosen at this stage for *S. commune* because it is easy to obtain and prepare for the submerged cultivation of mushrooms (Kim et al. 1999, Kizilcik et al. 2010) and can be reproduced in any microbiology laboratory.

The submerged cultures were carried out at 30 °C for 9 days and kept with reciprocal stirring (110 rpm) in the Shaker Certomat® HK - B.Braun. The cultures were performed in triplicate, using 50 mL Falcon tubes, with 30 mL of MCM and inoculated with 2 small agar discs (12 mm) containing fungal mycelium. The mycelium was then macerated with a tissue homogenizer (ULTRA-80I).

The broth and biomass were transferred to 100 mL Beckers and capped with aluminum foil to avoid evaporation. The biomass suspension in the culture broth was boiled in a heating plate for 3 h to extract the polysaccharides. Subsequently, the biomass was vacuum-filtered on filter paper and the supernatant was 10-time concentrated by evaporation. The residue was transferred to 50 mL Falcon tubes and three volumes of 95% ethanol was added. The mixture was kept under refrigeration (4 °C) for 16 h. Then, the polysaccharides were separated by centrifugation (2748 x g, 10 min), lyophilized, quantified by gravimetry and the total sugar content was determined through the phenol-sulfuric method using calibration curve with glucose as standard (0.01 to 0.1 g/L) (Dubois et al. 1956).

Polysaccharide characterization

The polysaccharides obtained from wild and transformants *S. commune* were analyzed by Fourier-transform infrared spectroscopy (FT-IR) (Nicolet iS10 FT-IR, Thermo Scientific) and nuclear magnetic resonance (NMR) spectroscopy in a 400 MHz Bruker Avance DRX400 spectrometer (Bruker Instruments).

FT-IR was used for identification of characteristic absorption bands of carbohydrates. A small amount of polysaccharide fractions was placed in the sample compartment of attenuated total reflectance cell (ATR) and the spectra were obtained at 25 °C in the absorbance mode in the range of 4000 to 500 cm^{-1} from 64 scans. Data were analysed using the OMNIC 8.0 software.

For NMR analyses, the samples were solubilized in deuterated dimethyl sulfoxide (20 mg/mL). Mono- and bi-dimensional spectra were acquired at 70 °C. The chemical shifts were relative to the the Me_2SO-d6 (^{13}C δ 39.7/^{1}H δ 2.60).

The high-pressure size-exclusion chromatography (HPSEC) was carried out using the equipment Waters 2410 with a differential refractometer (RI) detector to check the homogeneity of polysaccharides. Waters Ultrahydrogel 2000/500/250/120 columns were connected in series and coupled to the equipment and 0.1 mol/L $NaNO_2$ solution, containing NaN_3 (0.5 g/L) was used as eluent. The samples were filtered (0.22 µm; Millipore) and analyzed at 1 mg/mL. The data were collected and processed by the Wyatt Technology ASTRA software.

The neutral monosaccharide composition was determined with 2 mg of polysaccharide, hydrolyzed with 2M TFA, 120 ° C, for 2 h. Then the acid was evaporated to dryness and 5 mL of water and $NaBH_4$ (~ 5 mg, 16 h) were added to the tube to promote monosaccharide reduction (Wolfrom & Anno 1952). After this step, the samples were treated with acidic cationic resin for removal of Na^+ ions and then evaporated to dryness using a rotary evaporator (Fisatom). Later, 1 mL of methanol was added to remove trimethyl borate and it was evaporated to dryness (3x). The samples were subjected to acetylation with 0.5 mL acetic anhydride and 0.5 mL pyridine at 25 °C for 16 h (Wolfrom & Thompson 1963). Distilled water (1 mL) and chloroform (1 mL) were added to the final product with subsequent stirring. After the phase separation, the alditol acetates were extracted from the chloroform phase, washed with 5% $CuSO_4$ and distilled water (5x each). They were subsequently analyzed by gas-liquid chromatography (GLC) in a Thermo Scientific Trace GC Ultra gas chromatograph equipped with a fused silica capillary column (30 m x 0.25 mm internal diameter) DB-225 and a Ross injector. The flame ionization detector and injector temperatures were 300 ºC and 250 ºC, respectively. The oven temperature was programmed from 100 to 220 ºC at a rate of 60 ºC/min and a mixture of helium and nitrogen was used as the carrier gas (1.0 mL/min). The alditol acetates were identified by their retention times compared with standards.

