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Camel milk whey hydrolysate inhibits growth and biofilm formation of *Pseudomonas aeruginosa* PAO1 and methicillin-resistant *Staphylococcus aureus*

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Abstract

Pseudomonas aeruginosa PAO1 and Methicillin-Resistant *Staphylococcus aureus* (MRSA) are amongst the most virulent pathogens, causing chronic and life-threatening human infections. Thus, novel natural compounds able to inhibit these pathogens, reduce and/or eradicate their biofilms are in high demand. Camel milk has been demonstrated to contain many functional and bioactive molecules and has consequently been considered in various therapeutic applications. This study aimed to assess the antibacterial and antibiofilm activities of the camel milk whey proteins after hydrolysis by papain, and the obtained fractions from size exclusion chromatography (SEC) against PAO1 and MRSA. Antibacterial activity of camel milk whey against PAO1 and MRSA was enhanced by hydrolysis with papain. Size-exclusion fraction 2 (SEC-F2) had significantly ($P < 0.01$) the highest antibacterial activity against PAO1 and MRSA with a minimum inhibitory concentration of 0.156 and 0.3125 mg/mL, respectively. Additionally, SEC-F2 significantly ($P < 0.01$) decreased the biofilm biomass by 60.45 % and 85.48 % for PAO1 and MRSA, respectively. Moreover, SEC-F2 potentially reduced the PAO1 and MRSA biofilms depending on its concentrations. Scanning electron microscopy showed that the SEC-F2 fraction caused potential morphological changes in both PAO1 and MRSA, mostly represented in cell elongation and leakage of cytoplasmic content. In conclusion, this study has demonstrated that hydrolysis of camel milk whey with papain generates robust antibacterial and antibiofilm small-peptides against PAO1 and MRSA.

Key words: Camel milk whey; papain; antibacterial activity; antibiofilm

Abbreviations

MRSA, Methicillin-Resistant *Staphylococcus aureus*; PAO1, *Pseudomonas aeruginosa* PAO1; SEC-F1 & SEC-F2, Size-exclusion fraction 1 & 2; CMW, Camel milk whey; CMWH, Camel milk whey hydrolysates; MIC, Minimum inhibitory concentration; MBC, minimum bactericidal concentration.

1. Introduction

Extensive use and misuse of antibiotics in both human and animal medicine has led to an escalating challenge with circulating multidrug resistant bacterial strains. Amongst the most virulent and problematic pathogens, causing life-threatening chronic planktonic and biofilm related infections are *Pseudomonas aeruginosa* and *Staphylococcus aureus*. When living in a biofilm, these and other bacterial species protect themselves from environmental challenges, nutritional depletion and antibiotics (Bassetti, Vena, Croxatto, Righi, & Guery, 2018; Tong, Davis, Eichenberger, Holland, & Fowler, 2015), in part due to formation of dormant persister cells, not affected by conventional antibiotics. New treatment strategies affecting both resistant strains but also targeting persister cells and bacterial biofilms are therefore in crucial demand.

Inhibition of biofilm formation and reduction of pre-formed biofilms by the antimicrobial peptide have successfully been reported (Dawgul, MacIejewska, Jaskiewicz, Karafova, & Kamysz, 2014). It is known that milk proteins are a good source of antimicrobial peptides (Jenssen, 2005; Jenssen, & Hancock, 2009; Mohanty et al., 2016). In parallel to more studies human and bovine milk, camel milk also possesses a potent antimicrobial capacity due to its higher content of lactoferrin and lysozyme in particular (Al haj & Al Kanhal, 2010; Dheeb, Al-Mudallal, & Salman, 2016; Farnaud & Evans, 2003). Recent work has demonstrated that hydrolysis of camel milk proteins generates a mixture of bioactive peptides with activities including; antioxidant, anti-hypertensive, anti-diabetic and antimicrobial properties (Abdel-Hamid, Goda, De Gobba, Jenssen, & Osman, 2016; Alhaj et al., 2018; Jrad et al., 2014; Kumar, Chatli, Singh, Mehta, & Kumar, 2016). Hydrolysis by chymotrypsin, trypsin, proteinase K or papain enhanced the antibacterial activity of camel whey proteins against planktonic *Escherichia coli, S. aureus*, *Bacillus cereus,* and *Salmonella typhimurium* (Abdel-Hamid et al., 2016; Salami et al., 2010).

