Antibacterial and antibiofilm effects of α-humulene against Bacteroides fragilis

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Antibacterial and antibiofilm effects of α-humulene against Bacteroides fragilis

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Abstract

The rapid increase in antibiotic resistance has prompted the discovery of drugs that reduce antibiotic resistance or new drugs that are an alternative to antibiotics. Plant extracts have health benefits and may also exhibit antibacterial and antibiofilm activities against pathogens. This study determined the antibacterial and antibiofilm effects of α-humulene extracted from plants against enterotoxigenic Bacteroides fragilis (ETBF), which causes inflammatory bowel disease. The minimum inhibitory concentration (MIC) and biofilm inhibitory concentration (BIC) of α-humulene for B. fragilis were 2 μg/mL, and the biofilm eradication concentration (Pumbwe et al.) was in the range of 8-32 μg/mL. The XTT reduction assay confirmed that the cellular metabolic activity in biofilm rarely occurred at 8-16 μg/mL concentration. In addition, the biofilm inhibition by α-humulene was also detected via confocal laser scanning microscopy. Quantitative real-time polymerase chain reaction was also used to investigate the effect of α-humulene on the expression of resistance-nodulation-cell division (RND)-type multidrug efflux pump (bmeB1 and bmeB3) genes. As a result of qPCR, α-humulene significantly reduced the expression of bmeB1 and bmeB3 genes. This study demonstrates the potential therapeutic application of α-humulene to inhibit the growth of B. fragilis cells and biofilms, and contribute to the expansion of knowledge about biofilm medicine.

Key words: Bacteroides fragilis, α-humulene, antibiofilm, antibacterial, efflux pump genes.
**Introduction**

In 2018, deaths due to colorectal cancer (CRC) accounted for over 9% of all cancer deaths (Bray et al. 2018; Metz et al. 2019). The major CRC-induced bacteria found in more than 80% of CRC patient mucosa include enterotoxigenic *Bacteroides fragilis* (ETBF), which carries a *Bacteroides fragilis* toxin (Bft) known as fragilysin (Boleij et al. 2015; Nakano et al. 2007). ETBF is specifically associated with diarrhea, inflammatory bowel disease (IBD) and anaerobic bacteremia, and IBD is associated with biofilm formation (Housseau and Sears 2010; Metz et al. 2019; Zamani et al. 2017). Biofilm density was more than double in patients with IBD compared with healthy humans. *B. fragilis* accounted for more than 60% of the biofilms in IBD patients (Swidsinski et al. 2005). ETBF biofilms, which cause IBD and CRC, act as a defensive shield against host immune response and cause antibiotic resistance, suggesting the importance of control for pathogenic activity of ETBF biofilm (Li et al. 2017).

Natural extracts are generally used to remove these biofilms. Antibacterial and antibiofilm activities of natural extracts against human pathogens have been intensively studied to develop new food ingredients for potentially novel applications of pharmaceuticals (Shin and Eom 2019; Silva et al. 2016; Woo et al. 2017a). In this study, the focus is on α-humulene, one of the many sesquiterpenoids derived from aromatic plants (Helmig et al. 2007). The α-humulene is extracted from essential oils of plants such as *Salvia officinalis*, *Mentha spicata* and ginger family (Zingiberaceae) (Bouajaj et al. 2013; Chauhan et al. 2011; Suthisut et al. 2011). Furthermore, α-humulene was safe up to approximately 400 μg/mL in fibroblast cell line and microphage cells, but showed toxicity at concentrations above (Mazutti da Silva et al. 2018). It is well known that α-humulene has anti-inflammatory effects and essential oils containing α-humulene have with antibacterial effects (Fernandes et al. 2007; Pichette et al. 2006). However, studies investigating the antibacterial and antibiofilm effects of α-humulene against *B. fragilis* are still incomplete. Therefore, the purpose of this study was to evaluate the antibacterial and antibiofilm effects of α-humulene on *B. fragilis*.

