1 **Structure-function relationships of antifungal monohydroxy unsaturated fatty acids**

2 **(HUFA) of plant and bacterial origin**

3

4

5 Nuanyi Liang [a], Andrea Dacko [a], Alexander K. Tan [a], Sheng Xiang [a], Jonathan M. Curtis [a], and

6 Michael G. Gänzle [a *].

7

8 [a] *Department of Agricultural, Food and Nutritional Science, University of Alberta, Edmonton,*

9 *Canada*

10 * Correspondent footnote, phone: + 1-780-492-0774, E-mail: mgaenzle@ualberta.ca

11

12

13

14

Abstract

This study investigated the relationships between the structures of hydroxy unsaturated fatty acids (HUFA) and their antifungal activities. Structurally diverse HUFA, including four monohydroxy-18:1 isomers, two monohydroxy 18:2 isomers and two monohydroxy 18:2 isomers were extracted from seeds of plants (*Coriaria nepalensis*, *Thymus vulgaris*, *Mallotus philippensis* and *Dimorphotheca sinuata*) for which information was available on PlantFAdb database, and from culture supernatants of lactobacilli. They were purified by high-speed counter current chromatography (HSCCC) and identified by LC-MS/MS. The minimum inhibitory concentrations of HUFA were tested against a panel of five yeasts and five mycelial fungi. The membrane phase changes under HUFA treatment and the content of ergosterol were both measured to differentiate HUFA-sensitive and HUFA-resistant fungi. HUFA with a hydroxyl group near the center of the 18-carbon fatty acid chains were found to contribute strongly to HUFA antifungal activity. Antifungal HUFA targeted filamentous fungi but not yeasts. HUFA didn't alter the overall membrane fluidity of sensitive fungi, but the most HUFA-sensitive fungi had a lower average ergosterol content compared to the resistant yeasts. This indicates the possible interaction of HUFA with fungal membrane with low sterol content, which partially support the previous proposed mode of action. Findings here provide insight on further development of HUFA application in food products.

Keywords: antifungals, hydroxy unsaturated fatty acids (HUFA), HSCCC, plant seed oils, LAURDAN, ergosterol, membrane fluidity

37

38

39 **1. Introduction**

40 Fungal food spoilage contributes substantially to the 1.3 billion tons of annual global food lost
41 or wasted at the retail or household levels (FAO, 2019; Salas et al., 2017) and the associated
42 allergens and mycotoxin production additionally constitute food safety risks (Pitt & Hocking,
43 2009). Furthermore, the populations of spoilage fungi which are threats to food safety and food
44 security, may undergo changes due to increasingly complex food value chains (Oliveira, Zannini,
45 & Arendt, 2014), and due to climate change (Paterson & Lima, 2010).

46 Common perishable or processed foods that are susceptible to fungal spoilage include fruits,
47 vegetables, bread, cereals, nuts and refrigerated foods with long storage lives (Salas et al., 2017;
48 Pitt & Hocking, 2009). Currently used preservatives, such as propionic acid or sorbic acid, impact
49 food flavor and may be incompatible with the food industry's efforts to offer "clean-label"
50 products (Quattrini et al., 2018; Asioli et al., 2017). Thus, it is of interest to explore alternative
51 strategies for inhibiting fungal growth on agricultural commodities and food.

52 Plants provide a potential source of antifungal compounds for use in food and agriculture, since
53 the plant response against pathogenic fungi often involve the production of defense compounds
54 (Lacerda et al., 2014; Prost et al., 2005). In addition, bacterial plant symbionts are a valuable
55 sources of antifungal compounds (Farré-Armengol et al., 2016). Hydroxy-unsaturated fatty acids
56 (HUFA) contribute to plant defenses against fungi (Cantrell et al., 2008; Prost et al., 2005; Shimada
57 et al., 2014; Yara et al., 2008). The antifungal activities of specific HUFA against food spoilage
58 fungi is relatively strong when compared to most non-proteinaceous compounds of lactobacilli

59 that have been explored in food applications (Black et al., 2013; Siedler et al., 2019). HUFA are
60 also more chemically stable when compared to other oxylipins that contribute to plant defenses
61 against fungal pathogens (Prost et al., 2005). However, the structural determinants of the antifungal
62 activity of HUFAs and their inhibitory spectrum against yeasts and fungi that are relevant as
63 spoilage organisms in food and agricultural commodities are poorly documented.

64 The antifungal activity of HUFA relates to the presence of double bonds (Black et al., 2013)
65 and the number and the position of hydroxy groups (Granér et al., 2003; Prost et al., 2005).
66 Additionally, different fungal species show different levels of sensitivity toward HUFA; notably,
67 yeasts show high resistance when compared to filamentous fungi (Liang et al., 2017). However,
68 current knowledge on structure-function relationships is limited to HUFA with hydroxylation at
69 the Δ2-, Δ9-, and Δ13-positions, and to phytopathogens as indicator organisms (Prost et al., 2005).
70 Similarly, the application of HUFA as antifungals in food has also been limited to Δ10-, Δ12- and
71 Δ13-OH isomers (Black et al., 2013; Quattrini et al., 2018). The inhibitory spectrum against food
72 fermentation and food spoilage organisms is largely unexplored, as well as the implication of
73 active antifungal concentration of HUFA on other food quality parameters, such as flavor and
74 aroma.

75 Plant oxylipins are only produced in small quantities during plant defense activities (Yara et al.,
76 2008; Shimada et al., 2014), and so are difficult to extract in sufficient quantities for investigation
77 of their antifungal properties. In contrast, some plants seed oils contain high amount of HUFA
78 (Ohlrogge et al., 2018; Badami & Patil, 1980) which can be fractionated for further study, for
79 instance using high speed counter chromatography (HSCCC) (Liang et al., 2017). In addition,
80 lactobacilli convert linoleic acid into 10-hydroxy-12 octadecenoic acid and 13-hydroxy-9-

81 octadecenoic acid (Chen, Liang, Curtis, & Gänzle, 2016). These sources can provide diverse
82 HUFA analogues that can be used in studies of the HUFA structure-function relationships.
83 It has been proposed that the antifungal activity of HUFA relates to their partitioning into fungal
84 membranes, which alters membrane fluidity and disrupts membrane function. (Pohl, Kock, &
85 Thibane, 2011; Pohl, Voltchenko, & Rupprecht, 2008; Avis & Bélanger, 2001). However, how
86 this mechanism relates to the resistance of specific species of fungi to HUFA (Prost et al., 2005;
87 Liang et al., 2017) is not well understood. Fungal sensitivity towards the membrane active fatty
88 acid *cis*-9-heptadecenoic acid (C17:1) was accompanied by a lower fungal sterol content, which is
89 an important modulator of membrane-fluidity (Avis & Bélanger, 2001). It remains unknown
90 however, whether membrane composition, and particularly the distribution of sterols in fungal
91 membranes, also relate to fungal resistance to HUFA. The investigation on the difference between
92 HUFA-sensitive and –resistant fungi is essential to the development of the antifungal application
93 of these fatty acids, especially when developing methods to apply hydroxy fatty acids and in
94 combination with other antifungals.
95 To address these gaps in knowledge, this study aimed to obtain better understanding of the
96 effect of HUFA structure on their antifungal activities, and on their interaction with fungal
97 membranes. In order to acquire several HUFA with diverse structures, information on plant seed
98 oils containing C18 mono-HUFA was retrieved from the PlantFAdb database (Ohlrogge, Thrower,
99 Mhaske, Stymne, Baxter, Yang et al., 2017), which systematically documents fatty acid profiles
100 of plant species. Plant seed oils were extracted purification of HUFA was performed by HSCCC
101 after saponification of the lipids. In addition, HUFA were also obtained through bacterial
102 fermentation. The purified HUFA were characterized by LC-MS/MS and their antifungal activities

103 against food-related yeasts and molds were determined. The relationship between the inhibitory
104 spectrum of HUFA and their interaction with fungal membranes was assayed by determination of
105 the membrane fluidity and the sterol content of fungi.

106 2. Materials and Methods

107 *2.1 Chemical materials*

108 Plant seeds were purchased from the following sources: *Thymus vulgaris* L. (French Thymus)
109 seeds were purchased from Richters Herbs (Goodwood, ON, Canada); *Dimorphotheca sinuata*
110 DC. (African Daisy Seeds) seeds were obtained from High Country Gardens (Shelburne, VT, US)
111 and *Mallotus philippensis* (Lam.) Muell. Arg. were obtained from Rarepalmseeds.com (München,
112 Germany). *Coriaria nepalensis* Wall. seeds were purchased from XinTai Seed Production and
113 Wholesale Company (Jiangsu, China).

114 ACS-grade hexane, ethyl acetate and methanol were purchased from Sigma-Aldrich (St. Louis,
115 MO). Deionized ultra-filtered water was obtained from Fisher Scientific (Ottawa, Ontario,
116 Canada). Laurdan (6-dodecanoyl-2-dimethylaminonaphthalene) was purchased from Thermo
117 Scientific (Burlington, ON, Canada). Oleic acid and ricinoleic acid (purity >99%) were obtained
118 from Nu-Chek Prep, Inc. (Elysian, MN). Microbiological media were obtained from Fisher
119 Scientific.

120 *2.2 Microbial strains and culture conditions*

121 *Aspergillus niger* FUA5001 and *Penicillium roqueforti* FUA5004 were grown on malt extract
122 agar (MEA) at 25 °C for 7 days; *Pichia membranaefaciens* FUA4031, *Candida valida* FUA4030,
123 *Candida albicans* ATCC10231, and *Saccharomyces cerevisiae* FUA4011 were cultured in yeast

124 extract-peptone-dextrose (YPD) broth at 30 °C for 2 days and prepared for MIC test as previously
125 reported (Liang et al., 2017).

