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Inhibition of polymicrobial biofilm formation by saw palmetto oil, lauric acid and myristic acid

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Summary

Biofilms are communities of bacteria, fungi or yeasts that form on diverse biotic or abiotic surfaces, and play important roles in pathogenesis and drug resistance. A generic saw palmetto oil inhibited biofilm formation by *Staphylococcus aureus*, *Escherichia coli* O157:H7 and fungal *Candida albicans* without affecting their planktonic cell growth. Two main components of the oil, lauric acid and myristic acid, are responsible for this antibiofilm activity. Their antibiofilm activities were observed in dual-species biofilms as well as three-species biofilms of *S. aureus*, *E. coli* O157:H7 and *C. albicans*. Transcriptomic analysis showed that lauric acid and myristic acid repressed the expressions of haemolysin genes (*hla* and *hld*) in *S. aureus*, several biofilm-related genes (*csgAB*, *fimH* and *flhD*) in *E. coli* and hypha cell wall gene *HWP1* in *C. albicans*, which supported biofilm inhibition. Also, saw palmetto oil, lauric acid and myristic acid reduced virulence of three microbes in a nematode infection model and exhibited minimal cytotoxicity. Furthermore, combinatorial treatment of fatty acids and antibiotics showed synergistic antibacterial efficacy against *S. aureus* and *E. coli* O157:H7. These results demonstrate that saw palmetto oil and its main fatty acids might be useful for controlling bacterial infections as well as multispecies biofilms.

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Introduction

Most antimicrobial agents aim to inhibit microbial growth, ideally without harming the host or the environment. However, the overuse of these agents has caused worldwide emergence of drug-resistant pathogens. Bacterial and fungal biofilms play important roles in antimicrobial resistance and in a variety of device-related infections (Donlan, 2002; Hall-Stoodley *et al*., 2004; Handorf *et al*., 2019). Furthermore, various pathogenic microorganisms often form multispecies biofilms, which further increase tolerance to antimicrobial agents (Carlson and Johnson, 1985; Harriott and Noverr, 2011). Hence, alternative non-toxic approaches are needed to control single and polymicrobial biofilm formation. Unlike current antimicrobial strategies, it is important to identify biofilm inhibitors that do not inhibit planktonic cell growth (Wright, 2015), which is one of an antivirulence strategy that aims to reduce pathogenesis while its consequences not affecting bacterial growth, reducing the chance of the emergence of drug resistance (Cegelski *et al*., 2008).

Fatty acids are widespread in all forms of life, and more than 70 natural fatty acids have been identified (Kenar *et al*., 2017). The antimicrobial activities of various fatty acids, mostly above hundred $\mu g\ ml^{-1}$ doses, have been well reported against diverse microorganisms (Desbois and Smith, 2010; Yoon *et al*., 2018). However, several studies recently reported that fatty acids exhibit antibiofilm and antivirulence activities against bacteria and fungi at concentrations much lower than their MICs (Kumar *et al*., 2020).

Extract of the saw palmetto (*Seronoa repens*) berry is widely used as an alternative treatment of prostatic hyperplasia (Bent *et al*., 2006). The main components of saw palmetto products are fatty acids (more than 90%) and phytosterols, and the prominent fatty acids are caprylic, capric, lauric, myristic, palmitic, oleic, linolenic/linoleic and stearic acid (Penugonda and Lindshield, 2013; Booker *et al*., 2014). Various fatty acids, such as capric, lauric, palmitic, oleic, linoleic/linolenic and eicosadienoic acid, have been shown to possess antibiofilm activity against microbial biofilms of *S. aureus* and fungal *C. albicans* (Stenz *et al*., 2008; Davies and Marques, 2009; Murzyn *et al*., 2010; Thibane *et al*., 2012; Lee *et al*., 2017; Kim *et al*., 2018, 2019), and diverse fatty acid derivatives are important cell-to-cell signals in

various plant-associated bacteria (Ryan *et al*., 2015; Zhou *et al*., 2017). Hence, we hypothesized that fatty acid-rich saw palmetto products and their main components would have similar antibiofilm activities against other microbes and mixed microbes.

Most microorganisms exist as multispecies consortia and often form polymicrobial biofilms that have been shown to provide several advantages for the residing species such as increased tolerance against antimicrobial agents and protozoan grazing, as well as increased virulence in infections (Roder *et al*., 2016). For example, fungal *C. albicans* often forms biofilms with other bacteria like *S. aureus, P. aeruginosa* and *Enterococcus* species on host and environmental surfaces, which formed more rapidly and have a greater antimicrobial tolerance (Harriott and Noverr, 2009). While the significance of polymicrobial biofilms in human pathology has been increasingly acknowledged, biofilm inhibition of dual- and three-species has rarely been reported as of yet.

Hence, in the present study, the antibiofilm activity of saw palmetto oil was initially investigated against eleven microbes. GC-MS analysis revealed the presence of the main fatty acid components in the oil and the antibiofilm activity of the fatty acids were further tested against single strain of *S. aureus, E. coli* O157:H7 and *C. albicans*, as well as dual-species and three-species. To understand how fatty acids inhibit biofilm formation, confocal laser scanning microscopy (CLSM), scanning electron microscopy (SEM) and transcriptomic analysis were used to investigate morphological changes, biofilm formation, the hyphal growth of *C. albicans* and gene expression. Furthermore, a *Caenorhabditis elegans* model was used to investigate the antimicrobial effects of fatty acids and their cytotoxic nature. In addition, synergistic effects of fatty acids with antimicrobial agents were further studied.

Results

Saw palmetto oil and main fatty acid components inhibited biofilm formation of various microorganisms

The antibiofilm activity of saw palmetto oil was initially investigated against eleven microorganisms. Saw palmetto oil at concentrations of 50 $\mu g\ ml^{-1}$ significantly inhibited biofilm formation by four *S. aureus* strains including two methicillin-resistant strains, a *Staphylococcus epidermidis* strain, two pathogenic *E. coli* strains and two *C. albicans* strains, while it could not change biofilm formation by *A. baumannii* and *P. aeruginosa* (Fig. 1A). Most notably, saw palmetto oil inhibited biofilm formation by two *C. albicans* strains more than 85% even at 20 $\mu g\ ml^{-1}$.

GC-MS identified 25 fatty acids in saw palmetto oil (Table S3). Main components of saw palmetto oil were lauric acid (34.3%), myristic acid (14.3%), palmitic acid (9.6%) and oleic acid (29.1%) (Fig. 1B). The compositions of saw palmetto oil identified by GC-MS analysis concur with previous studies (Penugonda and Lindshield, 2013; Booker *et al*., 2014).

The antibiofilm potencies of four main fatty acids, lauric acid (C12:0), myristic acid (C14:0), palmitic acid (C16:0) and oleic acid (C18:1), were further investigated against three genera such as *S. aureus*, *E. coli* and *C. albicans*. It appears that lauric acid and myristic acid are most active against three microorganisms. For example, lauric acid and myristic acid at 20 $\mu g\ ml^{-1}$ inhibited biofilm formation by five *Staphylococcus*, *E. coli* O157:H7 and *C. albicans* strains more than 50% (Fig. 1C–F). For the *S. aureus* strain, oleic acid above 20 $\mu g\ ml^{-1}$ also appreciably decreased its biofilm formation, while palmitic acid did not (Fig. 1C). However, palmitic acid and oleic acid did not change the biofilm formation by *E. coli* O157:H7 and *C. albicans* (Fig. 1D and E). Similar to the result observed with saw palmetto oil, lauric acid and myristic acid at 5 $\mu g\ ml^{-1}$ mostly inhibited *C. albicans* biofilms (Fig. 1E), which is matched with previous reports (Prasath *et al*., 2019; Lee *et al*., 2020). The antibiofilm activity of oleic acid against *S. aureus* strains was previously reported (Stenz *et al*., 2008; Davies and Marques, 2009; Lee *et al*., 2017). However, it is the first observation that lauric acid and myristic acid also have the inhibitory effect on *S. aureus* biofilm formation. Also, we report for the first time that lauric acid and myristic acid exhibit antibiofilm activity against *E. coli* O157:H7.

