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**The antimicrobial peptide oranicin P16 isolated from
Trichosporon asahii ICVY021, found in camel milk's,
inhibits *Kocuria rhizophila*.**

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26 **ABSTRACT**

27 Six *Trichosporon asahii* strains designated ICVY021, ICVY022, ICVY023, ICVY024,
28 ICVY025 and ICVY026 were isolated from camel milk and studied for their inhibitory
29 activities against a large number of fungi and bacteria as target organisms. Only strain
30 ICVY021 was able to inhibit *Kocuria rhizophila* CIP 53.45, through production of an
31 extracellular heat-stable, proteinaceous antibacterial peptide, with partial amino acid
32 sequences of PPFPK and CTHV(L/I)(K/Q) or TCHV(L/I)(K/Q), determined using
33 LC/MS/MS. This peptide, named oranicin P16, was thought to impede the cell-division
34 mechanism. The anti- *K. rhizophila* activity was confirmed, *in situ*, using skim milk as a food
35 matrix. *T. asahii* ICVY021 did not show any hemolytic activity or any cytotoxicity effect
36 against eukaryotic Caco-2 cells. *T. asahii* ICVY021 was sensitive to azoles antifungal
37 compounds, but not to 5-fluorocytosine and anidulafungin.

38

39 **Keywords:** *Trichosporon asahii*; yeast antimicrobial peptide; *Kocuria rhizophila*; camel milk

40

1. Introduction

Isolation and characterization of non-conventional yeasts from foods and their applications as biopreservative agents has received limited investigation. Camel milk was recently targeted as a potential source of autochthonous non-conventional yeasts and was shown to have *Trichosporon asahii* strains (IDER et al., 2019). *Trichosporon* spp. are opportunistic organisms that are distributed throughout nature (Colombo et al., 2011), but their presence in the human microbiome seems to be limited (Mariné et al., 2015). These basidiomycetes yeast-like anamorphic organisms can be found in different ecological niches including the skin and gut of healthy individuals (Cho et al., 2015; Zhang et al., 2011), dairy products as spoilage yeasts (Garnier et al., 2017), soft churupi soup, Indian fermented foods, and camel milk (IDER et al., 2019; Rai et al. 2016), indicating their capabilities of adaptation and thriving in various host and environmental niches.

Fungal spoilage is a major concern for foods and causes between 5 and 10% of the world's food production losses (Garnier et al., 2017). The presence of such microorganisms in dairy and derivative products could lead to a visible growth of fungi or to the production of unwanted metabolites causing organoleptic deterioration, and possibly a safety hazard (Hymery et al., 2014; Ledenbach and Marshall, 2010). The use of biopreservation has become another way to handle/control the development of spoilage microorganisms as well as that of food-borne pathogens (Garnier et al., 2017).

T. asahii is also emerging as a biotechnological tool for its ability to decontaminate polluted environments by accumulating large amounts of oils (Ageitos et al., 2011), and also converting isoeugenol into vanillin (Ashengroph and Amini, 2017). Strains of *T. asahii* were reported to produce lipase (Singh and Gupta, 2016), aspartic-type peptidase (Valle et al., 2017), and antioxidant molecules (Zhang et al., 2016). Furthermore, the *T. asahii* GSY10 strain was able to produce lipid from molasses, and was suggested as a feed supplement in

dairy cattle (Paserakung et al., 2015). Chen and Chou (2017) reported the antagonistic effects of *T. asahii* strains against fungi through production of extracellular pH-dependent compounds.

In this study, the inhibitory properties of 6 *T. asahii* strains isolated from camel milk that had been collected from arid and semi-arid regions of Algeria (Ider et al., 2019), were tested against a range of target organisms including fungi and bacteria. Only *T. asahii* ICVY021 showed an inhibition against *Kocuria rhizophila* CIP 53.45 through production of an extracellular, heat-stable and proteinaceous peptide. *K. rhizophila*, which belongs to the *Micrococcaceae* family, was described as a contaminant of camel milk (Elhaj et al., 2013; Takarada et al., 2008) with enzymatic activities able to induce spoilage of dairy product (Pukančíková et al., 2016). Moreover *K. rhizophila* is responsible for human infection (Moissenet et al., 2012), and for diseases in rainbow trout or brown trout (Pekala et al., 2018). Therefore this study aimed at characterizing the antibacterial peptide, designated oranicin P16, produced by *T. asahii* strain ICVY021.

2. Materials and methods

2.1. Growth conditions and media

T. asahii strains ICVY021, ICVY022, ICVY023, ICVY024, ICVY025 and ICVY026 were isolated from camel milk in arid and semi-arid regions of Algeria (i.e., Béchar, Biskra, Djelfa, El-Bayadh and Ghardaïa (2), respectively) (Ider et al., 2019). *T. asahii* ICVY021 was chosen because of its antimicrobial activity, while the other 5 strains were randomly isolated among many other strains without antimicrobial activity. Strains were plated onto YEG agar (5.0 g.L⁻¹ yeast extract; 20.0 g.L⁻¹ glucose; 15.0 g.L⁻¹ agar-agar, Sigma Aldrich Co., St. Louis, MO, USA), supplemented with 0.1% (w/v) chloramphenicol (Sigma Aldrich). The plates were incubated for 3 days at 25 °C. Yeast colonies were purified by streaking onto YEG agar and

stored at -80 °C in YEG broth supplemented with 30% (v/v) glycerol for a maximum of three months.

2.2. Genomic DNA extraction

Total DNA was extracted using the Bustin's method (Harju et al., 2004). Cells from 1.5 mL of the overnight yeasts cultures in YEG broth were pelleted in a microcentrifuge tube at 8,000 g (8680 rpm in a FA-45-30-11 rotor, Model 5804R centrifuge, Eppendorf, Hamburg, Germany) for 10 min at 4°C. The cell pellets were suspended in a lysis buffer (2% Triton X-100; 1% SDS; 100 mM NaCl; 100 mM Tris-HCl at pH 8.0; 0.1 mM EDTA, Sigma Aldrich), and disrupted by two repetitive heat shock cycles at -80 °C (for two min) and 95 °C (for one min). DNA was extracted using the chloroform procedure as follows: 200 µL of chloroform (Sigma Aldrich) was added to the tubes and Vortexed (Vortex Genie 2, Dutscher, Brumath, France) for two min and centrifuged at 20,000 g, three min at room temperature (20-25 °C). The aqueous layer was transferred to a tube containing 400 µL of ice-cold 100% ethanol (Sigma Aldrich). The samples were precipitated for 5 min at room temperature, and centrifuged at 20,000 g for 5 min at room temperature. Supernatants were pipetted and the DNA pellets were washed with 0.5 mL of 70% ethanol followed by drying at 37 °C. DNA was re-suspended in 10 mM Tris-EDTA buffer (pH 8.0, Sigma Aldrich) and its concentration determined with a Nanodrop Biowave II (Biochrom, Harvard, UK), by measuring their absorbance at 260_{nm} UV wavelength.

2.3. Yeast identification using an API 20C AUX strip and molecular methods

The API 20C AUX identification procedure was done in accordance with the manufacturer's instructions (bioMérieux, Marcy-l'Étoile, France). Cultures of ~10⁶ colony forming units (CFU) mL⁻¹ were prepared in YEG broth, and 5 drops were dispersed in an API C medium vial provided by the manufacturer and homogenized by Vortexing. After homogenizing, the inoculum was deposited on the wells of the test strip, and incubated at 25°C for 48 h. The

wells with growth were allocated a plus sign, whereas those with the absence of growth were allocated a minus sign. The results were transformed into numerical codes using a correspondence table provided by the manufacturer. The isolates were identified using the 20C analytical profile index (API 20C) available online (<https://apiweb.biomerieux.com/>).

The sequences of the 26S rDNA and ITS1-5.8-ITS2 were determined using the polymerase chain reaction (PCR) products gathered from the genomic DNA of the yeast cells, extracted using the Wizard® Genomic DNA Purification Kit (Promega, Madison, WI, USA). The sequencing of the 26S rDNA and ITS1-5.8-ITS2 were done using primers and programs shown in [Table I](#). The PCR products were separated on a 1% agarose gel (Bio-Rad, Hercules, CA, USA) labelled with 0.05% GelRed (Thermo Fisher Scientific Co., Waltham, MA, USA) for 2 h at 100 V. The gels were analysed using PyElph 1.4 software available online (<https://pyelph.software.informer.com/1.4/>).

The PCR products were sequenced at Eurofins (Hamburg, Germany). A blast search of the nucleotide sequences was done through the nucleotide Blastn software (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) of the US National Center for Biotechnology Information (NCBI). The nucleotide sequences obtained for these samples were deposited at the NCBI database with the accession numbers given in [Table II](#).

2.4. Repetitive-element-palindromic-PCR (Rep-PCR) fingerprints

The Rep-PCR was done with the unique primer 5'-GTG GTG GTG GTG GTG-3' (GTG5, Eurofins) ([Ceugniet et al., 2015](#); [Ouoba et al., 2010, 2012](#)). The PCR program used included an initial denaturation at 94 °C for 4 min, followed by 30 cycles of denaturation at 94 °C for 1 min, hybridation at 40 °C for 1 min and elongation at 72 °C for 8 min. The final elongation was done for 16 min at 72 °C. For each reaction 150 ng of DNA, master mix PCR 2X (Thermo Fisher Scientific), ultra-pure water (Thermo Fisher Scientific) and 2 µM primer were used. The PCR products and 5 µL of DNA marker (Thermo Scientific, Vilnius, Lithuania), at

0.1 $\mu\text{g } \mu\text{L}^{-1}$, were deposited and separated on a 1% agarose gel labelled with 0.05% GelRed for 2 h at 100 V. The gels were visualized and photographed with a Gel-Doc 2000® (Bio-Rad), and the DNA profiles (size and number of amplicons) were established with GelCompar II (v6.6.11) software (Biosystematica, Ceredigion, Wales, UK).

