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ORIGINAL PAPER

Antimicrobial and antibiofilm activities of *Clostridium butyricum* supernatant against *Acinetobacter baumannii*

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Abstract

Acinetobacter baumannii is the major nosocomial pathogen that causes serious infections such as ventilator-associated pneumonia and bacteremia due to its biofilms. Hence, this study investigated the antimicrobial and antibiofilm potentials of cell-free supernatants (CFS) obtained from *Clostridium butyricum*, as probiotic, against *A. baumannii*. Our results demonstrated that *C. butyricum* CFS inhibited *A. baumannii* cell growth in planktonic culture. Also, *C. butyricum* CFS not only inhibited the biofilm development and dispersed mature biofilms, but also suppressed the metabolic activity of biofilm cells, showing antibiofilm activity. The biofilm components reduced by *C. butyricum* CFS were observed via confocal laser scanning microscopy. In addition, *C. butyricum* CFS exhibited antivirulence effect by inhibiting the motility of *A. baumannii*. Furthermore, *C. butyricum* CFS significantly downregulated the expression of efflux pump-related genes including *adeA*, *adeB* and *adeC* in *A. baumannii*. Our data demonstrate that *C. butyricum* CFS showed antimicrobial and antibiofilm effects on *A. baumannii*. These effects are closely associated with suppression of motility and efflux pump-related genes in *A. baumannii*. The findings suggest that *C. butyricum* CFS can be used as a new therapeutic alternative against biofilm-associated infection caused by multidrug-resistant *A. baumannii*.

Keywords *Acinetobacter baumannii* · *Clostridium butyricum* · Probiotic · Biofilm · AdeABC efflux pump

Introduction

Acinetobacter baumannii, an opportunistic bacterial pathogen, causes a variety of nosocomial infections with high mortality rates including ventilator-associated pneumonia (VAP), bacteremia, wound and urinary tract infections in patients using contaminated catheters (Dijkshoorn et al. 2007). According to the Infectious Diseases Society of America (Boucher et al. 2009; Antunes et al. 2014), *A. baumannii* is one of the multidrug-resistant (MDR) ESKAPE pathogens (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter* species), associated with antimicrobial resistance. The excessive use of antibiotics has promoted the emergence of antibiotic-resistant *A. baumannii*, resulting in serious public health challenges worldwide (Komolafe 2003). It is extremely difficult to treat the nosocomial infection caused by biofilms due to antibiotic tolerance (Lewis 2001).

Biofilm is described as a microbial aggregate enclosed within self-released extracellular polymeric substances including proteins, polysaccharides and extracellular DNA (Hall-Stoodley et al. 2004). Biofilm is also formed in a variety of biotic or artificial abiotic surfaces and is very difficult to eradicate, resulting in severe chronic bacterial infections (Lewis 2001). Also, biofilm formation is strongly resistant to conventional antibiotics, the host immune system, and stressful environment compared with free-living cells (Gunn et al. 2016). In addition, in the case of *A. baumannii*, virulence factors such as the biofilm-associated protein (Bap), efflux system (AdeABC, AdeFGH and AdeIJK), quorum sensing system, motility by pili and porins are closely involved in biofilm formation and the strong pathogenicity of

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A. baumannii (Eze et al. 2018). Therefore, the development of new therapeutic alternatives is necessary to prevent and treat biofilm-associated infections attributed to multidrug-resistant *A. baumannii*.

Probiotics have attracted attention as safe and beneficial therapeutics (Reid et al. 2003). According to United Nations Food and Agricultural Organization (FAO)/World Health Organization (WHO) (FAO/WHO 2001), probiotics are indicated as live microorganisms that provide health benefits when administered in appropriate amounts. Previous studies demonstrated that probiotics regulate the host immune system by stimulating cytokine production and cellular activity, and inhibit the clustering of pathogens (Mendonca et al. 2012; Roy et al. 2014; Hager et al. 2019). Among the numerous probiotics, *Clostridium butyricum*, an anaerobic butyric-acid-forming bacterium, is usually found in the intestines of healthy humans or animals. *C. butyricum* is used to treat non-antimicrobial- or antimicrobial-associated diarrhea and is widely available commercially in Asia because of its probiotic properties (Cassir et al. 2016). *C. butyricum* is highly resistant to antibiotics and the lower pH of gastric acid by producing endospores (Courvalin 2006). *C. butyricum* also produces a large amount of short-chain fatty acids (SCFAs), mostly butyric acid, which promote regulatory T cell generation (Kashiwagi et al. 2015) and downregulate the expression levels of virulence genes in pathogenic bacteria (Gantois et al. 2006). Furthermore, *C. butyricum* shows antimicrobial activities against *Helicobacter pylori*, enterotoxigenic *E. coli* (ETEC), *Vibrio* spp. and *Clostridium difficile* (Kuroiwa et al. 1990; Takahashi et al. 2000, 2004).

Although various studies have investigated the probiotic effect of *C. butyricum*, the potential effects of *C. butyricum* on *A. baumannii* biofilm have yet to be reported. The aim of this study was to determine the antagonistic activity of metabolites derived from *C. butyricum* against planktonic cell growth, biofilm development and RND (resistance–nodulation–cell division)-type efflux pump-related genes of *A. baumannii*. Our findings advance the boundaries of knowledge on the potential effects of *C. butyricum* against biofilm-associated infection by multidrug-resistant *A. baumannii*.

