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Soufian Ider, Yanath Belguesmia, Guillaume Cazals, Rabah Boukherroub, Françoise Coucheney, et al.. The antimicrobial peptide oranicin P16 isolated from *Trichosporon asahii* ICVY021, found in camel milk's, inhibits *Kocuria rhizophila*. *Food Bioscience*, 2020, 36, pp.100670. <10.1016/j.fbio.2020.100670>. <hal-03090013>

HAL Id: hal-03090013

<https://hal.science/hal-03090013v1>

Submitted on 15 Jul 2022

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1 **The antimicrobial peptide oranicin P16 isolated from**
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4
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25

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

41 1. Introduction

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

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

60 *T. asahii* is also emerging as a biotechnological tool for its ability to decontaminate polluted
61 environments by accumulating large amounts of oils (Ageitos et al., 2011), and also
62 converting isoeugenol into vanillin (Ashengroph and Amini, 2017). Strains of *T. asahii* were
63 reported to produce lipase (Singh and Gupta, 2016), aspartic-type peptidase (Valle et al.,
64 2017), and antioxidant molecules (Zhang et al., 2016). Furthermore, the *T. asahii* GSY10
65 strain was able to produce lipid from molasses, and was suggested as a feed supplement in

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

69 In this study, the inhibitory properties of 6 *T. asahii* strains isolated from camel milk that had
70 been collected from arid and semi-arid regions of Algeria (Ider et al., 2019), were tested
71 against a range of target organisms including fungi and bacteria. Only *T. asahii* ICVY021
72 showed an inhibition against *Kocuria rhizophila* CIP 53.45 through production of an
73 extracellular, heat-stable and proteinaceous peptide. *K. rhizophila*, which belongs to the
74 *Micrococcaceae* family, was described as a contaminant of camel milk (Elhaj et al., 2013;
75 Takarada et al., 2008) with enzymatic activities able to induce spoilage of dairy product
76 (Pukančíková et al., 2016). Moreover *K. rhizophila* is responsible for human infection
77 (Moissenet et al., 2012), and for diseases in rainbow trout or brown trout (Pekalaa et al.,
78 2018). Therefore this study aimed at characterizing the antibacterial peptide, designated
79 oranicin P16, produced by *T. asahii* strain ICVY021.

80

81 **2. Materials and methods**

82 *2.1. Growth conditions and media*

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

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

93 *2.2. Genomic DNA extraction*

94 Total DNA was extracted using the Bust n'Grab method (Harju et al., 2004). Cells from 1.5
95 mL of the overnight yeasts cultures in YEG broth were pelleted in a microcentrifuge tube at
96 8,000 g (8680 rpm in a FA-45-30-11 rotor, Model 5804R centrifuge, Eppendorf, Hamburg,
97 Germany) for 10 min at 4°C. The cell pellets were suspended in a lysis buffer (2% Triton X-
98 100; 1% SDS; 100 mM NaCl; 100 mM Tris-HCl at pH 8.0; 0.1 mM EDTA, Sigma Aldrich),
99 and disrupted by two repetitive heat shock cycles at -80 °C (for two min) and 95 °C (for one
100 min). DNA was extracted using the chloroform procedure as follows: 200 µL of chloroform
101 (Sigma Aldrich) was added to the tubes and Vortexed (Vortex Genie 2, Dutscher, Brumath,
102 France) for two min and centrifuged at 20,000 g, three min at room temperature (20-25 °C).
103 The aqueous layer was transferred to a tube containing 400 µL of ice-cold 100% ethanol
104 (Sigma Aldrich). The samples were precipitated for 5 min at room temperature, and
105 centrifuged at 20,000 g for 5 min at room temperature. Supernatants were pipetted and the
106 DNA pellets were washed with 0.5 mL of 70% ethanol followed by drying at 37 °C. DNA
107 was re-suspended in 10 mM Tris-EDTA buffer (pH 8.0, Sigma Aldrich) and its concentration
108 determined with a Nanodrop Biowave II (Biochrom, Harvard, UK), by measuring their
109 absorbance at 260_{nm} UV wavelength.

110

111 *2.3. Yeast identification using an API 20C AUX strip and molecular methods*

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

117 wells with growth were allocated a plus sign, whereas those with the absence of growth were
118 allocated a minus sign. The results were transformed into numerical codes using a
119 correspondence table provided by the manufacturer. The isolates were identified using the
120 20C analytical profile index (API 20C) available online (<https://apiweb.biomerieux.com/>).

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

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

134 135 *2.4. Repetitive-element-palindromic-PCR (Rep-PCR) fingerprints*

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

143 0.1 $\mu\text{g } \mu\text{L}^{-1}$, were deposited and separated on a 1% agarose gel labelled with 0.05% GelRed
144 for 2 h at 100 V. The gels were visualized and photographed with a Gel-Doc 2000® (Bio-
145 Rad), and the DNA profiles (size and number of amplicons) were established with
146 GelCompar II (v6.6.11) software (Biosystematica, Ceredigion, Wales, UK).

147

148 2.5. Assessment of antagonistic properties of *T. asahii* strains

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

168

169

170 *2.6. Purification of the inhibitory compound from T. asahii ICVY021*

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

182

183 *2.7. Minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC)*
184 *of the purified inhibitory compound oranicin P16 produced by T. asahii ICVY021*

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

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

194 $\geq 99.9\%$, as determined by counting the cells on MH plates, compared to the untreated
195 inoculum tested using the same conditions.

196 2.8. *In situ activity of oranicin P16 in skim milk*

197 To assess the *in situ* activity of the purified active compound, a time-killing experiment was
198 done. Tubes containing a commercial sterilized skim milk (Carrefour®, Carrefour group,
199 France, Boulogne-Billancourt, France) were supplemented with 10% (v/v) of PBS, or nisin
200 (Sigma Aldrich) at a final concentration of 1 $\mu\text{g mL}^{-1}$, or with the active compound oranicin
201 P16 at the MIC value. A volume of 20 μL of a *K. rhizophila* CIP 53.45 suspension of $\sim 2 \times 10^9$
202 CFU mL^{-1} in PBS, as determined by cell-counting, was introduced in the prepared tubes
203 containing 2 mL of the supplemented skim milk with PBS (control), nisin or oranicin P16,
204 and incubated at 37 °C for 6 h. The killing kinetics of the nisin and the oranicin P16 were
205 monitored using viable bacterial counts on agar plates compared to the control. Aliquots were
206 taken at 0, 2, 4 and 6 h, and serially diluted in saline solution for determination of viable
207 counts. Diluted samples (100 μL) were plated on the MH agar plates and colonies were
208 counted after overnight incubation at 37 °C.