2.5. Assessment of antagonistic properties of *T. asahii* strains

The inhibitory properties of culture supernatants of *T. asahii* ICVY021, ICVY022, ICVY023, ICVY024, ICVY026 and ICVY026 strains were assessed against different fungi and Gram-negative and Gram-positive bacteria, as target organisms. These included *Candida albicans* ATCC 10231, *Fusarium oxysporum* (Charles Viollette Institute collection, Lille University, Villeneuve d'Ascq, France), *Escherichia coli* ATCC 8739, *Salmonella enterica* subs. Enterica serotype Newport (Bioecoagro Lab collection, Lille University), *Staphylococcus aureus* ATCC 25923, *Listeria monocytogenes* 162 (Al Seraih et al., 2017). *K. salsicia* and 8 other *K. rhizophila* strains isolated from a meat grinder in a Danish Slaughterhouse, and kindly provided by Professor J. Herschend (Copenhagen, Denmark) were also tested. *T. asahii* strains were grown in YEG broth for 48 h at 25 °C with shaking (160 rpm). Culture supernatants (CS) were obtained by centrifugation at 6,000 g (6,690 rpm in a FA-45-6-30 rotor), for 10 min at 4 °C, and stored at this same temperature for a maximum of 2 days before use. One mL of target strain suspension in Mueller Hinton (MH) broth (Sigma Aldrich), previously adjusted at OD_{600nm} to 0.0025, was flooded on MH agar plates. After drying the plates at room temperature in a laminar flow hood (ADS Laminaire, Aulnay-sous-bois, France) for <20 min, wells of ~6 mm were made with sterile pipette tips (VWR, Radnor, PA, USA) in each plate, and filled with 50 μL of CS. After 2 h of incubation at 4 °C, allowing diffusion of CS, plates were incubated at 37 °C for 48 h. In all these experiments, the presence or absence of inhibition zones was visually determined.

2.6. Purification of the inhibitory compound from *T. asahii* ICVY021

The active CS obtained after the culture of *T. asahii* ICVY021 was purified using Amberlite® XAD®-2 polymeric adsorbent resin (Sigma Aldrich). CS (50 mL) were mixed with the XAD resin (2 g) and incubated overnight at 20 °C with orbital agitation at 100 rpm. The mixture was vacuum filtrated through a 0.2 µm filter (VWR) and the filtrate removed. The XAD resin was resuspended and washed twice with 20 mL of ultrapure water (Thermo Fisher Scientific), and incubated at 20 °C with agitation at 100 rpm for 20 min. The XAD resin containing the active compound(s) was separated again by vacuum filtration as described above and resuspended in a mix of 10 mL of ultrapure water + 10 mL methanol, and incubated at 100 rpm, 20 °C for 20 min. The water/methanol phase containing the active compound, designated oranicin P16, was recovered using a syringe after the resin settled, and the solvent was removed using a speed vacuum concentrator (SpeedVac, Thermo Fisher Scientific).

2.7. Minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) of the purified inhibitory compound oranicin P16 produced by *T. asahii* ICVY021

Following the growth of *K. rhizophila* CIP 53.45 in MH medium at 37 °C, 1 colony was randomly chosen and added to 10 mL of MH broth. Samples of 10 µL of this bacterial suspension were added to the 96 wells of bioassay microplates containing serial dilutions of active compound prepared as indicated above. The MIC was defined as the lowest concentration of this active compound that inhibited the visible growth of the target strain after overnight incubation at 37 °C.

The MBC was determined by subculturing the inoculated samples, used for the MIC assay, in MH agar without oranicin P16. The MBC was defined as the concentration of active compound that reduced the viability of the initial inoculum of *K. rhizophila* CIP 53.45 by

≥99.9%, as determined by counting the cells on MH plates, compared to the untreated inoculum tested using the same conditions.

2.8. *In situ activity of oranicin P16 in skim milk*

To assess the *in situ* activity of the purified active compound, a time-killing experiment was done. Tubes containing a commercial sterilized skim milk (Carrefour®, Carrefour group, France, Boulogne-Billancourt, France) were supplemented with 10% (v/v) of PBS, or nisin (Sigma Aldrich) at a final concentration of 1 µg mL⁻¹, or with the active compound oranicin P16 at the MIC value. A volume of 20 µL of a *K. rhizophila* CIP 53.45 suspension of ~2×10⁹ CFU mL⁻¹ in PBS, as determined by cell-counting, was introduced in the prepared tubes containing 2 mL of the supplemented skim milk with PBS (control), nisin or oranicin P16, and incubated at 37 °C for 6 h. The killing kinetics of the nisin and the oranicin P16 were monitored using viable bacterial counts on agar plates compared to the control. Aliquots were taken at 0, 2, 4 and 6 h, and serially diluted in saline solution for determination of viable counts. Diluted samples (100 µL) were plated on the MH agar plates and colonies were counted after overnight incubation at 37 °C.

2.9. *Molecular mass and amino acids sequence of the purified inhibitory compound*

Liquid chromatography - mass spectrometry (LC/MS) analyses, using positive mode with electrospray ionisation (ESI) and high resolution time of flight (TOF) mass analyzer, were done using an Acquity H-Class (Waters Corp., Milford, MA, USA) system equipped with a Kinetex EVO C18 column (1.9 mm particle size, 150 × 2.1 mm), (Phenomenex, Torrance, CA, USA). The mobile phase A consisted of water while mobile phase B was acetonitrile both containing 0.1% formic acid. The gradient started at 5% mobile phase B and increased to 100% mobile phase B over 40 min. The flow rate was 0.5 mL min⁻¹ and the eluent was directed to the atmospheric pressure ionisation source of the Synapt G2-S (Waters) tandem hybrid Quadrupole-TOF mass spectrometer operating in positive electrospray ionization

mode. Capillary and cone voltages were 3000 and 30 V, respectively. Source and desolvation temperatures were 120 and 450 °C, respectively. The molecular weight of the purified peptide was determined by the deconvolution of the resulting signal. MS/MS analyses were done using collision induced dissociations (CID) fragmentation at 40 eV. All data were acquired and processed using Masslynx 4.2 software (Waters). Then the sequences were blasted using the NCBI protein database using BlastP online software (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>). The matching peptides, with highest sequences homology, were then compared manually.

2.10. Transmission electron microscopy (TEM) of K. rhizophila CIP53.45 treated with the inhibitory compound

K. rhizophila CIP 53.45 was grown in 2 mL MH medium overnight and divided equally in two different tubes after Vortexing. After centrifugation at 8,000 g at 4 °C for 10 min, the supernatants were discarded and the cells pellets were washed twice with PBS. In the first tube, the pellets were resuspended and treated with the oranicin P16 at MBC, determined as described previously, and resuspended in sterile ultrapure water. In the second tube (control tube), the pellets were resuspended in sterile ultrapure water, and the samples were incubated overnight at 37 °C.

For TEM, samples were fixed in 2.5% glutaraldehyde (Merck Co., Kenilworth, NJ, USA) buffered with 0.1 M sodium cacodylate (Merck), post-fixed in 1% osmium tetroxide (EMS, Hatfield, PA, USA) in the same buffer and “en bloc” stained with 2% uranyl acetate (VWR). After washing with acetonitrile and air-drying, samples were embedded in epon-like resin (EMbed-812, EMS). Ultrathin sections of ~90 nm were cut using a Leica UC7 ultramicrotome (Wetzlar, Germany) and collected on 150 mesh hexagonal barred copper grids. The samples were observed at 200 kV using a Jeol JEM-2100 microscope (Jeol Ltd., Tokyo, Japan)

coupled with a Gatan Orius SC200D camera (Gatan Inc., Oxford, UK) with magnification of ~20,000 to 40,000x

2.11. Hemolysis and resistance to antifungal drugs of *T. asahii* ICVY021 antagonistic strain

The hemolytic activity of antagonistic strain was determined by streaking fresh colonies on blood agar plate (VWR). The plate was incubated at 37 °C for 24-48 h. The development of a clear zone around the colonies was considered as a positive result. The *in vitro* studies of sensitivity to amphotericin B, 5-fluorocytosine, fluconazol, voriconazol, caspofungin, posaconazol and anidulafungin (bioMérieux) were done using the E-test method (bioMérieux) according to CLSI (Clinical Laboratory Standards Institute) guidelines. Briefly, an inoculum of *T. asahii* ICVY021 was prepared and adjusted to $\sim 10^6$ cells mL⁻¹. A sample of 0.5 mL of this suspension was inoculated onto plates containing RPMI 1640 agar (1.5%) (Sigma Aldrich) with 2% glucose (Sigma Aldrich) using a sterile cotton swab. After a period of 15 min, the E-test strips (5.7×0.5 cm) containing the antifungal drugs, with concentrations ranging from 0.002 to 256 µg mL⁻¹, were applied on the plates (9 cm diameter). The MIC which is the lowest concentration allowing the complete inhibition of yeast growth was obtained after 48 h of incubation at 25° C.