The presence of uronic acids was investigated using the colorimetric method of Blumenkrantz & Asboe-Hansen (1973) and by anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD). After hydrolysis (2M TFA, 120 ° C, for 2 h), drying and repeated washing until total removal of the acid, the samples (1 mg/mL) were filtered through a membrane of 0.22 µm, injected in a Thermo Scientific Dionex ICS-5000 chromatograph (Thermo Fisher Scientific, USA) with CarboPac PA20 column (3 × 150 mm) using gradient of 1 M NaOH and 1 M NaOAc as eluent (Nagel et al. 2014) in N_2 atmosphere in a flow of 0.2 ml/min at 30 ºC. Data were collected and processed using the ChromeleonTM 7.2 Chromatography Data System software.

RESULTS

Plasmid construction

The plasmid used in this study was subjected to restriction enzymes to confirm that it was correctly constructed by CPEC. Figure S2 a exhibits the digestion profile (left) of pUCHYG-GPDGLS

and simulation of digestion (right) with J5 (https://j5.jbei.org/). Figure S2 b shows plasmid map pUCHYG-GPDGLS obtained by SnapGene. The CPEC method was highly efficient in the plasmid construction and can be easily applied as a genetic tool for fungi. The enzyme *Bam*HI was used as a negative control since there is no restriction site for it on the plasmid (uncut). As predicted in J5, the last two fragments cut with *Sal*I are similar in size, consequently, it is not possible to see both.

Characterization of polysaccharides extracted from cell wall of wild (ScW) and mutants of *S. commune*

S. commune protoplasts were transformed with plasmid pUCHYG-GPDGLS and five transformants were selected for further studies. Although we could not find overexpression of glucan synthase, we observed various phenotypical characteristics on cultures of the transformants, which deviated from the wild type. Mutants turned out to show morphological characteristics typical of mutants in the thin gene (Fowler & Mitton 2000). Since it is not possible to know the exact location where the mutation was caused in *S. commune*, we decided to investigate whether the cell wall chemical composition remained the same in the thin phenotype mutant compared to the wild fungus. In order to understand if mutants had similar cell wall composition, FT-IR from five mutants (T1, T2, T3, T4 and T5) were analyzed (Table II). Comparing the main absorption bands related to the functional groups of the polysaccharides extracted from ScW and mutants (T1, T2, T3, T4 and T5) it was possible to notice that some FT-IR signals were not present in the mutants.

The O-H axial absorption bands were observed with intermolecular hydrogen bonds between 3600 and 3000 cm^{-1} and, at 1644 cm^{-1} (Mohacek-Grosev et al. 2001). Asymmetric stretch bands of the CH_2 groups were found at 2900 to 2950 cm^{-1}. The characteristic carbohydrate region appears between 900 and 1200 cm^{-1}, confirming the C-O-C bond, due to the ether function of the

Table II. Wavenumber (cm^{-1}) and assignments for polysaccharides extracted from the wild (ScW) and mutants (T1, T2, T3, T4, T5) from *S. commune*.

Wave number (cm^{-1})						Assignments
ScW	T1	T2	T3	T4	T5	
3307	3294	3285	3285	3287	3327	Axial deformation OH
2927	-	-	2927	2931	2924	CH_2 stretch
1644	1644	1643	1644	1644	1650	Angular strain OH
-	-	1532	1532	1535	-	Amide II (chitin or proteins)
1423	-	-	-	-	-	Angular deformation C-O-H and CH_2
1368	-	-	-	-	-	Angular deformation C-O-H and CH_2
1323	-	-	-	-	-	Angular deformation C-O-H and CH_2
1252	-	-	-	-	-	Angular deformation C-O-H and CH_2
1020	1048	1051	1052	1047	1052	Axial deformation C-C + C-O + C-H
890	891	891	890	891	887	β-D-glucans

Table III. Monosaccharide composition of the hot-extracted polysaccharides of wild (ScW) and mutant (ScT4) *S. commune*. Values are presented as a mean of duplicates ± standard deviation.

Monosaccharide	ScW (%)	ScT4 (%)
Glucose	65.0±0.72	39.8±0.38
Galactose	29.0±0.58	50.0±0.65
Mannose	4.2±0.06	7.3±0.29
Arabinose	0.9±0.01	1.6±0.01
Rhamnose	0.2±0.08	0.3±0.02
Xylose	0.3±0.01	0.5±0.01
Fucose	0.4±0.01	0.5±0.01

bond between monomers in the polymer. Near to 890 cm^{-1}, typical bands from β configuration of the anomeric were found (Synytsya & Novak 2014) and 1020 cm^{-1} (Robert et al. 2005).

The signals between 1423 and 1252 cm^{-1} were found only in ScW and were attributed to the angular deformation of C-O-H and CH_2 (Mohaček-Grošev et al. 2001). Bands at frequencies of 1051–1047 cm^{-1} observed in mutants were typically for the presence of arabinose, mannose, rhamnose. In this region, galactans could interfere in the chemical shifts (Ho et al. 2020).