Hence, it can be concluded that the antibiofilm activities of saw palmetto oil against *S. aureus*, *E. coli* O157:H7 and *C. albicans* were largely due to the presence of lauric acid and myristic acid, which were focused later on in the study. However, the MICs of saw palmetto oil and two active fatty acids against all eleven microorganisms were found to be $> 500\ \mu g\ ml^{-1}$ (Table S1) and three fatty acids up to 200 $\mu g\ ml^{-1}$ did not much affect the planktonic cell growth (Fig. S1). These results indicate that saw palmetto oil, lauric acid and myristic acid inhibited microbial biofilm formation without affecting planktonic cell growth.

Biofilm inhibition on the bottom of 96-well plate was analysed by CSLM. While three microorganisms formed dense biofilms (thickness $> 40\ \mu m$ and almost 100% surface coverage) in non-treated controls of three microbes, saw palmetto oil at 100 $\mu g\ ml^{-1}$ dramatically reduced biofilm densities and thicknesses (Fig. 1G). Also, both lauric acid and myristic acid at 20 $\mu g\ ml^{-1}$ significantly inhibited biofilm formation by *S. aureus*, *E. coli* O157:H7 and *C. albicans*. However, saw palmetto oil and two fatty acids, myristoleic and lauric acids, at concentrations up to 100 $\mu g\ ml^{-1}$ could not eradicate

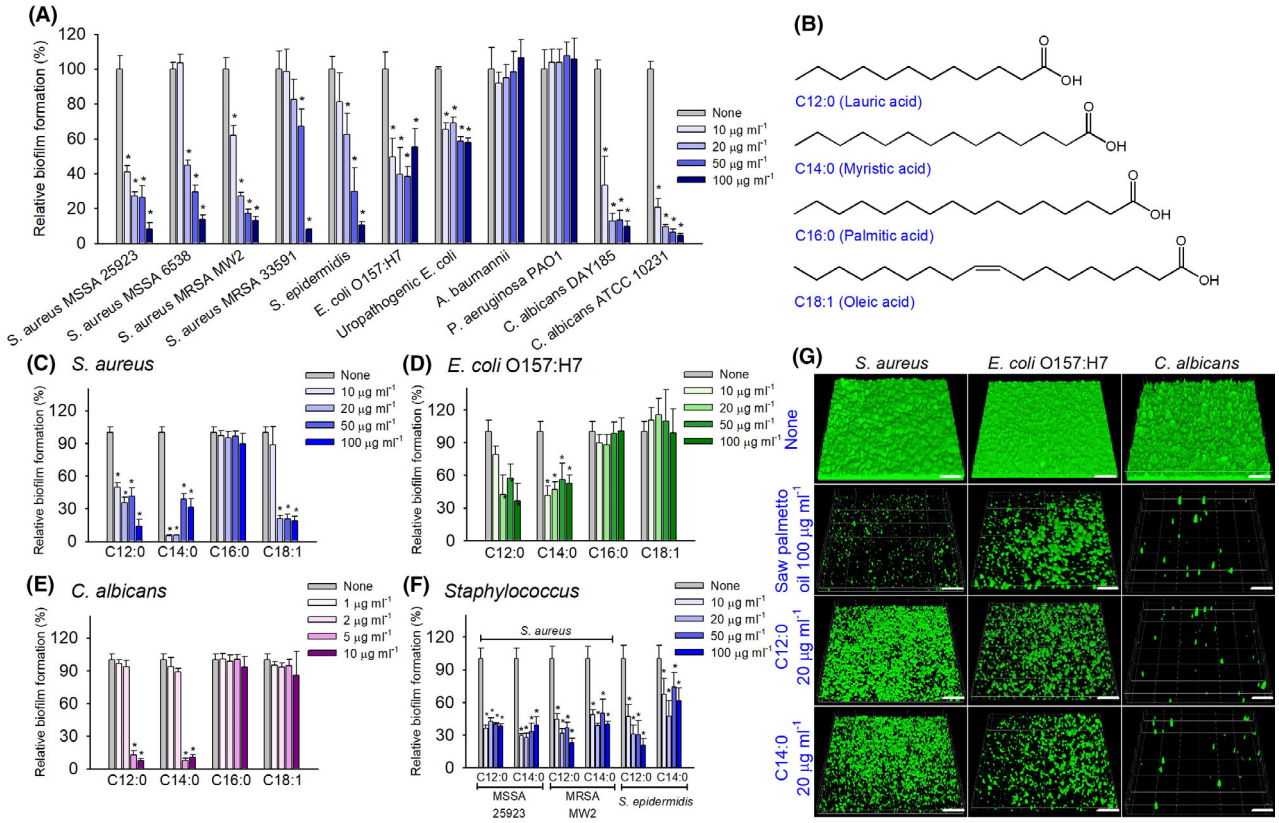

Fig. 1. Antibiofilm activities of saw palmetto oil and its main fatty acids. Biofilm formations by individual microbes were quantified in the presence of saw palmetto oil after incubation for 24 h in 96-well plates (A). Chemical structures of main fatty acids in the saw palmetto oil (B). Biofilm inhibition by fatty acids of *S. aureus* MSSA 6538 (C), *E. coli* O157:H7 (D), *C. albicans* DAY185 (E) and other *Staphylococcus* strains (F) were quantified in the presence of lauric acid (C12:0), myristic acid (C14:0), palmitic acid (C16:0) and oleic acid (C18:1) after incubation for 24 h in 96-well plates at 37 °C. n = 3 biologically independent samples. Error bars indicate standard deviations. $*P < 0.05$ versus non-treated controls. Biofilm formations on polystyrene plates were observed in the presence of saw palmetto oil at 100 μg ml^{-1}, C12:0 or C14:0 at 20 μg ml^{-1}, by confocal laser microscopy (G). Scale bars represent 100 μm. At least two independent cultures were performed for microscopic experiments, and at least 10 random positions were assayed.

the pre-existing biofilms of *S. aureus*, *C. albicans* and *E. coli* O157:H7 (Fig. S2).

Antibiofilm activities of saw palmetto oil and main fatty acids against polymicrobial biofilms of S. aureus*,* E. coli *O157:H7 and* C. albicans

The efficacies of saw palmetto oil and fatty acids on dual and three-species biofilms of *S. aureus*, *E. coli* O157:H7 and *C. albicans* were further studied. We have found proper media and inoculum to form biofilms of three sets of dual species and one set of three species, such as *S. aureus* and *E. coli* O157:H7 in LB medium, and *S. aureus* and *C. albicans, E. coli* O157:H7 and *C. albicans* in the 1:1 mixture of PDB and LB, and three species together in the 1:2 mixture of PDB and LB. In the non-treated controls of two- or three-species, decent biofilm formation (1 ~ 2 at OD_{570}) was observed in all four cases (Fig. 2A–D). As expected, both lauric acid and myristic acid dose-dependently inhibited biofilm formation of dual- and three-species biofilms (Fig. 2A–D). Most notably, lauric acid and myristic acid at 10 μg ml^{-1} inhibited three-species biofilm formation by 90% after culture for 24 h. To the best of our knowledge, it is the first report of three-species biofilm inhibition of *S. aureus*, *E. coli* O157:H7 and *C. albicans*. Importantly, saw palmetto oil dose-dependently inhibited biofilm formation of these four cases. Similar to single biofilms, it appears that saw palmetto oil and two fatty acids most significantly inhibited multispecies biofilms including *C. albicans* (Fig. 2B–D).