126 ***2.3 Extraction and saponification of oils from plant seeds rich HUFA***

127 In order to investigate the structure-function relationship of HUFA, mono-HUFA with various
128 molecular structures were obtained via extraction and saponification of plant seed oil rich in
129 HUFA. The distribution information of mono-HUFA in plant materials was obtained from
130 PlantFAdb database (https://plantfadb.org/, Ohlrogge et al., 2018) (Table 1). The extraction and
131 saponification of plants seed oil followed the protocols for the production of coriolic acid from
132 *Coriaria nepalensis* Wall. seed (Liang et al., 2017). Briefly, 5g of plant seeds (*C. nepalensis, D.*
133 *sinuata*, *M. philippensis* or *T. vulgaris*) were finely ground with liquid nitrogen and their total lipid
134 fractions were then extracted using a Soxhlet extractor. Oils of *C. nepalensis*, *D. sinuata* and *T.*
135 *vulgaris* were each extracted with 200 mL hexane, 450 rpm for 6 h. For *Mallotus philippensis*, the
136 same extraction conditions were applied except that petroleum ether (6 h) and diethyl ether (6 h)
137 were used to extract this oil subsequently (Gupta, Gupta, & Aggarwal, 1954), instead of using
138 hexane. Then every 1g of extracted oil was saponified with 10mL 0.1g/mL ethanolic KOH solution
139 at 70°C, 450rpm, followed by the extraction of free fatty acid mixture described previously (Liang
140 et al., 2017).

141 ***2.4 Purification and analysis of HUFA***

142 A solvent system was developed for the high-speed countercurrent chromatography (HSCCC)
143 purification of individual HUFA. The partition coefficient (*K* value) of various hexane/ethyl
144 acetate/methanol/water solvent systems was tested for each of the HUFA-containing saponified
145 plant oils with modifications to optimize the separation for each analysis (Liang et al., 2017).

146 Briefly, a 100 g/L stock solution of saponified oil in methanol was prepared, a 50 μL aliquot of
147 which was added into 5 mL of a solvent system consist of hexane, ethyl acetate, methanol and
148 water. Then, 200 μL aliquots of both the upper phase and the lower phase were put into separate
149 2mL HPLC vials, each of which dried under nitrogen and re-dissolved with 1mL methanol. For
150 each compound, the peak area from LC-ESI-MS/MS analysis in the MRM mode was recorded (as
151 explained below) and a partition coefficient (*K* value) was calculated using the formula K
152 $=A_{upper}/A_{lower}$, where A_{upper} and A_{lower} stands for the peak area of an analyte in the upper and lower
153 phases, respectively.
154 Saponified plant oil (200 mg) was then separated via HSCCC (TBE-300B HSCCC system,
155 Tauto Biotech, Shanghai, China) with the selected solvent system using the published protocols
156 (Liang et al., 2017). Briefly, approximately 2 L of the selected solvent systems were made in
157 separation funnel and equilibrated overnight. The upper and lower phases were then separately
158 collected. The upper phase (stationary phase) was first pumped (PrimeLine solvent delivery
159 module, Analytical Scientific Instruments, El Sobrante, CA) in the system to fill up the HSCCC
160 column (260 mL, β = 0.5−0.8, 1.9 mm i.d. tubing). Following that, the mobile phase (lower phase)
161 was pumped into the HSCCC column at 3mL/min with the column rotating (1000 rpm). Once the
162 mobile phase started constantly eluting out from the outlet, 200-250 mg of a saponified oil sample
163 dissolved in 5mL of each of the 2 phases was injected into the HSCCC system. Fractions from the
164 outlet were then collected every 3 min (CHF 122SC fraction collector, Avantec Toyo Kaisha Ltd.,
165 Tokyo, Japan).
166 Each HSCCC fraction was then analyzed by flow-injection (FIA)-ESI-MS/MS analysis (Liang
167 et al., 2017). The specific ion transitions of various HUFA analogues used to monitor the elution

168 of HUFA in the purification process are given in Table 1. This allowed identification of the
169 fractions, which contained the target HUFA; these were collected, combined, dried, re-dissolved
170 and analyzed for purity by LC-APPI-MS/MS.

171 In order to quantify HUFA in the saponified oil samples, and to measure the *K* value for HUFA
172 partitioned between immiscible phases, LC-ESI-MS/MS was used in the MRM mode using the
173 ion transitions indicated in Table 1. Each sample (2 μL) was separated on the Ascentis Express C8
174 column (15 cm × 2.1 mm, 3 μm; Sigma, St. Louis, MO) at 25 °C within an Agilent 1200 series LC
175 system (Agilent Technologies, Palo Alto, CA). The fatty acids were eluted at a flow rate of 0.25
176 mL/min with a gradient of mobile phases (A) water with 10 mM ammonium acetate and 0.2%
177 acetic acid and (B) 98% acetonitrile with 10 mM ammonium acetate and 0.2% acetic acid. The
178 gradient was 70 to 100% B in 23 min, followed by re-equilibration to 70% B, with a total run time
179 of 30 min. A 3200 QTRAP triple-quadrupole mass spectrometer (AB SCIEX, Concord, ON)
180 coupled with a turbospray electrospray-ionization ion source was used to perform negative-ion
181 ESI-MS/MS. GS1, GS2, and the curtain gas were nitrogen and set at 40, 60, and 25 arbitrary units,
182 respectively. The ion-spray voltage, ion-source temperature and dwell time were set at −4500 V,
183 450 °C and 40 ms respectively. The declustering potential (DP), collision energy (CE), collision-
184 cell entrance potential (CEP) and collision-cell exit potential (CXP) were specific for each target
185 compound: for coriolic acid, DP -45.00, EP -7.00, CEP -18.00, CE -26.00, CXP -2.00; for
186 dimorphecolic acid, DP -60.00, EP -12.00, CEP -20.00, CE -36.00, CXP -1.00; for kamlolenic
187 acid, DP -60.00, EP -12.00, CEP -22.50, CE 36.00, CXP -1.00; for 2-hydroxy oleic acid, DP -
188 50.00, EP -7.00, CEP -14, CE -28.00, CXP 0.00.

189 Data acquisition and processing was performed using AB SCIEX Analyst 1.4.2 software. To
190 analyze the HSCCC fractions using FIA-ESI-MS/MS, the same method was used except the
191 gradient was kept at the starting point for 2 min, followed by a gradient of 70 to 100% B in 23 min
192 and re-equilibration to 70% B, with a total run time of 32 min.

193 To analyze the purified fractions by normal-phase LC-APPI-MS/MS,(Liang et al., 2017), 2 μL
194 of samples (50ppm in hexane) were separated by PVA-Sil column (Waters Ltd., Mississauga, ON)
195 at 25 °C with gradient consist of (A) 0.2% acetic acid in hexane and (B) 0.2% acetic acid in
196 isopropanol: 99% A at 0 min, 70% A at 20 min, and 99% A at 20.1 min (total run time 27min).
197 APPI-MS/MS detection was performed using a QStar Elite hybrid orthogonal Q-TOF mass
198 spectrometer equipped with a PhotoSpray source (Applied Biosystems/MDS Sciex, Concord,
199 ON). The nebulizer gas, auxiliary gas, curtain gas, ionspray voltage, source temperature,
200 declustering potential (DP), focusing potential (FP), and DP2 were set to 65, 10, 25, −1300 V, 325
201 °C, −35 V, −130 V and −13 V, respectively. MS full scan was obtained over the range of m/z
202 50−1000; for MS/MS, a product-ion scan at m/z 50−700 of the according molecular ion $[M-H]^-$
203 was also performed to characterize the purified HUFA. The data were analyzed using Analyst QS
204 2.0.

205 ***2.5 Antifungal activity of hydroxy fatty acids***

206 The collected purified HUFA was applied to challenge the growth of fungi that are relevant as
207 fermentation cultures of spoilage organisms in food. The selection of strains included two spoilage
208 yeasts, *Pichia membranefaciens* and *Candida valida*, two mycelial fungi, *Aspergillus niger* and
209 *Penicillium roqueforti,* one food fermenting yeast, *Saccharomyces cerevisiae* and one human
210 pathogenic yeast, *Candida albicans*. The minimum inhibitory concentration (MIC) of HUFA

211 against these fungal strains was tested using a critical dilution assay as described (Liang et al.,
212 2017). Briefly, stock solution of 42.67 g/L of mono-HUFA was made in ethanol. For each
213 individual experiment, 100 µL of stock solution was added to 100 µL mMRS media, mixed and
214 transferred to create a series of 2-fold dilution in 96-well plates. The 96-well plates were then dried
215 in a laminar flow with lid open, with a reference of 50 µL ethanol to indicate the drying time
216 required for complete evaporation of ethanol in the media. The suspension of fungal spores or cells
217 (10^4 cfu/mL, 33.3 µL) were then inoculated in the media, creating a final concentration of HUFA
218 in the range of 0.01-16 g/L. Fungal growth were observed daily; MIC were recorded one day after
219 visible fungal growth in positive control, which was performed by fungal inoculated in media
220 without the addition of fatty acids.

221 ***2.6 Extraction and GC-MS quantitation of fungal sterols***

222 To determine the content of sterol, a representative membrane fluidity modulator, the fungi
223 tissues were first prepared as follow: sub-culture was kept at 25 °C (filamentous fungi at mMRS
224 broth) or 30 °C (yeasts at YPD broth) at 150 rpm for 3 d. The fungi tissues were then washed
225 through centrifugation and rinsing with physiological water twice and sterile distilled water once
226 before freeze-drying. Non-saponifiable components of fungal biomass were then extracted as
227 described (Adams & Parks, 1968) with some modifications: 20 g of ground lyophilized tissues of
228 fungi were weighed, followed by the addition of the internal standard (12.5 µL of 1.25 g/L
229 5α-cholestane in hexane). Later, 37.5 µL of pyrogallol solution (0.5 % in ethanol, w/v), 25 µl
230 potassium hydroxide solution (60 %, w/w), 37.5 µL ethanol and 100 µL water were added into the
231 sample. After vortexing, saponification was performed at 90 °C, 30 min. The reaction mixture was
232 the cooled down, followed by the addition of 100 µL of water and 400 µL hexane. After vortexing

233 and centrifugation, the upper phase of the mixture was collected, and the lower aqueous phase was
234 re-extracted with another 400 µL of hexane twice. The hexane extracts were combined and dried
235 under nitrogen. In order to silylate the –OH in the fungal sterol for improved GC separation
236 (Duchateau, Janssen, & Louter, 2003), Mixture of BSA+TMCS+TMSI, 3:2:3 (Sylon BTZ) Kit
237 (Supelco Inc., Bellefonte, PA, USA) (20 µL) and anhydrous pyridine (20 µL) were added in to the
238 extracted non-saponifiable fractions and kept 60 °C, 15 min. Hexane (210 µL) was added into the
239 reaction mixture. The mixture was first injected neat into GC-MS for qualitative analysis, then a
240 1:4 (v/v) dilution in hexane was used in quantitative analysis.