209 2.9. *Molecular mass and amino acids sequence of the purified inhibitory compound*

211 Liquid chromatography - mass spectrometry (LC/MS) analyses, using positive mode with
212 electrospray ionisation (ESI) and high resolution time of flight (TOF) mass analyzer, were
213 done using an Acquity H-Class (Waters Corp., Milford, MA, USA) system equipped with a
214 Kinetex EVO C18 column (1.9 mm particle size, 150 \times 2.1 mm), (Phenomenex, Torrance,
215 CA, USA). The mobile phase A consisted of water while mobile phase B was acetonitrile
216 both containing 0.1% formic acid. The gradient started at 5% mobile phase B and increased to
217 100% mobile phase B over 40 min. The flow rate was 0.5 mL min^{-1} and the eluent was
218 directed to the atmospheric pressure ionisation source of the Synapt G2-S (Waters) tandem
219 hybrid Quadrupole-TOF mass spectrometer operating in positive electrospray ionization

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

228
229 *2.10. Transmission electron microscopy (TEM) of K. rhizophila CIP53.45 treated with the*
230 *inhibitory compound*

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

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

245 coupled with a Gatan Orius SC200D camera (Gatan Inc., Oxford, UK) with magnification of
246 ~20,000 to 40,000x

247

248

249 *2.11. Hemolysis and resistance to antifungal drugs of T. asahii ICVY021 antagonistic strain*

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

263 The interpretation of MIC values was done using the data given for *T. asahii* by [Kanafani and](#)
264 [Perfect \(2008\)](#), in accordance with the recommendations approved by the European
265 Committee on Antimicrobial Susceptibility Testing ([EUCAST, 2017](#)) and the CLSI ([CLSI,](#)
266 [2018](#)) using *Candida albicans* as a reference strain ([Cuenca-Estrella et al., 2012](#)). According
267 to these recommendations, the *T. asahii* was considered resistant to the antifungal agents
268 tested when their MIC were equal or higher to the following cut off concentrations: 2 mg L⁻¹
269 for amphotericin B, 50 mg L⁻¹ for 5-fluorocytosine, 8 mg L⁻¹ for fluconazol, 1 mg L⁻¹ for

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

272

273

274 2.12. Cytotoxicity assay

275 Intestinal human colorectal tumour Caco-2 cells (Sigma Aldrich) were grown in 96-well cell
276 culture plates and pre-incubated for 7 days at 37 °C, 5% CO₂ atmosphere in Dulbecco
277 modified Eagle medium (DMEM, Pan-Biotech GmbH, Aidenbach, Germany) supplemented
278 with 10% of fetal bovine serum (FBS, Thermo Fisher Scientific), 2 mM glutamine (Pan-
279 Biotech GmbH), 100 µg mL⁻¹ of penicillin and 100 µg mL⁻¹ of streptomycin (Pan-Biotech
280 GmbH). A 24 h old culture of *T. asahii* ICVY021 were diluted to 2×10⁸ CFU mL⁻¹ in
281 antibiotic and serum free DMEM. A suspension of 100 µL was added to each well containing
282 ~2.10⁵ Caco-2 cells. Furthermore, the cytotoxicity on the Caco-2 cells was measured after 24
283 h of contact by determining the dehydrogenase activity of mitochondria with the CCK-8
284 (Counting Kit-8) assay (Dojindo, Kumamoto, Japan). Briefly, after incubation cells were
285 washed with DMEM without any supplementation to remove non-adherent yeasts. To
286 eliminate adherent yeasts, 50 µL of amphotericin B (Sigma Aldrich), at a final concentration
287 of 5 mg L⁻¹ in PBS, were added to each well. After 2 h of incubation at 37 °C, the solution of
288 amphotericin B was removed and the wells were filled with 100 µL of DMEM supplemented
289 with 5 µL of CCK-8 (Dojindo) reagent and incubated for an additional 2 h at 37 °C. Wells
290 with Caco-2 cells treated with Triton X100 solution (Sigma Aldrich), diluted to a final
291 concentration of 0.1% in DMEM were used as a positive control of cytotoxicity whereas
292 wells with non-treated Caco-2 cells served as a negative control. An additional negative
293 control using wells with Caco-2 cells treated with amphotericin B, with the same conditions
294 as described before, but not exposed to yeast was also used.

295 The CCK-8 reagent was used at 5% (v/v) final concentration to evaluate the viability of Caco-
296 2 cells by determining the activity of cellular dehydrogenase, resulting in an orange colored
297 formazan from the water-soluble tetrazolium salt: WST-8(2-(2-methoxy-4nitrophenyl)-3-(4-
298 nitrophenyl)-5-(2,4-disulfophenyl)2H tetrazolium, monosodium salt)). The formation of
299 formazan was measured at 450 nm using a microplate reader (Xenius SAFAS, Monaco), and
300 compared with the absorbance of non-treated cells considered as the control.

301 302 2.13. Adhesion assay

303 The adhesion abilities of *T. asahii* ICVY021 on Caco-2 cells were determined as reported by
304 [Ait Seddik et al. \(2017\)](#). Caco-2 cells/well (4×10^4) were seeded in 24-well tissue culture plate,
305 incubated for 7 days at 37 °C (5% CO₂ and 95% air). Cells of *T. asahii* ICVY021 grown in
306 YEG broth for 48 h at 25 °C were harvested by centrifugation at 6,000 g at 4 °C for 10 min,
307 and resuspended in DMEM without FBS and antibiotics to a final concentration of 10^5
308 CFU/well. To determine the effect of proteins or carbohydrates on the adhesion process of *T.*
309 *asahii* ICVY021 with confluent Caco-2 monolayers, the yeast cells were treated with trypsin
310 from porcine pancreas (Sigma Aldrich) at a final concentration of 2.5 mg mL⁻¹ for 1 h at
311 37°C, or with 50 mM of periodic acid (Sigma Aldrich) in 10 mM PBS (pH 7.4) for 30 min at
312 37 °C ([Ait Seddik et al. \(2017\)](#)). Both samples were washed twice with PBS and resuspended
313 in DMEM without FBS and antibiotics. Untreated yeast-cells at $\sim 10^5$ CFU/well or treated
314 ones with trypsin or periodic acid were applied to confluent Caco-2 cells and incubated for 2
315 h at 37 °C (5% CO₂ and 95% air). The Caco-2 monolayers were washed twice with 500 µL of
316 PBS to remove non-adherent yeast cells. Lysates obtained after incubation with Triton X100
317 (Sigma Aldrich) at 0.1% (v/v) for 15 min, were serially diluted and plated on YEG agar to
318 determine the number of adherent yeast cells, untreated and treated with trypsin or periodic
319 acid.

320

321 *2.14. Statistical analysis*

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

326 **3. Results**

327
328 *3.1. API 20C AUX strips and molecular methods are reliable methods for identification of T.*
329 *asahii strains*

330 After 72 h at 25 °C colonies of *T. asahii* ICVY021, ICVY022, ICVY023, ICVY024,
331 ICVY025 and ICVY026 on YEG-agar plates were white, dried and wrinkled with irregular
332 margins (Fig. 1). All the strains were identified as *T. adahii* using API 20C AUX strips and
333 molecular methods. The scores obtained using the API 20C AUX system ranged from 90 to
334 100% (Table II), while those obtained after blasting 26 rDNA and ITS1-5.8-ITS2 regions
335 sequences ranged between 99 to 100% (Table II).

336
337 *3.2. T. asahii ICVY021 showed a slightly different DNA pattern*

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

341
342 *3.3. T. asahii ICVY021 is the only strain with inhibitory properties*

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

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

351 For the *in situ* assessment of this activity, killing curves were obtained in a commercial skim
352 milk using *K. rhizophila* CIP 53.45 as a target organism, treated or not with nisin at 1 µg mL⁻¹
353 ¹, and the purified oranicin P16, used at the MIC value (215 µg mL⁻¹). Microbial counts were
354 measured as CFU mL⁻¹ and the resulting averaged data were transformed to Log CFU mL⁻¹.
355 The cell number in the treated skim milk with oranicin P16 decreased by ~1 Log CFU mL⁻¹,
356 whereas the untreated sample was unaffected showing a growing population, which reached
357 6.61 Log CFU mL⁻¹ (Fig. 4). Nevertheless, nisin showed the strongest inhibitory activity with
358 a rapid decreasing number of the viable and cultivable cells, by 3 Logs after 6 h, therefore
359 killing >99.9% of the initial bacterial populations.