CLSM analysis confirmed that saw palmetto oil or two fatty acids at 20 μg ml^{-1} significantly decreased three-species biofilm formation on the bottom of 96-well plates (Fig. 2E). SEM observation also revealed the inhibitory effect of saw palmetto oil, lauric acid or myristic acid on biofilm formation of three-species (Fig. 2F). We observed polymicrobial associations between *S. aureus*, *E. coli*

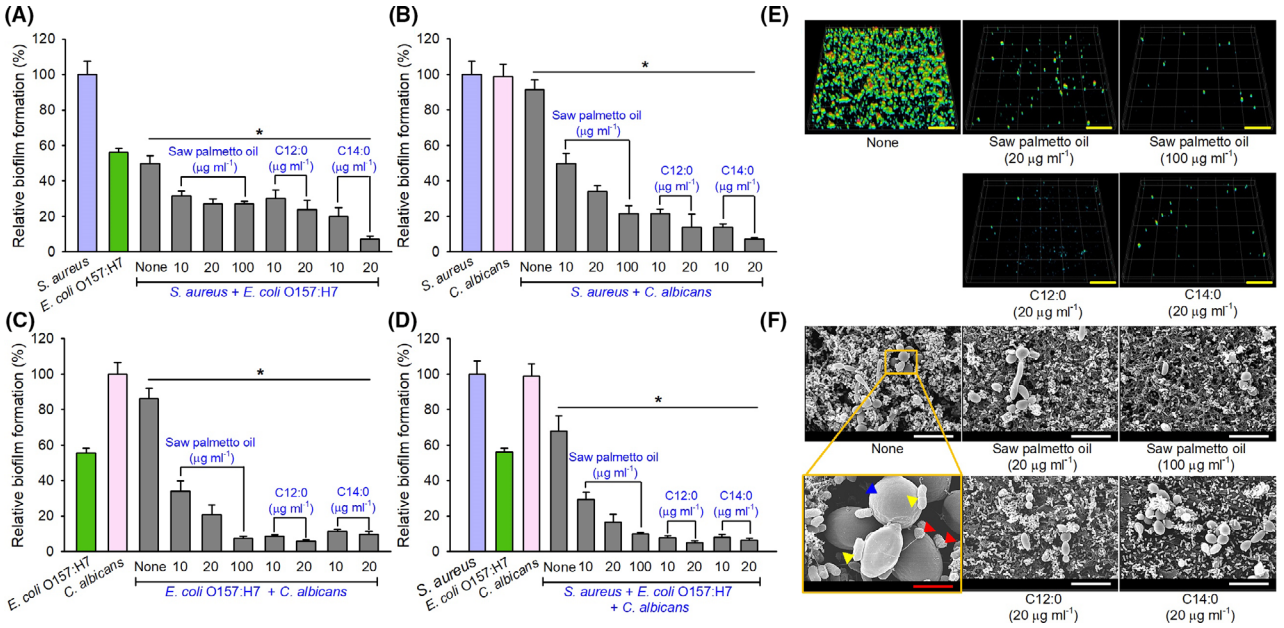

Fig. 2. Biofilm inhibition by saw palmetto oil, lauric acid and myristic acid in the polymicrobial models. Inhibitory effects of saw palmetto oil, lauric acid (C12:0) and myristic acid (C14:0) against dual biofilms of *S. aureus* MSSA 6538 and *E. coli* O157:H7 in LB medium (A), dual biofilms of *S. aureus* 6538 and *C. albicans* DAY185 in a 1:1 mixture of PDB and LB media (B), dual biofilms of *E. coli* O157:H7 and *C. albicans* in a 1:1 mixture of PDB and LB media (C) and three-species biofilms of *S. aureus*, *E. coli* O157:H7 and *C. albicans* biofilms in a 1:2 mixture of PDB and LB media (D) were determined after culture for 24 h at 37 °C. $n = 3$ biologically independent samples. $^*P < 0.05$ versus non-treated controls. CLSM (E) and SEM (F) observation of three-species biofilms of *S. aureus*, *E. coli* O157:H7 and *C. albicans*. In the inset, the large cells are *C. albicans* (indicated by blue triangle), the small round cells are *S. aureus* (indicated by red triangle), and rod type *E. coli* O157:H7 cells (indicated by yellow triangle). The yellow scale bar represents 100 μm, the white scale bar represents 15 μm, and the red scale bar in inset represents 3 μm. At least two independent cultures were performed for microscopic experiments, and at least 10 random positions were assayed.

O157:H7 and *C. albicans* in non-treated control samples. It appears that *C. albicans* formed large hyphae and a few yeast cells that were much larger than round *S. aureus* cells and rod type *E. coli* O157:H7 cells. Notably, saw palmetto oil, lauric acid or myristic acid obviously reduced hyphal formation of *C. albicans* and *S. aureus* and *E. coli* O157:H7 cells were widely distributed.

Differential gene expressions induced by saw palmetto oil, lauric acid and myristic acid

To investigate the molecular basis responsible for biofilm inhibition by saw palmetto oil, lauric acid and myristic acid, qRT-PCR was performed against three individual microorganisms with various biofilm-related genes.

In *S. aureus* cells, saw palmetto oil and lauric acid, but not myristic acid, dramatically reduced the expression of alpha-haemolysin *hla* gene by 20- and 32-fold, respectively, and downregulated delta-haemolysin (*hld*, also known as *RNAIII*), while other genes, such as *agrA*, *aur*, *icaA* and *sigB*, were not affected (Fig. 3A). It has been reported that *hld* (*RNAIII*) stimulates *hla* translation (Morfeldt *et al*., 1995). Hence, *hla* repression is partially due to the down-regulation of *hld* by saw palmetto oil and lauric acid. Interestingly, saw palmetto oil and lauric acid upregulated *sarA*, a positive regulator of *S. aureus* biofilm development (Beenken *et al*., 2003; Valle *et al*., 2003). Previously, two omega fatty acids repressed the expression of *hla* and *hld* (Kim *et al*., 2018). Hence, it appears that antibiofilm activities of lauric acid and other omega fatty acids are partially due to down-regulating the gene expression of the *hla* and *hld*.

In *E. coli* O157:H7 cells, the effects of lauric acid and myristic acid on the expression of biofilm-related genes were more significant than that of saw palmetto oil. Notably, lauric acid and myristic acid repressed the expression of two fimbriae genes (*csgAB*), three motility genes (*fimH*, *flhD* and *motB*) and two quorum-sensing genes (*luxRS*) (Fig. 3B).