Experiments evaluating the α-humulene susceptibility were conducted to confirm the antibacterial effect, and the antibiofilm effect of α-humulene on *B. fragilis* was investigated using crystal violet assay.
and XTT [2,3-bis (2-methoxy-4-nitro-5-sulfo-phenyl)-2H-tetrazolium-5-carboxanilide] reduction assay. In addition, confocal laser scanning microscopy (CLSM) was performed to visualize and confirm the biofilm affected by α-humulene. To investigate the antibiofilm effect at the gene level in \textit{B. fragilis}, resistance-nodulation-cell division (RND) type efflux pump-related \textit{Bacteroides} multidrug efflux (\textit{bmeB1} and \textit{bmeB3}) genes were investigated using real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR). Since the efflux pump promotes the formation of the biofilm matrix, the decrease of \textit{bmeB1} and \textit{bmeB3} gene expression is a significant antibiofilm indicator (Alav et al. 2018). Furthermore, based on the study suggesting that changes in \textit{bmeB1} and \textit{bmeB3} genes controlled the minimum inhibitory concentrations (Pumbwe et al. 2006b), qRT-PCR of \textit{bmeB1} and \textit{bmeB3} genes was performed. Since resistance to existing drugs is associated with serious clinical complications, the development of new therapeutic approaches may contribute to the treatment of disease caused by \textit{B. fragilis}. Therefore, the aim of this study was to determine the antibacterial and antibiofilm activity of α-humulene against \textit{B. fragilis}.
Materials and methods

Bacterial strains and culture conditions

In this study, three types of bacterial strain were used: wild-type enterotoxigenic \( B. \) fragilis 86-5443-2-2 (WT-ETBF; \( bft-2 \)), wild-type non-enterotoxigenic \( B. \) fragilis NCTC 9353 (WT-NTBF), and a recombinant strain overexpressing \( bft \) in WT-NTBF created by the addition of \( bft-2 \) to WT-NTBF (rETBF; \( bft-2 \)) (Myers et al. 1989; Rhee et al. 2009). \( B. \) fragilis strains used in the experiment were cultured in brain heart infusion broth (BHI; Difco, Becton-Dickinson and Company, USA) supplemented with 1% glucose, hemin, yeast extract and L-cystine (BHIS) (Cuchural et al. 1986; Rosenblatt and Stewart 1975) in a 37°C incubator for 24 h under anaerobic conditions. The BHIS broth supplemented with 20% glycerol was used as a cryoprotective agent and stored at -80°C until experimental use. Experimental results were measured using a Multiskan GO plate reader (Thermo Fisher Scientific, MA, USA). BD GasPak™EZ CO\(_2\) container system sachets (Becton–Dickinson, MD, USA) were used to create anaerobic environment. The \( \alpha \)-humulene used in this study was purchased from Sigma-Aldrich (St. Louis, MO, USA). The final ethanol concentration did not exceed 1% in all experiments.

Evaluation of antibacterial activity

The antibacterial activity was assessed by identifying the minimum inhibitory concentration (MIC). The MIC was determined by broth microdilution method, in accordance with the clinical and laboratory standards institute (CLSI 2018) guidelines. Briefly, bacterial suspensions at an optical density equal to 0.5 McFarland standard in BHIS media were prepared for each strain. At a final concentration of \( 1 \times 10^6 \) CFU/mL, the bacteria in the BHIS media were distributed in 96-well microtiter plates at a concentration of 0.125 to 4 \( \mu \)g/mL \( \alpha \)-humulene in a total volume of 200 \( \mu \)l. Growth control was inoculated with BHIS microbial suspension and 1% ETOH. MIC values were read after 24 h of incubation at 37°C under anaerobic condition. The inhibition of planktonic cell growth was measured...
with a Multiscan GO plate reader (Thermo Fisher Scientific, MA, USA).

**Evaluation of biofilm inhibition activity**

In order to confirm the biofilm inhibitory activity, the biofilm formation was induced as previously described (Jang and Eom 2019b; Kim et al. 2019) with minor modifications. In brief, after culturing the bacteria under anaerobic conditions, as in antibacterial activity, a 96-well polystyrene plates (BD, Falcon) was added to the BHIS medium containing different concentrations of α-humulene (0.125-4 μg/mL) and bacteria in a total volume of 200 μl. After 24 h of incubation, the supernatant was removed from the well to remove the planktonic cells except for the biofilm, and washed three times with sterilized phosphate-buffered saline (PBS). Plates were dried by fixing at 60°C for 1 h. To quantify the biofilm biomass, 0.5% of the crystal violet was stained for 5 minutes and then washed three times with sterilized distilled water and dried at 60°C for 1 h. After drying, 150 μl of 33% acetic acid was added to each well for 20 min to destain the biofilm biomass. Finally, 150 μl of acetic acid was transferred from each well to a new 96 well polystyrene plate. The absorbance was analyzed at 570 nm (OD$_{570}$) using a Multiscan GO plate reader (Thermo Fisher Scientific, MA, USA). Samples without α-humulene were used as controls.