Since the mutants had the same profile in FT-IR and thin phenotype, one of them was randomly selected for further investigation. The T4 mutant, now called ScT4, was selected in order to understand the changes in the mutant cell wall. Figures S3 and S4 exhibit ScW and ScT4 spectra, respectively.-

Figure 1 shows the chromatograms from HPSEC of the fractions extracted from the broth and mycelium of wild (ScW) and mutant *S. commune* (ScT4) after hot extraction, filtration and precipitation with ethanol 1:3 (v/v). It was possible to identify that both fractions had polymodal elution profiles, with four main peaks. For ScT4, the peak eluting before 40 min was shifted toward lower elution time compared to ScW, and that at elution time ~ 50 min was shifted toward higher elution time compared to the profile from wild *S. commune*. Differences in the peak intensities were also observed.

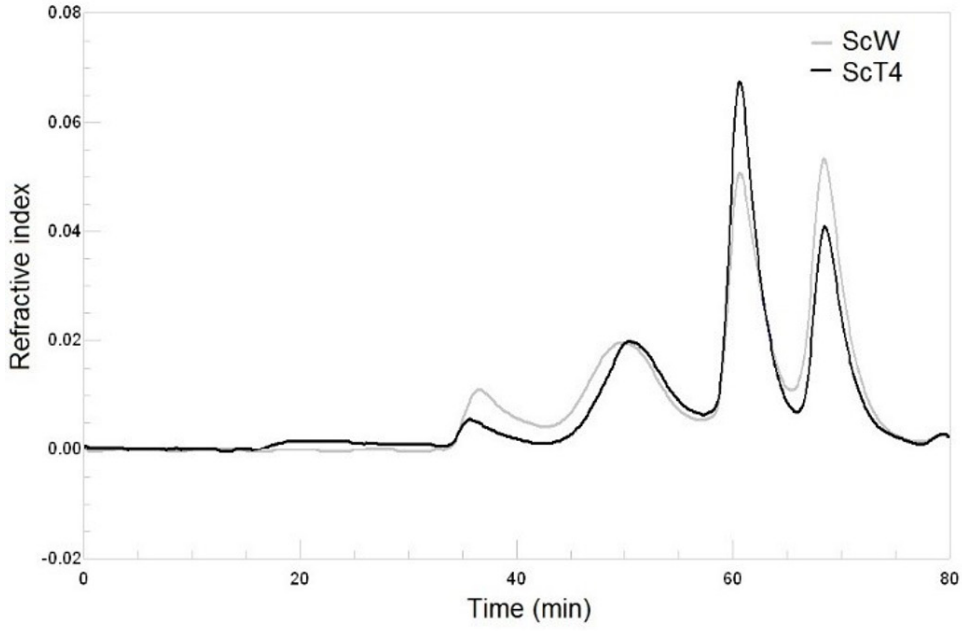

Figure 1. Elution profile of the fractions extracted from the broth and mycelium of wild (ScW) and mutant *S. commune* (ScT4).

The concentration of ScW and ScT4 polysaccharides at 9 days of culture was of 0.75 ± 0.22 and 0.76 ± 0.16 g/L, respectively. The values observed for total sugar of polysaccharides by the phenol-sulfuric method using glucose as standard were of 65.9%±7.2 (ScW) and 54.5%±2.0 (ScT4). The predominance of carbohydrates was also confirmed by FT-IR. In the present study, no uronic acids were found in the extracted polysaccharides.

The monosaccharide composition of the hot-extracted polysaccharides obtained from ScT4 was determined and compared with that of the *S. commune* wild type (ScW) as shown in Table III. The monosaccharide composition demonstrated inversion of composition in terms of glucose and galactose, indicating that the mutation caused by pUCHYG-GPDGLS insertion might have changed the organization of the polysaccharides present in the cell wall of *S. commune* T4 genome. Galactose (50%) was the main component in the hot-soluble fraction from cell wall of ScT4. The Man/Gal ratio found for ScT4 and ScW was about 1:7 suggesting the presence of mannogalactans. According to the results, mannogalactans were the main component of ScT4 instead of glucans as found for ScW, which was composed of 65% glucose.

^{13}C NMR spectra were also compared. Figure 2 shows NMR ^{13}C-DEPT for ScW (Figure 2a) and ScT4 (Figure 2b) samples. Signals indicative of linked C-3 (δ 86.66 to 80.70) and linked C-6 (δ 67.60) from β-D-Glucans were observed for ScW (Kang et al. 2018). The signals inverted in the DEPT experiment (δ 61.43 to 60.26 for ScW and δ 60.22 for ScT4), characterize the free C-6 (-CH_2) of hexoses units and non-reducing terminals (Falk & Stanek 1997), while three of those signals were presented only for ScW and one for ScT4.