In *C. albicans* cells, it was notable that both saw palmetto oil and lauric acid repressed the expression of *HWP1* gene (Fig. 3C) that is essential for hyphal development and their expressions have been shown to be correlated with cell elongation and biofilm formation (Nobile *et al*., 2006). Also, notably, the expression of *CHT4* encoding chitinase was induced by saw palmetto oil and the two fatty acids. It was reported that *CHT4* transcription was decreased upon yeast-to-hyphal switch (Prasath *et al*., 2019), which suggested that saw palmetto oil and two fatty acids could inhibit hyphal

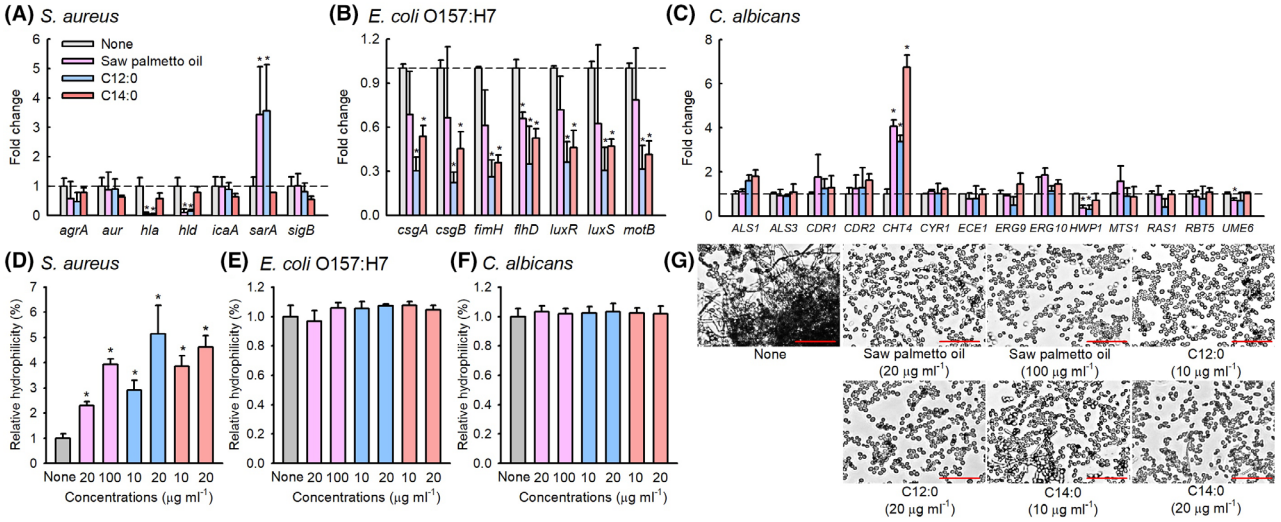

Fig. 3. Effects of fatty acids on transcriptional profiles, cell hydrophobicity and hyphal formation. *S. aureus* MSSA 6538 (A), *E. coli* O157:H7 (B) or *C. albicans* DAY185 (C) was incubated with or without saw palmetto oil at 100 μg ml^{-1}, lauric acid (C12:0) at 20 μg ml^{-1} or myristic acid (C14:0) at 20 μg ml^{-1} for 6, 4 or 6 h at 37 °C with shaking at 250 rpm respectively. Transcriptional profiles were acquired by qRT-PCR. Fold changes represent change in the transcriptions of treated *vs.* non-treated controls. The experiment was conducted in duplicate (four qRT-PCRs were tested per gene). *$P < 0.05$ *vs.* non-treated controls (None). The cell surface hydrophobicity was determined after incubation for 24 h at 37 °C with or without saw palmetto oil, lauric acid (C12:0) and myristic acid (C14:0) in *S. aureus* 6538 (D), *E. coli* O157:H7 (E) or *C. albicans* DAY185 (F). Inhibition of hyphal filamentation by fatty acids in *C. albicans* DAY185 after incubation for 24 h at 37 °C in PDB medium (G). The scale bar represents 50 μm. At least two independent cultures were performed for microscopic experiments, and at least 10 random positions were analysed.

formation. Also, it was recently reported that medium-chain fatty acids repressed the expression of *HWP1* gene via mimicking of the quorum-sensing molecule farnesol (Lee *et al.*, 2020). Current results partially support the hypothesis of the reduction of biofilm formation (Fig. 1E) and hyphae formation (Fig. 2F).

Changes of hydrophobicity in S. aureus *and hyphal inhibition in* C. albicans *by fatty acids*

Cell surface hydrophobicity and biofilm formation are closely linked in several bacteria as hydrophobic cells adhere more to hydrophobic surfaces (Krasowska and Sigler, 2014). Hence, cell surface hydrophobicity was measured in the presence of fatty acids in the three strains (Fig. 3D–F). Interestingly, saw palmetto oil, lauric acid and myristic acid increased hydrophilicity, which means cells are less hydrophobic in *S. aureus*, while they did not change the hydrophilicity in either *E. coli* O157:H7 or *C. albicans* cells (Fig. 3E and F). These results partially explain the inhibition of *S. aureus* biofilm formation by fatty acids in hydrophobic polystyrene surfaces.

Since the yeast-to-hypha transition is known to be a prerequisite of biofilm development by *C. albicans* (Chandra *et al.*, 2001), the effect of fatty acids on hyphal formation was observed. Saw palmetto oil, lauric acid and myristic acid substantially suppressed the hyphal transition, while untreated control cells were predominantly hyphal cells (Fig. 3G). Recently, several medium-chain fatty acids were reported to inhibit hyphal growth in *C. albicans* (Lee *et al.*, 2020). These results and transcriptomic results support that lauric acid and myristic acid potently inhibit hypha formation and reduce biofilm formation by *C. albicans* and even multispecies biofilm formation including that of *C. albicans*.

Antivirulence activities of saw palmetto oil, lauric acid and myristic acid in the nematode model with minimal cytotoxicity

Biofilm formation and cell adhesion are key virulence factors in animal hosts. *Caenorhabditis elegans* model is viewed as an alternative to the use of rodent models in various studies in biology, especially in the field of bacterial infection. Infections of pathogenic microorganisms, such as *S. aureus* (Sifri *et al.*, 2003), *E. coli* O157:H7 (Chou *et al.*, 2013) or *C. albicans* (Pukkila-Worley *et al.*, 2009) could kill the nematodes.

As expected, *C. elegans* was well survived with *E. coli* OP50, which is a common food source for the nematode, while the lifespan of *C. elegans* was much decreased in the presence of each pathogen. Saw palmetto oil, lauric acid and myristic acid were found to markedly prolong *C. elegans* survival in the presence of *S. aureus* (Fig. 4A), *E. coli* O157:H7 (Fig. 4B) or *C.*