**Confirmation of preformed mature biofilm eradication activity**

Modified biofilm eradication concentration assays (Ceri et al. 2001; Qu et al. 2016; Woo et al. 2017b) was performed to confirm the effect of α-humulene on the removal of mature biofilms in 96-well plates. Briefly, bacterial cell suspensions at a concentration of 1 × 10$^6$ CFU/mL were incubated at 37°C without α-humulene treatment for 24 h under anaerobic conditions. When the biofilm was formed by incubating the bacteria for 24 h, the supernatant was removed and washed with sterilized PBS to eradicate the planktonic cells. Subsequently, 200 μl of BHIS medium containing various concentrations of α-humulene (0.25 to 64 μg/mL) was added to each well of the plate and incubated for 24 h. After incubation, the bacterial supernatant was removed and the plates washed three times with 200 μl of
sterilized PBS. Finally, the crystal violet staining assay was performed as described above, and the absorbance was analyzed at 570 nm (OD<sub>570</sub>). The sample without α-humulene was used as a control.

**Assessment of metabolic activity of cells in biofilm**

The cellular metabolic activity of *B. fragilis* (WT-ETBF, rETBF, and WT-NTBF) biofilms was analyzed using the XTT reduction assay as described previously (Chaieb et al. 2011; Kim et al. 2018; Xu et al. 2016). Biofilm was preformed as described above. The same process was conducted before crystal violet staining in BEC assay. After treatment with α-humulene, the plate was washed with sterilized PBS, and XTT Cell Proliferation Assay Kit (ATCC, Manassas, VA, USA) was used to confirm the metabolic activity of the cells in the biofilm. The XTT reagent and the activation reagent were mixed at a ratio of 50:1 (v/v) before use, and 50 μl was dispensed into each well, followed by incubation of the plates at 37°C in the dark for 3 h. The changes in chromaticity associated with the metabolic activity of the cells in the biofilm were measured at 475 nm and 650 nm using the Multiskan GO microtiter plate reader (Thermo Fisher Scientific, MA, USA).

**Biofilm observation with confocal laser scanning microscopy (CLSM)**

CLSM can be used to study not only biofilm matrix studies, but also the amount of bound biomass, and can be used to determine the localization and relative amounts of matrix components. In addition, the spatiotemporal effects of antibacterial treatment can be confirmed (Jang and Eom 2019a; Lawrence and Neu 1999; Reichhardt and Parsek 2019). Briefly, *B. fragilis* strains (WT-ETBF, rETBF, and WT-NTBF) and α-humulene were dispensed on 24-well glass bottom imaging plates (Eppendorf AG, Hamburg, Germany) and incubated for 24 h with shaking at 120 rpm under anaerobic conditions. Only BHIS medium and *B. fragilis* were added to the control group. After incubation, the supernatant was removed and each well was washed three times with PBS to remove the planktonic cells. The biofilms were fixed with 3.7% (v/v) formaldehyde for 1 h at room temperature before fluorescent staining. After drying for 1 hour, the biofilms were dyed with three different fluorescent dyes. First, the amino group
of *B. fragilis* biofilm was stained with fluorescein isothiocyanate isomer I (FITC, 10 μg/μl; Sigma-Aldrich, Germany) for 1 h to visualize the proteins of biofilm. Concanavalin A-Alexa Fluor 594 conjugate (Con A, 0.1 μg/μl; C-11253, Molecular Probes, Eugene, OR, USA) was used to stain the carbohydrates of *B. fragilis* biofilm for 30 min. To visualize extracellular nucleic acids, 4,6-diamidino-2-phenylindole dihydrochloride (DAPI, 1 mg/L; Molecular Probes, Eugene, OR, USA) was used to stain for 45 min. The wells were rinsed three times with PBS at each stage of staining in the dark to remove any remaining dye. According to the Zeiss LSM-710 confocal laser microscope (Carl Zeiss, Thornwood, NY, USA) instructions, the wavelengths used for FITC, Con A and DAPI detection were 488 nm, 561 nm and 405 nm, respectively, to study the fluorescence-stained biofilm. The ZEN software (Carl Zeiss, Thornwood, NY, USA) was used to obtain confocal images of the biofilm.