360
361 *3.4. Resistance to antifungal drugs, hemolytic and cytotoxic effects of T. asahii ICVY021*

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

370 The adhesion ability was also investigated using the human cell line Caco-2. As shown in Fig.
371 5B, the results showed than 10% of *T. asahii* ICVY021 cells were adhesive. This score is
372 weak compared to those for dairy yeasts (Živković et al., 2015), but remains higher than that

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

375
376 *3.5. The inhibitory proteinaceous sample contains a peptide with a molecular weight of 4,607*

377 *Da.*

378 To identify the compound(s) responsible for the antimicrobial activity, the inhibitory
379 proteinaceous sample was analysed using LC/MS/MS with a reversed-phase gradient
380 designed to separate a wide range of organic compounds. Among all the chromatographic
381 peaks detected, one of them was found to show the features of a peptide sequence. Indeed, the
382 corresponding mass spectrum obtained in the positive mode with ESI and high resolution
383 TOF mass analyser showed multiple charged molecular ions ranging from +3 up to +6
384 protonations, as seen in Fig. 6 (top). These MS signals were deconvoluted to obtain the
385 molecular weight of 4,607 Da as shown in Fig. 6 (bottom). The sequencing of such a long
386 peptide was directly attempted using LC/MS/MS with CID without any proteolytic step.
387 During this experiments, the selected cations from the quadrupole (Q) collide with argon gas
388 in the collision cell to induce fragmentation. Products ions derived from CID were then mass
389 analysed by TOF analyser. Although partial sequence coverage was expected from
390 fragmentation of a very large peptide with these low energy CID conditions (Mouls et al.,
391 2007), the aim of such identification study was to propose a sequence tag that can be used for
392 further search of known peptides and proteins in databases. For that purposes, all protonated
393 molecular ions were dissociated but only the penta-charged ion $(M+5H)^{5+}$ detected at m/z
394 922.6 provided many fragments that allowed the identification of the PFPF(K/Q) sequence
395 tag. The first series of fragment ions without decarbonylation (Fig. 7) were attributed to y ions
396 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
397 mass of 128 can be attributed to either K or Q. Nevertheless, the detection of the m/z 84 ion
398 related to the dehydrated immonium ion of K might favour the PFPFK sequence than PFPFQ.

399 Two other ion series were related to the *b* ions due the loss of CO and formation of the
400 corresponding *a* ions. The two sequences V(L/I)(K/Q) or (L/I)V(K/Q) can be envisaged since
401 two possibilities may be obtained for the b_{n+1} ion at either *m/z* 441 or 455. The residue
402 detected at a mass difference of 128 Da can be attributed as previously to isobaric K or Q. In
403 the same way, a mass difference of 113 Da corresponds to isomeric L or I. Moreover, the
404 immonium ion of H was detected at *m/z* 110 so this peptide contained this residue. Taking
405 into account that three other ions at *m/z* 159, 170 and 187 were assigned as *a/b* ions and have
406 experienced neutral losses (H₂O, NH₃ noted ° and *, respectively) are often observed in CID.
407 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*
408 205 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
409 a_{n-1}° at *m/z* 159 were detected). These result implied that the position of H can be proposed
410 after V or after (L/I) giving the partial sequence HV(L/I)(K/Q) or H(L/I)V(K/Q). Finally, the
411 residual mass corresponding to be the smallest *b* ion (*m/z* 205) can be the dipeptide CT or TC.
412 Four high probable sequences can be suggested CTHV(L/I)(K/Q), CTH(L/I)V(K/Q), or
413 TCHV(L/I)(K/Q), and TCH(L/I)V(K/Q). The detection of an internal ion at *m/z* 237 related to
414 the VH or HV chain could favour the two possibilities CTHV(L/I)(K/Q) or TCHV(L/I)(K/Q).
415 In conclusion, two putative sequence tags were evidenced with PPFPK and CTHV(L/I)(K/Q)
416 or TCHV(L/I)(K/Q). These sequences were compared to those found in the NCBI protein
417 database, and showed a partial homology with proteins, with undefined function, from the
418 plant *Lupinus angustifolius* and the bacterium *Candidatus curtissbacteria*. More information
419 could be deduced from peptide proteolysis prior to MS/MS sequencing to dissociate shorter
420 peptides more amenable to CID fragmentations.

421 422 3.6. TEM

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

425 packets. When, these cells were treated with the inhibitory compound, oranicin P16 at the
426 $MBC = 860 \mu\text{g mL}^{-1}$, from *T. asahii* ICVY021, they showed altered walls with apparent
427 stoppage of the cell division process. The cells seem stuck at the same stage with widespread
428 mortality with difficulties to distinguish the plasma membrane compared to the untreated cells
429 (Fig. 8A, B).

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4. Discussion

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

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

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

459 *T. asahii* ICVY021 produces an antimicrobial peptide, designated oranicin P16, in reference
460 to Oran, an Algerian city, where the strain was isolated and characterized for its antibacterial
461 function. Oranicin P16, of 4,607 Da, was produced uniquely after 48 h of growth, and
462 consisted of two sequence tags of PPFPK and CTHV(L/I)(K/Q) or TCHV(L/I)(K/Q).
463 Moreover, TEM analysis of *K. rhizophila* CIP 53.45 strain treated with oranicin P16 indicated
464 a possible action on its cell-division process. The size, stability to high temperatures or
465 alteration by proteases of oranicin P16 are typical of bacteriocins from Gram-positive and
466 Gram-negative bacteria (Drider and Rebuffat, 2011). *T. asahii* ICVY021 was not hemolytic,
467 and was sensitive to clinical antifungal drugs. Mechanisms by which *Trichosporon* species
468 resist antifungal drugs remain to be better understood, although similarities with *Candida* and
469 *Aspergillus* species were reported (Kushima et al., 2015). Moreover, the genes *ERG11*
470 encoding the lanosterol 14- α -demethylase enzyme, which is the primary target of azoles
471 (Kushima et al., 2015), can be lost with a single amino acid substitution (Kushima et al.,
472 2017). *T. asahii* ICVY021 was slightly resistant to 5-fluorocytosine and anidulafungin but this
473 may not be a critical point because the *in vitro* MIC values are not always systematically
474 consistent with the therapeutical doses (Rex and Pfaller, 2002). Therefore, more accurate
475 methods such as the minimum effective concentration (MEC) are needed (Kurtz et al., 1994).