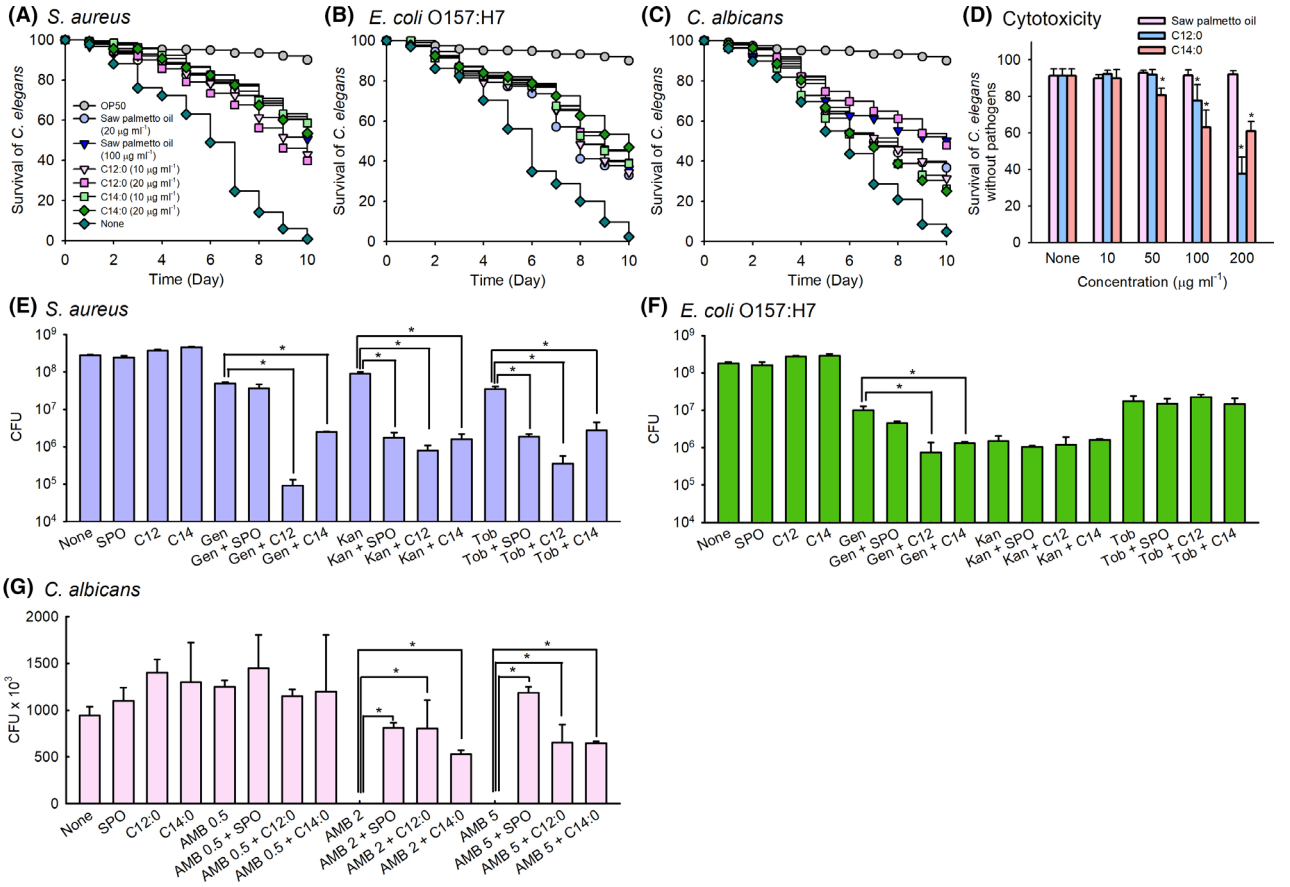

Fig. 4. Anti-virulence activities of fatty acids and co-administration of fatty acids and antimicrobial agents. Nematode survivals after exposure to *S. aureus* MSSA 6538 (A), *E. coli* O157:H7 (B) or *C. albicans* DAY185 (C) cells in the presence of saw palmetto oil, lauric acid (C12:0) or myristic acid (C14:0). Chemical toxicities were determined by treating non-infected *C. elegans* for 4 days (D). OP50 indicates *E. coli* OP50-fed controls, and none indicates non-treated controls. $*P < 0.05$ *vs.* non-treated controls (None). OP50 represents *E. coli* OP50. Cell survivals were measured in the presence of antibiotic gentamicin (10 μg ml^{-1}), kanamycin (50 μg ml^{-1}) or tobramycin (10 μg ml^{-1}) against *S. aureus* (E) or *E. coli* O157:H7 (F) and antifungal amphotericin B (0.5, 2 and 5 μg ml^{-1}) against *C. albicans* (G). $*P < 0.05$ *vs.* non-treated controls. None: non-treated control. SPO: saw palmetto oil (100 μg ml^{-1}), fatty acids C12:0 and C14:0 (20 μg ml^{-1}), Gen: gentamicin, Kan: kanamycin, Tob: tobramycin, AMB: amphotericin B. $n = 2$ biologically independent samples.

albicans (Fig. 4C). For example, nematode survival was almost 100 per cent without pathogen for 10 days, whereas in the presence of saw palmetto oil at 100 μg ml^{-1}, or myristic acid at 20 μg ml^{-1}, more than 55% of the nematodes survived. This result is in line with the observed downregulation of biofilm formation (Fig. 1) and repression of haemolysin genes (Fig. 3A). Similar antivirulence characteristics of saw palmetto oil, lauric acid and myristic acid are observed in the infection of *E. coli* O157:H7 (Fig. 4B) or *C. albicans* (Fig. 4C).

In addition, to examine the cytotoxicity of saw palmetto oil, lauric acid and myristic acid, *C. elegans* survival was investigated with feeding only *E. coli* OP50, but without any pathogens. No toxic effects were observed when non-infected nematodes were exposed to saw palmetto oil at concentrations up to 200 μg ml^{-1}, while lauric acid and myristic acid at 100 and 200 μg ml^{-1} were found to be a mild titre for nematodes (Fig. 4D). These cytotoxicity results of fatty acids partially matched with a previous report (Lima *et al*., 2002). However, it is notable that lauric acid and myristic acid exhibit antibiofilm activity at concentrations of 10 or 20 μg ml^{-1}, much less than the level of their cytotoxicity.

Combinatory efficacies of fatty acids and antimicrobial agents

Since combinatorial approaches have been proposed to enhance antimicrobial efficacy (Wright, 2016), combinatory efficacies of fatty acids and antimicrobial agents on microbial growth were investigated against individual microorganisms. First, antibiotic gentamicin alone at 10 μg ml^{-1} was tested against *S. aureus* or *E. coli* O157:H7 and antifungal amphotericin B alone at 0.5, 2

or 5 μg ml^{-1} was tested against *C. albicans*. As expected, aminoglycoside gentamicin partially killed more than 90% of the two bacteria within 1 h (Fig. 4E and F), which matched with previous reports (Weinstein *et al*., 1963; Yoshizawa *et al*., 1998). Also, amphotericin B above 2 μg ml^{-1} nearly abolished *C. albicans* growth (Fig. 4G), which also matched with a previous report (Brajtburg *et al*., 1990). Second, the combinations of fatty acids and antimicrobial agents were tested against three individual microorganisms. It is notable that the addition of lauric acid (C12:0) or myristic acid (C14:0) at 20 μg ml^{-1} caused much less survival of *S. aureus* (Fig. 4E) and *E. coli* O157:H7 (Fig. 4F), while lauric acid or myristic acid alone at 20 μg ml^{-1} did not affect their cell growth at all (MICs $>$ 500 μg ml^{-1}). It appears that the antibacterial efficacy of gentamicin was significantly enhanced in the presence of lauric acid or myristic acid, while saw palmetto oil at 100 μg ml^{-1} showed a marginal effect on the antibiotic efficacy. Further combinatorial assays with kanamycin and tobramycin led to different results as the efficacy of kanamycin and tobramycin in the presence of fatty acids was enhanced only in *S. aureus* (Fig. 4E), but not in *E. coli* O157:H7 (Fig. 4F).

In the case of *C. albicans*, unexpected results were obtained as the addition of saw palmetto oil, lauric acid or myristic acid significantly increased the survival of *C. albicans* (Fig. 4G). It was confirmed with two different concentrations of amphotericin B at 2 or 5 μg ml^{-1}. Hence, it appears that fatty acids negatively affect the antifungal efficacy, which should be further investigated.

Discussion

This study presents for the first time that lauric acid and myristic acid from saw palmetto oil suppress three-species biofilm formation by *S. aureus*, *E. coli* O157:H7 and *C. albicans* with minimal cytotoxic effect. The mode of action of the active fatty acids was partially revealed, and the synergistic antimicrobial efficacies of fatty acids were observed.