Materials and methods

Bacterial strains, media and growth conditions

Acinetobacter baumannii ATCC 19606 and *Clostridium butyricum* ATCC 19398 (NCTC 7423, a non-toxigenic strain) were purchased from the American Type Culture Collection (ATCC). Multidrug-resistant *A. baumannii* clinical isolates P2713 (respiratory isolate) and P3000 (blood isolate) strains were obtained from the Gyeongsang National University Hospital Culture Collection. *A. baumannii* strains were cultured in trypticase soy broth (TSB; Difco, Becton–Dickinson and Company, USA) at 37 °C. *C. butyricum* ATCC 19398 was cultured in reinforced clostridial medium (RCM; Difco, Becton–Dickinson and Company, USA) at 37 °C under anaerobic conditions. All bacterial strains were preserved in a broth containing 20% (v/v) glycerol at − 80 °C until experimental use.

To prepare the cell-free supernatant (CFS), 1 mL of 1×10^8 CFU mL^{-1} *C. butyricum* culture was added to the reinforced clostridial medium and incubated anaerobically at 37 °C for 24 h. The broth was centrifuged at 3,000 rpm for 15 min at 4 °C, and the supernatant was filtered through a 0.2-µm pore size syringe filter (Advantec, Tokyo, Japan). To neutralize the effect of organic acid on the antimicrobial activity, *C. butyricum* CFS was adjusted to pH 6.5 with 1 N NaOH as previously described (Wasfi et al. 2018).

Susceptibility testing of *C. butyricum* CFS on *A. baumannii* in planktonic culture

The antimicrobial activity of CFS extracted from *C. butyricum* was evaluated via the broth microdilution method described by the Clinical and Laboratory Standards Institute (CLSI) guidelines (Clinical and Laboratory Standards Institute 2018) with a few modifications. Briefly, the overnight culture of *A. baumannii* strains was prepared in TSB medium. A reference to *A. baumannii* strains was adjusted to 5×10^5 CFU mL^{-1} and dispensed into a 96-well microtiter plate (BD Falcon™, BD, NJ, USA) with the various concentrations of *C. butyricum* CFS or adjusted *C. butyricum* CFS. The *C. butyricum* CFS or adjusted *C. butyricum* CFS was serially diluted twofold in the TSB medium. A 96-well microtiter plate was incubated at 37 °C for 24 h, followed by the analysis of cell growth in planktonic culture at 600 nm (A_{600}) wavelength through a Multiskan GO plate reader (Thermo Fisher Scientific, USA). Overnight-cultured *A. baumannii* strains served as the control.

Biofilm formation and *C. butyricum* CFS treatment

The biofilm formation assay and biofilm dispersal assay were performed in a 96-well microtiter plate as mentioned previously (Kaur et al. 2018; Kim et al. 2018), with some modifications. To determine the inhibitory effect of CFS derived from *C. butyricum* on biofilm formation, overnight-cultured bacterial suspensions were diluted to 5×10^5 CFU mL^{-1} in TSB medium. Diluted bacterial suspensions were inoculated with twofold serial dilutions of *C. butyricum* CFS into a 96-well microtiter plate, followed by incubation at 37 °C for 24 h.

To investigate the dispersal effect of *C. butyricum* CFS on mature biofilms, the bacterial suspensions were adjusted to

5×10^5 CFU mL^{-1}, dispensed into a 96-well microtiter plate and incubated at 37 °C for 24 h. Non-adherent cells were removed by washing with phosphate-buffered saline (PBS). Next, fresh TSB medium containing the twofold serial dilutions of *C. butyricum* CFS was added to the mature biofilms and incubated at 37 °C for an additional 24 h. The control was used as described above. To evaluate quantitatively the antibiofilm activity of *C. butyricum* CFS, the crystal violet assay was conducted as described in the following.

Crystal violet assay

The crystal violet assay was conducted to investigate the total biomass of biofilm quantitatively as mentioned previously (Cady et al. 2012), with a few modifications. Briefly, biofilms formed in a 96-well microtiter plate were rinsed with PBS to remove planktonic cells. Biofilms were dried at 60 °C for 1 h and stained with 1% crystal violet for 5 min. To remove the remaining dye, the stained biofilms were washed with sterile distilled water and dried at 60 °C for 1 h. At the end of drying, the stained biofilms were dissolved by adding 33% acetic acid for 15 min to extract the crystal violet. The total biomass of biofilm was quantitatively analyzed at 570 nm (A_{570}).

Quantitative analysis of biofilm metabolic activity via XTT reduction assay

The XTT [2,3-bis(2-methoxy-4-nitro-5-sulfo-phenyl)-2*H*-tetrazolium-5-carboxanilide] reduction assay was performed to evaluate the metabolic activity of *A. baumannii* biofilm cells as described previously (Pierce et al. 2008; Fischer et al. 2010), with minor modifications. Briefly, biofilms were established in a 96-well microtiter plate, as mentioned above. The twofold serial dilutions of *C. butyricum* CFS were inoculated into the well of a 96-well microtiter plate containing the established biofilms. Following incubation, *A. baumannii* biofilms were washed with PBS to remove weakly attached cells. The XTT Cell Proliferation Assay Kit (ATCC, Manassas, VA, USA) was employed to analyze the metabolic activity of *A. baumannii* biofilm cells quantitatively. The XTT and activation reagents were dispensed at a ratio of 50:1 (v/v) into the well of a 96-well microtiter plate and incubated at 37 °C in the dark for 3 h. To quantify the biofilm metabolic activity, the specific absorbances were measured at a test wavelength of 475 nm (A_{475}) and a reference wavelength of 655 nm (A_{655}), subtracting the mean background values from the data.