241 The GC-MS analysis was performed on a 6890N GC system coupled with 5975B mass
242 spectrometry in electron ionization mode. A column capillary column (30.0 m × 320.00 µm × 0.25
243 µm) was used. Helium was the carrier gas. Injection temperature was 290 °C and the solvent delay
244 time set at 1.80 min. Split mode was used and the split ratio was set to 12.5:1, with injection
245 volume 1.00 µL. The oven temperature was set at 245 °C initially with initial hold of 0.50 min,
246 then increased to 265 °C at a rate of 2.00 °C/min, followed by a ramp to 290 °C at a rate of 3.60
247 °C/min, and final hold of 10.00 min. The total run time was 27.64 min. An MS scan range of *m/z*
248 50-1000 was applied.

249 ***2.7 Measurement of fungal membrane fluidity using Laurdan assay***

250 The fluorescent probe Laurdan was applied in the spectroscopic measurement of the fluidity
251 changes of fungal membrane, as described previously with some modifications (Sánchez-
252 Maldonado, Schieber, & Gänzle, 2016). Briefly, 3-day fungal sub-culture was harvested via
253 centrifugation and washed with 30mL physiological water twice. Washed fungal tissue (10 mg)
254 was weighed then 300µL saline (9 g/L NaCl and 1 g/L Tween 80) was then added to re-suspend

255 the fungal tissue. Laurdan solution (2mM in ethanol) were added to the fungal tissue suspensions
256 to a final concentration of 80 µM; they were then incubated in the dark for 3 hours at 25 °C. After
257 incubation, fungal tissues were washed twice with saline (9 g/L NaCl and 1 g/L Tween 80) and re-
258 suspended in saline with/without treatment of fatty acid (4 g/L) for 30min (25 °C). The fluorescent
259 intensity of treated sample at emission wavelength from 400nm to 600nm was recorded with
260 excitation wavelength of 360 nm. Generalized polarization (GP) was calculated as GP = $(I_{440}-$
261 $I_{490})/(I_{440}+I_{490})$, where I_{440} and I_{490} were the fluorescent intensity at the wavelength of 440 nm and
262 490 nm respectively.

263 ***2.8 Statistical analysis***

264 Significant differences of the sensitivity of fungal strains against different isomers of HUFA
265 were analysed by two way analysis of variance (ANOVA) with Tukey's post hoc analysis using
266 SPSS Statistics Software. Significant differences were determined at an error levels of 5%
267 ($P<0.05$).

268 **3. Results**

269 **3.1 Extraction, HSCCC purification, and identification of antifungal HUFA from plant seed**
270 **oil.**

271 Sources of HUFA that cover a range of structural characteristics were identified using the
272 PlantFAdb database (https://plantfadb.org, Ohlrogge et al., 2018). Of the plants containing
273 hydroxylated C18 fatty acids, those shown in Table 1 were commercially available in seed form,
274 as an oil extract or in free fatty acid form. In addition, Table 1 includes hydroxylated fatty acids
275 produced by the bacteria *L. hammesii* and *L. plantarum* TMW1.460Δ*lah* (Liang et al., 2017). The
276 oil yield (g oil / g seed) from the seeds of *Coriaria nepalensis, Dimorphotheca sinuata*, *Thymus*

277 *vulgaris* and *Mallotus philippensis* was 67%, 22%, 32% and 34%, respectively, which is similar
278 to previous studies (Jones & Earle, 1966; Smith & Wolff, 1969; Gupta et al., 1954; Liang et al.,
279 2017).

280 After saponification and conversion to free fatty acids, HUFA were purified by HSCCC. The
281 hexane/ethyl acetate/methanol/water system with ratio of 7:3:7:4 (v/v/v/v) was selected for the
282 *Dimorphotheca sinuata* fatty acids due to the anticipated similarity in the partition behavior of the
283 conjugated hydroxy diene isomers, dimorphecolic acid (9-OH C18:2) and coriolic acid (13-OH
284 C18:2). Suitable solvent systems were selected based on the partition coefficient criterion of $0.4 \leq$
285 $K \leq 2.5$, for fatty acids extracted from both *Thymus* and *Mallotus*. The K values of fatty acids
286 present in these oils in various solvent systems were measured as Table 2 and Table 3. For the
287 separation of 2-OH C18:3, the solvent system 7:3:7:3 with a K value 0.96 for HUFA was chosen;
288 for separation of 18-OH C18:3, the solvent system 6:4:6:4, providing a K value 0.79 for the analyte,
289 was chosen. Fractions containing the analyte (Figure 1) were combined. The combined fractions
290 were then analyzed by LC-MS/MS to confirm their identities (Figure 2), and to determine their
291 purities.

292 In the LC-APPI-MS total ion current chromatograms, single peaks were observed in the purified
293 fractions (Figure S1 - A1, B1, C1 and D1). The fragment ions seen in the LC-MS/MS spectra of
294 these purified fractions (Figure 2) were consistent with those reported in previous literature: A)
295 13-OH C18:2 (*m/z* 295.2322 $[M-H]^-$, 277.2137 $[M-H-H_2O]^-$, and 195.1371 [α-cleavage of 13-OH
296 group]) (Liang et al., 2017; Murphy, 2015; Wheelan, Zirrolli, & Murphy, 1993); B) 9-OH C18:2
297 (*m/z* 295.2241 $[M-H]^-$, 277.2127 $[M-H-H_2O]^-$, and 171.1008 [α-cleavage of 9-OH group])
298 (Murphy, 2015; Wheelan et al., 1993); and D) 2-OH C18:3 (*m/z* 293.2150 $[M-H]^-$, 275.2052 [M-

299 H-H_2O]$^-$, 247.2090 [α-cleavage of 2-OH group], 191.1819 and 139.1152) (Gaquerel, Steppuhn, &
300 Baldwin, 2012; Wang et al., 2013). However, no LC-MS/MS data was found for kamlolenic acid
301 18-OH C18:3 (*m/z* 293.2084, 275.1982, 263.1986) (Figure 2C), but the observed fragmentation
302 matched the expected interpretation for HUFA of [M-H]-, [M-H-H_2O]$^-$ and [α-cleavage of 18-OH
303 group], respectively.

304 **3.2 Antifungal activity of HUFA**

305 In order to relate the antifungal properties to the molecular structures of HUFA, the antifungal
306 activity of purified HUFA were determined against 6 food-associated fungi (Table 4). Due to the
307 limited relevance of double bond positions in the antifungal activities of HUFA, investigation here
308 mainly focused on the position of hydroxy groups (Kobayashi et al., 1987; Prost et al., 2005).
309 Mono- and diunsaturated fatty acids with hydroxylation at position 9, 10, 12 and 13 exhibited a
310 similar fungal static activity; their MIC against *A. niger* and *P. roqueforti* ranged from 0.23-0.50
311 g/L. Unsaturated fatty acids with hydroxylation at position 2 or 18 exhibited a lower antifungal
312 activity against *P. roqueforti* with MICs of 2 g /L or higher. Yeasts were resistant to all of the
313 HUFA tested; only 2-OH C18:3 displayed inhibitory activity against yeasts with MICs in the range
314 of 1 – 5 g/L, 2-OH C18:1 inhibited *Pichia membranaefaciens* with an MIC of less than 1 g/L.

315 **3.3 Effect of HUFA on the fluidity of fungal membranes monitored by Laurdan**

316 In order to evaluate the effect of HUFA treatment on the fluidity of fungal membranes in
317 presence or absence of HUFA, General Polarization (GP) values were measured by Laurdan assay.
318 A decease of GP indicates the transition of a membrane from the gel-phase to a liquid-crystalline
319 (liquid disorder)-phase, and thus an increase in membrane polarity (Prasassi, Krasnowska,
320 Bagatolli, & Gratton, 1998).

321 In order to validate this LAURDAN method, we first monitored the fungal membrane fluidity
322 change after addition of ethanol, due to its known effect on membrane fluidity in *S. cerevisiae*
323 (Alexandre, Berlot, & Charpentier, 1994). In the controls without ethanol, the GP value decreased
324 in the order *A. niger* > *S. cerevisiae* > *P. roqueforti* and was thus not related to the resistance of
325 the organisms to HUFA. The GP value of all three organisms decreased with increasing ethanol
326 concentrations, indicating an increased fluidity of the membrane (Figure 3A). The impact of the
327 antifungal ricinoleic acid on GP values of *S. cerevisiae* and *P. roqueforti* was not different from
328 oleic acid, which has no antifungal activity, further indicating that membrane fluidity as measured
329 by the GP is not related to the resistance to hydroxyl fatty acids (Fig. 3B).

330 **3.4 Comparison of the sterol level in HUFA-resistant and HUFA-sensitive fungi**

331 Sterols are known to play a role in the maintenance of membrane fluidity (Benyagoub,
332 Willemot, & Bélanger, 1996; Dufourc, 2008) and sterol levels have previously been associated
333 with fungal sensitivity towards an antifungal fatty acids produced by *Pseudozyma flocculosa* (Avis
334 & Bélanger, 2001). Here, ergosterol was quantified by GC/MS (Duchateau et al., 2003) in order
335 to investigate whether the sterol content of the fungal tissues relates to fungal sensitivity to HUFA.
336 Ergosterol is the main constituent of membrane sterols in all samples tested, accounting for more
337 than 85% of the total peak area of possible sterol peaks in the GC-MS chromatograms. The highest
338 sterol content was identified in *W. anomalus* (Fig. 4A); mycelial fungi had a low ergosterol content.
339 *C. albicans* was the only yeast with an ergosterol content of less than 4 mg/g in fungal biomass
340 (Fig. 5A). A plot of the HUFA MIC values against the measured sterol content of fungal biomass
341 revealed a clear distinction between yeasts and mycelial fungi. Yeasts can be seen to have high
342 MIC values and high ergosterol contents, with the exception of *C. albicans*, which has a low

343 ergosterol contents. *In contrast,* all of the mycelial fungi tested were found to have low MIC values
344 and low sterol contents (Figure 3B*)*.

345 **4. Discussion**

346 In the present study, HUFA from plant seed oils were identified in the PlantFAdb database,
347 extracted, purified and then tested with respect to their inhibitory activity against food-associated
348 fungi. The antifungal properties of mono-OH HUFA were found to depend on their specific
349 structure, with most activity when their hydroxyl groups are located in the middle (C9-C13) of the
350 C18 HUFA chain. Studies on their mode of action suggested that HUFA are membrane active, and
351 that fungal resistance to HUFA relates to the sterol content of the fungi biomass. The relationship
352 of HUFA molecular structure and their antifungal activities provide insight on their application in
353 food and their impact on food quality.