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

480

481 **5. Conclusion**

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

490

491 **Conflicts of interests**

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

494

495 **Acknowledgements**

496 SI received a short term Ph.D. grant from the Algerian Government. Experiments done at
497 Lille University were supported by la région des Hauts-de-France through the Contrat-Plan-
498 Etat-Région (CPER) Alibiotech [2016-2020]. The authors would like to thank Dr. Xavier
499 Lepoutre from Hôpital Victor Provo de Roubaix for his kind assistance in assessing the
500 antifungal drugs. Authors would like to thank Anne-Sophie Lacoste and Loïc Brunet of the
501 UMS2014/US41 PLBS-BICeL Campus Cité Scientifique facility for access to instruments

502 and technical advices. The TEM preparations and observations were done on devices acquired
503 through funding from the Agence Nationale de la Recherche ANR 10-EQPX-0004

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Fig. 1

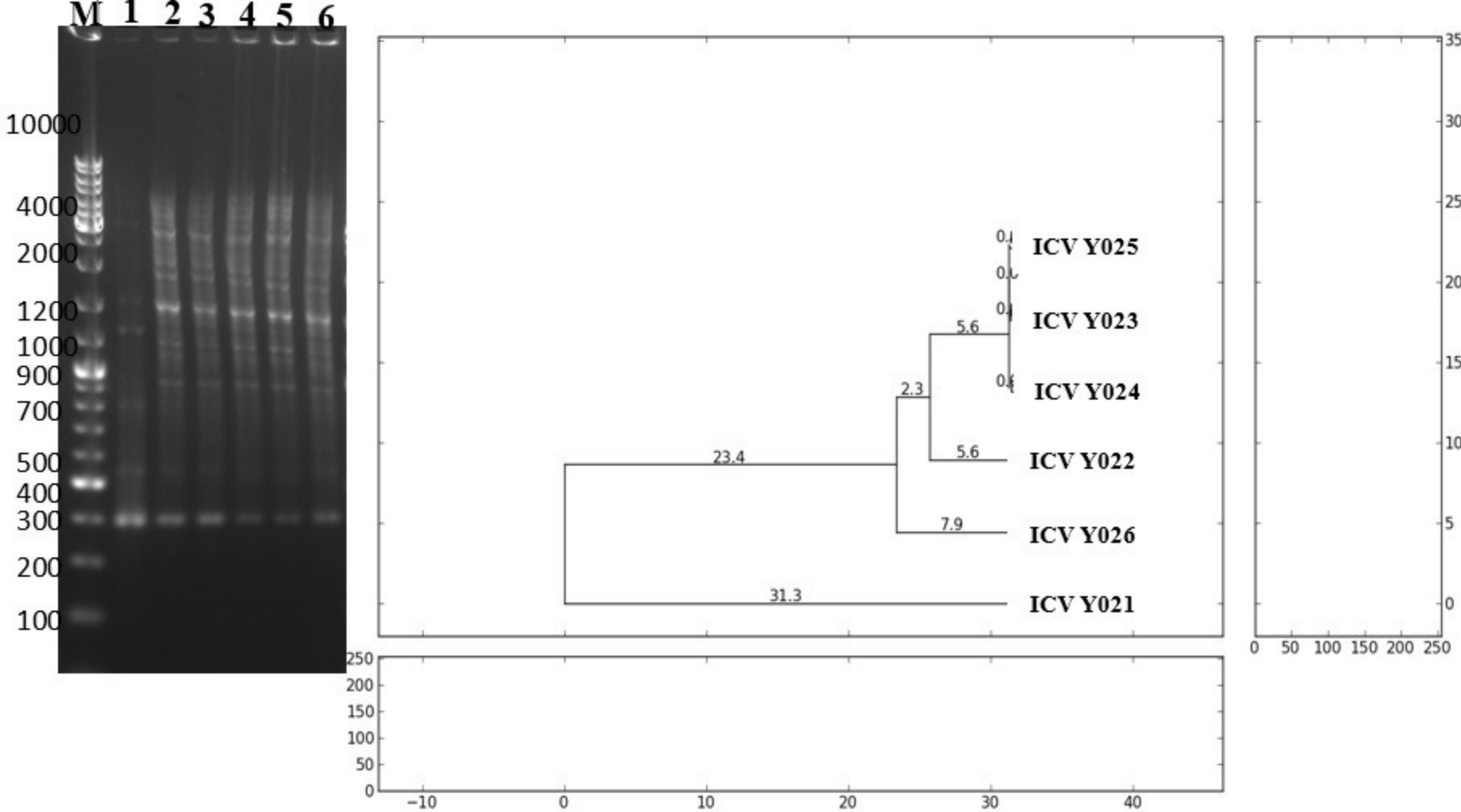


Fig. 2

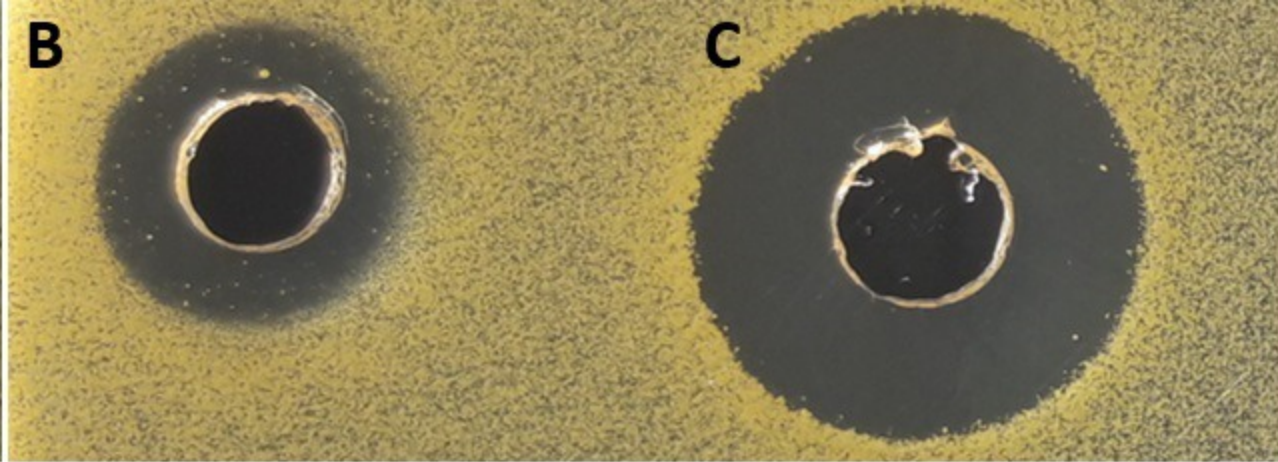


Fig. 3

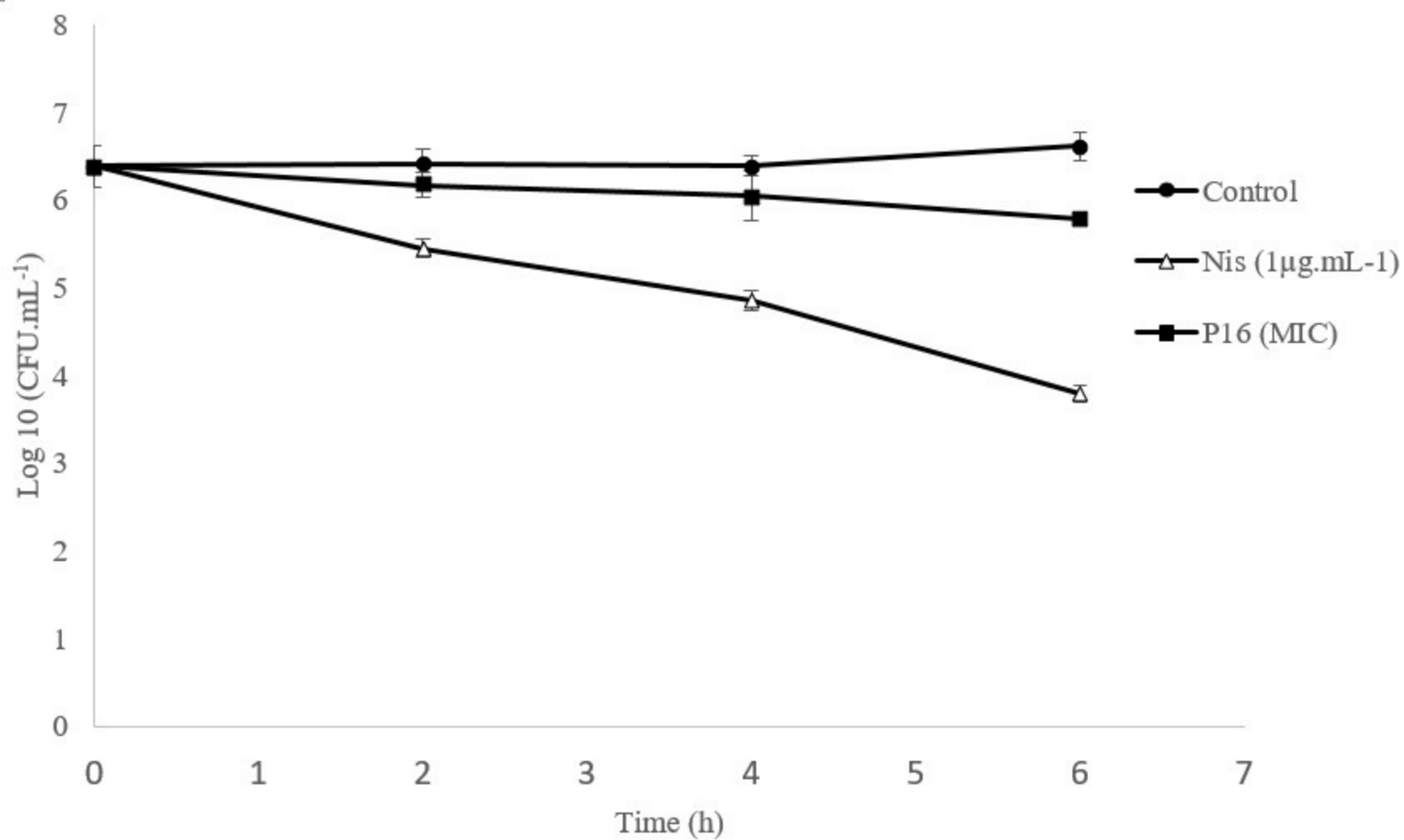


Fig. 4

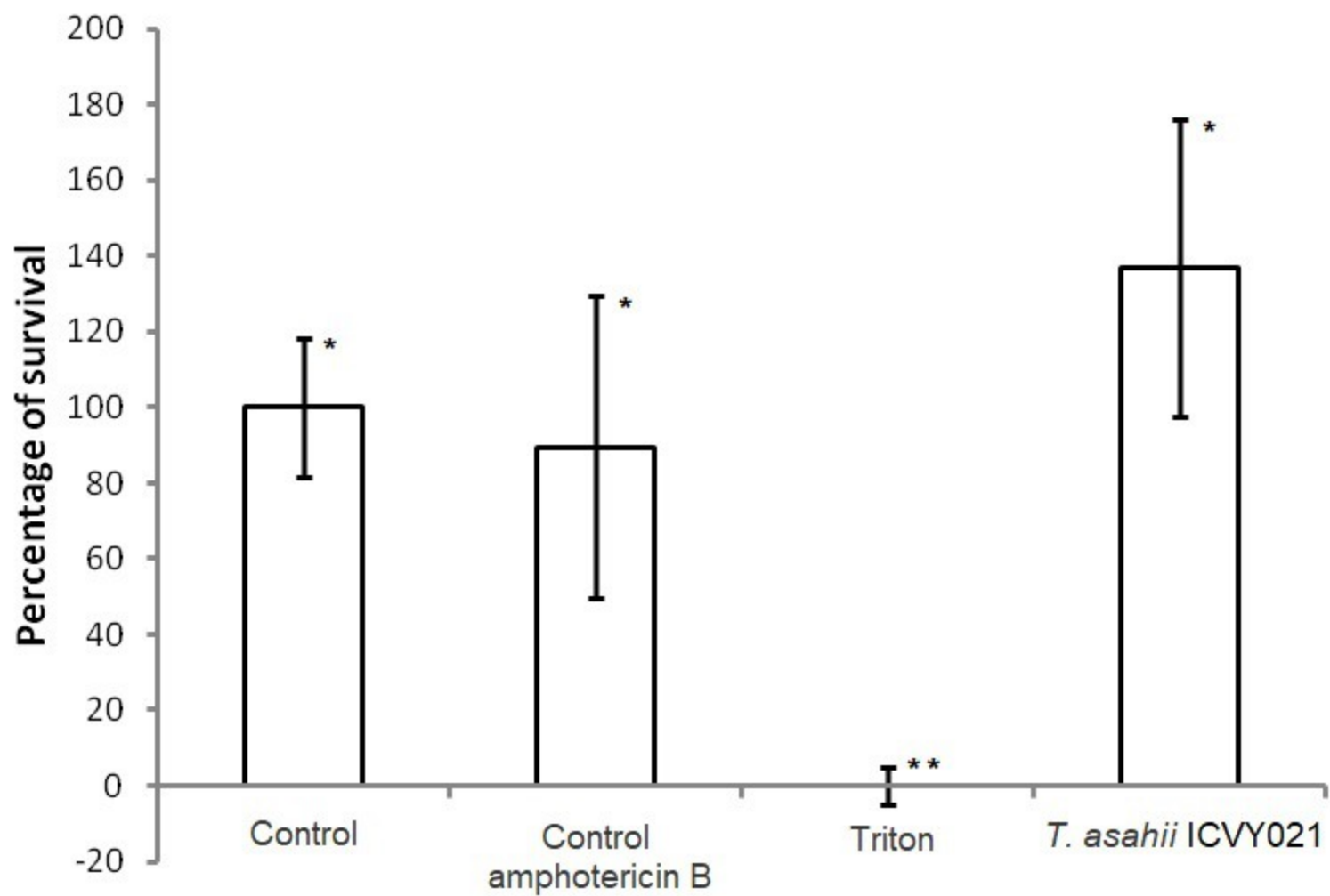


Fig. 5A

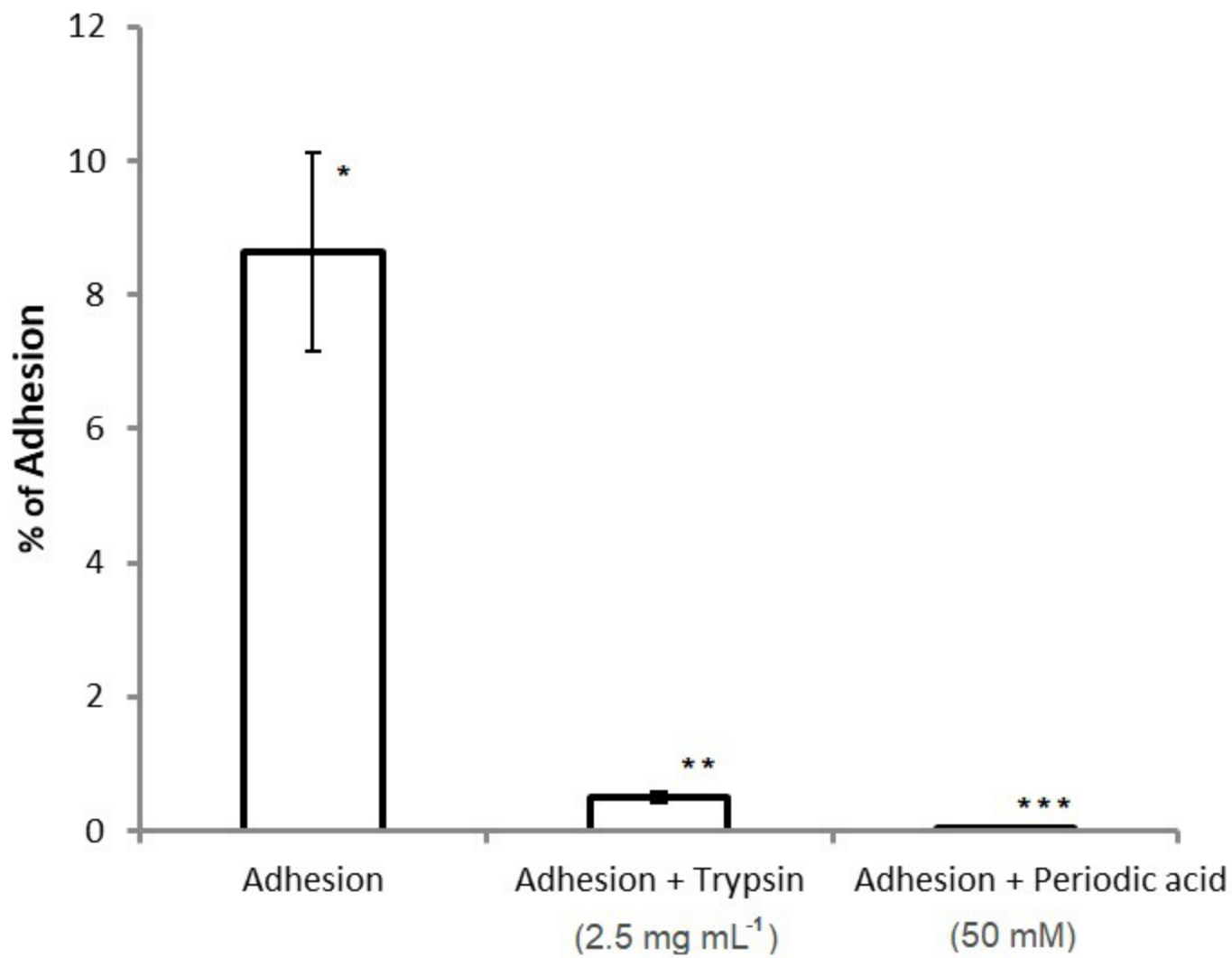


Fig. 5B

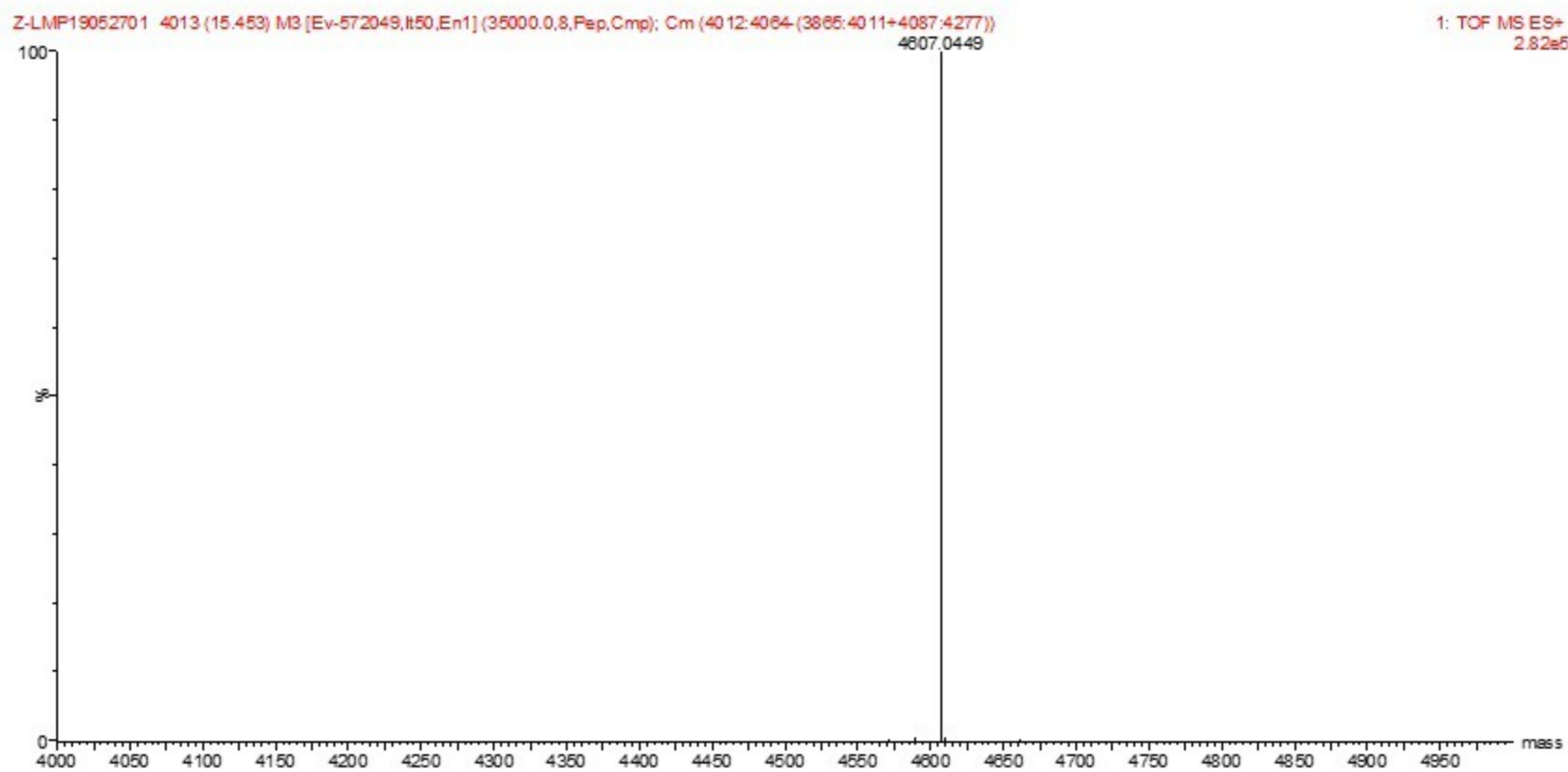
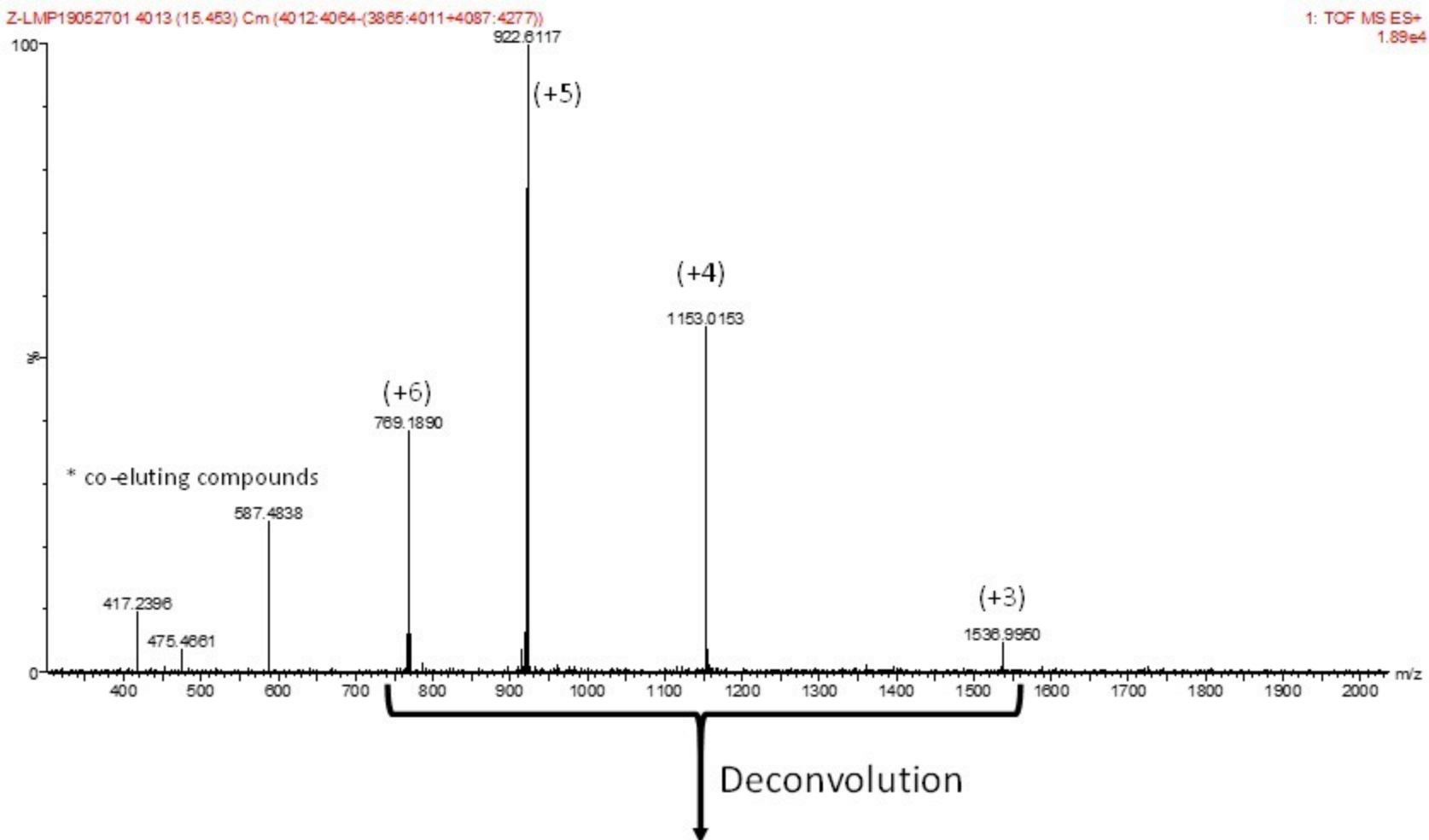