**RNA isolation**

The NucleoSpin RNA mini Kit (Macherey-Nagel, Düren, Germany) was used for RNA extraction of *B. fragilis* strains (WT-ETBF, rETBF, and WT-NTBF). First, *B. fragilis* cells were added to the BHIS medium containing different concentrations of α-humulene (0.25-1 μg/mL) and incubated at 37°C for 24 h under anaerobic conditions. After incubation, pellets were obtained by centrifuging 2 mL of the bacterial culture at 25,000 g for 90 s at 4°C in each sample. RNA was extracted from the pellets according to the manufacturer's instructions in the NucleoSpin RNA mini Kit. RNA was filtered using silica columns and treated with DNase to prevent DNA contamination. The quality and concentration of the extracted RNA samples were evaluated using BioDrop µLITE (BioDrop Ltd., Cambridge, UK).

**Real-time Quantitative Reverse Transcription PCR (qRT-PCR)**

qRT-PCR was used to quantify the expression of RND efflux pump genes in *B. fragilis* cells (WT-ETBF, rETBF, and WT-NTBF) according to the degree of α-humulene treatment. The efflux pump genes used were *bmeB1* and *bmeB3* (Pumbwe et al. 2008; Pumbwe et al. 2006b). First, the extracted RNA was reverse-transcribed to cDNA depending on the instructions provided by ReverTraAce qPCR
RT Master Mix with gDNA Remover (Toyobo, Osaka, Japan). The qRT-PCR was performed using StepOnePlus™ Real-Time PCR System (Applied Biosystems, Foster City, CA) with the cDNA and amplified using Power SYBR® Green PCR Master Mix (Applied Biosystems, Warrington, UK). The 16S rRNA primers and *bmeB1* and *bmeB3* primers were referenced and well described in Table 1 (Bundgaard-Nielsen et al. 2019; Pumbwe et al. 2006b). The qRT-PCR were performed as following cycling conditions: at 95°C for 10 min for denaturation and 15 sec at 95°C, 1 min at 58°C for 16S rRNA genes (55°C for *bmeB1* and *bmeB3* genes) and 40 cycles of annealing stage. Melting curve analysis (95°C for 15 sec, 60°C for 1 min, and 95°C for 15 sec) was performed at the end of the qRT-PCR cycles. The transcription levels of the target genes were calculated using the 2^ΔΔCT method and the transcription level of 16S rRNA was used for standardization as an internal housekeeping control gene.

**Statistical analysis**

Data are expressed as means ± standard deviation (SD). Statistical analyses were performed to determine significant differences between the treated samples and the control sample via one-way analysis of variance (ANOVA) followed by Dunnett’s test using SPSS version 20 (SPSS Inc., Chicago, IL, USA). Statistical significance was considered at *p* value < 0.05, **P** value < 0.01, ***P** value < 0.001, and indicated.
Results

The effect of α-humulene on bacterial cell growth

The microbroth dilution assay was used to investigate the susceptibility of *B. fragilis* to α-humulene. The antibacterial activity of α-humulene against *B. fragilis* strains (WT-ETBF, rETBF, and WT-NTBF) was measured as absorbance at 600 nm (OD$_{600}$). Bacterial growth was gradually inhibited with increasing α-humulene concentrations. All *B. fragilis* strains (WT-ETBF, rETBF, and WT-NTBF) were inhibited by more than 90% at an α-humulene concentration of 2 μg/mL (Fig. 1). The MIC$_{90}$ is defined as the lowest concentration of each agent that inhibits cell growth by up to 90% compared with control (Drago et al. 2017; Schwarz et al. 2010). The MIC$_{90}$ of α-humulene against *B. fragilis* strains was 2 μg/mL. However, bacteria are significantly inhibited even at 0.5 μg/mL. Therefore, α-humulene represents an effective antibacterial agent against *B. fragilis*.

The effect of α-humulene on biofilm prevention

The effect of α-humulene on *B. fragilis* biofilm production was studied using the crystal violet assay. As a result, α-humulene significantly inhibits *B. fragilis* biofilm formation. BIC$_{90}$ was defined as the lowest concentration of α-humulene that inhibits biofilm formation by up to 90% compared with the control without α-humulene (Moskowitz et al. 2011), and therefore, the BIC$_{90}$ of all *B. fragilis* strains (WT-ETBF, rETBF, and WT-NTBF) was 2 μg/mL (Fig. 2). These results were similar to the results at MIC$_{90}$. In particular, WT-ETBF was markedly reduced compared with other *B. fragilis* strains. Three strains were found to be significantly reduced even at 0.5 μg/mL. When α-humulene concentrations exceeded MIC$_{90}$ concentrations, the biofilms of *B. fragilis* were completely inhibited. These results suggested that α-humulene inhibited the formation of *B. fragilis* biofilm.