354 Previous studies have indicated antifungal properties of HUFA (Prost et al., 2005; Pohl et al.,
355 2011), especially 13-OH C18:2 (Yara et al., 2008), 9-OH C18:2 (Cantrell et al., 2008), and 2-OH
356 C18:3 (Shimada et al., 2014) but provided only limited information on structure-function
357 relationship (Prost et al., 2005). This lack of knowledge is linked to the small quantities of HUFA
358 that were available in past studies. The present study focused on plant seed oils that contain high
359 amounts of HUFA which, in combination with an efficient purification technique, enabled
360 additional observations on the structure-function relationship of HUFA. Long-chain saturated
361 hydroxy fatty acids exhibit no antifungal activity (Black et al., 2013), possibly due to their high
362 hydrophobicity and low solubility (Pohl et al., 2011). The present study showed that unsaturated
363 HUFA with hydroxylation at the C9-C13 position of the C18 chain exhibited comparable
364 antifungal activity while their 2- and 18-OH analogues were less active. This might be attributed

365 to differences in the dissociation constants between fatty acids (pK_A) (Pohl et al., 2008), and/or
366 their different interactions with fungal cellular membrane (Pohl et al., 2011; Benyagoub et al.,
367 1996; Avis & Bélanger, 2001), including the translocation across the cell membrane (Kamp &
368 Hamilton, 1992). The –OH position will also alter the monolayer packing properties of HUFA
369 when these integrate into phospholipid bilayers (Negelmann, Pisch, Bornscheuer, & Schmid,
370 1997). In contrast to other HUFA analogues, 2-OH C18:3 showed a broad spectrum of weak
371 inhibition against all of the species tested (MIC ≥ 1g/L). This indicates that different HUFA have
372 different modes of antifungal action.
373 Due to the diversity of possible fungal strains, it is useful to understand the structure-function
374 relationships of HUFA targeting food-related fungi. The observed differences in antifungal activity
375 between HUFA containing different hydroxylation patterns, and the difference between the
376 resistance to HUFA antifungal activity of yeasts compared to filamentous fungi, expands prior
377 knowledge obtained with coriolic acid (Liang et al., 2017). Similar to the food spoilage fungi *A.*
378 *niger* and *P. roqueforti*, phyto-pathogenic filamentous fungi like *Botrytis cinerea* and
379 *Cladosporium herbarum* exhibited similar specificity to HUFA structures. Specifically, the latter
380 fungi were highly inhibited by 9-OH and 13-OH C18:2 or C18:3 HUFA, but not 2-OH C18:3 or
381 C18:1 (Prost et al., 2005). Some previous studies have also indicated the sensitivity of phyto-
382 pathogenic fungi to HUFA. For example, for HUFA with –OH in the center of the 13-OH C18:3
383 fatty acid chain, the isomer 13(*S*)-hydroxy-9(*Z*),11(*E*),15(*Z*)-octadecatrienoic acid) inhibited *B.*
384 *cinerea* and *C. herbarum* at a concentration of 0.03 g/L, while another isomer 13(*S*)-Hydroxy-
385 6(*Z*),9(*Z*),11(*E*)-octadecatrienoic acid, was not active (Prost et al., 2005). In addition, other
386 filamentous fungi like *Alternaria brassicicola, Fusarium oxysporum,* and *Rhizopus* sp. were

387 resistant to any of the HUFA tested, at a concentration of around 0.03 g/L (Prost et al., 2005;
388 Cantrell et al., 2008). In a study targeting the phytopathogenic fungi *Leptosphaeria maculans* and
389 *Alternaria brassicae*, 0.3 g/L of 13-OH C18:3 HUFA, but not 13-OH C18:2 (coriolic acid)
390 inhibited growth of hyphae and 0.3 g/L of coriolic acid inhibited spore germination of
391 *Leptosphaeria maculans* in certain growth conditions (Granér et al., 2003). Different
392 phytopathogens may have their specific resistance mechanisms, which results in the diverse levels
393 of sensitivity to HUFA.
394 The delay of cell growth and inhibition of germ sporulation caused by antifungal fatty acids has
395 been attributed to their partitioning into fungal membranes and the associated increase in
396 membrane fluidity (Pohl et al., 2011; Pohl et al., 2008; Avis & Bélanger, 2001). Partitioning of
397 both HUFA and their non-OH counterparts into membranes is rapid without assistance of
398 membrane protein (Pohl et al., 2008; Ek-von Mentzer, Zhang, & Hamilton, 2001), and thus does
399 not explain differences in their antifungal activities (Liang et al., 2017; Chen et al., 2016). The
400 present study documented that GP values of fungal membranes were similar after treatments with
401 HUFA and non-hydroxylated fatty acids, so it is concluded that there is no specific impact of
402 HUFA on the overall membrane fluidity. Of note, GP values of fungal membranes were also not
403 altered by addition of 0 – 20% ethanol while the same ethanol concentrations strongly influenced
404 the fluidity of bacterial membranes (Chen et al., 2016), which may relate to the presence of sterols
405 in fungal membranes. HUFA may target specific regions or lipid rafts of the fungal membrane
406 without changing the overall membrane fluidity. Similarly, it has been reported that ricinoleic acid
407 and oleic acid exhibited different effects on membrane disorder but show the same effect on phase
408 transition in a dimyristoylphosphatidylcholine (DMPC) lipid membrane model (Jenske,

409 Lindström, Gröbner, & Vetter, 2008). Furthermore, intracellular fatty acid binding proteins
410 (FABP) in the cytosol, in the presence of phospholipid bilayers, exhibited binding preference
411 towards HUFA (13-OH C18:2) over their non-OH FA precursors; in addition, liver FABP, in the
412 presence of vesicles, specifically showed higher efficiency while binding 13-OH C18:2 compared
413 to intestinal FABP (Ek-von Mentzer et al., 2001). These different characteristics may at least
414 partially contribute to the antifungal activity of HUFA compared to their non-OH counterparts.
415 Clearly, more studies are needed to investigate the interaction between HUFA and fungal
416 membranes in order to explain the observed structure-function differences.
417 Fungal membrane lipid compositions, including phospholipids, unsaturated fatty acids and sterols,
418 have been associated with their sensitivity towards antifungal fatty acids (Benyagoub et al., 1996;
419 Avis & Bélanger, 2001). In particular, sterols maintain the structure of the membrane during stress
420 (Avis & Bélanger, 2001; Dufourc, 2008) and their content is altered in fungi that alternate between
421 unicellular and a mycelial/pseudo-mycelial state (Ghannoum, Janini, Khamis, & Radwan, 1986;
422 Morozova, Kozlov, Tereshina, Memorskaya, & Feofilova, 2002). Ergosterol is the major sterol in
423 fungal membranes and thus representative of the total sterol content. For example, >90% of sterol
424 was ergosterol in *C. albicans* ATCC 10231 (Shrestha, Garzan, & Garneau-Tsodikova, 2017). In
425 the present study, the minor sterols were not quantified because they had a very low abundance
426 (data not shown).
427 We observed that yeasts were resistant to HUFA and tended to have high sterol content, while
428 mycelial fungi were sensitive to HUFA and had a lower sterol content. *S. cerevisiae* has a higher
429 sterol content when compared to *A. niger* and some *Penicillium* spp. (Appleton, Kieber, & Payne,
430 1955). The yeast strain *Pseudozyma rugulosa* also had a higher sterol content compared to other

431 mycelial fungi tested, and showed higher resistance towards antifungal fatty acids (Avis &
432 Bélanger, 2001). However, we observed one exception, i.e. the pathogenic yeast *C. albicans*, a
433 HUFA-resistant strain with a sterol content that was comparable to or lower than that of HUFA-
434 sensitive mycelial fungi. Data obtained in the present study by GC/MS confirmed the prior data
435 on *A. niger, Penicillium spp.*, and *C. albicans* that was obtained by the Liebermann-Burchard
436 reaction (Kieber, Payne, & Appleton, 1955; Appleton et al., 1955). *C. albicans* alters the sterol
437 content of its membrane to adjust its cellular morphology (Ghannoum et al., 1986). In addition,
438 the sterol profile of membranes of *C. albicans* is also modified in response to antifungal
439 compounds (Hitchcock, Barrett-Bee, & Russell, 1986). Therefore, HUFA-resistant species may
440 employ different stress responses under HUFA treatment. Further studies related to the adaptation
441 of yeasts and fungi to antifungal HUFA are necessary to confirm the role of sterols in resistance
442 of food-associated fungi and phytopathogens.

443 The better understanding on the structure-function relationship and the mode of action of
444 antifungal HUFA provides important insights on their applications in foods. Previously, the
445 efficacy *in situ* of HUFA has been determined in only in bread (Black et al., 2013; Quattrini et al.,
446 2018). However, these applications of HUFA in food were limited to Δ10-, Δ12- and Δ13-OH
447 isomers. Results here indicated the application HUFA can also expand to other HUFA with –OH
448 located in the middle of the fatty acid chain, and in some cases, Δ2-. In addition, plant seed oil
449 extract may provide a novel source of antifungal HUFA, for example, lipids from thyme, a
450 common culinary material. Additionally, the application of bacterial fermentation extract or plant
451 lipids that are hydrolysed by food-grade lipases can provide advantages in the development of
452 "clean-label" products. In addition, since data suggests that the sensitivity of fungi to HUFA may