The interpretation of MIC values was done using the data given for *T. asahii* by [Kanafani and Perfect \(2008\)](#), in accordance with the recommendations approved by the European Committee on Antimicrobial Susceptibility Testing ([EUCAST, 2017](#)) and the CLSI ([CLSI, 2018](#)) using *Candida albicans* as a reference strain ([Cuenca-Estrella et al., 2012](#)). According to these recommendations, the *T. asahii* was considered resistant to the antifungal agents tested when their MIC were equal or higher to the following cut off concentrations: 2 mg L⁻¹ for amphotericin B, 50 mg L⁻¹ for 5-fluorocytosine, 8 mg L⁻¹ for fluconazol, 1 mg L⁻¹ for

voriconazol, 1 mg L⁻¹ for caspofungin, 0.06 mg L⁻¹ for posaconazol and 1 mg L⁻¹ for anidulafungin.

2.12. Cytotoxicity assay

Intestinal human colorectal tumour Caco-2 cells (Sigma Aldrich) were grown in 96-well cell culture plates and pre-incubated for 7 days at 37 °C, 5% CO₂ atmosphere in Dulbecco modified Eagle medium (DMEM, Pan-Biotech GmbH, Aidenbach, Germany) supplemented with 10% of fetal bovine serum (FBS, Thermo Fisher Scientific), 2 mM glutamine (Pan-Biotech GmbH), 100 µg mL⁻¹ of penicillin and 100 µg mL⁻¹ of streptomycin (Pan-Biotech GmbH). A 24 h old culture of *T. asahii* ICVY021 were diluted to 2×10⁸ CFU mL⁻¹ in antibiotic and serum free DMEM. A suspension of 100 µL was added to each well containing ~2.10⁵ Caco-2 cells. Furthermore, the cytotoxicity on the Caco-2 cells was measured after 24 h of contact by determining the dehydrogenase activity of mitochondria with the CCK-8 (Counting Kit-8) assay (Dojindo, Kumamoto, Japan). Briefly, after incubation cells were washed with DMEM without any supplementation to remove non-adherent yeasts. To eliminate adherent yeasts, 50 µL of amphotericin B (Sigma Aldrich), at a final concentration of 5 mg L⁻¹ in PBS, were added to each well. After 2 h of incubation at 37 °C, the solution of amphotericin B was removed and the wells were filled with 100 µL of DMEM supplemented with 5 µL of CCK-8 (Dojindo) reagent and incubated for an additional 2 h at 37 °C. Wells with Caco-2 cells treated with Triton X100 solution (Sigma Aldrich), diluted to a final concentration of 0.1% in DMEM were used as a positive control of cytotoxicity whereas wells with non-treated Caco-2 cells served as a negative control. An additional negative control using wells with Caco-2 cells treated with amphotericin B, with the same conditions as described before, but not exposed to yeast was also used.

The CCK-8 reagent was used at 5% (v/v) final concentration to evaluate the viability of Caco-2 cells by determining the activity of cellular dehydrogenase, resulting in an orange colored formazan from the water-soluble tetrazolium salt: WST-8(2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)2H tetrazolium, monosodium salt)). The formation of formazan was measured at 450 nm using a microplate reader (Xenius SAFAS, Monaco), and compared with the absorbance of non-treated cells considered as the control.

2.13. Adhesion assay

The adhesion abilities of *T. asahii* ICVY021 on Caco-2 cells were determined as reported by Ait Seddik et al. (2017). Caco-2 cells/well (4×10^4) were seeded in 24-well tissue culture plate, incubated for 7 days at 37 °C (5% CO₂ and 95% air). Cells of *T. asahii* ICVY021 grown in YEG broth for 48 h at 25 °C were harvested by centrifugation at 6,000 g at 4 °C for 10 min, and resuspended in DMEM without FBS and antibiotics to a final concentration of 10^5 CFU/well. To determine the effect of proteins or carbohydrates on the adhesion process of *T. asahii* ICVY021 with confluent Caco-2 monolayers, the yeast cells were treated with trypsin from porcine pancreas (Sigma Aldrich) at a final concentration of 2.5 mg mL⁻¹ for 1 h at 37°C, or with 50 mM of periodic acid (Sigma Aldrich) in 10 mM PBS (pH 7.4) for 30 min at 37 °C (Ait Seddik et al. (2017)). Both samples were washed twice with PBS and resuspended in DMEM without FBS and antibiotics. Untreated yeast-cells at $\sim 10^5$ CFU/well or treated ones with trypsin or periodic acid were applied to confluent Caco-2 cells and incubated for 2 h at 37 °C (5% CO₂ and 95% air). The Caco-2 monolayers were washed twice with 500 µL of PBS to remove non-adherent yeast cells. Lysates obtained after incubation with Triton X100 (Sigma Aldrich) at 0.1% (v/v) for 15 min, were serially diluted and plated on YEG agar to determine the number of adherent yeast cells, untreated and treated with trypsin or periodic acid.

2.14. Statistical analysis

Data of cytotoxicity and adhesion assays were expressed as a mean \pm standard error calculated over three independent experiments. Analysis of statistical significance was done using one way ANOVA and the post-hoc Tukey Test ($P < 0.05$) using XL-STAT (Addinsoft, Bordeaux, France).

3. Results

3.1. API 20C AUX strips and molecular methods are reliable methods for identification of *T. asahii* strains

After 72 h at 25 °C colonies of *T. asahii* ICVY021, ICVY022, ICVY023, ICVY024, ICVY025 and ICVY026 on YEG-agar plates were white, dried and wrinkled with irregular margins (Fig. 1). All the strains were identified as *T. asahii* using API 20C AUX strips and molecular methods. The scores obtained using the API 20C AUX system ranged from 90 to 100% (Table II), while those obtained after blasting 26 rDNA and ITS1-5.8-ITS2 regions sequences ranged between 99 to 100% (Table II).

3.2. *T. asahii* ICVY021 showed a slightly different DNA pattern

The analysis of rep-PCR DNA fingerprints and their related dendrogram delineated two main groups of yeasts. The first is composed of ICVY022, ICVY023, ICVY024, ICVY025 strains, and the second contains only *T. asahii* ICVY021 (Fig. 2).

3.3. *T. asahii* ICVY021 is the only strain with inhibitory properties

The inhibitory properties of *T. asahii* strains were tested against different organisms including fungi, and both Gram positive and Gram negative bacteria. CS were tested as sources of inhibitory compound(s). Only *T. asahii* ICVY021 showed an inhibition zone against *K. rhizophila* CIP 53.45 (Fig. 3). The CS was heat-treated for 10 min at 60, 80 and 100 °C, and tested against *K. rhizophila* CIP 53.45. The antibacterial activity had slightly decreased with

treatment at 100 °C and 80 °C but not at 60 °C (data not shown). Protease treatments abolished the anti- *K. rhizophila* CIP 53.45 activity, suggesting a proteinaceous active compound.

For the *in situ* assessment of this activity, killing curves were obtained in a commercial skim milk using *K. rhizophila* CIP 53.45 as a target organism, treated or not with nisin at 1 µg mL⁻¹, and the purified oranicin P16, used at the MIC value (215 µg mL⁻¹). Microbial counts were measured as CFU mL⁻¹ and the resulting averaged data were transformed to Log CFU mL⁻¹. The cell number in the treated skim milk with oranicin P16 decreased by ~1 Log CFU mL⁻¹, whereas the untreated sample was unaffected showing a growing population, which reached 6.61 Log CFU mL⁻¹ (Fig. 4). Nevertheless, nisin showed the strongest inhibitory activity with a rapid decreasing number of the viable and cultivable cells, by 3 Logs after 6 h, therefore killing >99.9% of the initial bacterial populations.

3.4. Resistance to antifungal drugs, hemolytic and cytotoxic effects of *T. asahii* ICVY021

T. asahii ICVY021 was tested against 6 antifungal drugs routinely used in treatment of yeast infections. The MIC values obtained were 0.125 mg L⁻¹ for amphotericin B, 200 mg L⁻¹ for 5-fluorocytosine, 0.380 mg L⁻¹ for fluconazole, 0.02 mg L⁻¹ for voriconazole, 0.047 mg L⁻¹ for posaconazole and 32 mg L⁻¹ for anidulafungin. *T. asahii* ICVY021 do not show hemolytic activity, as no blood hemolysis was observed around the grown colonies after 24-48 h of growth at 25 °C. Likewise, no cytotoxic effect of *T. asahii* ICVY021 was observed with Caco-2 cells after 24 h of contact with *T. asahii* (Fig. 5A). Taken together, these results began to establish the safety of *T. asahii* ICVY021.

The adhesion ability was also investigated using the human cell line Caco-2. As shown in Fig. 5B, the results showed that 10% of *T. asahii* ICVY021 cells were adhesive. This score is weak compared to those for dairy yeasts (Živković et al., 2015), but remains higher than that

observed for *Candida* species (Al Seraih et al., 2015). Moreover, the treatment with trypsin or periodic acid led to a decrease of the percentage of adhesive *T. asahii* ICVY021 to <1%.

3.5. The inhibitory proteinaceous sample contains a peptide with a molecular weight of 4,607 Da.

To identify the compound(s) responsible for the antimicrobial activity, the inhibitory proteinaceous sample was analysed using LC/MS/MS with a reversed-phase gradient designed to separate a wide range of organic compounds. Among all the chromatographic peaks detected, one of them was found to show the features of a peptide sequence. Indeed, the corresponding mass spectrum obtained in the positive mode with ESI and high resolution TOF mass analyser showed multiple charged molecular ions ranging from +3 up to +6 protonations, as seen in Fig. 6 (top). These MS signals were deconvoluted to obtain the molecular weight of 4,607 Da as shown in Fig. 6 (bottom). The sequencing of such a long peptide was directly attempted using LC/MS/MS with CID without any proteolytic step. During this experiments, the selected cations from the quadrupole (Q) collide with argon gas in the collision cell to induce fragmentation. Products ions derived from CID were then mass analysed by TOF analyser. Although partial sequence coverage was expected from fragmentation of a very large peptide with these low energy CID conditions (Mouls et al., 2007), the aim of such identification study was to propose a sequence tag that can be used for further search of known peptides and proteins in databases. For that purposes, all protonated molecular ions were dissociated but only the penta-charged ion $(M+5H)^{5+}$ detected at m/z 922.6 provided many fragments that allowed the identification of the PPFP(K/Q) sequence tag. The first series of fragment ions without decarbonylation (Fig. 7) were attributed to y ions starting at m/z 358.21 (noted y_n) up to m/z 924.5 (y_{n+5}). The last residue of this series with a mass of 128 can be attributed to either K or Q. Nevertheless, the detection of the m/z 84 ion related to the dehydrated immonium ion of K might favour the PPFPK sequence than PPFPQ.