HSQC spectrum (Figure 2c and 2d) was used mainly to observe the correlation between the anomeric carbon and their respective hydrogens, as shown in Table SI. ScW (Figure 2c) presented signals at δ 4.64/102.92 to δ 4.40/102.14 referring to anomeric carbons (H-1/C-1) with β-glycosidic bonds from glucose (Silveira et al. 2014), and δ 4.54/101.75 corresponding to glucose (H-1/C-1) in α-type glycosidic bonds (Synytsya & Novak 2013). Signal at δ 5.17/97.05 refers to the anomeric carbons (H-1/C-1) with α-type glycosidic bonds from galactose (Maity et al. 2014). The signals δ 3.60/85.70 denote H-3/C-3 of glucose units →3,6-β-D-Glcp-1→ linked (Silveira et al. 2014). ScT4 (Figure 2d) showed signals at δ 4.41/101.7 that refers to anomeric carbons (H-1/C-1) with α-type glycosidic bonds from glucose (Synytsya & Novak 2013) and signals in the region of δ 5.17/97.0 and δ 4.40/96.22 referring to the anomeric carbons (H-1/C-1) with α-type glycosidic bonds from galactose (Maity et al. 2014, Meng et al. 2018). Some researchers attribute signals δ 101.7 ~ 101.8 to anomeric carbon (C-1) of β-D-mannose in mannogalactan (Maity et al. 2014, Silveira et al. 2015), but the monosaccharide is in low concentration (4.2 and 7.3%) in polysaccharides extracted from ScW and ScT4, respectively, making the interpretation difficult.

Phenotype analysis and plasmid integration

Figure 3a and b show hypothetical differences between the outer layer (flexible) and phenotypes observed for *S. commune*. The presence of less thick hyphae (Figure 3a – ScT4) and normal hyphae (Figure 3b - ScW) was observed. The five transformants selected for this study (T1, T2, T3, T4 and T5) showed differences in the production of extracellular polysaccharides compared to the wild type after precipitation of the culture medium with ethanol. In the liquid medium, ScT4 (Figure 3c) had a restricted and incipient production of extracellular polysaccharides in comparison with the wild-type phenotype (Figure 3d). As expected, when the presence of

the hygromycin resistance gene was assessed by PCR amplification, all transformants turned out to contain the gene (Figure 3e).

It was possible to observe that mutants with less thick hyphae were unable to maintain the production of extracellular polysaccharides (bright gel covering the biomass in Figure 3d). This could be due to the insertion of the pUCHYG-GPDGLS plasmid used in the *S. commune* transformation was ectopically inserted, resulting in random mutations in the fungus genome. After crossbreeding between the compatible B strain of *S. commune* and the ScT4 mutant, the progeny showed a 1:1 segregation ratio for wild type vs. thin phenotype. There was no association between the hygromycin resistance gene and thin phenotype.

DISCUSSION

Usually, the protocols to construct plasmids lack details, preventing success in the reproduction of the correct fusion of DNA fragments. In the present study, a step-by-step protocol based on CPEC (Quan & Tian 2009) was described to be used as a genetic tool in fungi. The effect of pUCHYG-GPDGLS plasmid integration in *S.*