Confocal laser scanning microscopy (CLSM)

To perform confocal laser scanning microscopy (CLSM), the biofilm of *A. baumannii* strain was developed in the presence or absence of *C. butyricum* CFS on a tissue culture-treated 24-well glass bottom imaging plate (Eppendorf AG, Hamburg, Germany, cat. no.: 0030741021) as reported previously (Nosyk et al. 2008), with minor modifications. The biofilms were fixed using 3.7% (v/v) formaldehyde for 1 h. Subsequently, the amino groups, carbohydrates and extracellular nucleic acids were stained with fluorescein isothiocyanate isomer I (FITC, 10 µg μL^{-1}; Sigma-Aldrich, Germany), Concanavalin A-Alexa Fluor 594 conjugate (Con A, 0.1 µg μL^{-1}; C-11253, Molecular Probes, Eugene, OR, USA) and 4,6-diamidino-2-phenylindole dihydrochloride (DAPI, 1 mg L^{-1}; Molecular Probes, Eugene, OR, USA), respectively. After each staining, the biofilms were rinsed with PBS to remove the excess dye. All staining procedures were carried out in the dark room. The stained biofilms of *A. baumannii* strain were observed at laser beam wavelengths of 495, 590 and 358 nm to detect FITC, Con A and DAPI, respectively. Zeiss LSM-710 confocal laser microscope (Carl Zeiss, Thornwood, NY, USA) and ZEN software (Carl Zeiss, Thornwood, NY, USA) were utilized to visualize and image the biofilms of *A. baumannii* strain.

Motility assay

A motility assay was performed as described previously (Nait Chabane et al. 2014; Raorane et al. 2019), with a few modifications. *A. baumannii* strain was inoculated onto modified LB broth (tryptone 10 g L^{-1}; NaCl 5 g L^{-1}; yeast extract 5 g L^{-1}) containing 0.2% agar supplemented with or without twofold serial dilutions of *C. butyricum* CFS. Following inoculation, polystyrene petri dishes (SPL 10060, Pocheon, Korea, 60 × 15 mm) were incubated at 37 °C overnight. Motility was determined by measuring the diameters of turbid zone formed by bacterial cells traveling across agar plates.

Quantitative polymerase chain reaction (qPCR)

To isolate the total RNA, *A. baumannii* strains were incubated at a density of 5×10^5 CFU mL^{-1} at 37 °C for 8 h. At the end of incubation, the twofold serial dilutions of *C. butyricum* CFS were inoculated into the bacterial culture and incubated at 37 °C for additional 10 h. Each bacterial culture was centrifuged at 25,000 × g for 90 s at 4 °C to collect the *A. baumannii* cells. NucleoSpin RNA Mini Kit (Macherey–Nagel, Düren, Germany) was used to extract and purify the total RNA of *A. baumannii* strains. The redundant DNA in RNA samples was treated with RNase-free DNase to prevent DNA contamination. The concentration and quality of isolated total RNA were identified through BioDrop µLITE (BioDrop Ltd., Cambridge, UK). Total RNA (1 µg) was reverse-transcribed into cDNA, using ReverTra Ace qPCR RT Master Mix with gDNA Remover (Toyobo,

Japan) in compliance with manufacturer's recommendations. To evaluate the expression of RND-type efflux pump-related genes in *A. baumannii*, qPCR was performed using a StepOnePlus™ Real-Time PCR System (Applied Biosystems, USA). The qPCR amplification products were detected by Power SYBR® Green PCR Master Mix (Applied Biosystems, USA). The primers used this study are listed in Table 1 (Peleg et al. 2007; Coyne et al. 2010). The *16S rRNA* gene, a housekeeping gene, was used for normalization. The qPCR data were analyzed using the $2^{-\Delta\Delta CT}$ method.

Statistical analysis

All results are presented as means ± standard deviations (SD) of triplicate from three independent experiments. To compare the significant differences between the treated groups and the untreated control group, statistical analyses were carried out via one-way analysis of variance (ANOVA) with Dunnett's test using the GraphPad Prism, version 5 (GraphPad Software, CA, USA). The Student's *t* test was used to analyze the qPCR data. A value of $*p<0.05$, $**p<0.01$ and $***p<0.001$ was considered statistically significant.

Results

Antimicrobial activity of *C. butyricum* CFS on planktonic cell growth of *A. baumannii*

The antimicrobial activity of *C. butyricum* CFS against *A. baumannii* strains was evaluated at 600 nm (A_{600}). In the presence of 50% *C. butyricum* CFS, the planktonic cell growth of *A. baumannii* ATCC 19606, clinical isolates P2713 and P3000 strains was drastically inhibited by 98.51%, 99.22% and 98.6%, respectively (Fig. 1a–c). Although 12.5% and 25% *C. butyricum* CFS were less

Table 1 Primer sequences used for qPCR

Primer	Primer sequence (5′–3′)	References
16S rRNA	Forward CAGCTCGTGTCGTGAGATGT Reverse CGTAAGGGCCATGATGACTT	Peleg et al. (2007)
adeA	Forward ATCGCTAACAAAGGCTTGAA Reverse CGCCCCCTCAGCTATAGAA	Coyne et al. (2010)
adeB	Forward CTTGCATTTACGTGTGGTGT Reverse GCTTTTCTACTCCACCCAAA	Coyne et al. (2010)
adeC	Forward TACACATGCGCATATTGGTG Reverse CGTAAAATAACTATCCACTCC	Coyne et al. (2010)