The effect of α-humulene on preformed mature *B. fragilis* biofilms

Usually, bacterial biofilms are already established in chronic wounds before treatment. Therefore, it
is important to confirm the ability of α-humulene to remove established biofilms produced by *B. fragilis* strains. BEC<sub>90</sub> is defined as the lowest concentration of the compound that eliminates up to 90% of the established biofilm compared with the control (Nostro et al. 2007). The three *B. fragilis* strains differed in that WT-ETBF was eradicated more than 90% at 8 μg/mL, rETBF at 16 μg/mL and WT-NTBF at 32 μg/mL (Fig. 3). WT-ETBF, rETBF and WT-NTBF showed significant reduction in preformed biofilm, but the most dramatic effect was observed with WT-ETBF, which was the most biofilm-forming. The inhibitory effect occurred at lower concentrations than the biofilm removal effect. Thus, α-humulene treatments were more effective against early biofilm formation than preformed mature biofilms.

**The effect of α-humulene on cell viability within *B. fragilis* biofilms**

The removal of the biofilm was evaluated using the crystal violet assay, which was used to stain all the biofilm components. However, this assay cannot be used to assess the viability of cells remaining in the biofilm structure (Smith and Hunter 2008). To determine cell viability, an XTT reduction assay was used to measure the viability of cells in the biofilm. The metabolic activity of the cells was measured according to the changes in chromaticity of the XTT reducing reagent. The viability of remaining cells on the biofilm after α-humulene treatment is shown in Figure 4. In general, the cell viability decreased in proportion to biofilm biomass removal. In addition, the metabolic activity of the *B. fragilis* strain was decreased as the concentration of α-humulene increased. WT-ETBF and rETBF showed a dramatic decrease in metabolic activity at the concentration of 8 μg/mL compared with the control. WT-NTBF showed limited cellular metabolic activity at 16 μg/mL (Fig. 4). This finding suggests that α-humulene influenced not only the biofilm structure but also the cellular metabolic activity.

**Visualizing the effects of α-humulene on *B. fragilis* biofilms**

To evaluate the effect of α-humulene on *B. fragilis* biofilm, the biofilm stained with a fluorescent dye was observed using CLSM, which can be used in biofilm investigations to determine the
localization and the relative level of matrix components and to identify the spatial effects of therapeutic agents (Carvalho et al. 2012). The *B. fragilis* strains (WT-ETBF, rETBF and WT-NTBF) treated with 1% ETOH showed a thick and dense biofilm (Fig. 5A, C and E). Each biofilm treated with 1 μg/mL α-humulene was found to be significantly reduced compared with the control (Fig. 5B, D and F). As a result, the CLSM images of α-humulene-treated biofilms showed a decrease in protein, carbohydrate, and nucleic acid levels as well as cell density and thickness due to α-humulene inhibitory activity against *B. fragilis* biofilm. The quantification of the CLSM image is shown in supplementary figure 1. Thus, biofilm monitoring using CLSM confirmed that α-humulene treatment reduced all of the major components of biofilm, including proteins, carbohydrates and nucleic acids.