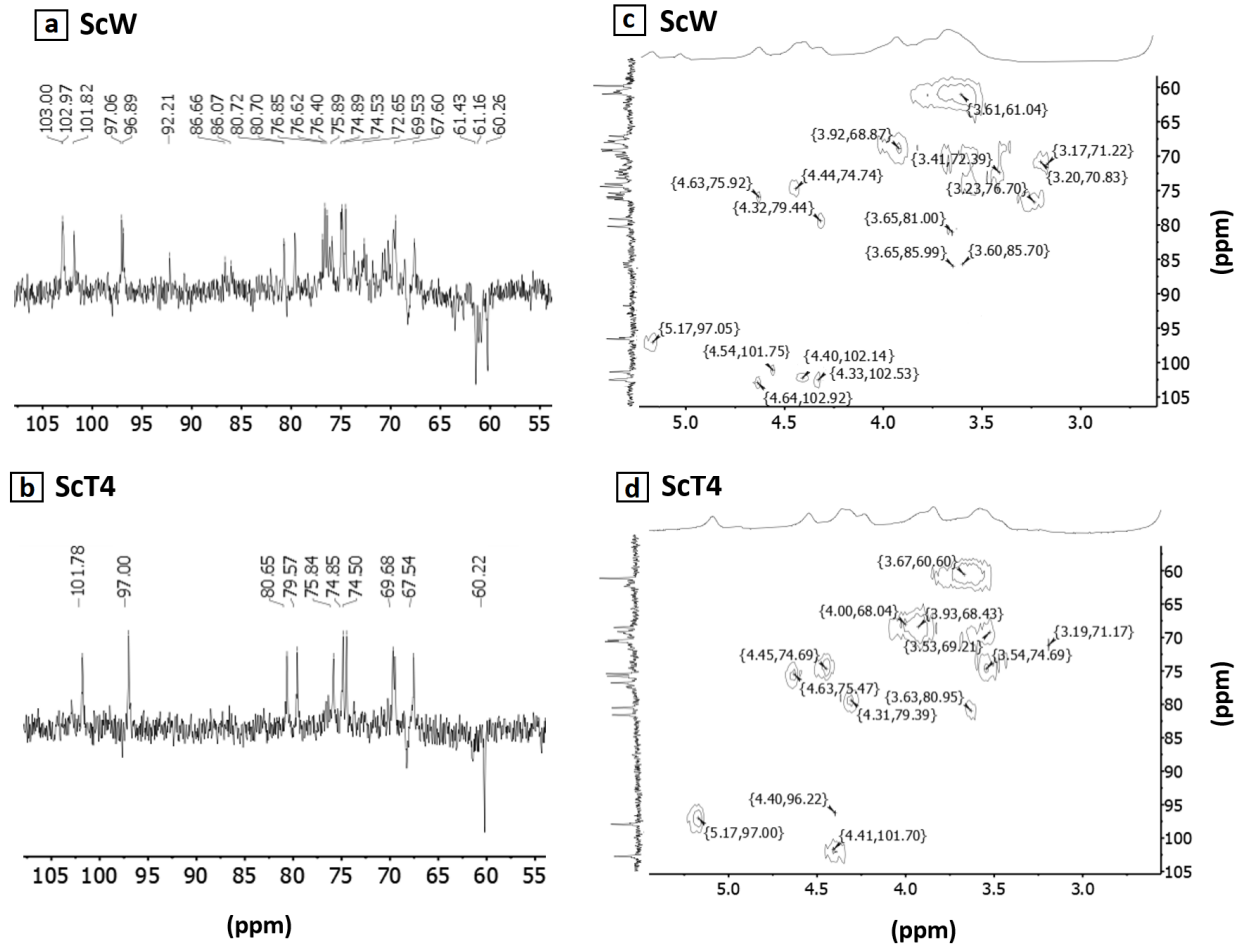

Figure 2. a - ^{13}C-DEPT NMR spectra for wild (ScW). b - ^{13}C-DEPT NMR spectra for T4 mutant *S. commune* (ScT4) polysaccharides. c - HSQC spectrum of the ScW fraction in deuterated dimethyl sulfoxide. d - HSQC spectrum of the ScT4 fraction in deuterated dimethyl sulfoxide. Chemical shifts relative to the ^{13}C NMR-DEPT signals were expressed as δ (ppm).

commune phenotype and the resulting changes in the hot-soluble polysaccharides from cell wall were investigated.

The differences found in the chemical structure of polysaccharides extracted with hot water from wild (ScW) and mutant *S. commune* (ScT4) reflect the unique characteristics of phenotypes after plasmid integration. The mutant selected for study (ScT4) had a thin phenotype, and these characteristics have also been described in other studies (Erdmann et al. 2012, Van Peer et al. 2009).

Sietsma & Wessels (1977) found a water-soluble mucilage of β-(1→3),(1→6)-glucans in the outer layer (flexible) of the *S. commune* cell wall. Recently, Ehren et al. (2020) proposed a cell wall model for basidiomycete with a rigid core and a flexible network. The rigid fraction of the cell wall of *S. commune* consist of chitin, α- and β-glucans while the flexible fraction is composed of β-glucans with α-linked reducing and non-reducing ends and polymeric mannose. Based on our findings, mannogalactans might be included as a flexible component to *S. commune* cell wall, as proposed in Figure 3a and b.