Two other ion series were related to the *b* ions due the loss of CO and formation of the corresponding *a* ions. The two sequences V(L/I)(K/Q) or (L/I)V(K/Q) can be envisaged since two possibilities may be obtained for the b_{n+1} ion at either *m/z* 441 or 455. The residue detected at a mass difference of 128 Da can be attributed as previously to isobaric K or Q. In the same way, a mass difference of 113 Da corresponds to isomeric L or I. Moreover, the immonium ion of H was detected at *m/z* 110 so this peptide contained this residue. Taking into account that three other ions at *m/z* 159, 170 and 187 were assigned as *a/b* ions and have experienced neutral losses (H₂O, NH₃ noted ° and *, respectively) are often observed in CID. They can be related to the b_n ion (*m/z* 342) by the mass difference of H (expected b_{n-1} at *m/z* 205 and a_{n-1} at *m/z* 177 but were not detected, but the b_{n-1}° at *m/z* 187, $b_{n-1}^{\circ*}$ at *m/z* 170 and a_{n-1}° at *m/z* 159 were detected). These result implied that the position of H can be proposed after V or after (L/I) giving the partial sequence HV(L/I)(K/Q) or H(L/I)V(K/Q). Finally, the residual mass corresponding to be the smallest *b* ion (*m/z* 205) can be the dipeptide CT or TC. Four high probable sequences can be suggested CTHV(L/I)(K/Q), CTH(L/I)V(K/Q), or TCHV(L/I)(K/Q), and TCH(L/I)V(K/Q). The detection of an internal ion at *m/z* 237 related to the VH or HV chain could favour the two possibilities CTHV(L/I)(K/Q) or TCHV(L/I)(K/Q). In conclusion, two putative sequence tags were evidenced with PPFPK and CTHV(L/I)(K/Q) or TCHV(L/I)(K/Q). These sequences were compared to those found in the NCBI protein database, and showed a partial homology with proteins, with undefined function, from the plant *Lupinus angustifolius* and the bacterium *Candidatus curtissbacteria*. More information could be deduced from peptide proteolysis prior to MS/MS sequencing to dissociate shorter peptides more amenable to CID fragmentations.

3.6. TEM

The observation of the control sample by TEM showed the typical shape of the *K. rhizophila* cells, consisting of spherical cells of 1.0-1.5 μm diameter, occurring in pairs, tetrads and

packets. When, these cells were treated with the inhibitory compound, oranicin P16 at the MBC = 860 $\mu\text{g mL}^{-1}$, from *T. asahii* ICVY021, they showed altered walls with apparent stoppage of the cell division process. The cells seem stuck at the same stage with widespread mortality with difficulties to distinguish the plasma membrane compared to the untreated cells (Fig. 8A, B).

4. Discussion

The presence of *Trichosporon* spp strains in food and food derivative products was recently reported for French foods (Ceugniet et al., 2017; Garnier et al., 2017), and also in Algerian camel milk (Ider et al., 2019). *T. asahii* strains ICVY021, ICVY022, ICVY023, ICVY024, ICVY025 and ICVY026 from Algerian camel milk (Ider et al., 2019) were studied for their ability to inhibit other microorganisms and characterized for different aspects. No differences were observed in their morphologies aspects following their growth on the YEG agar medium. All the colonies from these different strains were white, dried and wrinkled with irregular margins (Fig. 1). Morphological changes have been reported for clinical strains (Ichikawa et al., 2016), but not for food ones. These changes in the morphological aspects can be associated with different factors such as production of β -N-acetylhexosaminidase (Ichikawa et al., 2004), or exposure to oxidants (Zong et al., 2012). Additional studies are necessary to say whether these morphological changes constitute a general metabolic scheme, or are uniquely related to pathogenic phenotypes. The data and statistical analysis enabled us to categorize *T. asahii* strains ICVY021, ICVY022, ICVY023, ICVY024, ICVY025, and ICVY026 from camel milk origin into two main branches. The first contained only strain ICVY021, and the second contained the other ones (Fig. 2). *T. asahii* ICVY021 and ICVY022

isolated in the Ghardaïa region, have similar DNA patterns, unlike the other strains isolated in different locations.

T. asahii is an opportunistic basidiomycetous yeast-like anamorphic organism thriving in different ecological niches because of their metabolic adaptation and genome plasticity. The strains from food sources need further assessments to determine their safety and ability to produce antimicrobial substances. These are two important traits that can help promote these strains for industrial applications.

T. asahii ICVY021 produces an antimicrobial peptide, designated oranicin P16, in reference to Oran, an Algerian city, where the strain was isolated and characterized for its antibacterial function. Oranicin P16, of 4,607 Da, was produced uniquely after 48 h of growth, and consisted of two sequence tags of PPFPK and CTHV(L/I)(K/Q) or TCHV(L/I)(K/Q). Moreover, TEM analysis of *K. rhizophila* CIP 53.45 strain treated with oranicin P16 indicated a possible action on its cell-division process. The size, stability to high temperatures or alteration by proteases of oranicin P16 are typical of bacteriocins from Gram-positive and Gram-negative bacteria (Drider and Rebuffat, 2011). *T. asahii* ICVY021 was not hemolytic, and was sensitive to clinical antifungal drugs. Mechanisms by which *Trichosporon* species resist antifungal drugs remain to be better understood, although similarities with *Candida* and *Aspergillus* species were reported (Kushima et al., 2015). Moreover, the genes *ERG11* encoding the lanosterol 14- α -demethylase enzyme, which is the primary target of azoles (Kushima et al., 2015), can be lost with a single amino acid substitution (Kushima et al., 2017). *T. asahii* ICVY021 was slightly resistant to 5-fluorocytosine and anidulafungin but this may not be a critical point because the *in vitro* MIC values are not always systematically consistent with the therapeutical doses (Rex and Pfaller, 2002). Therefore, more accurate methods such as the minimum effective concentration (MEC) are needed (Kurtz et al., 1994).

T. asahii ICVY021 was able to adhere to human Caco-2 cells, and this adhesion was reduced by ~90% and almost abolished when proteinase and periodic acid, respectively, were added. These data show the involvement of proteins and polysaccharides at the cell-surface of *T. asahii* ICVY021 in the adhesion to eukaryotic cells.

5. Conclusion

The capability of a non-conventional yeast from camel milk, namely *T. asahii* strain ICVY021 to produce an extracellular antimicrobial peptide of 4,607 Da, with two putative sequence tags of PPFPK and CTHV(L/I)(K/Q) or TCHV(L/I)(K/Q) were studied. This peptide is active only against *K. rhizophila* CIP 53.45 and likely acts as an amitotic peptide. From a biotechnology point of view, the purified peptide, named oranicin P16, may be a valid alternative to antibiotics against *K. rhizophila* human infections or outbreaks of the diseases in rainbow or brown trout. Therefore, the determination of the whole sequence of this antibacterial peptide as well as its expression in heterologous hosts need to be done.

Conflicts of interests

The authors confirm that they have no conflicts of interest with respect to the work described in this manuscript.