Fig. 6

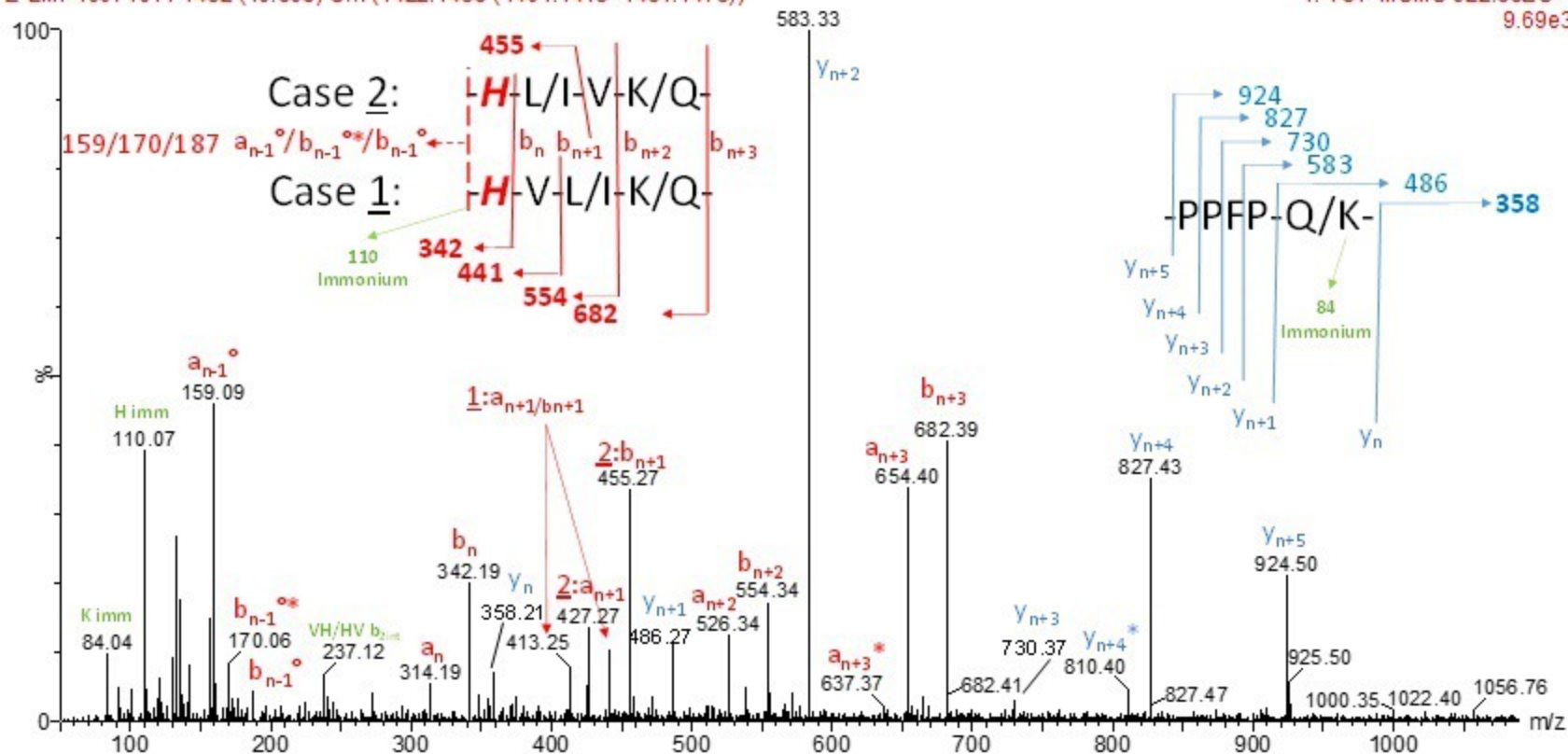
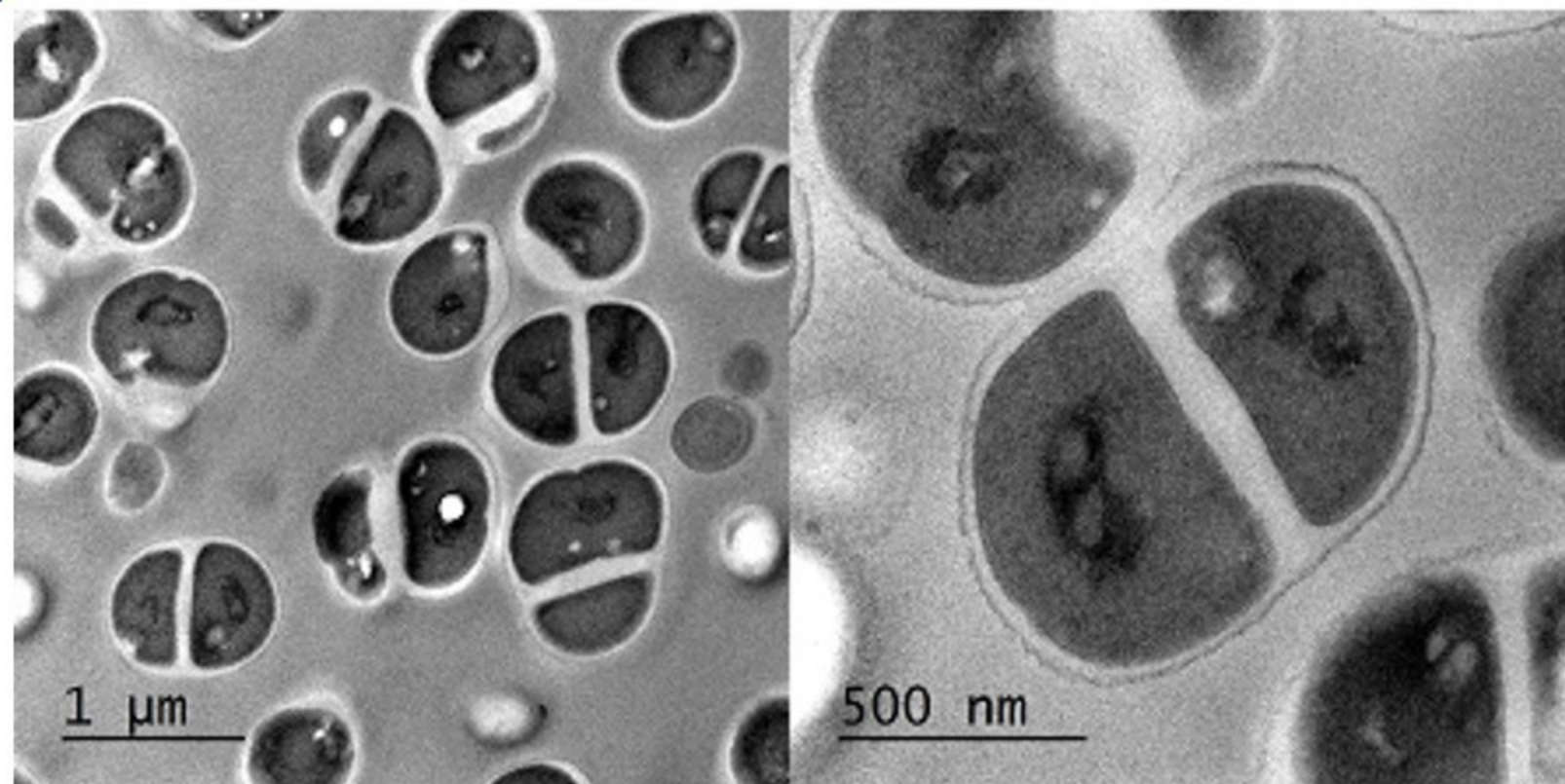