Mutations in the *thn1* gene are often associated with morphological changes in *S. commune*. The predicted product of *thn1*

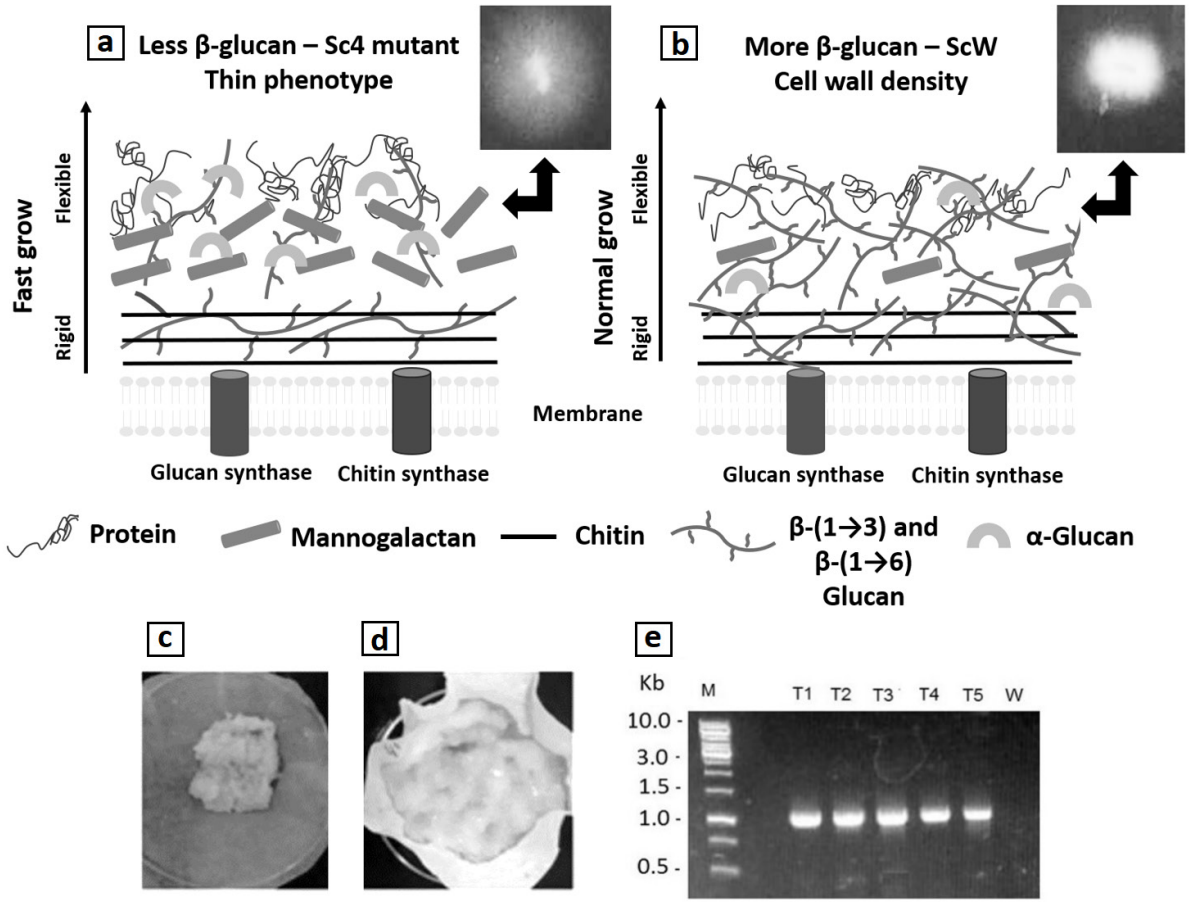

Figure 3. Hypothetical representation of cell wall structure and phenotypic characteristics of *S. commune*. a - ScT4 mutant phenotype presenting less thick mycelium; b - Phenotype of wild mycelium; c - Biomass of hygromycin resistant mutant phenotype after liquid culture; d - Wild phenotype biomass; e - PCR amplification of the hygromycin resistance gene for the *S. commune* mutants T1, T2, T3, T4, T5 and for the wild type (W).

is a putative regulator of G protein and a transposition of a class II transposon into this gene has been shown to be responsible for the thin phenotype. A disruption of the *thn1* has a pleiotropic effect on vegetative growth and sexual development of *S. commune* (Fowler & Mitton 2000). Cellular pathways involved in the sexual development of *S. commune* have been investigated (Erdmann et al. 2012). These authors evaluated the influence of the *thn1* gene by the expression profile of a homokaryotic mutant strain presenting a thin phenotype. The 114 regulated genes (72 positively and 42 negatively) showed involvement in cellular responses potentially initiated by G protein signaling, the node where Thn1 is expected to act. Positively regulated genes include those that influence posttranslational processing and modification or genes with a stress-related protein function, various chitinase candidates and other glycoside hydrolases, suggesting functions associated with cell wall reorganization. Proteins involved in cell wall biogenesis or membrane turnover were identified between the positively and negatively regulated genes, suggesting the reprogramming of the cells by genetic pathways.