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Fig. 1

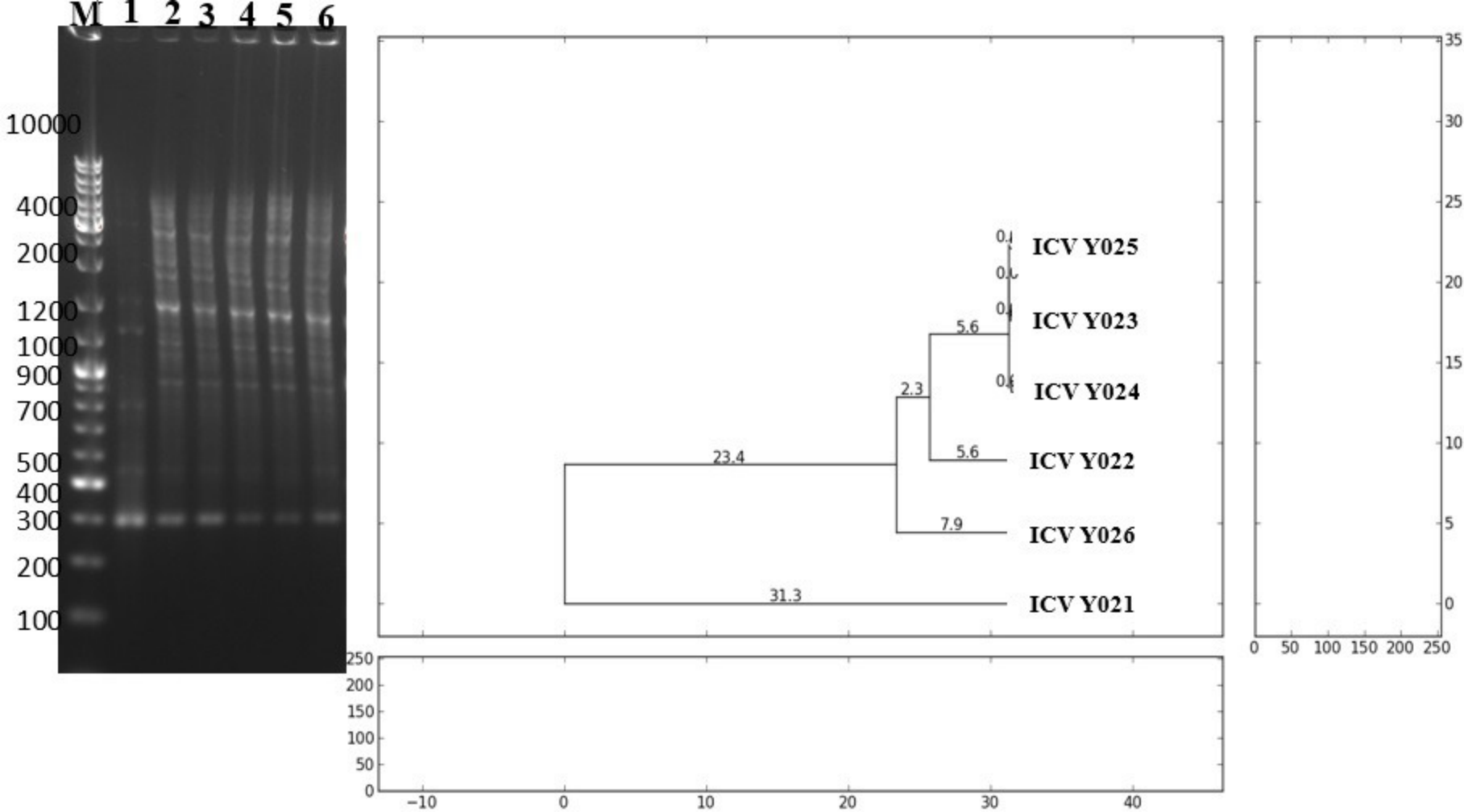


Fig. 2

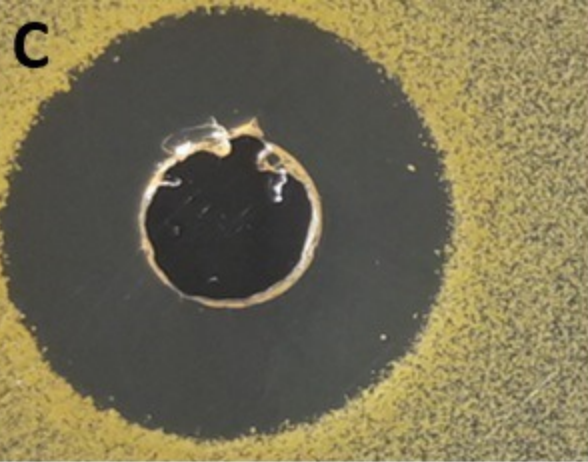
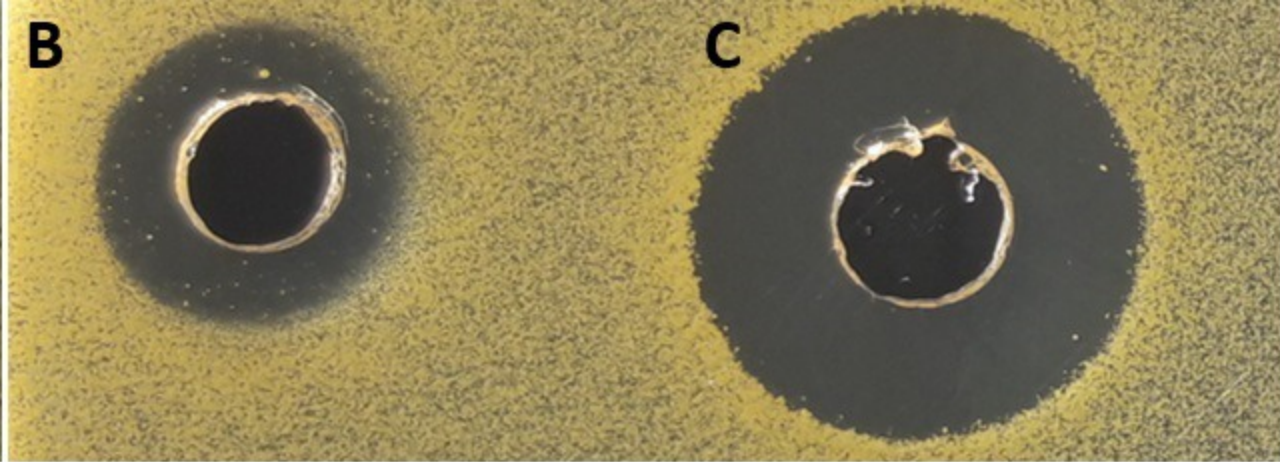