While broad-spectrum antimicrobial activities of fatty acids at high doses were well documented due to its amphipathic nature (Desbois and Smith, 2010; Yoon *et al*., 2018), recent studies reported that fatty acids at concentrations much lower than their MICs exhibit antibiofilm and antivirulence activities against bacteria and fungi (Kumar *et al*., 2020). Current study demonstrates that lauric acid and myristic acid abundant in saw palmetto oil could inhibit biofilm formation of single-, dual- and three-species biofilm models of *S. aureus*, *E. coli* O157:H7 and *C. albicans* without affecting their planktonic cell growth. Lauric acid is mainly found in coconut oil, laurel oil and palm kernel oil, and myristic acid is found in nutmeg butter, coconut oil, laurel oil, palm kernel oil, saw palmetto oil and even breast milk (Beare-Rogers *et al*., 2001). The wide-ranging distributions of lauric acid and myristic acid indicate the importance of protective roles as not only antimicrobial components but also antibiofilm inhibitors against single or multispecies consortia. The action mechanisms of lauric acid and myristic acid are varied in three tested microbes since the three species have different systems of controlling their biofilm formation.

In *S. aureus*, several fatty acids such as palmitic acid (C16:0), oleic acid (C18:1) (Stenz *et al*., 2008), linoleic acid (C18:2), eicosadienoic acids (C20:1) (Lee *et al*., 2017) and omega-3 fatty acids, such as *cis*-4,7,10,13,16,19-docosahexaenoic acid (DHA) and *cis*-5,8,11,14,17-eicosapentaenoic acid (EPA) (Kim *et al*., 2018; Kumar *et al*., 2020) at sub-MIC levels, inhibited biofilm formation and haemolytic activity in *S. aureus*. Alpha-haemolysin (Hla) is a major cytotoxic agent and is positively associated with biofilm formation in *S. aureus* (Caiazza and O'Toole, 2003), which is supported by the current results of the repression of *hla* and *hld* by fatty acids (Fig. 3A). Also, it has been suggested that both saturated and unsaturated fatty acids at high doses can be incorporated through a kinase-dependent pathway, ultimately influencing the membrane fluidity in *S. aureus* (Liaw *et al*., 2004). Therefore, it appears that the decrease of haemolysin production and membrane fluidity is possible mechanism of lauric acid and myristic acid to control *S. aureus* biofilm formation.

In *E. coli* O157:H7, both lauric acid and myristic acid influenced the expression of various biofilm-related genes, such as fimbriae genes (*csgAB*), motility genes (*fimH*, *flhD* and *motB*) and quorum-sensing genes (*luxRS*) (Fig. 3B). Fimbriae are important for *E. coli* O157:H7 biofilm formation (Ryu and Beuchat, 2005; Rendón *et al*., 2007), motility plays a role in *E. coli* biofilm formation (Pratt and Kolter, 1998; Ren *et al*., 2001), and autoinducer-2 (AI-2) quorum sensing also positively influences the development of biofilm of commensal *E. coli* (González Barrios *et al*., 2006; Hardie and Heurlier, 2008) and of the *E. coli* O157:H7 strain (Bansal *et al*., 2008; Vikram *et al*., 2010). Therefore, the antibiofilm activities of lauric acid and myristic acid are due to the downregulation of the gene expression of fimbriae, motility and AI-2 genes. Previously, palmitic acid showed antibiofilm activity against *E. coli* with an IC_{50} of 7.1 μM (Sandai *et al*., 2016; Kumar *et al*., 2020). Also, *cis*-2-decenoic acid induced biofilm dispersal of *E. coli, S. aureus, C. albicans* and other microbes (Davies and Marques, 2009) and *cis*-2-decenoic acid combined with antibiotics and disinfectants was found to effectively remove more than 80% of pre-formed biofilms of *E. coli* (Rahmani-Badi *et al*., 2014).

In *C. albicans*, the switching of yeast cells to hyphal cells is considered to play an important role in biofilm formation and the pathogenesis of fungal infections (Douglas, 2003). It was recently reported that lauric acid inhibited *C. albicans* biofilm formation by downregulation of several hyphal and biofilm-related genes (Lee *et al*., 2020). Also, it was reported that myristic acid inhibited biofilm and hyphal formation by *C. albicans*, probably targeting several proteins involved in ergosterol synthesis, sphingolipid metabolism, multidrug resistance and the oxidative stress (Prasath *et al*., 2019). Transcriptomic results in the current study partially matched with the previous reports as the expression of *HWP1* downregulated by fatty acids (Fig. 3C). Furthermore, the gene expression of chitinase *CHT4* was induced by fatty acids (Fig. 3C) that also might play a role in the inhibition of hyphal and biofilm formation. While speculative, it appears inhibition of the hyphae formation and biofilm formation by various fatty acids is not rare in the ecosystem.

Biofilms often consist of multiple species of bacteria residing in close proximity to one another in natural environmental conditions (Elias and Banin, 2012), but current knowledge of the polymicrobial biofilms is still very limited (Roder *et al*., 2016). Especially, biofilm controls of three species are rarely reported, while various studies of dual-species biofilm have been reported. For example, the efficacy of disinfectants against *S. aureus*, *C. albicans* and *P. aeruginosa* in multispecies biofilms was reported and multispecies biofilms were always less susceptible than single species biofilms (Kart *et al*., 2014). In several dual-species biofilm studies including *C. albicans*, hyphal formation is a dominant factor as bacteria like *S. aureus* and *E. coli* can adhere to hyphae (Peters and Noverr, 2013; De Brucker *et al*., 2015; Kong *et al*., 2016). Recently, linoleic acid from centipede oil (Kim *et al*., 2020) and nepodin (Lee *et al*., 2019) was reported to reduce hyphal formation and biofilm formation by *C. albicans* and the numbers of *S. aureus* adhered. In this study, we developed for the first time an *in vitro* three-species biofilm model including *S. aureus*, *E. coli* O157:H7 and *C. albicans* and further demonstrated that lauric acid and myristic acid could inhibit biofilm formation of three-species biofilms (Fig. 2D–F). Based on SEM observation, *S. aureus* and *E. coli* O157:H7 can adhere to larger *C. albicans* cells in the form of yeast or hyphae cells (Fig. 2F) and saw palmetto oil and myristic acid repressed the expression of *HWP1* (hyphal wall protein 1 gene) (Fig. 3C). Therefore, in polymicrobial biofilms including *C. albicans*, the ability of hyphal morphogenesis and its culture conditions are crucial factors for overall biofilm formation. Since several medium-chain fatty acids also repressed the expression of *HWP1* gene via mimicking of the quorum-sensing molecule farnesol (Lee *et al*., 2020), it is highly possible that lauric acid and myristic acid would also interfere the quorum sensing of *C. albicans*. Notably, lauric acid and myristic acid repressed the expression of quorum-sensing genes (*luxRS*) in *E. coli* (Fig. 3B), but not any quorum-sensing gene (*agrA*) in *S. aureus* (Fig. 3A). It is intriguing that fatty acids may affect inter-kingdom signalling systems in mono- and multi-species biofilms.