453 relate to their sterol content, food components that affect the sterol synthesis of fungi (OuYang et
454 al., 2016; Parveen et al., 2004) may enhance the antifungal activity of HUFA in situ. Food
455 applications of antifungal HUFA must also consider their impact on flavor. For example, coriolic
456 acid has bitter taste but the MIC of coriolic acid is lower than its bitter taste threshold (2.25 g/L-
457 2.52 g/L) (Biermann & Grosch, 1979) which indicates that active concentration of antifungal
458 HUFA can be applied without negatively impacting flavor. Additionally, HUFA are precursors for
459 aromatic lactone (Romero-Guido et al., 2011), which may provide additional functionality in food.
460 In addition to their potential role as antifungal compounds in foods, the production of HUFA has
461 been closely related to a board spectrum of biological functions, such as signaling (Weber, 2002;
462 Spite, Clària, & Serhan, 2014), virulence (Volkov et al., 2010) and response mechanisms towards
463 biotic stresses (Weber, 2002; Prost et al., 2005). In particular, 2-OH C18:3 has been classified as
464 phytoalexin due to its *in vivo* production in plant associated with phytopathogenic fungal infection
465 (Shimada et al., 2014). In addition, it exhibited a tissue-protective effect against phytopathogenic
466 bacterial infection (Hamberg, Sanz, Rodriguez, Calvo, & Castresana, 2003), but this protective
467 effect could not be attributed to bactericidal activity (Hamberg et al., 2003; Prost et al., 2005). This
468 suggests that 2-OH C18:3 may be associated with other plant defense and/or signaling mechanisms
469 (Gaquerel et al., 2012). The dual functions of HUFA as direct antifungals and plant signal
470 molecules may lead to further applications in plant protection in agriculture (Prost et al., 2005;
471 Weber, 2002). The general methods used in this study to source, extract and purify diverse HUFA
472 species could be beneficial in an expanded investigation of the diverse bioactivities of HUFA.
473 In conclusion, the present study has identified specific plant seed oils as a source of antifungal
474 hydroxy unsaturated fatty acids, and showed distinct antifungal properties of HUFA with hydroxyl

475 groups located towards the center of the fatty acid chain, at about the C9-C13 positions, compared
476 to those with hydroxyl groups near either terminus, at C2 or C18 positions. The HUFA with –OH
477 in C9-C13 position inhibited the growth of food spoilage fungi *P. roqueforti* and *A. niger*, but did
478 not interfere with yeast growth. In contrast, 2-OH linolenic acid (2-OH C18:3) showed minor
479 inhibition against both mycelial fungi and yeasts. HUFA-resistant yeasts tended to possess a higher
480 sterol content than in HUFA-sensitive fungi. The present study made use of HSCCC for
481 purification of HUFA; since this is a scalable technique it could be used in developing HUFA-
482 based antifungal methods for food and agricultural applications, as well as in further studies of
483 their antifungal properties. Although the resistance of yeasts prevents applications of HUFA in
484 food where spoilage by yeasts is of concern, at the same time it enables applications in foods where
485 yeasts are used for desired fermentations.

486 **Acknowledgements**

487 Western Grains Foundation and the Alberta Wheat Commission are acknowledged for the
488 funding of this project. Nuanyi Liang acknowledges the financial support from Mitacs. Alexander
489 K. Tan and Sheng Xiang thank the funding of University of Alberta Research Experience (UARE)
490 2018 Winter Program. Michael Gänzle acknowledges financial support from the Canada Research
491 Chair program.

492 **References**

493 Adams, B. G., & Parks, L. W. (1968). Isolation from yeast of a metabolically active water-
494 soluble form of ergosterol. *The Journal of Lipid Research, 9*, 8-11.

495 Alexandre, H., Berlot, J. P., & Charpentier, C. (1994). Effect of ethanol on membrane fluidity of
496 protoplasts from *Saccharomyces cerevisiae* and *Kloeckera apiculata* grown with or without

497 ethanol, measured by fluorescence anisotropy. *Biotechnology Techniques, 8*, 295-300.
498 https://doi.org/10.1007/Bf02428970.
499 Appleton, G. S., Kieber, R. J., & Payne, W. J. (1955). The sterol content of fungi. II. Screening
500 of representative yeasts and molds for sterol content. *Applied Microbiology, 3*, 249-251.
501 Asioli, D., Aschemann-Witzel, J., Caputo, V., Vecchio, R., Annunziata, A., Næs, T., & Varela,
502 P. (2017). Making sense of the "clean label" trends: A review of consumer food choice behavior
503 and discussion of industry implications. *Food Research International, 99*, 58-71.
504 https://doi.org/10.1016/j.foodres.2017.07.022.
505 Avis, T. J., & Bélanger, R. R. (2001). Specificity and mode of action of the antifungal fatty acid
506 *cis*-9-heptadecenoic acid produced by *Pseudozyma flocculosa*. *Applied and Environmental*
507 *Microbiology, 67*, 956-960. https://doi.org/10.1128/Aem.67.2.956-960.2001.
508 Badami, R. C., & Patil, K. B. (1980). Structure and occurrence of unusual fatty acids in minor
509 seed oils. *Progress in Lipid Research, 19*, 119-153.
510 Benyagoub, M., Willemot, C., & Bélanger, R. R. (1996). Influence of a subinhibitory dose of
511 antifungal fatty acids from *Sporothrix flocculosa* on cellular lipid composition in fungi. *Lipids,*
512 *31*, 1077-1082. https://doi.org/10.1007/bf02522465.
513 Biermann, U., & Grosch, W. (1979). Bitter-tasting monoglycerides from stored oat flour.
514 *Zeitschrift für Lebensmittel-Untersuchung und Forschung, 169*, 22-26. https://doi.org/Doi
515 10.1007/Bf01353409.
516 Black, B. A., Zannini, E., Curtis, J. M., & Gänzle, M. G. (2013). Antifungal hydroxy fatty acids
517 produced during sourdough fermentation: microbial and enzymatic pathways, and antifungal

518 activity in bread. *Applied and Environmental Microbiology, 79*, 1866-1873.
519 https://doi.org/10.1128/AEM.03784-12.
520 Cantrell, C. L., Case, B. P., Mena, E. E., Kniffin, T. M., Duke, S. O., & Wedget, D. E. (2008).
521 Isolation and identification of antifungal fatty acids from the basidiomycete *Gomphus floccosus*.
522 *Journal of Agricultural and Food Chemistry, 56*, 5062-5068. https://doi.org/10.1021/jf8008662.
523 Chen, Y. Y., Liang, N. Y., Curtis, J. M., & Gänzle, M. G. (2016). Characterization of linoleate
524 10-hydratase of *Lactobacillus plantarum* and novel antifungal metabolites. *Frontiers in*
525 *Microbiology, 7*, 1561. https://doi.org/10.3389/fmicb.2016.01561.
526 Duchateau, G. S. M. J. E., Janssen, H. G. M., & Louter, A. J. H. (2003). Plant sterol analysis in
527 relation to functional foods. In P. C. Dutta (Ed.), *Phytosterol as Functional Food Components*
528 *and Nutraceuticals* (pp. 75-132). New York: Marcel Dekker, Inc.
529 Dufourc, E. J. (2008). Sterols and membrane dynamics. *Journal of Chemical Biology, 1*, 63-77.
530 https://doi.org/10.1007/s12154-008-0010-6.
531 Ek-von Mentzer, B. A., Zhang, F. L., & Hamilton, J. A. (2001). Binding of 13-HODE and 15-
532 HETE to phospholipid bilayers, albumin, and intracellular fatty acid binding proteins -
533 Implications for transmembrane and intracellular transport and for protection from lipid
534 peroxidation. *Journal of Biological Chemistry, 276*, 15575-15580.
535 https://doi.org/10.1074/jbc.M011623200.
536 FAO. Save food: global initiative on food loss and waste reduction—key findings. (2019).
537 Retrieved from http://www.fao.org/save-food/resources/keyfindings/en/ Accessd 12 August 2019

538 Farré-Armengol, G., Filella, I., Llusia, J., & Peñuelas, J. (2016). Bidirectional interaction
539 between phyllospheric microbiotas and plant volatile emissions. *Trends in Plant Science, 21*,
540 854-860. https://doi.org/10.1016/j.tplants.2016.06.005.
541 Gaquerel, E., Steppuhn, A., & Baldwin, I. T. (2012). *Nicotiana attenuata* ɑ-DIOXYGENASE1
542 through its production of 2-hydroxylinolenic acid is required for intact plant defense expression
543 against attack from *Manduca sexta* larvae. *New Phytologist, 196*, 574-585.
544 https://doi.org/10.1111/j.1469-8137.2012.04286.x.
545 Ghannoum, M. A., Janini, G., Khamis, L., & Radwan, S. S. (1986). Dimorphism-associated
546 variations in the lipid composition of *Candida albicans*. *Journal of General Microbiology, 132*,
547 2367-2375. https://doi.org/10.1099/00221287-132-8-2367.
548 Granér, G., Hamberg, M., & Meijer, J. (2003). Screening of oxylipins for control of oilseed rape
549 (*Brassica napus*) fungal pathogens. *Phytochemistry, 63*, 89-95. https://doi.org/10.1016/S0031-
550 9422(02)00724-0.
551 Gupta, S. C., Gupta, S. S., & Aggarwal, J. S. (1954). The component acids of Kamala oil
552 (*Mallotus philippinensis*, Muell. Agr.). *Journal of the American Oil Chemists' Society, 31*, 287-
553 289. https://doi.org/10.1007/Bf02639158.
554 Hamberg, M., Sanz, A., Rodriguez, M. J., Calvo, A. P., & Castresana, C. (2003). Activation of
555 the fatty acid ɑ-dioxygenase pathway during bacterial infection of tobacco leaves - Formation of
556 oxylipins protecting against cell death. *Journal of Biological Chemistry, 278*, 51796-51805.
557 https://doi.org/10.1074/jbc.M310514200.

558 Hitchcock, C. A., Barrett-Bee, K. J., & Russell, N. J. (1986). The lipid composition of azole-
559 sensitive and azole-resistant strains of *Candida albicans*. *Journal of General Microbiology, 132*,
560 2421-2431. https://doi.org/10.1099/00221287-132-9-2421.
561 Jenske, R., Lindström, F., Gröbner, G., & Vetter, W. (2008). Impact of free hydroxylated and
562 methyl-branched fatty acids on the organization of lipid membranes. *Chemistry and Physics of*
563 *Lipids, 154*, 26-32. https://doi.org/10.1016/j.chemphyslip.2008.03.010.
564 Jones, Q., & Earle, F. R. (1966). Chemical analyses of seeds II: oil and protein content of 759
565 species. *Economic Botany, 20*, 127-155.
566 Kamp, F., & Hamilton, J. A. (1992). pH gradients across phospholipid membranes caused by fast
567 flip-flop of un-ionized fatty acids. *Proceedings of the National Academy of Sciences of the*
568 *United States of America, 89*, 11367-11370. https://doi.org/10.1073/pnas.89.23.11367.
569 Kieber, R. J., Payne, W. J., & Appleton, G. S. (1955). The sterol content of fungi. I. Methods for
570 disrupting cells, extracting and determining sterols. *Applied Microbiology, 3*, 247-248.
571 Lacerda, A., Vasconcelos, É., Pelegrini, P., & Grossi-de-Sa, M. F. (2014). Antifungal defensins
572 and their role in plant defense. *Frontiers in Microbiology, 5*, 116.
573 https://doi.org/10.3389/fmicb.2014.00116.
574 Liang, N., Cai, P., Wu, D., Pan, Y., Curtis, J. M., & Gänzle, M. G. (2017). High-speed counter-
575 current chromatography (HSCCC) purification of antifungal fatty acids from plant-seed oil and
576 *Lactobacillus* cultures. *Journal of Agricultural and Food Chemistry, 65*, 11229-11236.
577 https://doi.org/10.1021/acs.jafc.7b05658.
578 Morozova, E. V., Kozlov, V. P., Tereshina, V. M., Memorskaya, A. S., & Feofilova, E. P.
579 (2002). Changes in lipid composition and carbohydrate composition of *Aspergillus niger* conidia

580 during germination. *Applied Biochemistry and Microbiology, 38*, 129-133.