Fig. 3

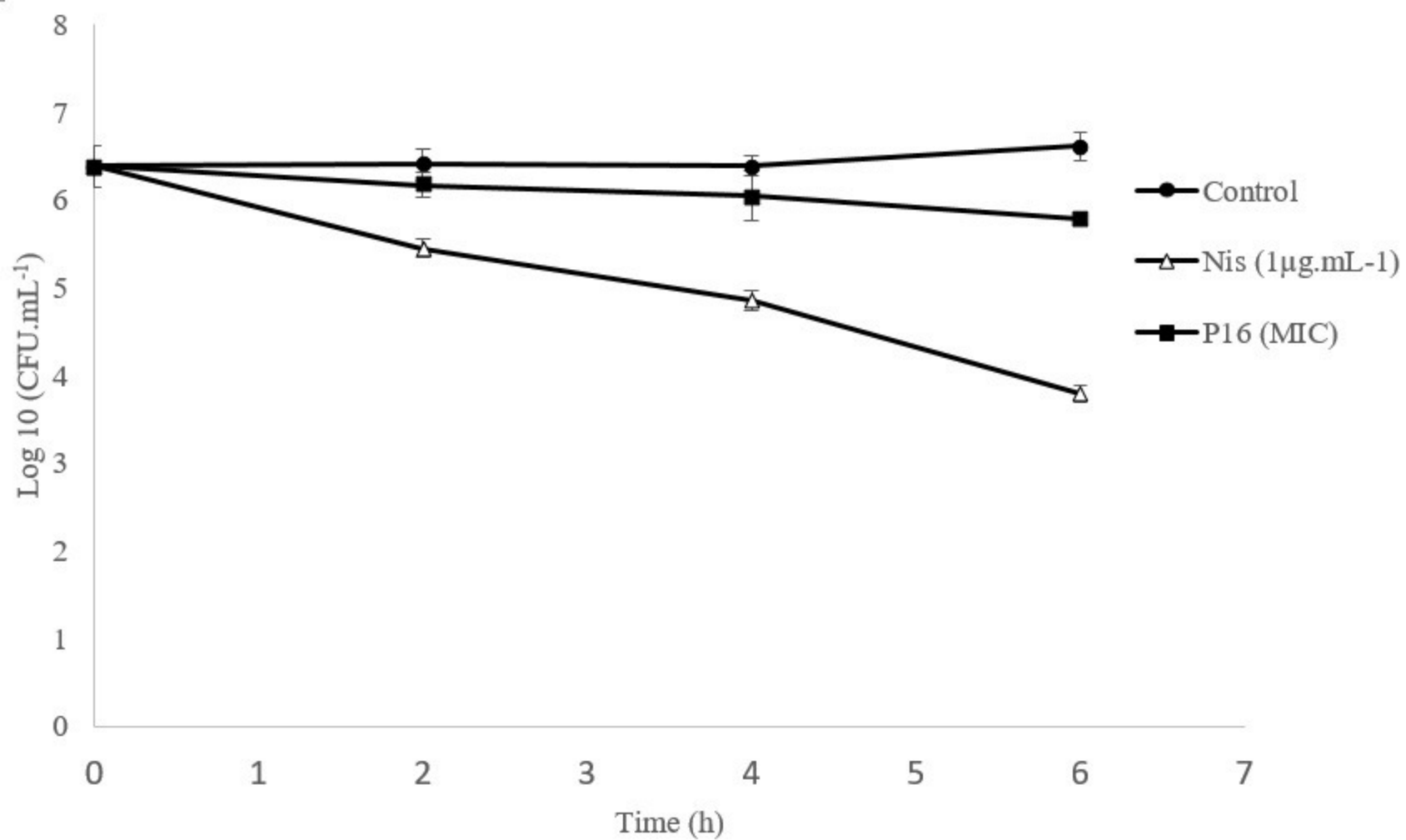


Fig. 4

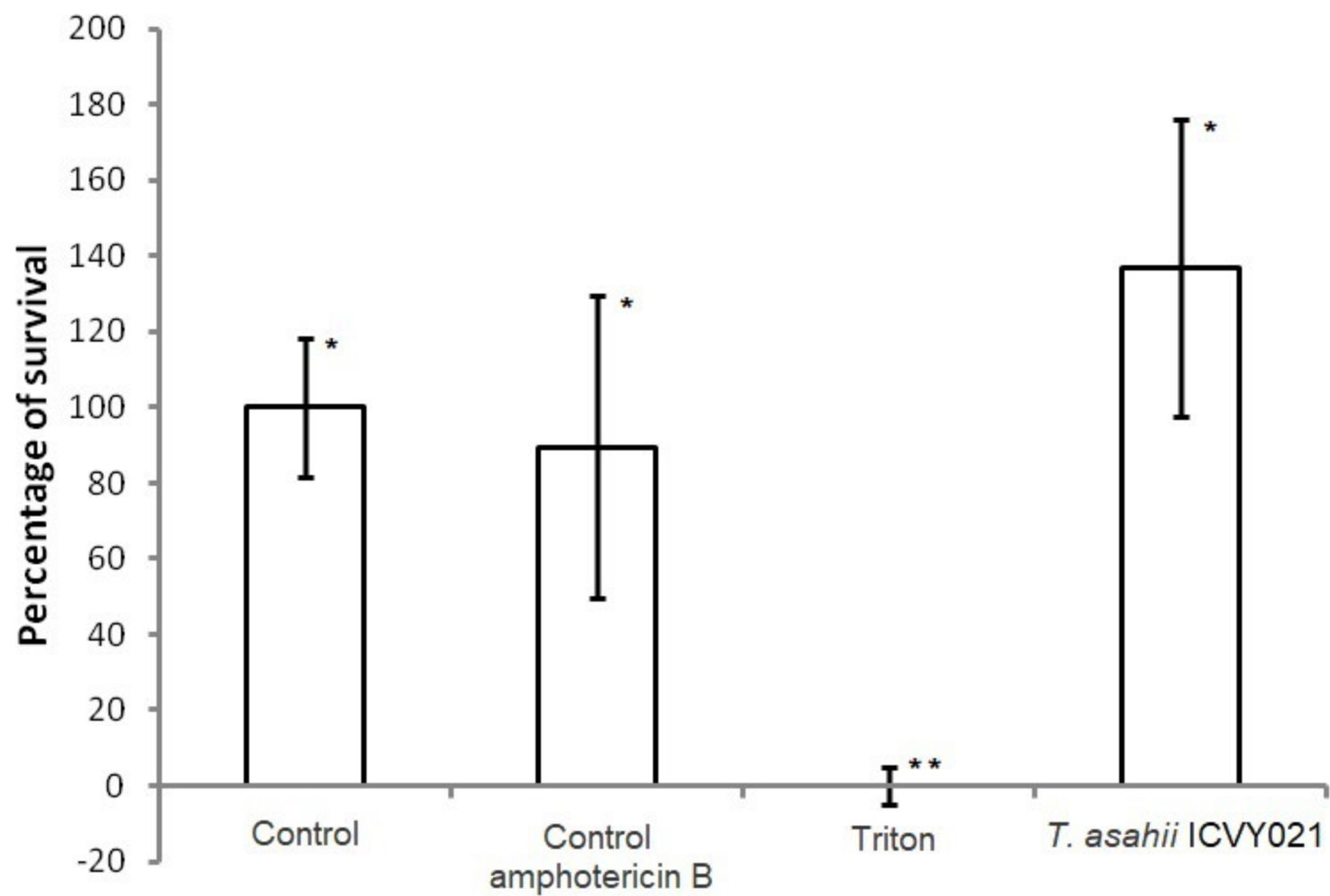


Fig. 5A

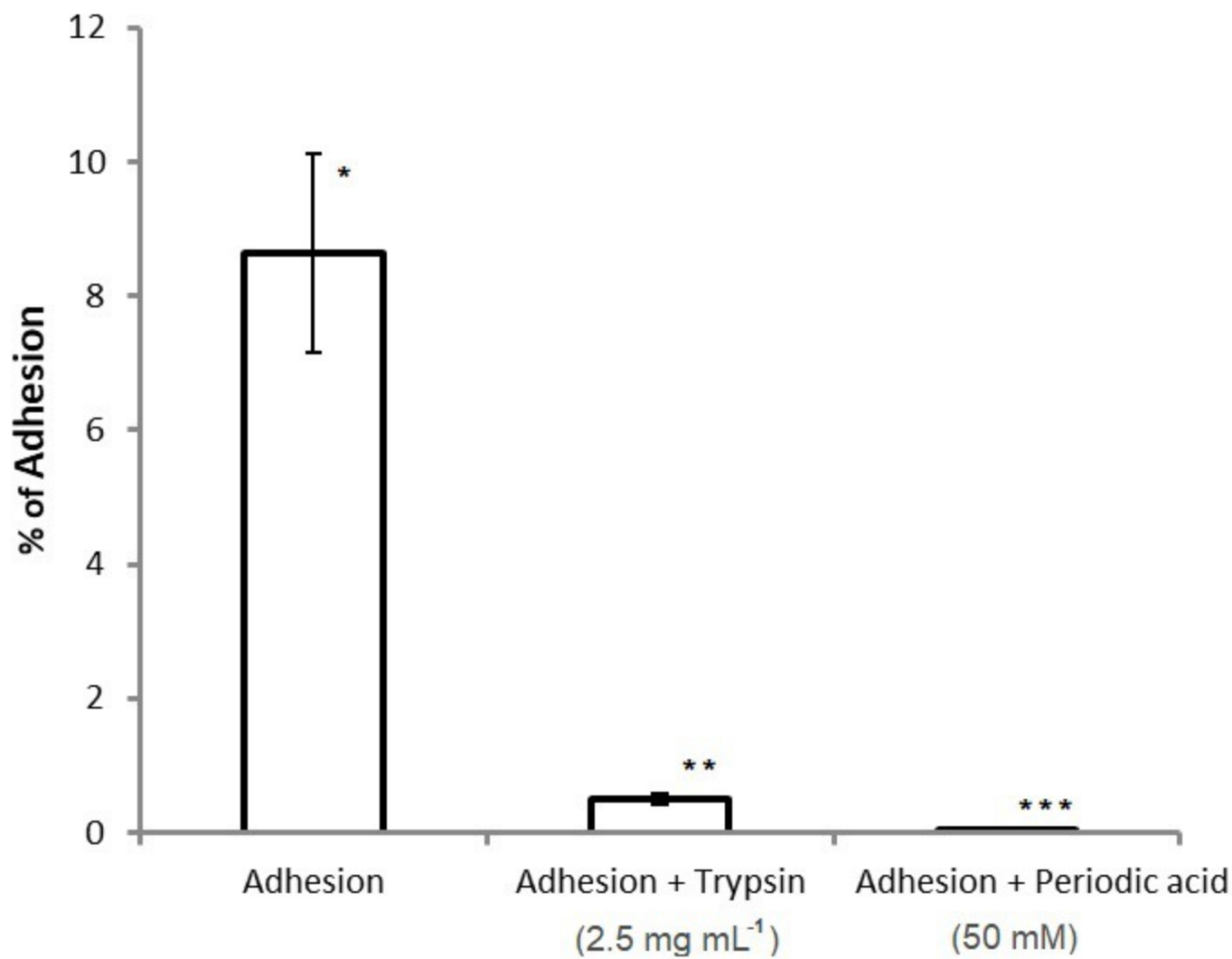


Fig. 5B