Combinatorial therapy has gained importance due to the limited efficacy of antimicrobial agents against drug-resistant pathogens and their poor penetration abilities within biofilms. Several reports showed that fatty acids can be used as an antibiotic adjuvant since combinatorial therapy of fatty acids and antimicrobial agents synergistically diminished biofilm formation and decreased bacterial pathogenesis (Ells *et al*., 2009; Rahmani-Badi *et al*., 2014; Sepehr *et al*., 2014; Kumar *et al*., 2020), probably due to change of phospholipid composition and membrane permeability (Eder *et al*., 2017; Hobby *et al*., 2019). In the current study, synergistic antimicrobial efficacies of fatty acids were observed in two bacteria, *S. aureus* and in *E. coli* O157:H7, but not in fungal *C. albicans* (Fig. 4E–G). For *C. albicans* cells, antifungal amphotericin B binds with ergosterol, a component of fungal cell membranes, forming pores that cause rapid leakage of monovalent ions (K^+, Na^+, H^+ and Cl^-) and produced reactive oxygen species and subsequent fungal cell death (Brajtburg *et al*., 1990; Haraguchi *et al*., 1996). In this study, antifungal activity of amphotericin B was reversed by lauric acid and myristic acid. Since lauric acid and myristic acid inhibited hyphal formation (Fig. 3), while speculative, amphotericin B is likely to be more effective against hyphal cells than yeast cells. To enhance the antibiotic efficacy, combinatorial therapy and fatty acids can be simultaneously used in tandem or combination and also drug delivery systems using chitosan, natural polymers, liposomes or nanoparticles, further enhancing the bioavailability and decrease drug cytotoxicity.

The emergence of drug-resistant microorganisms has driven the development of novel antibiotics, antifungals and even adjuvants. Control of drug-resistant biofilm formation is important in pathogens such as *S. aureus*, *E. coli* O157:H7 and *C. albicans*. This study shows the antibiofilm activities of saw palmetto oil and its main fatty acids, lauric acid and myristic acid, against each pathogen and mixture of two or three species. The efficacies were confirmed *in vivo* in a *C. elegans* model with minimal cytotoxicity. Since many saw palmetto oils are commercially available as health supplements and lauric acid and myristic acid are naturally present in various plant oils, these fatty acid products could be relatively safe to be applied in multispecies infections as potent antibiofilm agents and antibiotic adjuvants.

Furthermore, fatty acid-rich plant oils can be used to control pathogenic biofilms of single and multiple microbes by coating medical devices, synthesizing composites of polymer fatty acids, and producing emulsion with fatty acids for medical and cosmetic purposes. Also, our findings indicate that other fatty acids could be utilized for combinatorial therapy against drug-resistant bacteria. Experiments such as bacterial adhesion and antivirulence efficacy using epithelial cells or animal model could be further investigated.

Experimental procedures

Strains, chemicals and culture materials

Eleven microorganisms used in the present study, medium, minimum inhibitory concentrations (MICs) and inoculum size for MIC measurement are listed in Table S1. All media (Luria-Bertani (LB), tryptic soy broth (TSB), potato dextrose broth (PDB), nutrient broth and agar) were purchased from Becton Dickinson (Franklin Lakes, NJ, USA). Minimum inhibitory concentrations (MICs) were determined (Table S1) according to the Clinical Laboratory Standards Institute (CLSI) for bacteria and yeasts (CLSI, 2015; CLSI, 2017). MIC was defined as the lowest concentration that inhibited cell growth by 80% as assessed by spectrophotometry and colony counting. Experiments were performed using at least three independent cultures.

A generic saw palmetto oil was purchased from Amazon (Seattle, USA). Four fatty acids, namely dodecanoic acid (lauric acid), tetradecanoic acid (myristic acid), hexadecanoic acid (palmitic acid), and 9-octadecenoic acid (oleic acid), and antimicrobial agents, gentamicin and amphotericin B, were purchased from Sigma-Aldrich (St. Louis, USA) or TCI Co. (Tokyo, Japan). Dimethyl sulfoxide (DMSO) was used to dissolve all fatty acids, and 0.1% (v/v) DMSO was used as the negative control; at this concentration, it did not affect bacterial growth or biofilm formation.

Biofilm assay in 96-well plate

Biofilm formation of various microbes was produced on 96-well polystyrene plates, as previously described (Lee *et al*., 2019). Briefly, overnight cultures at an initial turbidity of OD 0.05 (~ 10^8 or 10^7 CFU ml^{-1}) for bacteria and OD 0.1 (~ 10^5 CFU ml^{-1}) for *Candida* at 600 nm (Table S1) were then inoculated into a proper culture media (final volume 300 μl) with or without fatty acids and incubated for 24 h without shaking at 37 °C. Biofilm cells that adhered to 96-well plates (SPL Life Sciences, Pocheon, Korea) were stained with 0.1% crystal violet Sigma-Aldrich (St. Louis, USA) for 20 min, washed repeatedly with sterile distilled water and resuspended in 95% ethanol. Plates were read at 570 nm, and results are presented as the means of at least six repetitions from three independent cultures. The percentage of inhibition ratio represents the relative biofilm formation (100 × biofilm formation with chemical/biofilm formation of untreated control).

Gas chromatograph/mass spectroscopy (GC-MS)

The components of saw palmetto oil were separated and analysed by gas chromatography-mass spectroscopy (GC-MS) using an Agilent 6890N GC and SP-2560 (Supelco, Sigma-Aldrich, St. Louis, USA) with a silica capillary column (100 m × 0.25 mm i.d., film thickness 0.25 mm). Column temperature conditions and the derivatization (methylation) of fatty acids were as previously described (Kim *et al*., 2018). Triundecanoin (C11:0) was used as the internal standard, and quantifications were performed by integrating areas and correcting for fatty acid methylation. Supelco 37 components FAME Mix (Supelco) were used as the reference standard.

Biofilm observations by CSLM and SEM

Single, dual or triple biofilms were formed on 96-well polystyrene plates with or without fatty acids without shaking for 24 h at 37 °C. Planktonic cells were then removed by washing with distilled water three times, and single or dual biofilms were stained with carboxyfluorescein diacetate succinimidyl ester (Invitrogen, Molecular Probes, Inc., Eugene, USA) (Lee *et al*., 2016). Plate bases were then visualized using a 488 nm Ar laser (emission 500 to 550 nm) under a confocal laser microscope (Nikon Eclipse Ti, Tokyo, Japan). Two independent cultures were performed under each experimental condition and at least 10 random positions were assayed.

Scanning electron microscopy was also used to observe multispecies biofilm formation on nylon membranes, as previously described (Kim *et al*., 2016). Briefly, a nylon membrane (Merck Millipore, Burlington, USA) was cut into 0.5 × 0.5 cm pieces and placed in 96-well plates containing single or mixed species grown with or without fatty acids and incubated for 24 h at 37 °C. PDB medium was used for *C. albicans* biofilm, a 1:1 mixture of PDB and LB media was used for the dual *C. albicans* and *S. aureus* biofilms, a 1:1 mixture of PDB and LB media was used for the dual biofilms of *C. albicans* and *E. coli* O157:H7, or a 1:2 mixture of PDB and LB media was used for the triple biofilms of *S. aureus*, *C. albicans* and *E. coli* O157:H7. Cells that adhered to the nylon membrane were fixed with glutaraldehyde (2.5%) and formaldehyde (2%) for 24 h and then post-

fixed using osmium and dehydrated using an ethanol series (50, 70, 80, 90, 95 and 100%) and isoamyl acetate. After critical-point drying, cells were examined and imaged using an S-4100 scanning electron microscope (Hitachi, Tokyo, Japan) at a voltage of 15kV according to the manufacturer's instructions.