581 https://doi.org/10.1023/A:1014302314834.

582 Murphy, R. C. (2015). Chapter 1 Fatty acids. In*Tandem Mass Spectrometry of Lipids: Molecular*

583 *Analysis of Complex Lipids* (pp. 1-39). United Kingdom: The Royal Society of Chemistry.

584 Negelmann, L., Pisch, S., Bornscheuer, U., & Schmid, R. D. (1997). Properties of unusual

585 phospholipids. III: Synthesis, monolayer investigations and DSC studies of hydroxy

586 octadeca(e)noic acids and diacylglycerophosphocholines derived therefrom. *Chemistry and*

587 *Physics of Lipids, 90*, 117-134. https://doi.org/10.1016/S0009-3084(97)00085-6.

588 Ohlrogge, J., Thrower, N., Mhaske, V., Stymne, S., Baxter, M., Yang, W. L., . . . Matthäus, B.

589 (2018). PlantFAdb: a resource for exploring hundreds of plant fatty acid structures synthesized

590 by thousands of plants and their phylogenetic relationships. *The Plant Journal, 96*, 1299-1308.

591 https://doi.org/10.1111/tpj.14102.

592 Oliveira, P. M., Zannini, E., & Arendt, E. K. (2014). Cereal fungal infection, mycotoxins, and

593 lactic acid bacteria mediated bioprotection: from crop farming to cereal products. *Food*

594 *Microbiology, 37*, 78-95. https://doi.org/10.1016/j.fm.2013.06.003.

595 OuYang, Q. L., Tao, N. G., & Jing, G. X. (2016). Transcriptional profiling analysis of

596 *Penicillium digitatum*, the causal agent of citrus green mold, unravels an inhibited ergosterol

597 biosynthesis pathway in response to citral. *BMC Genomics, 17*, 599.

598 https://doi.org/10.1186/s12864-016-2943-4.

599 Parasassi, T., Krasnowska, E. K., Bagatolli, L., & Gratton, E. (1998). Laurdan and Prodan as

600 polarity-sensitive fluorescent membrane probes. *Journal of Fluorescence, 8*, 365-373.

601 https://doi.org/10.1023/A:1020528716621.

602 Parveen, M., Hasan, M. K., Takahashi, J., Murata, Y., Kitagawa, E., Kodama, O., & Iwahashi, H.
603 (2004). Response of *Saccharomyces cerevisiae* to a monoterpene: evaluation of antifungal
604 potential by DNA microarray analysis. *Journal of Antimicrobial Chemotherapy, 54,* 46-55.
605 https://doi.org/10.1093/jac/dkh245.
606 Paterson, R. R. M., & Lima, N. (2010). How will climate change affect mycotoxins in food?
607 *Food Research International, 43*, 1902-1914. https://doi.org/10.1016/j.foodres.2009.07.010.
608 Pitt, J. I., & Hocking, A. D. (2009). *Fungi and food spoilage.* (3rd ed.). New York: Springer
609 Dordrecht Heidelberg London New York.
610 Pohl, C. H., Kock, J. L. F., & Thibane, V. S. (2011). Antifungal free fatty acids: a review. In A.
611 Méndez-Vilas (Ed.), *Science against microbial pathogens: current research and technological*
612 *advances* (pp. 61-71).
613 Pohl, E. E., Voltchenko, A. M., & Rupprecht, A. (2008). Flip-flop of hydroxy fatty acids across
614 the membrane as monitored by proton-sensitive microelectrodes. *Biochimica et Biophysica Acta*
615 *- Biomembranes, 1778*, 1292-1297. https://doi.org/10.1016/j.bbamem.2008.01.025.
616 Prost, I., Dhondt, S., Rothe, G., Vicente, J., Rodriguez, M. J., Kift, N., Fournier, J. (2005).
617 Evaluation of the antimicrobial activities of plant oxylipins supports their involvement in defense
618 against pathogens. *Plant Physiology, 139*, 1902-1913. https://doi.org/10.1104/pp.105.066274.
619 Quattrini, M., Liang, N., Fortina, M. G., Xiang, S., Curtis, J. M., & Gänzle, M. (2018).
620 Exploiting synergies of sourdough and antifungal organic acids to delay fungal spoilage of bread.
621 *International Journal of Food Microbiology*. https://doi.org/10.1016/j.ijfoodmicro.2018.09.007.

622 Salas, M. L., Mounier, J., Valence, F., Coton, M., Thierry, A., & Coton, E. (2017). Antifungal
623 microbial agents for food biopreservation-a review. *Microorganisms, 5*.
624 https://doi.org/10.3390/microorganisms5030037.
625 Sánchez-Maldonado, A. F., Schieber, A., & Gänzle, M. G. (2016). Antifungal activity of
626 secondary plant metabolites from potatoes (*Solanum tuberosum* L.): Glycoalkaloids and phenolic
627 acids show synergistic effects. *Journal of Applied Microbiology, 120*, 955-965.
628 https://doi.org/10.1111/jam.13056.
629 Shimada, T. L., Takano, Y., Shimada, T., Fujiwara, M., Fukao, Y., Mori, M., . . . Hara-
630 Nishimura, I. (2014). Leaf oil body functions as a subcellular factory for the production of a
631 phytoalexin in *Arabidopsis*. *Plant Physiology, 164*, 105-118.
632 https://doi.org/10.1104/pp.113.230185.
633 Shrestha, S. K., Garzan, A., & Garneau-Tsodikova, S. (2017). Novel alkylated azoles as potent
634 antifungals. *European Journal of Medicinal Chemistry, 133*, 309-318.
635 https://doi.org/10.1016/j.ejmech.2017.03.075.
636 Smith, C. R., & Wolff, I. A. (1969). Characterization of naturally occurring α-hydroxylinolenic
637 acid. *Lipids, 4*, 9-14. https://doi.org/10.1007/Bf02531787.
638 Spite, M., Clària, J., & Serhan, C. N. (2014). Resolvins, specialized proresolving lipid mediators,
639 and their potential roles in metabolic diseases. *Cell Metabolism, 19*, 21-36.
640 https://doi.org/10.1016/j.cmet.2013.10.006.
641 Volkov, A., Liavonchanka, A., Kamneva, O., Fiedler, T., Goebel, C., Kreikemeyer, B., &
642 Feussner, I. (2010). Myosin cross-reactive antigen of *Streptococcus pyogenes* M49 encodes a
643 fatty acid double bond hydratase that plays a role in oleic acid detoxification and bacterial

644 virulence. *The Journal of Biological Chemistry, 285*, 10353-10361.
645 https://doi.org/10.1074/jbc.M109.081851.
646 Wang, X., Chen, J., Xu, J., Chen, H., Yan, X., & Zhou, C. (2013). Quantitative analysis of
647 oxylipins in *Laminaria japonica* by LC–MS. *Chinese Journal of Pharmaceutical Analysis, 33*,
648 1656-1664.
649 Weber, H. (2002). Fatty acid-derived signals in plants. *Trends in Plant Science, 7*, 217-224.
650 https://doi.org/10.1016/S1360-1385(02)02250-1.
651 Wheelan, P., Zirrolli, J. A., & Murphy, R. C. (1993). Low-energy fast atom bombardment
652 tandem mass spectrometry of monohydroxy substituted unsaturated fatty acids. *Biological Mass*
653 *Spectrometry, 22*, 465-473. https://doi.org/10.1002/bms.1200220808.
654 Yara, A., Yaeno, T., Montillet, J. L., Hasegawa, M., Seo, S., Kusumi, K., & Iba, K. (2008).
655 Enhancement of disease resistance to *Magnaporthe grisea* in rice by accumulation of hydroxy
656 linoleic acid. *Biochemical and Biophysical Research Communications, 370*, 344-347.
657 https://doi.org/10.1016/j.bbrc.2008.03.083.
658 Zheng, J., Wittouck, S., Salvetti, E., Franz, C.M.A.P., Harris, H.M.B., Mattarelli, P., O'Toole,
659 P.W., Pot, B., Vandamme, P., Walter, J., Watanabe, K., Wuyts, S., Felis, G.E., Gänzle, M.G.,
660 Lebeer, S. A taxonomic note on the genus *Lactobacillus*: Description of 23 novel genera,
661 emended description of the genus *Lactobacillus* Beijerinck 1901, and union of *Lactobacillaceae*
662 and *Leuconostocaceae*. *International Journal of Systematic and Evolutionary Microbiology*, in
663 press. https://doi.org/10.1099/ijsem.0.004107.
664

Table 1. HUFA analogues extracted from plant oils and *Lactobacillus* fermentation, or purchased, and their LC-MS/MS (multiple reaction monitoring, MRM) ion transitions, which were for construction of HSCCC chromatogram and the measurement of K value

HUFA	Molecular structures	Sources	Q_1 (m/z)	Q_3 (m/z)
Kamlolenic acid (18-OH C18:3) [a]		*Mallotus philippensis*	293.2	263.2
2-OH linolenic acid (2-OH C18:3)		*Thymus vulgaris*	293.4	191.3
Dimorphecolic acid (9-OH C18:2)		*Dimorphotheca sinuata*	295.2	171.1
Coriolic acid (13-OH C18:2)		*Coriaria nepalensis*	295.2	195.1
Ricinoleic acid (12-OH C18:1)		Castor bean oil	297.2	183.1
13-OH C18:1		*Lactiplantibacillus plantarum* TMW1.460Δ*lah* [b]	297.2	197.1
10-OH C18:1		*Levilactobacillus hammesii* [b]	297.2	185.1
2-Hydroxy oleic acid (2-OH C18:1)		Commercial standard	297.6	251.4

[a] Aggarwal, 1955.