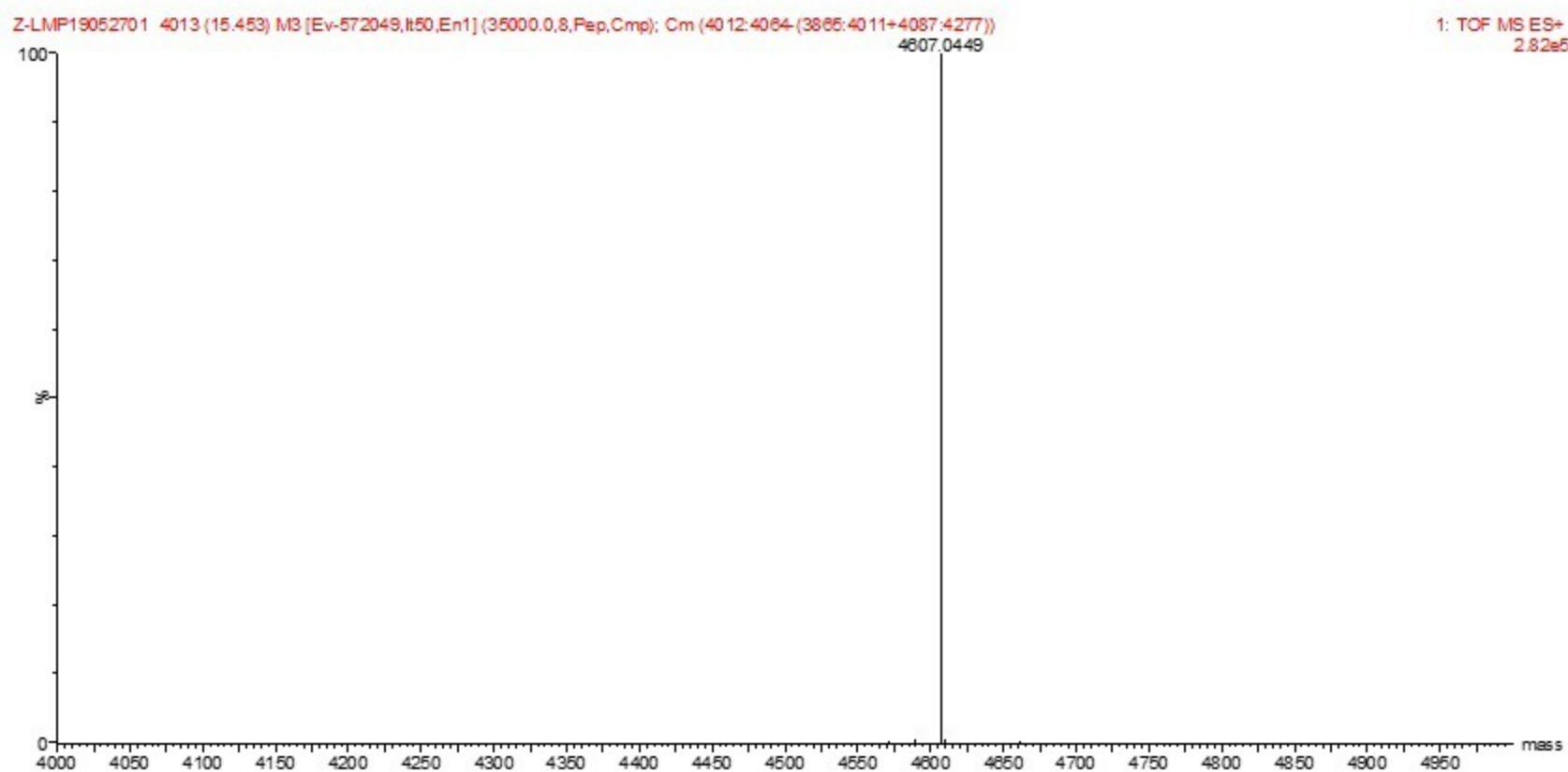
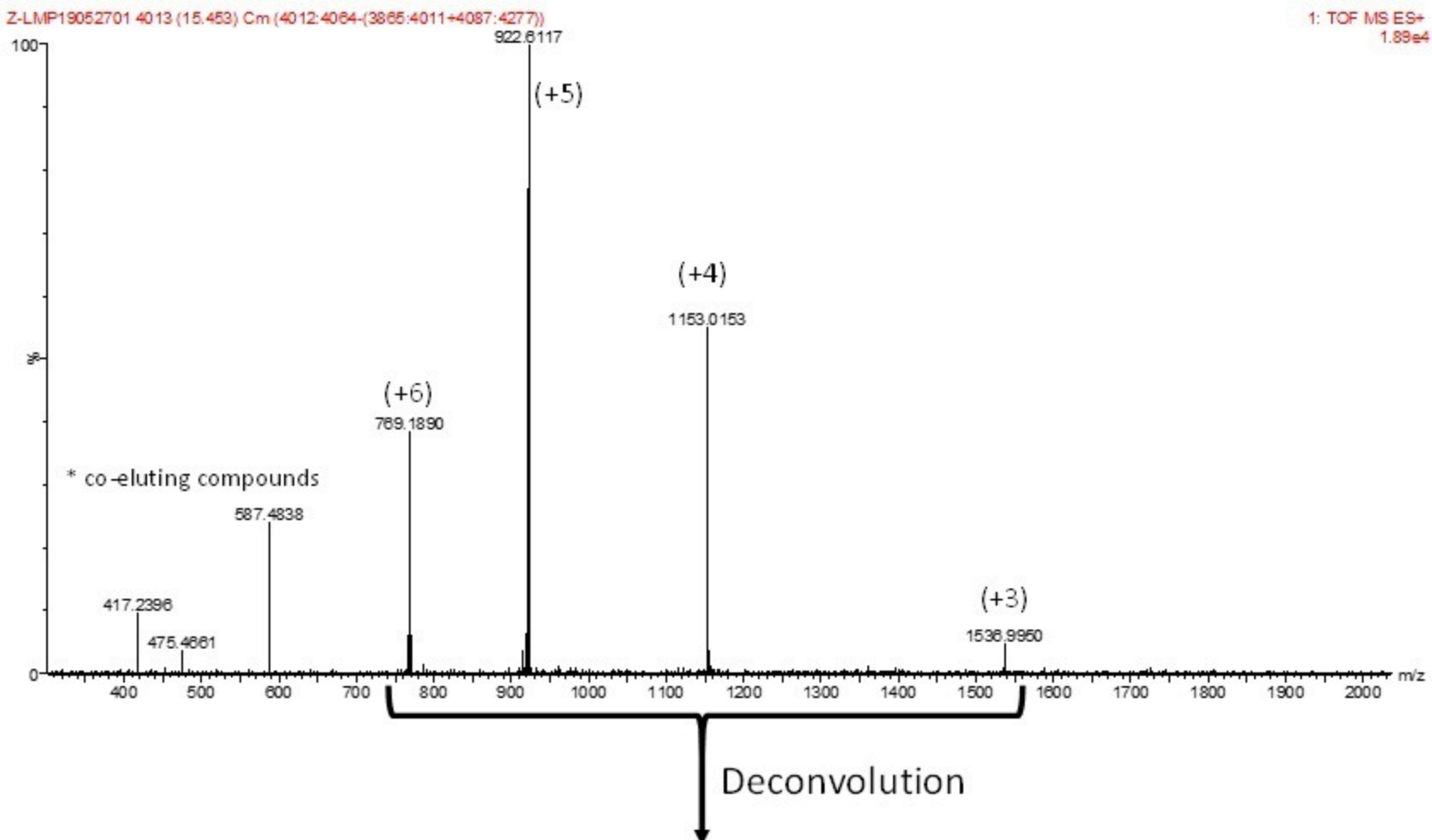


Fig. 6

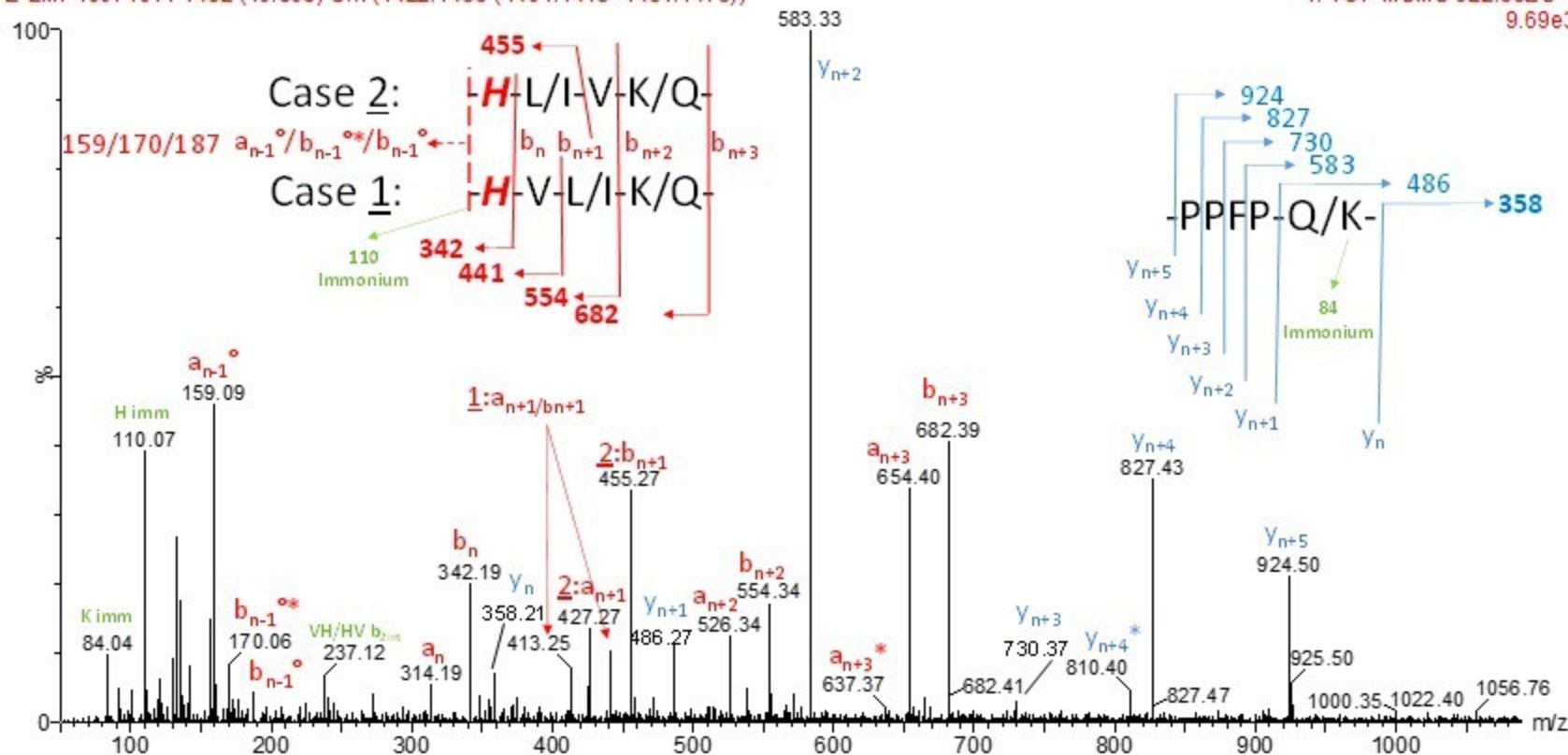
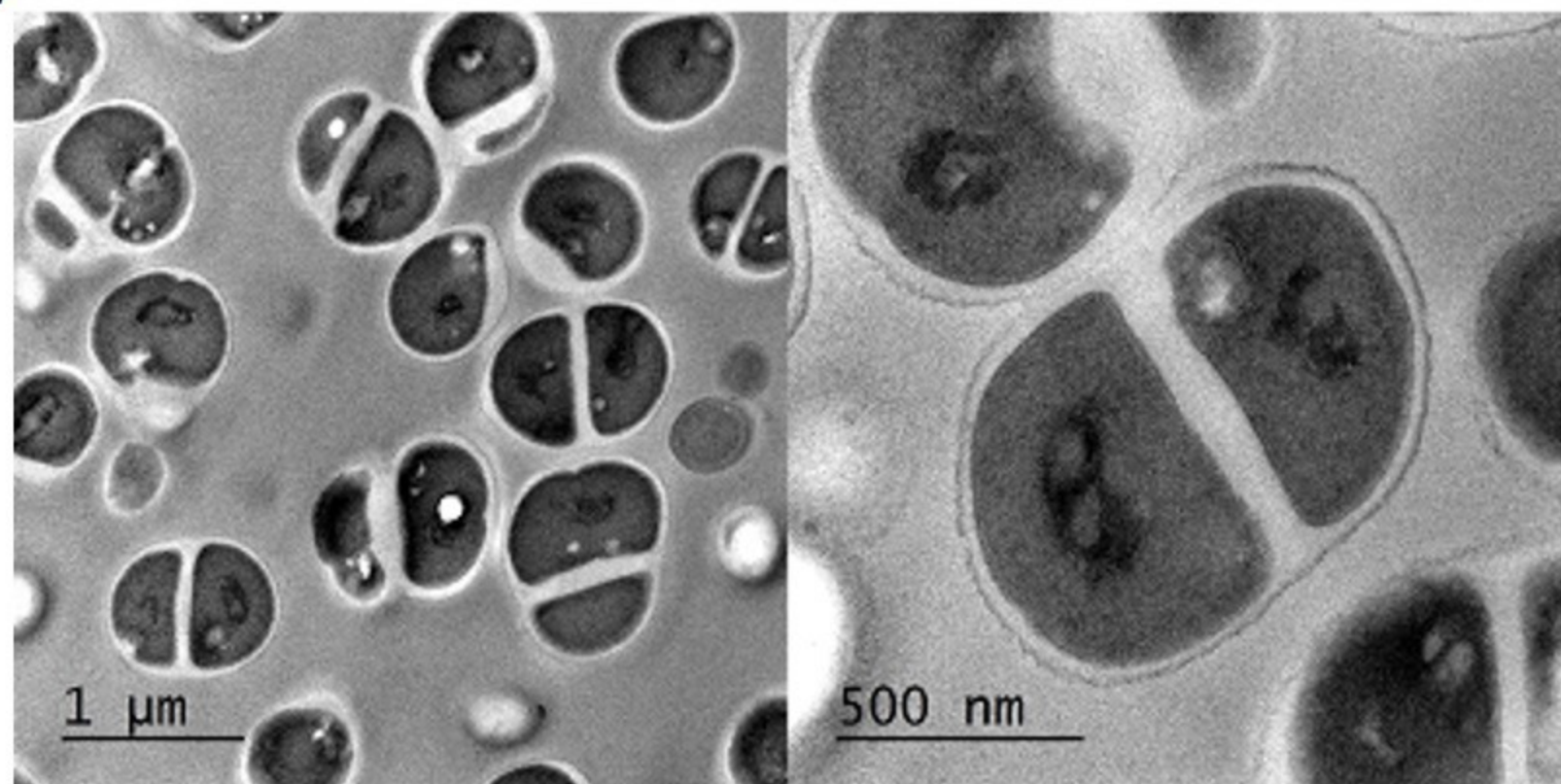