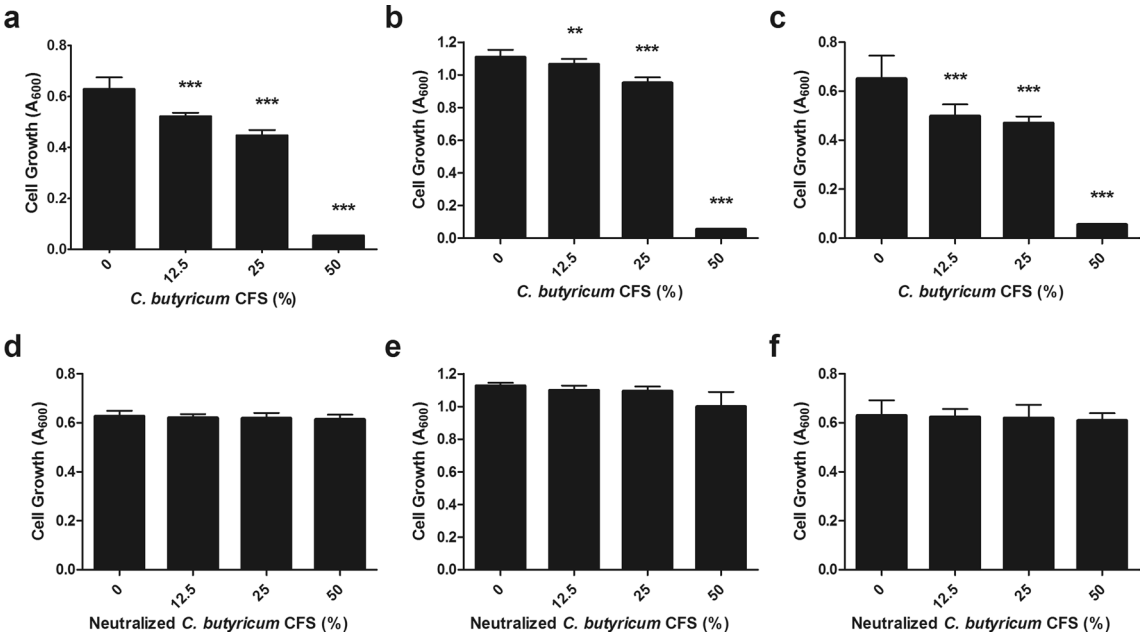

Fig. 1 Antimicrobial activity of *C. butyricum* CFS against the growth of *A. baumannii* in planktonic culture. *A. baumannii* ATCC 19606 (**a**, **d**), clinical isolates P2713 (**b**, **e**) and P3000 (**c**, **f**) strains were incubated following treatment with *C. butyricum* CFS (**a**–**c**) or adjusted *C. butyricum* CFS to pH 6.5 (**d**–**f**) at 37 °C for 24 h. The growth of *A. baumannii* in planktonic culture was measured at A_{600} using microplate spectrophotometers. The results are expressed as means ± standard deviations (SD). ** and *** denote significant differences at $p<0.01$ and $p<0.001$, respectively

effective, these concentrations showed a statistically significant reduction in planktonic cell growth of *A. baumannii* strains. Cassir et al. reported that a bacteriocin-like inhibitory substance produced by *C. butyricum* exhibited antimicrobial effects against several organisms (Cassir et al. 2016). Thus, our study investigated the antimicrobial effect of bacteriocin-like inhibitory substance on *A. baumannii*. According to a previous study, neutralized probiotic CFS had a similar effect to bacteriocin-like inhibitory substance (Kim and Kang 2019). Based on previous study, *C. butyricum* CFS was neutralized to pH 6.5 to eliminate the action of organic acids. Unexpectedly, the treated *C. butyricum* CFS did not show any significant effects against planktonic cell growth of *A. baumannii* strains (Fig. 1d–f). The data showed that the neutralized *C. butyricum* CFS exhibited less antimicrobial activity than *C. butyricum* CFS against *A. baumannii* (Fig. 1), indicating that the bacteriocin-like inhibitory substance was not involved in significant effects against planktonic cell growth of *A. baumannii*.

Inhibitory effect of *C. butyricum* CFS on *A. baumannii* biofilm formation

The inhibitory effect of *C. butyricum* CFS on biofilm development of *A. baumannii* strains was quantitatively determined using the crystal violet assay. As shown in Fig. 2a, ATCC 19606 strain was suppressed by 33.97%, 43.17% and 99.65% following treatment with 12.5%, 25% and 50% *C. butyricum* CFS, respectively. Biofilm formation of clinical isolate P2713 strain was inhibited 24.43%, 28.29% and 93.69% (Fig. 2b) and that of P3000 strain was also suppressed by 30.92%, 36.45% and 97.06% (Fig. 2c) in the presence of 12.5%, 25% and 50% *C. butyricum* CFS, respectively. The results showed that *C. butyricum* CFS significantly inhibited the biofilm development of all *A. baumannii* strains used in this study in a dose-dependent manner (Fig. 2).

Dispersal effect of *C. butyricum* CFS on mature biofilms of *A. baumannii*

The dispersal of mature biofilm is an important strategy for controlling biofilms. The *C. butyricum* CFS had the potential to disperse mature biofilms as well as to inhibit biofilm development of *A. baumannii*, as described above. The *C. butyricum* CFS (12.5–25%) disrupted the mature biofilms of *A. baumannii* ATCC 19606, clinical isolates P2713 and P3000 strains by 33.57–66.5%, 24.02–28.44% and 63.27–84%, respectively. Notably, the mature biofilms of ATCC 19606, clinical isolates P2713 and P3000 strains were significantly eradicated by 82.47%, 80.52% and 92.66% following exposure to 50% *C. butyricum* CFS, respectively (Fig. 3).