**α-humulene induced changes in relative gene expression of *B. fragilis* gene associated with efflux pump**

The qRT-PCR was used to investigate the molecular mechanisms based on the antibiofilm action of α-humulene against *B. fragilis* strains (Price et al. 2007). The α-humulene significantly altered the expression of *bmeB1* and *bmeB3* in the RND efflux pump genes of *B. fragilis* strains (Pumbwe et al. 2006b; Venter et al. 2015). In line with previous studies, our data also showed that α-humulene reduced the *bmeB1* and *bmeB3* gene expression of *B. fragilis* strains in a concentration-dependent manner. The α-humulene reduced the *bmeB1* gene expression of WT-ETBF at concentrations of 0.25, 0.5 and 1 μg/mL (by 1.39-, 1.44- and 1.50-fold, respectively), rETBF (by 1.30-, 1.32- and 1.92-fold, respectively) and WT-NTBF (by 1.13-, 1.46- and 1.80-fold, respectively). Likewise, at concentrations of 0.25, 0.5 and 1 μg/mL, α-humulene reduced the *bmeB3* gene expression by WT-ETBF (by 1.28-, 2.17- and 2.72-fold, respectively), rETBF (by 1.79-, 4.12- and 4.19-fold, respectively) and WT-NTBF (by 1.50-, 1.63- and 1.76-fold, respectively) (Fig. 6). These results demonstrated that α-humulene reduces the expression of efflux pump genes *bmeB1* and *bmeB3*. In particular, *bmeB1* was found to be more effective against WT-ETBF and rETBF with *bft* gene.
Discussion

The role of ETBF biofilms in CRC has already been reported (Jang and Eom 2019a; Li et al. 2017; Raskov et al. 2018). Therefore, it is necessary to find a substance that removes the ETBF biofilms. The α-humulene is a member of a well-known family of sesquiterpenoids that reduce inflammation and tumorigenesis (Zhang et al. 2009). In addition, many studies have reported the antimicrobial and antibiofilm effects of essential oils containing α-humulene against Bacillus cereus, Staphylococcus aureus (Ho et al. 2010) and Salmonella typhimurium (Miladi et al. 2016). However, the antimicrobial and antibiofilm efficacy of only α-humulene against B. fragilis has been inadequately investigated. Therefore, this study analyzed the efficacy of α-humulene against three strains of WT-ETBF, rETBF, and WT-NTBF.

Antibacterial and antibiofilm experiments were conducted to investigate B. fragilis strains using α-humulene, an essential oil component. As a result, an α-humulene concentration of 2 μg/mL inhibited cell growth and biofilm formation of B. fragilis strains by more than 90%. The MIC of α-humulene against Staphylococcus aureus was already established as 2.6 μg/mL (Pichette et al. 2006), which was similar to ours, suggesting that α-humulene has antibacterial effects regardless of gram-negative or gram-positive bacteria. Furthermore, ETBF carrying the bft gene tended to form additional robust biofilms than other B. fragilis strains without bft gene (Pierce and Bernstein 2016). In line with previous study, cell growth was similar in the three strains, biofilm formation was higher in WT-ETBF than WT-NTBF. However, α-humulene treatment more dramatically inhibited WT-ETBF with bft gene.

Experiments involving preformed mature biofilm eradication were also conducted using a crystal violet assay. More than 90% of the WT-ETBF, rETBF, and WT-NTBF biofilms were eradicated at 8 μg/mL, 16 μg/mL and 32 μg/mL, respectively. These results confirm that α-humulene is more effective against WT-ETBF carrying bft genes. In addition, eradication of mature biofilms showed that higher α-humulene concentrations were required than those required to inhibit the formation of early biofilms. These results are consistent with Miladi et al. who showed that essential oils with α-humulene affect the early stages of biofilm attachment (Miladi et al. 2016). Thus, these data indicate that α-humulene is
more effective in inhibiting biofilms at an early stage. The XTT reduction assay of the cellular metabolic
activity of the biofilm confirmed that the metabolic activity of all *B. fragilis* strains was reduced at α-
humulene concentrations higher than BIC. Because α-humulene affects the early stages of biofilm, cells
in preformed biofilm may have decreased metabolic activity at higher concentrations than BIC.

CSLM was used to analyze the biofilm inhibition. The biofilm matrix consists of diverse compounds
that make up the extracellular polymeric substances (EPSs) (Donlan 2002). EPS is made up of
polysaccharides, a wide variety of proteins, glycoproteins, and glycolipids. In some cases, it consists of
large amounts of extracellular DNA (e-DNA) (Flemming et al. 2007). Therefore, proteins,
carbohydrates, and nucleic acids, which are biofilm components, were stained with a fluorescent dye.
The control group untreated with α-humulene showed a thick biofilm with a dense structure. However,
at sub-MICs, biofilms exhibit decreased cell density due to a significant decrease in protein,
carbohydrates and DNA content in biofilm matrix. Later, we will consider using the LIVE/DEAD
staining to check if the membrane was damaged.