Fig. 7

A)



B)

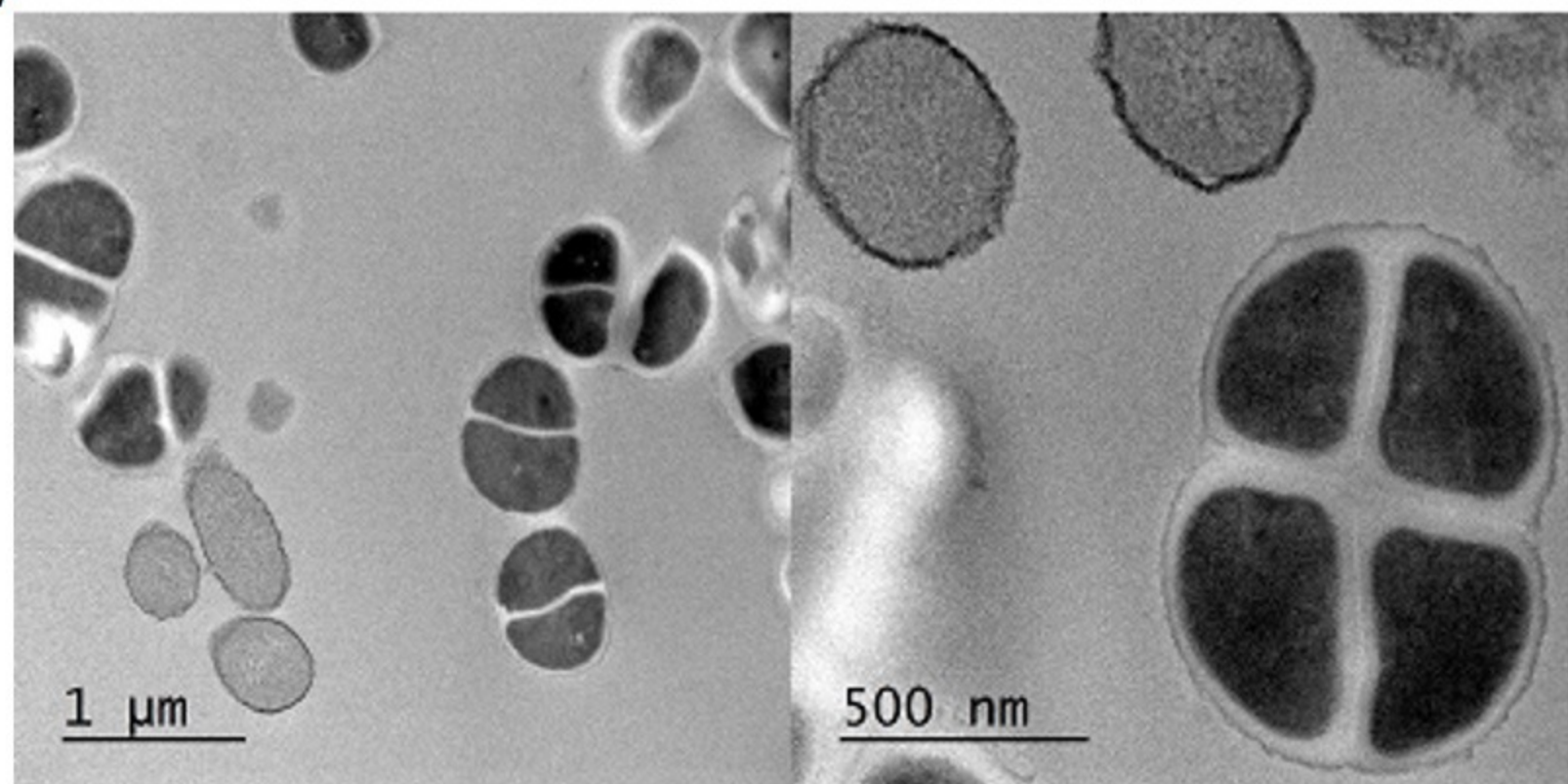


Fig. 8

Table I

Primers and PCR programmes used in this work

PCR objective	Primers sequences	Source of primers	PCR programme
Amplification 26S rDNA	NL-1 (Forward) 5'-CGC CCG CCG CGC GCG GCG GGC GGG GGG GGG GCC ATA TCA ATA AGC GGA GGA AAA G-3' LS-2 (Reverse) 5'- ATT CCC AAA CAA CTC GAC TC-3' 5'- ATT CCC AAA CAA CTC GAC TC-3'.	Eurofins Germany	Initial denaturation 95 °C, 5 min. 30 cycles <ul style="list-style-type: none"> • Denaturation 95 °C, 1 min • Annealing 52 °C, 45 s • Elongation 72 °C, 1 min Final elongation 72 °C, 1 min
Amplification ITS1-5.8S-ITS2	ITS1 (Forward) 5'- TCC GTA GGT GAA CCT GCG G-3' ITS4 (Reverse) 5'- TCC TCC GCT TAT TGA TAT GC-3'.	Eurofins Germany	Initial denaturation 95 °C, 5 min 35 cycles <ul style="list-style-type: none"> • Denaturation 94 °C, 30 s • Annealing 57 °C, 30 s, • Elongation at 72 °C, 1 min Final elongation 72 °C, 5 min

Table II

API 20C AUX and molecular identifications of *Trichosporon asahii* strains from camel milk

Isolate	Strain	API 20C (% identity)	26 rDNA (% identity) / Accession number	ITS1-5.8-ITS2 region (% identity) / Accession number	26 rDNA Accession number	ITS1-5.8- ITS2 region Accession number
P16	ICVY021	<i>T. asahii</i> 100%	<i>T. asahii</i> 100%/ (MG707737.1)	<i>T. asahii</i> 100%/ (KP162247.1)	MH193364	MH188544
PF1	ICVY022	<i>T. asahii</i> 100%	<i>T. asahii</i> 100%/ (JQ665245.1)	<i>T. asahii</i> 100%/ (KP162247.1)	MH193366	MH188546
KH4(4)	ICVY023	<i>T. asahii</i> 99.3%	<i>T. asahii</i> 100%/ (MG707737.1)	<i>T. asahii</i> 100%/ (KC127676.1)	MH193368	MH188548
D1(11)	ICVY024	<i>T. asahii</i> 90.5%	<i>T. asahii</i> 99%/ (NG_055732.1)	<i>T. asahii</i> 100%/ (KP162247.1)	MH193365	MH188545
SA1(4)	ICVY025	<i>T. asahii</i> 100%	<i>T. asahii</i> 99%/ (MG707737.1)	<i>T. asahii</i> 100% / (KC127676.1)	MH193367	MH188547
SA	ICVYO26	<i>T. asahii</i> 100%	Nd	Nd	Nd	Nd

- **Legend.** Nd: not determined



Camel Milk



Trichosporon asahii



Inhibition of
Kocuria rhizophila by
a **peptidic compound**
(Oranicin P16)



No cytotoxicity



No hemolysis