Fig. 7

A)



B)

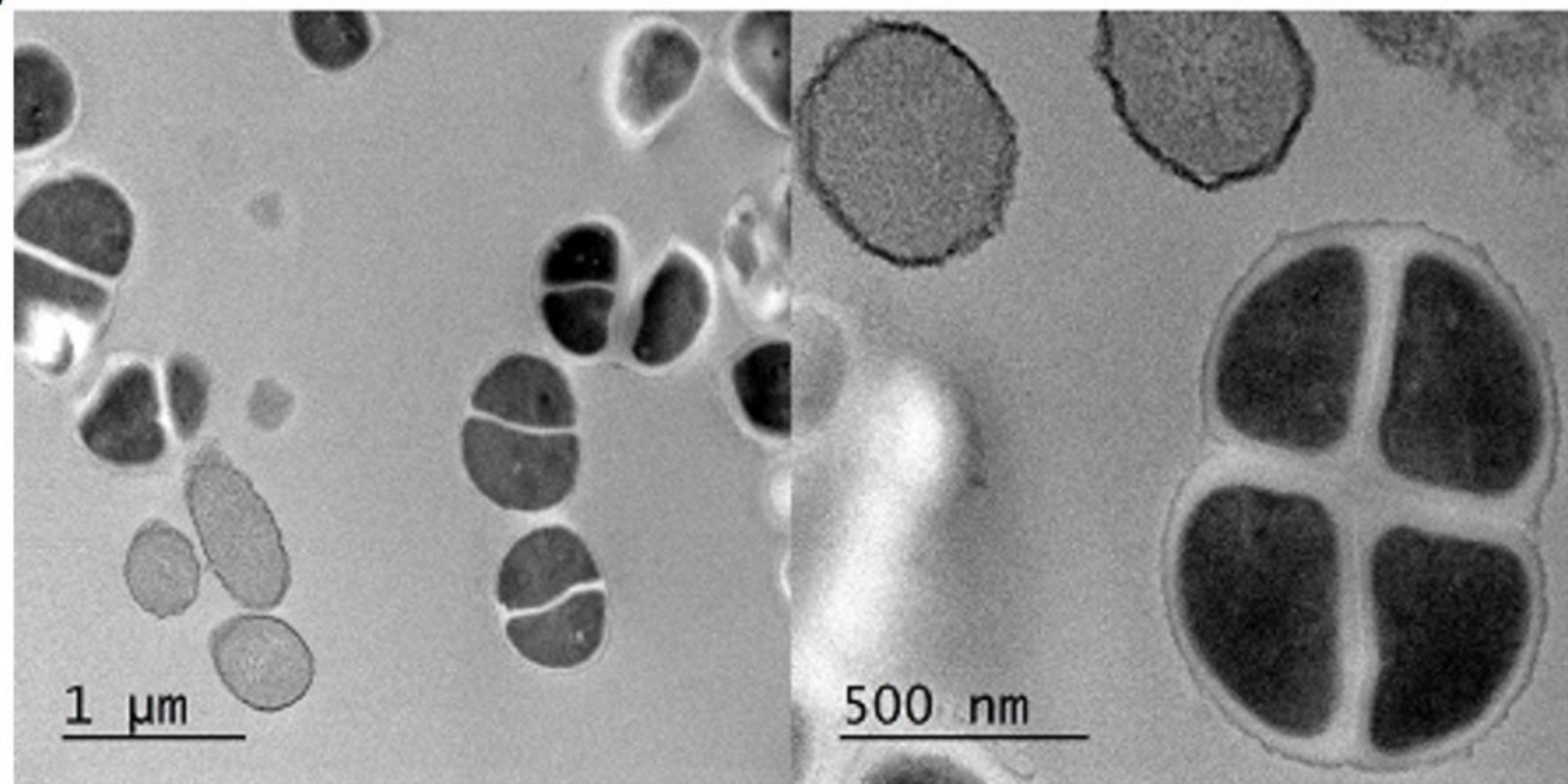


Fig. 8

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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

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27 **Table II**28 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	ICVY026	<i>T. asahii</i> 100%	Nd	Nd	Nd	Nd

29 • **Legend.** Nd: not determined

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Camel Milk



Trichosporon asahii



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



No cytotoxicity



No hemolysis