In gram-negative bacteria, including *Pseudomonas*, the RND efflux pump system induces multi-drug
resistance (MDR) because antibiotics protrude from inside the cell into the extracellular environment
(Poole and Srikumar 2001; Pumbwe et al. 2006a). In addition, increasing evidence suggests that the
efflux pumps play a role in biofilm formation in many studies (Alav et al. 2018; Gilbert et al. 2002).
The qRT-PCR results showed that the expression of *bmeB1* and *bmeB3* genes decreased with α-
humulene in a concentration-dependent manner. Thus, α-humulene treatment of *B. fragilis*, which
reduces the expression of *bmeB1* and *bmeB3* genes, resulted in suppression of biofilm formation as well
as reduced the risk of antibiotic resistance.

In summary, these data show that α-humulene not only inhibits biofilm formation significantly but
also eradicates preformed mature biofilms by *B. fragilis*. However, this study does not explain the
mechanism of action. The α-humulene has been shown to induce the destruction of membrane structure
and cell wall in oral bacteria (Azizan et al. 2017). It is assumed that α-humulene may disrupt the cell
membrane structure of *B. fragilis*, which also affects the efflux pump system in the cell membrane.
Recent attention has been focused on the need for antibacterial and antibiofilm agents that can be used as alternatives to antibiotics. In particular, the development of plant extracts with antibacterial properties has attracted increased attention academically and industrially because of their safety and effectiveness. This study demonstrated effective biofilm inhibition of ETBF by plant-derived $\alpha$-humulene. The encouraging results from this work indicate that future studies in vivo would be justified.
Conflict of Interest

All authors declare no conflict of interest relevant to this article.

Acknowledgements

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Ethics approval

Not required.

Authorship

We declare that all listed authors have made substantial contributions to:

• the research design, or the acquisition, analysis or interpretation of data; and to

• drafting the paper or revising it critically;

• and that all authors have approved the submitted version.

We also declare that nobody who qualifies for authorship has been excluded from the list of authors.
References


### Table 1. Primers for real-time quantitative reverse transcription PCR

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<td>Forward 5'- AGT AGA GGT GGG CGG AAT TC -3'</td>
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<tr>
<td></td>
<td>Reverse 5'- GTG TCA GTT GCA GTC CAG TG -3'</td>
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<tr>
<td><strong>bmeB1</strong></td>
<td>Forward 5'- ACA TTG ATG TCG TCC GAT CC -3'</td>
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<tr>
<td></td>
<td>Reverse 5'- CGA TAT TCG ACA CAC GTC CT -3'</td>
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<tr>
<td><strong>bmeB3</strong></td>
<td>Forward 5'- GTA CCG GAA GTT CAA GGT GT -3'</td>
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<td>Reverse 5'- GAG CAG CCT CGA TAT TCT GT -3'</td>
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**Figure captions**

**Fig. 1.** The effect of α-humulene on bacterial cell growth. WT-ETBF, rETBF and WT-NTBF strains were grown in BHIS broth and measured according to the absorbance at 600 nm (OD$_{600}$). Data are representative of three independent experiments. Error bars are expressed as means ± standard deviation (SD). Asterisks indicate different levels of significance compared with the control (ETOH): (*) $P$ value ≤ 0.05, (**) $P$ value ≤ 0.01, (***) $P$ value ≤ 0.001 **Note:** WT-ETBF, wild-type enterotoxigenic *B. fragilis*; WT-NTBF, wild-type non-enterotoxigenic *B. fragilis* NCTC 9353; rETBF, recombinant strain overexpressing *bft* in WT-NTBF generated by the addition of *bft*-2 to WT-NTBF.
Fig. 2. The effect of α-humulene on biofilm formation. WT-ETBF, rETBF and WT-NTBF strains were grown in BHIS broth, stained with 0.5% crystal violet, and their absorbance was measured at 570 nm (OD$_{570}$). Data are representative of three independent experiments. Error bars are expressed as means ± standard deviation (SD). Asterisks indicate significant differences compared with the control (ETOH) as follows: (*) $P$ value $\leq 0.05$, (**) $P$ value $\leq 0.01$, (***) $P$ value $\leq 0.001$ Note: WT-ETBF, wild-type enterotoxigenic *B. fragilis*; WT-NTBF, wild-type non-enterotoxigenic *B. fragilis* NCTC 9353; rETBF, recombinant strain overexpressing *bft* in WT-NTBF generated by the addition of *bft*-2 to WT-NTBF.
Fig. 3. The effect of α-humulene on preformed mature *B. fragilis* biofilms. WT-ETBF, rETBF and WT-ETBF strains were grown in BHIS broth. The absorbance at 570 nm (OD$_{570}$) was measured after staining with 0.5% crystal violet to evaluate the inhibitory effect of α-humulene against preformed mature biofilms. Data are representative of three independent experiments. Error bars are indicated as means ± standard deviation (SD). Asterisks indicate significant differences compared with the control (ETOH): (*) $P$ value $\leq$ 0.05, (**) $P$ value $\leq$ 0.01, (***) $P$ value $\leq$ 0.001  