Effect of *C. butyricum* CFS on the metabolic activity of *A. baumannii* biofilm cells

To demonstrate the antibiofilm effect of *C. butyricum* CFS, the metabolic activity of *A. baumannii* biofilm cells was analyzed via XTT reduction assay. The specific absorbance values were calculated using the results of testing samples and the background blank (Huyck et al. 2012). In the presence of 25% *C. butyricum* CFS, the metabolic activity of the clinical isolate P2713 was inhibited by only 47%, less than that of the other strains. However, 50% *C. butyricum* CFS decreased the metabolic activity of all *A. baumannii* strains by 92.93–100% (Fig. 4). As illustrated in Fig. 4, the data suggested that the *C. butyricum* CFS effectively inhibited the metabolic activity of *A. baumannii* biofilm cells as well as suppressed and dispersed the biofilm by *A. baumannii*.

Confocal laser scanning microscopy

To evaluate the antibiofilm activity of *C. butyricum* CFS against *A. baumannii*, the most abundant biofilm of the

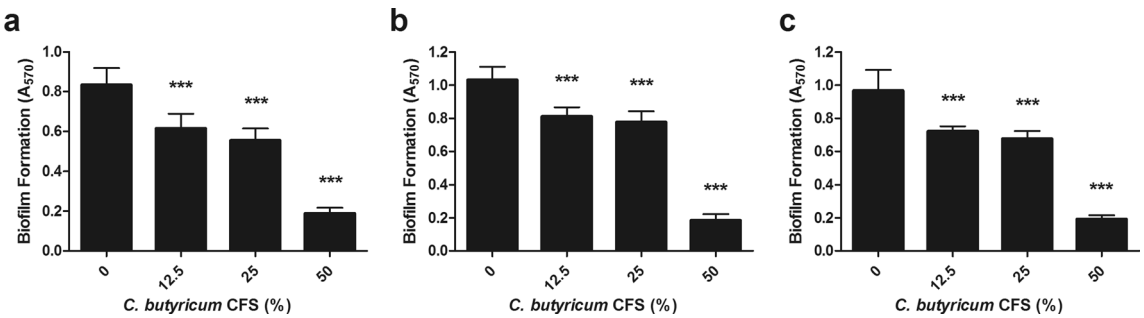

Fig. 2 Inhibitory effect of *C. butyricum* CFS on biofilm development of *A. baumannii*. *A. baumannii* ATCC 19606 (**a**) and clinical isolates P2713 (**b**) and P3000 (**c**) strains were incubated with *C. butyricum* CFS at 37 °C for 24 h. Biofilms of *A. baumannii* strains were stained with 1% crystal violet and analyzed by measuring the absorbance at A_{570}. The results are expressed as means ± standard deviations (SD). *** denotes significant differences at $p < 0.001$

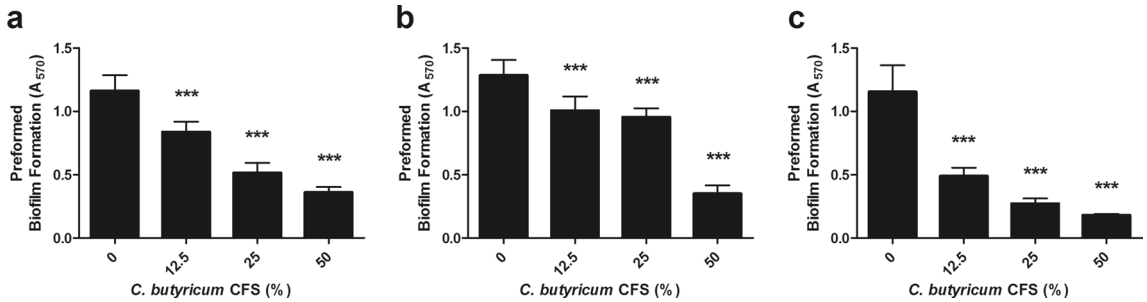

Fig. 3 Dispersal effect of *C. butyricum* CFS on mature biofilms of *A. baumannii*. Following exposure to *C. butyricum* CFS, the mature biofilms of *A. baumannii* ATCC 19606 (**a**) and clinical isolates P2713 (**b**) and P3000 (**c**) strains were incubated at 37 °C for 24 h. The mature biofilms of *A. baumannii* strains were stained with 1% crystal violet and assessed by measuring at A_{570}. The results are expressed as means ± standard deviations (SD). *** denotes significant differences at $p < 0.001$

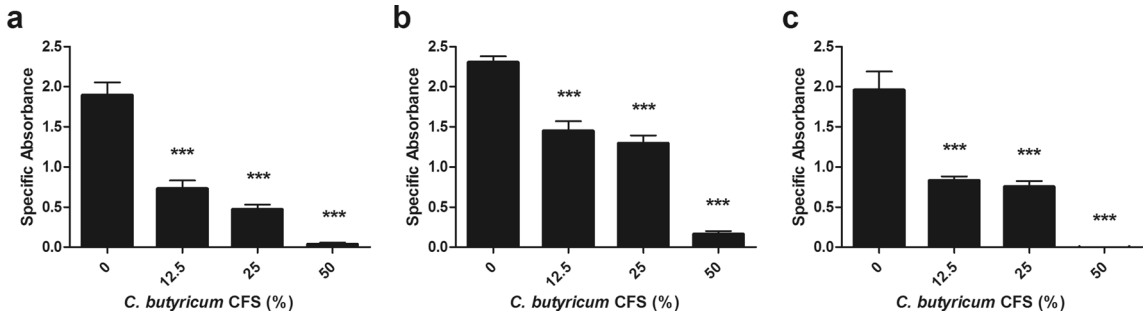

Fig. 4 Effect of *C. butyricum* CFS on the metabolic activity of *A. baumannii* biofilm cells. The established biofilms of *A. baumannii* ATCC 19606 (**a**) and clinical isolates P2713 (**b**) and P3000 (**c**) strains were incubated in the presence of *C. butyricum* CFS at 37 °C for 24 h. The metabolic activity of *A. baumannii* biofilm cells was analyzed via XTT reduction assay. Specific absorbance was expressed as A_{475} (Test)—A_{475} (Blank)—A_{655} (Test). The results are expressed as means ± standard deviations (SD). *** describes significant differences at $p < 0.001$