Quantitative Real-Time PCR (qRT-PCR)

Three sets of transcriptomic analyses were performed with *S. aureus*, *E. coli* O157:H7 and *C. albicans* cells. For the *S. aureus*, the previous method was used (Kim *et al*., 2018). *S. aureus* MSSA 6538 cells were inoculated into 25 ml of LB broth at 37 °C in 250-ml flasks at a starting OD_{600} of 0.05 and then incubated for 6 h with shaking at 250 rpm in the presence or absence of saw palmetto oil (100 $\mu g\ ml^{-1}$), lauric acid (20 $\mu g\ ml^{-1}$) and myristic acid (20 $\mu g\ ml^{-1}$). For the *E. coli* O157:H7, the previous method was used (Kim *et al*., 2015). *E. coli* O157:H7 cells were inoculated into 25 ml of LB broth at 37 °C in 250-ml flasks at a starting OD_{600} of 0.05 and then incubated for 4 h with shaking at 250 rpm in the presence or absence of saw palmetto oil (100 $\mu g\ ml^{-1}$), lauric acid (20 $\mu g\ ml^{-1}$) and myristic acid (20 $\mu g\ ml^{-1}$). RNase inhibitor (RNAlater, Ambion, TX, USA) was then added, and cells were immediately chilled for 30 s in a dry ice bath having 95% ethanol to prevent RNA degradation. Cells were then harvested by centrifugation at 16 600 *g* for 1 min, and total RNA was isolated using a Qiagen RNeasy mini Kit (Valencia, USA) according to the manufacturer's instructions. For the *C. albicans*, another previous method was used (Lee *et al*., 2018). 25 ml of *C. albicans* DAY185 at an initial turbidity of 0.1 at OD_{600} (~ 10^5 CFU ml^{-1}) was inoculated into PDB broth in 250 ml and incubated for 6 h at 37 °C with agitation (250 rpm) in the presence or absence of saw palmetto oil (100 $\mu g\ ml^{-1}$), lauric acid (20 $\mu g\ ml^{-1}$) and myristic acid (20 $\mu g\ ml^{-1}$). To prevent RNA degradation, RNase inhibitor (RNAlater, Ambion, TX, USA) was added to cells and cells were immediately chilled for 30 s in a dry ice bath having 95% ethanol to prevent RNA degradation. Total RNA was isolated using a hot acidic phenol method, and RNA was purified using a Qiagen RNeasy mini Kit (Valencia, USA) according to the manufacturer's instructions.

qRT-PCR was used to determine the expressions of various biofilm-related genes (*agrA*, *aur, hla, hld, icaA, sarA* and *sigB* in *S. aureus, csgA, csgB, fimH, flhD, luxR, luxS* and *motB* in *E. coli* O157:H7, *ALS1, ALS3, CDR1, CDR2, CHT4, CYR1, ECE1, ERG9, ERG10, HWP1, MTS1*, *RAS1*, *RBT5* and *UME6,* in *C. albicans*). The specific primers used for qRT-PCR are listed in Table S2. Three housekeeping genes, *16s rRNA*, *rrsG* or *RDN18*, were used, respectively, and the expression of housekeeping genes was not affected by fatty acids. The qRT-PCR was done using SYBR Green master mix (Applied Biosystems, Foster City, USA) and an ABI StepOne Real-Time PCR System (Applied Biosystems) according to the manufacturer's instructions. At least two independent cultures were used.

Cell-Surface hydrophilicity assay

Cell-surface hydrophobicity was analysed as previously reported (Rosenberg *et al*., 1980). Briefly, cell cultures were harvested by centrifugation at 7000 *g* for 5 min after 20-h incubation in LB with shaking at 250 rpm at 37 °C. Harvested cells were mixed with hexadecane (TCI chemicals, Tokyo, Japan) at a ratio of 6:1 by vortexing for 90 s. Mixtures were allowed to stand for 30 min at room temperature to enable phase separation. Aqueous phase absorbance was measured at OD_{600}. Experiments were performed using four independent cultures per each material.

Observation of C. albicans *hyphae*

To observe hyphal formation, microscopic imaging system was used as previously described (Lee *et al*., 2020). Briefly, *C. albicans* DAY185 cells were re-inoculated at 1:50 dilution in 2 ml of PDB medium with or without fatty acids (0, 10, 20 or 100 $\mu g\ ml^{-1}$) and incubated for 24 h at 37 °C without shaking. After the incubation, cells were mixed and visualized using an iRiS™ Digital Cell Imaging System (Logos Bio Systems, Anyang, Korea) according to the manufacturer's instructions. At least four independent cultures were used.

Antivirulence and cytotoxicity assays of fatty acids in the nematode model

To investigate the effects of fatty acids on the virulence of *S. aureus*, *E. coli* O157:H7 or *C. albicans*, we used *C. elegans* strain *fer-15(b26)*; *fem-1(hc17)*, as previously described (Kim *et al*., 2020). Briefly, approximately 30 nematodes were added to each well of 96-well plates containing M9 for *S. aures* and *E. coli* O157:H7 or PDB:M9 (20:80) mixture for *C. albicans* infection. Also, the untreated control and saw palmetto oil (20 or 100 $\mu g\ ml^{-1}$), lauric acid (10 or 20 $\mu g\ ml^{-1}$) or myristic acid (10 or 20 $\mu g\ ml^{-1}$)-treated *S. aures*, *E. coli* O157:H7 (~ 10^8, 10^7 CFU ml^{-1} respectively) or *C. albicans* cells (~ 10^5 CFU ml^{-1}) were added into the wells containing nematodes. Plates were then incubated for 10 days at 25 °C without shaking. For cytotoxicity assays, 30 non-infected nematodes were pipetted into the single wells of a 96-well plate containing M9 buffer and fatty acid (saw palmetto oil, lauric acid or myristic

acid) at final concentrations of 0, 10, 50, 100 or 200 μg ml^{-1} without pathogens. Plates were then incubated for 4 days at 25 °C without shaking. Three independent experiments were performed in triplicate. Results are expressed as percentages of live nematodes (survival), determined by responses to platinum wire touching. Observations were made using an iRiS™ Digital Cell Imaging System (Logos Bio Systems, Anyang, Korea) according to the manufacturer's instructions.

Combinatory treatment of fatty acids and antimicrobial agents

Combinatorial efficacies of fatty acids and antimicrobial agents were analysed on *S. aureus*, *E. coli* O157:H7 and *C. albicans*. Briefly, overnight cultures at an initial turbidity of OD 0.05 for bacteria and OD 0.1 for *Candida* at 600 nm were inoculated into a proper culture media (final volume 1000 μl) with antimicrobial agents, gentamicin (10 μg ml^{-1}), kanamycin (50 μg ml^{-1}), tobramycin (10 μg ml^{-1}) or amphotericin B (0.5, 2, 5 μg ml^{-1}) and/or fatty acids (saw palmetto oil at 100 μg ml^{-1}, C12 or C14 at 20 μg ml^{-1}). Cells were incubated for 1 h with shaking at 37 °C. Cell mixtures were diluted serially in PBS and plated on proper culture media. CFU was determined by colony counting after 24-h incubation at 37 °C. At least four repetitions from two independent cultures were performed.

Statistical analysis

Replication numbers for assays are provided above, and results are expressed as means ± standard deviations. The statistical analysis was performed using one-way ANOVA followed by Dunnett's test using SPSS version 23 (SPSS Inc., Chicago, IL, USA). P values of < 0.05 were considered significant, and asterisks indicate significant differences between treated and untreated samples.

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Conflict of interest

None declared.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Fig. S1. Planktonic cell growth in the presence of fatty acids.

Fig. S2. Dispersal of established biofilms by fatty acids.

Table S1. Strains and culture media used in this study and MICs of saw palmetto oil, lauric acid, and myristic acid.

Table S2. Primer sequences used for qRT-PCR.

Table S3. GC-MS analysis of saw palmetto oil.