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Fig. 4. The effect of α-humulene on cell viability within *B. fragilis* biofilms. WT-ETBF, rETBF and WT-NTBF strains were grown in BHIS broth. The cell viability of biofilm treated with α-humulene was evaluated using the XTT reduction assay. Specific absorbance was expressed as OD 475 nm (Test) – OD 475 nm (Blank) – OD 650 nm (Test). Data are representative of three independent experiments. Error bars are indicated as means ± standard deviation (SD). Asterisks indicate significant differences compared with the control (ETOH) as follows: (*) *P* value ≤ 0.05, (**) *P* value ≤ 0.01, and (***) *P* value ≤ 0.001. Note: WT-ETBF, wild-type enterotoxigenic *B. fragilis*; WT-NTBF, wild-type non-enterotoxigenic *B. fragilis* NCTC 9353; rETBF, recombinant strain overexpressing *bft* in WT-NTBF generated by the addition of *bft*-2 to WT-NTBF.
Fig. 5. Confocal laser scanning microscopy (CLSM) showing the effects of α-humulene on *B. fragilis* biofilms. (a) WT-ETBF treated with 1% ETOH; (b) WT-ETBF treated with α-humulene at 1 μg/mL; (c) rETBF treated with 1% ETOH; (d) rETBF treated with α-humulene at 1 μg/mL; (e) WT-NTBF treated with 1% ETOH; (f) WT-NTBF treated with α-humulene at 1 μg/mL. Proteins, carbohydrates, and nucleic acids of biofilms were stained with FITC (green fluorescent staining), Concanavalin A (red fluorescent staining), and DAPI (blue fluorescent staining), respectively. CLSM images were observed at 40 × magnification. The scale bars represent 50 μm.
**Fig. 6.** α-humulene induced transcriptional changes in the *B. fragilis* gene associated with efflux pump.

The qRT-PCR results of (a) *bmeB1* (b) *bmeB3* genes. The transcription levels of the target genes were calculated using the $2^{-\Delta\Delta C_T}$ formula and the transcription level of 16S rRNA was used to standardize an internal housekeeping control gene. Data are representative of three independent experiments. Error bars are expressed as means ± standard deviation (SD). Levels of gene expression are indicated as fold changes with respect to non-treated cells. Asterisks indicate significant differences compared with the control (ETOH) as follows: (*) $P$ value $\leq 0.05$, (**) $P$ value $\leq 0.01$, and (***) $P$ value $\leq 0.001$ **Note:** WT-ETBF, wild-type enterotoxigenic *B. fragilis*; WT-NTBF, wild-type non-enterotoxigenic *B. fragilis* NCTC 9353; rETBF, recombinant strain overexpressing *bft* in WT-NTBF generated by the addition of *bft*-2 to WT-NTBF.
Supplementary Fig. 1. Quantification of CLSM Images. Typical images of CLSM were analyzed by Image J program. Data are representative of three independent experiments. Error bars are indicated as means ± standard deviation (SD). Asterisks indicate significant differences compared with the control (ETOH) as follows: (*) $P$ value $\leq 0.05$, (**) $P$ value $\leq 0.01$, and (***) $P$ value $\leq 0.001$
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Table 1. Primers for real-time quantitative reverse transcription PCR

<table>
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<th>Genes</th>
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| 16S rRNA | Forward: 5'-AGT AGA GGT GGG CGG AAT TC -3'  
Reverse: 5'-GTG TCA GTT GCA GTC CAG TG -3' |
| bmeB1 | Forward: 5'-ACA TTG ATG TCG TCC GAT CC -3'  
Reverse: 5'-CGA TAT TCG ACA CAC GTC CT -3' |
| bmeB3 | Forward: 5'-GTA CCG GAA GTT CAA GGT GT -3'  
Reverse: 5'-GAG CAG CCT CGA TAT TCT GT -3' |