[b] previously *Lactobacillus plantarum* and *Lactobacillus hammesii*, respectively (Zheng et al., 2020)

Table 2. Partition coefficient (*K* value) of 2-OH linolenic acid in the hexane/ethyl acetate/methanol/water (HEMWat) systems with different solvent ratios. Fatty acids, C16:0, C17:0, C18:0, C18:1, C18:2, C18:3, 2-OH C18:1, 2-OH C18:3 were measurement with LC-MS/MS (MRM) with the following ion transition: 255.2/255.2, 269.2/269.2, 283.2/283.2, 281.2/281.2, 279.2/279.2, 277.2/277.2, 297.2/251.2, 293.4/191.3.

HEMwat System Ratio (*v/v/v/v*)	C16:0	C17:0	C18:0	C18:1	C18:2	C18:3	2-OH C18:1	2-OH C18:3
9:1:9:1	2.29	2.75	>=3	2.48	1.72	1.22	<0.5	<0.5
8:2:8:2	>=3	>=3	>=3	>=3	>=3	3.00	0.79	<0.5
7:3:7:3	>=3	>=3	>=3	>=3	>=3	>=3	2.66	0.96
7:3:7:4	>=3	>=3	>=3	>=3	>=3	>=3	>=3	1.67
7:3:6:4	>=3	>=3	>=3	>=3	>=3	>=3	>=3	2.29
7:3:5:5	>=3	>=3	>=3	>=3	>=3	>=3	>=3	>=3

Table 3. Partition coefficient (K) of kamlolenic acid in hexane-ethyl acetate-methanol-water (HEMWAT) systems with different solvent ratios. Fatty acids, beside the fatty acid mentioned above, 13-OH C18:2, mono-OH C18:0 and 18-OH C18:3 were monitored with the following ion transition and retention time: 295.2/195.1 (5.14 min), 299.2/299.2 (7.83 min), 293.2/263.2 (3.71 min).

HEMwat System Ratio (*v/v/v/v*)	C16 :0	C17:0	C18:0	C18:1	C18:2	C18:3	13-OH C18:2	Mono-OH C18:0	18-OH C18:3
7:3:7:3	>=3	>=3	>=3	>=3	>=3	>=3	<0.5	0.70	<0.5
7:3:7:4	>=3	>=3	>=3	>=3	>=3	>=3	1.29	1.46	<0.5
7:3:6:4	>=3	>=3	>=3	>=3	>=3	>=3	1.91	2.20	0.65
6:4:6:4	>=3	>=3	>=3	>=3	>=3	>=3	1.95	2.68	0.79
7:3:5:5	>=3	>=3	>=3	>=3	>=3	>=3	>=3	>=3	2.31

Table 4. Minimum inhibitory concentrations of various hydroxyl fatty acids against filamentous fungi. Results are presented as means ± standard deviation of triplicate independent experiments.

Fatty acids	Minimum inhibitory concentrations (g/L)[**]					
	Aspergillus niger	***Penicillium roqueforti***	***Candida albicans***	***Saccharomyces cerevisiae***	***Candida valida***	***Pichia membranae-faciens***
13-OH C18:2	$0.33\pm0.07^{B,c}$	0.33 ± 0.14^{Bd}	$\geq8^{Aa}$	$\geq8^{Aa}$	$\geq8^{Aa}$	$\geq8^{Aa}$
9-OH C18:2	0.23 ± 0.03^{Bc}	0.33 ± 0.14^{Bd}	≥8 A a	$\geq8^{Aa}$	$\geq8^{Aa}$	$\geq8^{Aa}$
13-OH C18:1	0.42 ± 0.14*^{Bc}	$0.42\pm0.14^{*Bcd}$	$\geq8^{Aa}$	$\geq8^{Aa}$	$\geq8^{Aa}$	$\geq8^{Aa}$
12-OH C18:1	0.38 ± 0.13^{Bc}	0.33 ± 0.14^{Bd}	$\geq8^{Aa}$	$\geq8^{Aa}$	$\geq8^{Aa}$	$\geq8^{Aa}$
10-OH C18:1	$0.50\pm0.00^{*Bc}$	$0.42\pm0.14^{*Bcd}$	$\geq8^{Aa}$	$\geq8^{Aa}$	$\geq8^{Aa}$	$\geq8^{Aa}$
18-OH C18:3	1.17 ± 0.29^{Bb}	$\geq8^{Aa}$	$\geq8^{Aa}$	$\geq8^{Aa}$	$\geq8^{Aa}$	$\geq8^{Aa}$
2-OH C18:3	1.50 ± 0.29^{Aab}	2.33 ± 1.53^{Abc}	3.00 ± 1.73^{Ab}	3.00 ± 1.73^{Ab}	2.00 ± 0.00^{Ab}	1.00 ± 0.00^{Ab}
2-OH C18:1	1.50 ± 0.87^{BCa}	2.67 ± 1.15^{Bb}	$\geq8^{Aa}$	$\geq8^{Aa}$	$\geq8^{Aa}$	0.83 ± 0.29^{Ca}

[*]data from Liang et al., 2017.

MIC data for different HUFA against the same indicator strain are significantly different (P<0.05) if they do not share the same lowercase superscript.

MIC data for the same HUFA against different indicator strains are significantly different (P<0.05) if they do not share the same uppercase superscript.

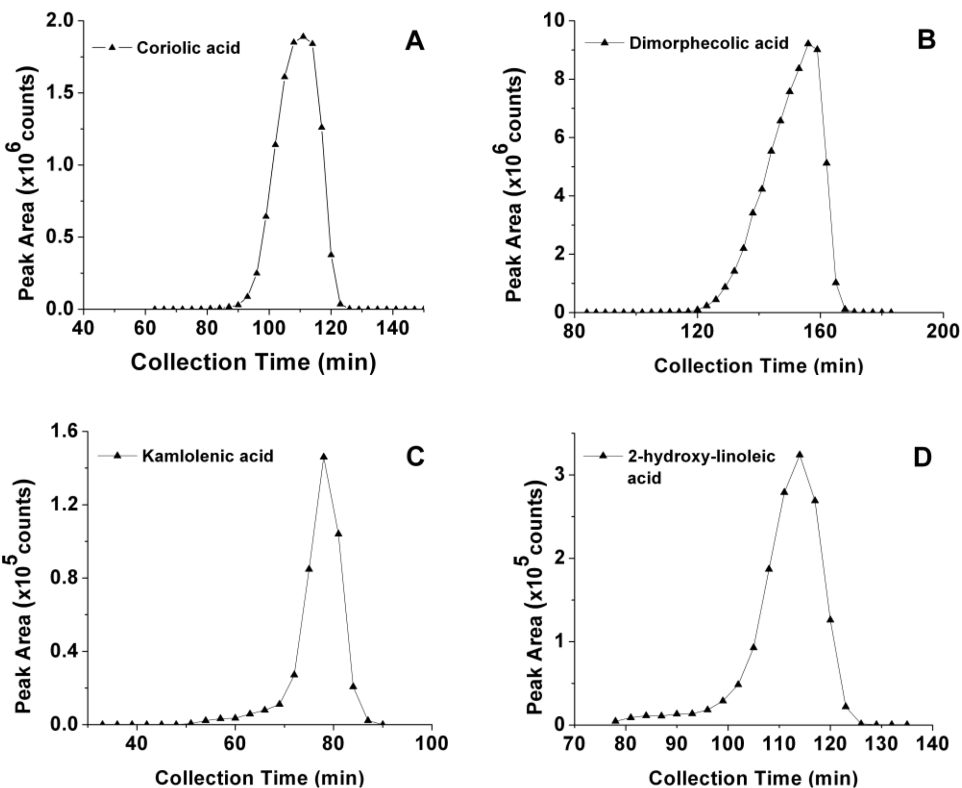
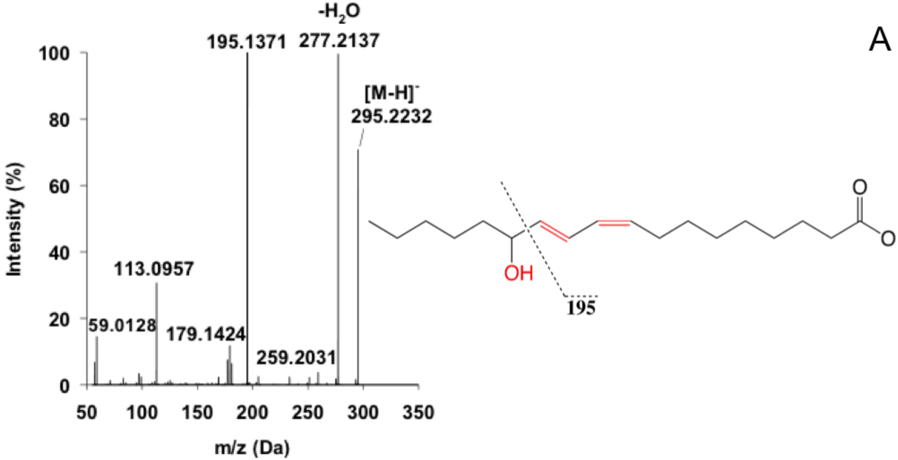
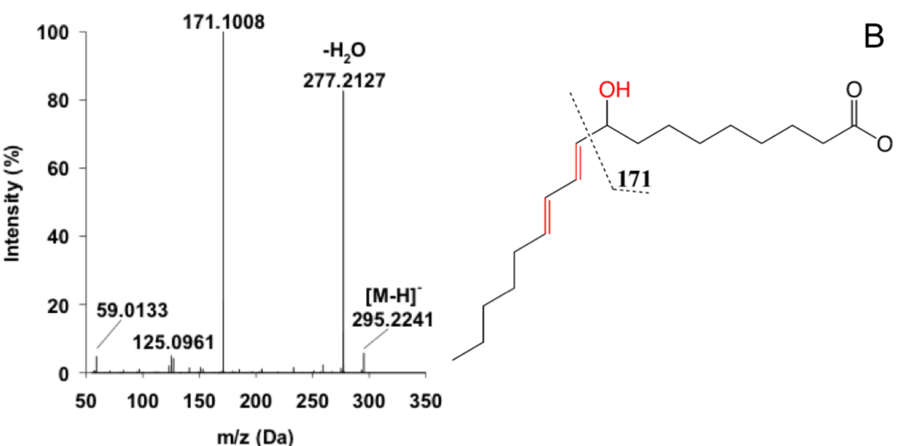