clinical isolate P2713 was analyzed using CLSM. As shown in Fig. 5a, the untreated control showed noticeable bacterial cell aggregates and massive amounts of extracellular matrix in *A. baumannii* biofilms. In the presence of 12.5% and 25% *C. butyricum* CFS, *A. baumannii* biofilms dose-dependently reduced the biomass concentration and thickness and showed a poorly developed architecture (Fig. 5b–c). As shown in Fig. 5, a significant reduction in biofilm integrity, especially carbohydrates and proteins, was observed upon treatment with *C. butyricum* CFS when compared with the untreated group. This result suggests that CFS collected from *C. butyricum* showed a reduction in biomass and thickness, and structural disintegration of *A. baumannii*.

Inhibition of motility by *C. butyricum* CFS treatment

The antivirulence effect of *C. butyricum* CFS on the motility of *A. baumannii* was determined using a semisolid agar. The clinical isolate P2713 strain formed the most abundant biofilm used in the motility assay. The clinical isolate P2713 was active and motile with a mean diameter of 5.45 ± 0.05 cm in the turbid zone for non-treated groups. Following exposure to 12.5% and 25% *C. butyricum* CFS, the diameters of turbid zone were 4.05 ± 0.15 cm and 1.95 ± 0.35 cm, respectively. In the presence of 50% *C. butyricum* CFS, the motility of *A. baumannii* was completely inhibited (Fig. 6). Results showed that *C. butyricum* CFS significantly suppressed bacterial migration, in a dose-dependent manner.

Effect of *C. butyricum* CFS on the expression of RND-type efflux pump-related genes in *A. baumannii*

qPCR was used to evaluate the changes in transcriptional levels of RND-type efflux pump-related genes in *A. baumannii*. In the presence of 50% *C. butyricum* CFS, the expression of *adeA* gene in *A. baumannii* ATCC 19606, clinical isolates P2713 and P3000 strains was decreased by 106.99-fold, 37.89-fold and 9.08-fold, respectively (Fig. 7a). Also, treatment with 50% *C. butyricum* CFS downregulated the expression of *adeB* gene in ATCC 19606, clinical isolates P2713 and P3000 strains by 173.67-fold, 88.39-fold and 16.66-fold, respectively (Fig. 7b). Furthermore, the expression of *adeC* gene in ATCC 19606, clinical isolates P2713