Another transcription factor that interferes in the basidiomycete cell wall density is PacC. It is a transcription factor important for adaptability to environmental changes such those from osmotic conditions, cell wall stress and oxidative stress. PacC-silenced strains of *Ganoderma lucidum* were about 25–30% thinner than those of the wild type strain and had β-1,3-glucan content decreased c. According to the authors, the ability of PacC to bind to the promoters of glucan synthase-encoding genes corroborate that PacC transcriptionally regulates these genes (Hu et al. 2020).

Small changes in the monosaccharide composition of structural polysaccharide can lead to noticeable impacts on fungal cell wall integrity (Kang et al. 2018, Ehren et al. 2020). Mutation in *LmIRFD* gene (*Leptosphaeria maculans* Interferon-Related Developmental Regulator) was reported to affect phenotype of the ascomycete *L. maculans* and increase the level of galactose in spores. The mutant had galactose (50.4%), glucose (38.6%) and mannose (9%), while for the wild type, glucose (84.1%) and mannose (13.5%) were found as the main components. The *LmIRFD* gene has been described to play a regulatory role in cell proliferation, stress response and differentiation. However, how mutations in this gene result in altered cell wall polysaccharides is still unknown (Wouw et al. 2009).

Analysis of the composition of the exopolysaccharides extracted from the submerged culture of *S. commune* were performed by Du et al. (2017). The exopolysaccharides were precipitated from the culture medium with ethanol (4 volumes) and were purified by chromatography (DEAE-52 and Sephadex G-150). These authors found a total sugar of 89%, with predominance of glucose (57.5%) and 26.8% mannose, in addition to 4.71% arabinose, 4.55% galactose, 3.79% ribose, 7.52% of uronic acids. The purified exopolysaccharide showed anti-inflammatory activity. In the present study, glucose (65.0 %) was also the major monosaccharide found for ScW.

Three fractions extracted from *S. commune* were identified and tested for antioxidant potential. Hot water extract (HWE), hot water-extracted polysaccharides (HWP), and hot alkali polysaccharides (HWAE) had α- and β-glucans, which were identified by the Megazyme enzymatic kit and confirmed by NMR and FT-IR. As for the monosaccharide composition determined by thin-layer chromatography, glucose was predominant in the three fractions, with small amounts of galactose, mannose and traces of fucose and xylose (Klaus et al. 2011),

thereby corroborating with monosaccharides found in the present research for ScW.

In the present study, initially, it was hypothesized that the transformed plasmid could increase the production of polysaccharides, since it contains a constitutive GPD promoter controlling the glucan synthase gene. However, this was not observed, either in RT-PCR experiments to confirm the GLS overexpression in the mutants (data not shown) or by the increase in glucan content. Surprisingly, we found higher amount of galactose (50.0%) in the mutant fungus (ScT4), while in the wild type (ScW), glucose is the major constituent. This might be associated with the disruption of a gene by the integrating plasmid that controls the production of the cell wall, and further studies involving transcription factors, other genes and regulation, are necessary to understand this issue.

The production of polysaccharides or exopolysaccharides varies according to the culture conditions, amount of inoculum and culture medium (Klaus et al. 2011, Du et al. 2017). For *S. commune*, exopolysaccharide (EPS) yields of 0.39 and 0.60 g/L after 10 and 15 days of cultivation, respectively, in the same medium (MCM) have been reported. In the present study, after 9 days of liquid culture, the wild *S. commune* showed higher yield (0.75 g/L) than that obtained by Kizilcik et al. (2010) after 10 days of cultivation (0.39 g/L). This was probably due to the fact that in the present study, the polysaccharides (ScW and ScT4) were extracted from both, mycelium biomass and culture broth.

Da et al. (2012) introduced mutations in the *S. commume* genome through exposure to UV light for 30 min (SCM1), 60 min (SCM2) and 90 min (SCM3) and investigated the influence on the EPS and biomass production in basal medium (6 g glucose, 1.6 g malt extracts, 2 g peptone, 1.2 g yeast extracts, 0.8 g KH_2PO_4, 0.4 g $MgSO_4.7H_20$, 0.4 g urea and pH adjusted to 5.8). The authors reported a higher mycelial biomass (4.42 g/100 mL) and EPS (460 mg/100 mL) yield for SCM1 grown at 28 °C in comparison with the other types under the same conditions. On the other hand, at the incubation temperature of 32 ºC, the SCM2 mutant strain produced the highest mycelial biomass and EPS yield, but at 36 ºC and 40 ºC the wild type of *S. commune* had the best performance in the mycelial biomass and EPS production.

Heterogalactans have already been found for some fungi of the *Pleurotus* (Carbonero et al. 2008, Rosado et al. 2003), *Inonotus* (Vinogradov & Wasser 2005), *Albatrellus* genera (Samuelsen et al. 2019), among others. The occurrence of α-D-galactose is mostly described for basidiocarps in the literature (Ruthes et al. 2016).