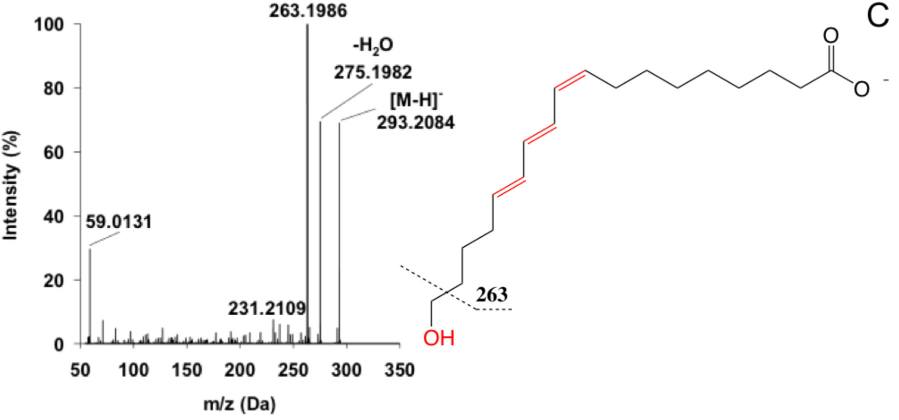
Figure 1. HSCCC chromatogram of saponified seed oil from various oil sources: (A) coriolic acid (13-hydroxy-9,11-octadecadienoic acid) from *Coriaria nepalensis* seed oil (data from 16); (B) dimorphecolic acid (9-hydroxy-10,12-octadecadienoic acid) from *Dimorphotheca sinuata* seed oil; (C) kamlolenic acid (18-hydroxy-9,11,13-octadecatrienoic acid) from *Mallotus philippensis* seed oil; (D) 2-hydroxy-linolenic acid (2-hydroxy-9,12,15-octadecatrienoic acid) from *Thymus vulgaris* seed oil.



-H2O
195.1371
277.2137
A
100
80
60
40
20
0
Intensity (%)
[M-H]-
295.2232
113.0957
59.0128
179.1424
259.2031
50 100 150 200 250 300 350
m/z (Da)
OH
195



171.1008
-H2O
277.2127
B
100
80
60
40
20
0
Intensity (%)
OH
171
59.0133
125.0961
[M-H]-
295.2241
50 100 150 200 250 300 350
m/z (Da)



263.1986
-H2O
275.1982
[M-H]-
293.2084
C
100
80
60
40
20
0
Intensity (%)
59.0131
231.2109
263
OH
50 100 150 200 250 300 350
m/z (Da)

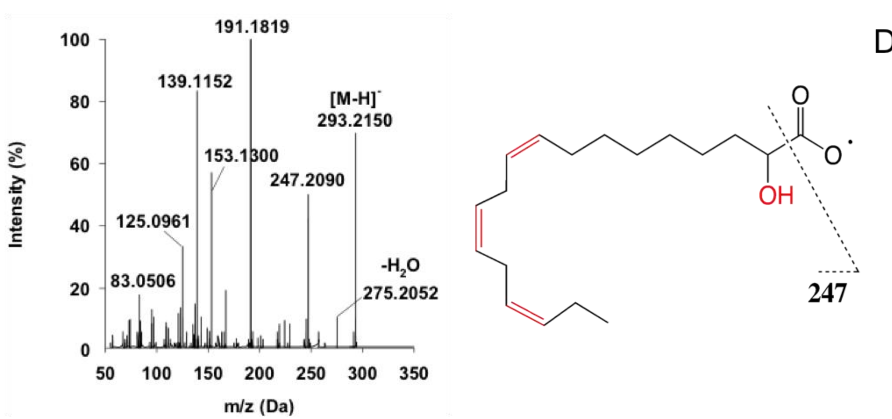
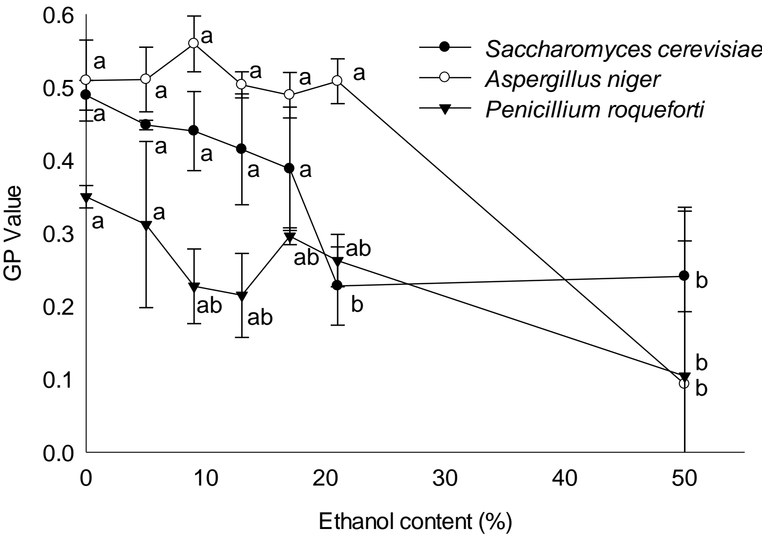


Figure 2. APPI-MS/MS spectra of [M-H]- ion and molecular structure of ionized purified hydroxyl fatty acids (A) coriolic acid; (B) dimorphecolic acid; (C) kamlolenic acid; and (D) 2-hydroxy-linolenic acid



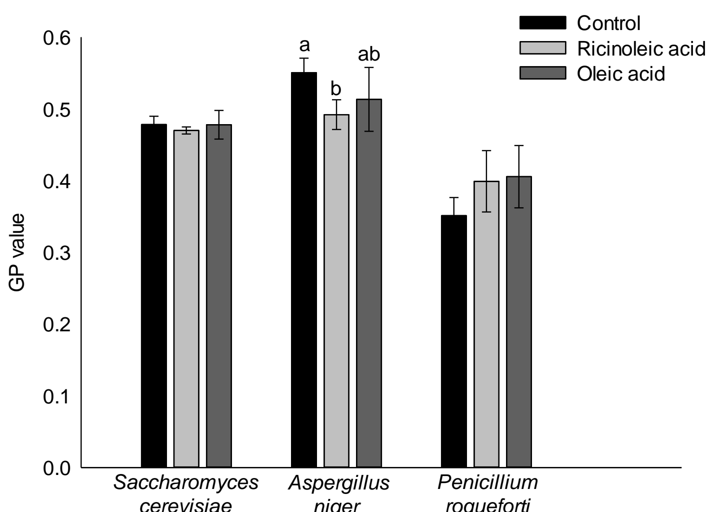


Figure 3. Upper figure) Fungal membrane fluidity change of in different concentration of ethanol treatments and fatty acid treatments, monitored by GP value using LAURDAN assay; Lower figure) The measurement of fungal cell membrane fluidity of *Saccharomyces cerevisiae*, *Aspergillus niger* and *Penicillium roqueforti* under fatty acid (ricinoleic and oleic acid) treatment. Generalized polarization GP= $(I_{440}-I_{490}) / (I_{440}+I_{490})$, where I_{440} and I_{490} stood for fluorescent intensity under wavelengths of 440 nm and 490 nm, respectively.

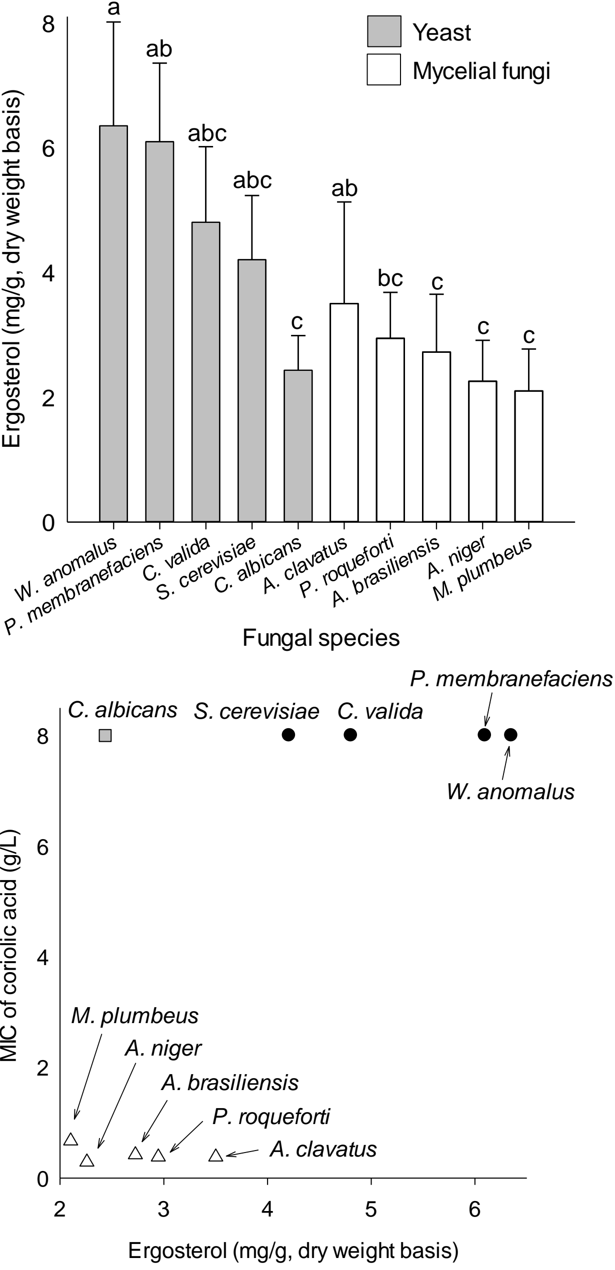


Figure 4. Upper figure) GC-MS quantitation of ergosterol extracted from food-related fungi (dry weight basis) and lower figure) relationship between fungal ergosterol content and fungal MIC of coriolic acid. The abbreviation of fungi species represented the following strains: *Candida valida* (*C.v*), *Pichia membranefaciens (P.m), Saccharomyces cerevisiae (S.c), Candida albicans (C.a), Wickerhamomyces anomalus (W.a), Penicillium roqueforti (P.r), Aspergillus brasiliensis (A.b),*

Mucor plumbeus (M.p), Aspergillus niger (A.n), and Aspergillus clavatus (*A.c*). The outliner (grey: *C.a)* was determined using Cook's distance >0.4 in SPSS software.