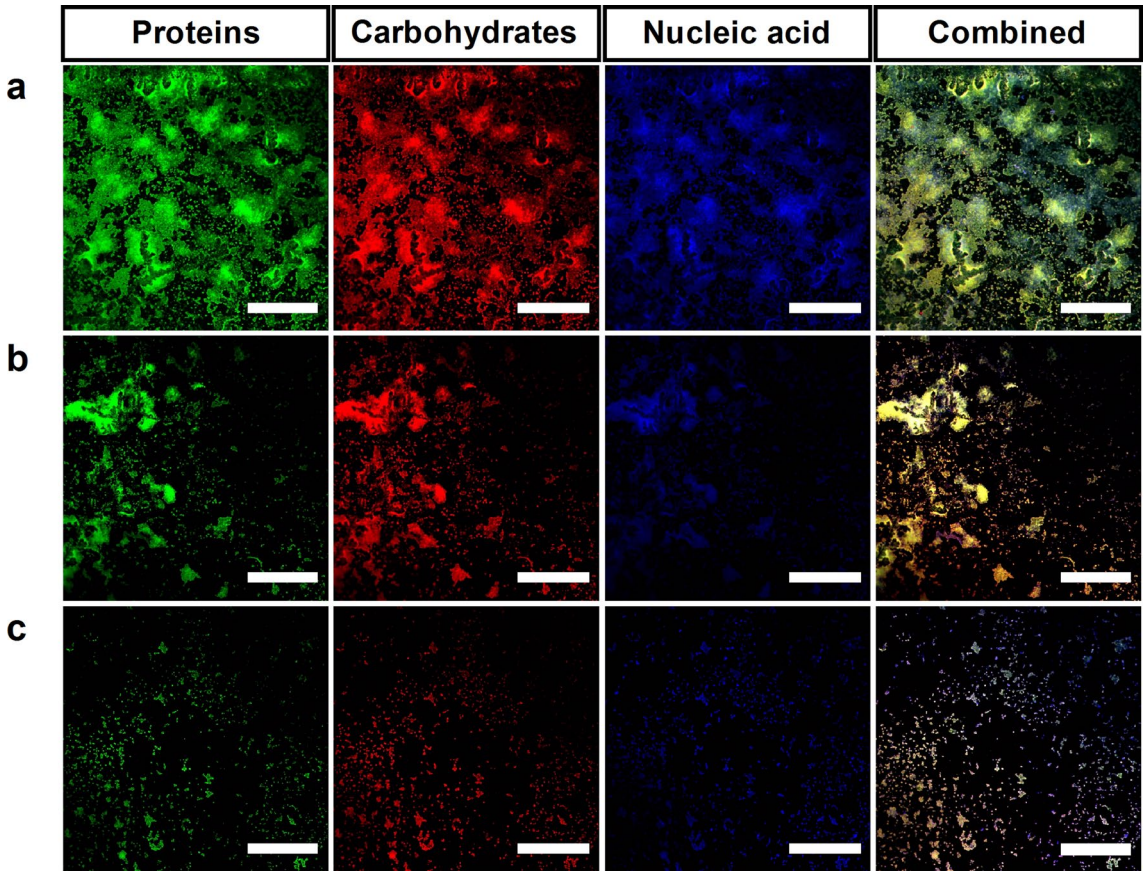

Fig. 5 Confocal laser scanning micrographs of *A. baumannii* biofilms. **a** Non-treated biofilms; (**b**) biofilms treated with 12.5% *C. butyricum* CFS; (**c**) biofilms treated with 25% *C. butyricum* CFS. Proteins, carbohydrates and nucleic acids in *A. baumannii* biofilm were visualized by staining with FITC (green fluorescent), Con A (red fluorescent), and DAPI (blue fluorescent), respectively. *A. baumannii* biofilms were observed at ×40 magnification. The scale bar indicates 50 µm

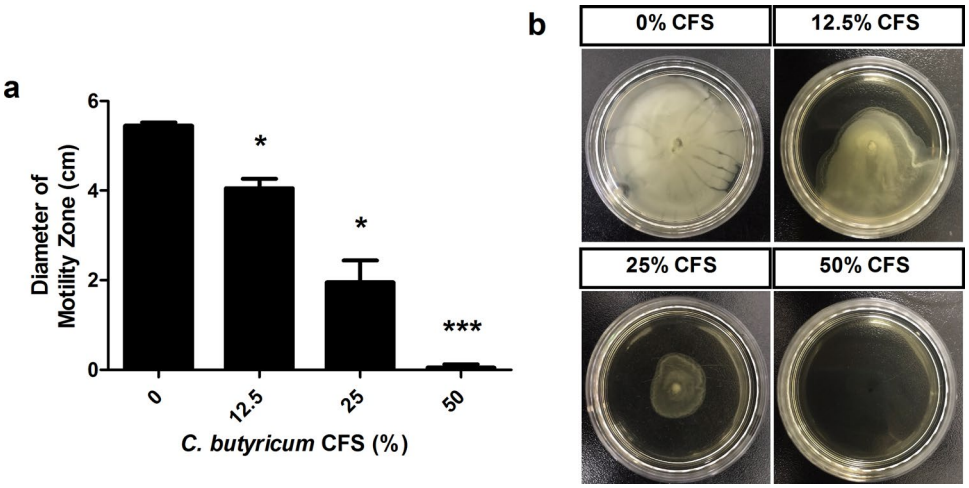

Fig. 6 Antivirulence effect of *C. butyricum* CFS on *A. baumannii* motility. **a** Mean diameters of twitch colonies (cm); **b** twitch colonies of *A. baumannii* cells. *A. baumannii* was inoculated onto LB plates containing 0.2% agar and *C. butyricum* CFS. The results are expressed as means ± standard deviations (SD). * and *** denote significant differences at $p < 0.05$ and $p < 0.001$, respectively

and P3000 strains was suppressed by 286.95-fold, 98.82-fold and 11.22-fold, respectively (Fig. 7c). As shown in Fig. 7, the expression of RND-type efflux pump-related genes in all strains used in the study was significantly downregulated by treatment with *C. butyricum* CFS in a dose-dependent

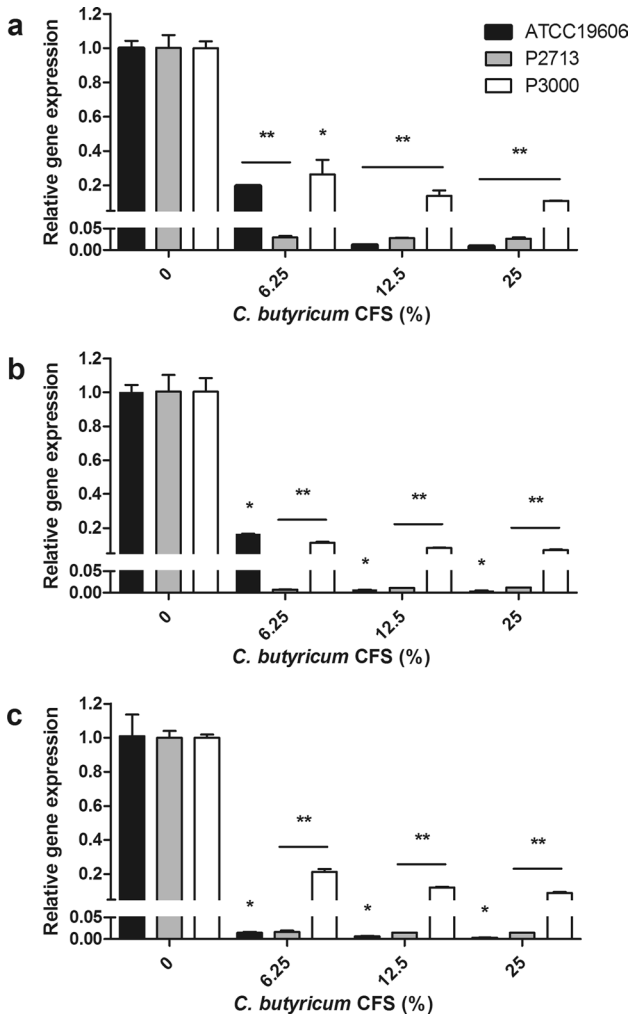

Fig. 7 qPCR analysis of RND-type efflux pump-related gene *adeA* (**a**), *adeB* (**b**) and *adeC* (**c**) in *A. baumannii*. Gene expression of *A. baumannii* ATCC 19606 (black bar) and clinical isolates P2713 (gray bar) and P3000 (white bar) strains. The samples were normalized to compare the relative expression levels using housekeeping gene, *16S rRNA*. The data are expressed as fold changes and analyzed by Student's *t*-test to compare the gene expression between treated and non-treated groups. The results are expressed as means ± standard deviations (SD). * and ** denote significant differences at $p < 0.05$ and $p < 0.01$, respectively

manner. The result was consistent with the results obtained above.