The EPS extracted and purified from *P. sajor-caju* was composed of mannose (37.0%), galactose (39.7%), and 3-*O*-methyl-galactose (23.3%) (Silveira et al. 2015). The main chain was identified like (1→6)-linked α-D-Gal*p* and 3-*O*-methyl-α-D-Gal*p* units and the α-D-Gal*p* units were substituted at *O*-2 by non-reducing end units of β-D-Man*p*. As the same Man/Gal ratio was found for the mutant ScT4 and wild *S. commune*, thus the results from the present study suggest the presence of mannogalactans in *S. commune*.

An α-(1→6)-linked D-galactan with α-D-(1→6)-linked Man*p* branches, attached to t-β-D-Glc*p* or t-α-D-Fuc*p* side chains was isolated from *Flammulina velutipes*. This heterogalactan induced macrophage activation mediated by autophagy via Toll-like receptor 4, having a promising application in the pharmacological area (Meng et al. 2018).

The role of galactans or mannogalactans in the fungal cell wall and how these molecules are synthesized have not been described yet. However, several biotechnological,

immunological and medicinal applications have been proposed for these biopolymers (Delattre et al. 2011, Meng et al. 2018, Samuelsen et al. 2019).

The possibility of extraction of different polysaccharides from mutant fungi brings new horizons to the chemistry of carbohydrates and instigates the discovery of new structures with biotechnological potential. Thus, studies with this scope should be encouraged. In the present work, the presence of a mannogalactan in the mutant ScT4 fungus, which presented a thin phenotype in comparison with the wild type of *S. commune*, was found. This finding regarding the chemical composition of the hot-soluble cell wall polysaccharides of a mutant fungus, reinforces the adaptation of the fungal cell wall. Cell wall density and thickness might be related to the higher glucan content in ScW (Figure 3b). This could partially explain the thin phenotype of ScT4, due to higher content of mannogalactan and relatively lower in β-glucan. The greater number of inverted signals in ^{13}C nuclear magnetic resonance (DEPT) for the C-6 (-CH_2) might suggest higher branching in polysaccharides extracted from the ScW.

Based on the observations of the phenotypes obtained from the ScT4 mutant, it was possible to conclude that hygromycin resistance gene was not associated with the absence of extracellular polysaccharides. Since it would be expected a normal production of extracellular polysaccharides in the hygromycin sensitive spore group, regardless of the phenotype, which was not observed for the ScT4 mutant.

To the best of our knowledge, this is the first report on the isolation of mannogalactans from *S. commune* liquid cultures. It is a great challenge to understand the role of this polysaccharide in the cell wall of filamentous fungi. In addition, previous reports concerning biological effects of fungal polysaccharides instigate the investigation on the biological activity of mannogalactans. As perspective, future studies are necessary to identify in the genome of mutant fungus where the plasmid pUCHYG-GPDGLS has been inserted and understand the mechanisms that may lead to the cell wall changes, in addition to comparing the chemical composition with other thin mutants.

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SUPPLEMENTARY MATERIAL

Table SI.
Figures S1-S4.

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NICOLE DALONSO[1]
https://orcid.org/0000-0001-7253-1888

CARMEN L.O. PETKOWICZ[2]
https://orcid.org/0000-0002-8255-0246

LUIS G. LUGONES[3]
https://orcid.org/0000-0002-5259-3739

MARCIA L.L. SILVEIRA[1]
https://orcid.org/0000-0003-4616-8645

REGINA M.M. GERN[1]
https://orcid.org/0000-0002-6492-4689

[1]Programa de Pós-Graduação em Saúde e Meio Ambiente, Universidade da Região de Joinville/ UNIVILLE, Rua Paulo Malschitzki, 10, Zona Industrial Norte, 89201-972 Joinville, SC, Brazil

[2]Universidade Federal do Paraná, Departamento de Bioquímica e Biologia Molecular, Centro Politécnico, Av. Coronel Francisco H. dos Santos, 100, Caixa Postal 19046, Jardim das Américas, 81531-980 Curitiba, PR, Brazil

[3]Utrecht University, Molecular Microbiology Department, Padualaan n° 8, Utrecht Science Park, 3584 CH, Utrecht, The Netherlands

Correspondence to: **Nicole Dalonso**
E-mail: nenidalo@yahoo.com.br

Author contributions

Nicole Dalonso: Conceptualization, writing and methodology; Carmen L. O. Petkowicz: Validation, methodology, writing and reviewing. Luis G. Lugones: Methodology. Marcia L. L. Silveira: Investigation and validation. Regina M. M. Gern: Supervision, writing, reviewing and editing.

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