Discussion

C. butyricum secretes a variety of antimicrobial substances, such as bacteriocins called butyricin, as well as organic acids, mainly butyric acid (Cassir et al. 2016). Bacteriocins are described as antibacterial peptides that possess killing or inhibiting action against the growth of closely related bacteria (Silva et al. 2018). Bacteriocins released by *C. butyricum* are bactericidal against a variety of bacteria, except gram-negative bacteria (Cassir et al. 2016). Consistent with previous studies, our study found that the bacteriocin of *C. butyricum* did not exhibit a strong antimicrobial effect against *A. baumannii*, gram-negative bacteria. Therefore, our findings suggest that planktonic cell growth of *A. baumannii* was inhibited by organic acids formed by *C. butyricum* rather than bacteriocin-like inhibitory substance.

Butyric acid, which constitutes the majority of the short-chain fatty acids (SCFAs) secreted by *C. butyricum*, exhibits amphipathic properties (Gill et al. 2018). The amphipathic interaction between the biofilm and butyric acid is facilitated by the significant water content (97%) of the biofilms (Cordeiro et al. 2019). CFS derived from *C. butyricum* is more effectively absorbed in biofilms of *A. baumannii*. Based on these studies, our findings suggest that the antimicrobial and antibiofilm effects of *C. butyricum* CFS are related to action of butyric acid.

The experiment used *A. baumannii* strains isolated from patients with respiratory system and bloodstream infections, which account for the largest proportion of *A. baumannii* infection. According to Saranya et al., the respiratory isolates formed robust and thicker biofilm compared with the blood isolates (Vijayakumar et al. 2016). Similarly, our data also showed that P2713 strain (a respiratory isolate) produced more biofilm than P3000 strain (a blood isolate).

The biofilm formation of *A. baumannii* is associated with various virulence factors. The biosynthesis of pili in *A. baumannii* is mediated via expression of csuA/BABCDE chaperone–usher assembly system, which is essential for twitching motility (Luo et al. 2015). The simultaneous expression of pili and twitching motility facilitates the adherence of *A. baumannii* to abiotic surfaces and occurs in the early stage of biofilm development (Tomaras et al. 2003; Luo et al. 2015). Based on these studies, our study suggests that the antibiofilm effect of *C. butyricum* CFS is closely associated with the inhibition of motility in *A. baumannii*. However, the mechanism of biofilm development and motility of *A. baumannii* has yet to be clearly identified and requires further study.

The RND-type efflux systems, another virulence factor, play key roles in gram-negative bacteria: (1) resistance to antibiotic and antibacterial substances; (2) modulation of virulence factors involved in the expression of quorum sensing systems; (3) neutralization of intracellular metabolites; and (4) regulation of cellular homeostasis and intercellular communication (Beceiro et al. 2013). The RND-type efflux systems in *A. baumannii* are classified into AdeABC, AdeFGH and AdeIJK types. Among the three RND-type efflux systems, the AdeABC is the major efflux pump system related to antibiotic resistance of *A. baumannii*. The AdeABC efflux pump system consists of three components: AdeA, which synthesizes the inner membrane fusion protein;

AdeB, which produces the *trans*-membrane segment; and AdeC, which generates the outer membrane protein channel (Marchand et al. 2004). The structural genes promote the efflux of the drug out of the cell across the inner and outer membranes, resulting in resistance to various antibiotics (Modarresi et al. 2015). A previous study reported that biofilm formation in *A. baumannii* closely involves the genes encoding RND-type efflux pump system (He et al. 2015). More specifically, the overexpression of AdeABC efflux pump genes contributed to increased biofilm development (He et al. 2015; Yoon et al. 2015), whereas the downregulation of AdeABC efflux pump genes was associated with decreased biofilm, along with the increase in antimicrobial susceptibility and reduction in virulence factors (Richmond et al. 2016). Consistent with previous studies, our findings suggest that *C. butyricum* CFS suppresses the *A. baumannii* biofilm by inhibiting the expression of RND-type efflux pump-related genes, indicating that the expression of AdeABC efflux pump genes is closely related to *A. baumannii* biofilm formation.

In conclusion, our findings demonstrate that CFS derived from *C. butyricum* exerts antimicrobial and antibiofilm effects on *A. baumannii*. Also, these effects are closely related to the inhibition of motility and RND-type efflux pump-related *adeABC* genes in *A. baumannii*. This study reinforced the value of *C. butyricum* as a probiotic and suggested the potential of *C. butyricum* as a new therapeutic alternative against *A. baumannii.* However, further studies are needed to determine the mechanisms of *C. butyricum* CFS regulating *A. baumannii* biofilms. Such studies expand our insight into the development of new antimicrobial and antibiofilm agents to treat biofilm-associated infection by multidrug-resistant *A. baumannii*.

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Compliance with ethical standards

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

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