Bovine lactoferrin have been reported to affect bacterial biofilms of *P. aeruginosa*. (Kamiya, Ehara, & Matsumoto, 2012), while donkey lactoferrin are active against *Serratia liquefaciens* (Mahdi, Zaki, Salman, & Zwain, 2017). Antibiofilm activity against *Candida parapsilosis* (Fais et al., 2017) and *Klebsiella pneumonia* (Morici et al., 2017) has also been reported for hLF1-11, a short N-terminal derived peptide from human lactoferrin. Xu *et al.* (2010) has reported that lactoferrin derived peptides and a lactoferricin chimera could inhibit *P. aeruginosa* biofilm formation. In addition, the κ-casein macropeptide at concentration down to 0.4 mg/mL could

inhibit the formation of biofilm by *Listeria monocytogenes* (Yun, Kim, Park, Kim, & Oh, 2014). Furthermore, lactoferrin and peptide derivatives have also been investigated for their potent *in vitro* and *in vivo* antimicrobial activities against MRSA (Yamauchi, Tomita, Giehl, & Ellison, 1993). However, the effect of camel milk whey proteins and hydrolysed peptide fragments on bacterial biofilms have not been investigated, despite the fact that it has already been demonstrated that papain hydrolysed camel whey protein possess antibacterial activity against Gram-positive and Gram-negative bacteria (Abdel-Hamid et al., 2016). Therefore, the aim of this work was to further evaluate the antibiofilm and antibacterial mechanisms of fractionated papain hydrolysed camel milk whey protein against *P. aeruginosa* and Methicillin-resistant *Staphylococcus aureus* (MRSA).

2. Material and Methods

2.1. Bacterial strains and chemicals

Pseudomonas aeruginosa PAO1 (H103 wild type) and methicillin-resistant *Staphylococcus aureus* (MRSA; C623) (Cherkasov et al., 2009) were obtained from the Department of Science and Environment, Roskilde University, Denmark. Ampicillin (A9518) was purchased from Sigma Aldrich (Brøndby, Denmark).

2.2. Camel milk whey hydrolysate and size exclusion fraction

Lyophilized samples of camel milk whey (CMW), camel milk whey hydrolysate (CMWH; 27 % degree of hydrolysis) and the two size exclusion chromatography fractions (SEC-F1 and SEC-F2) obtained from our previous study by Abdel-Hamid et al. (2016) were used for this study. In brief, the lyophilized CMW was hydrolyzed by papain (E/S ratio of 1:200, w/w) for 4 h at 37 °C and pH 6.0. The degree of hydrolysis was 27% as previously determined (Adler-Nissen, 1986). CMWH was fractionated by size exclusion chromatography (SEC) as described by Abdel-Hamid et al. (2016).

2.3. Antibacterial activity

The antibacterial activity of CMWH and its size exclusion fractions was assessed against PAO1 and MRSA using the disc diffusion assay as described by Abdel-Hamid et al. (2016). Briefly, the overnight cultures of bacteria were diluted to reach 6 log CFU/mL, and spread on Mueller Hinton agar plates, followed by deposition of fifteen µl drops of CMW, CMWH, SEC-

F1 and SEC-F2 at concentration 10 mg/mL. The plates were incubated at 37°C for 48 h before the diameter (mm) of the clear zone was recorded.

2.4. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

MIC and MBC were determined according to standard methods (Saporito, Vang Mouritzen, Løbner-Olesen, & Jenssen, 2018) in three biological replicates. PAO1 and MRSA were inoculated into 10 mL Mueller Hinton broth and incubated overnight at 37 °C in a shaking water bath. For the MIC assay, the overnight cultures were diluted 1:100 in fresh Mueller Hinton broth, incubated at 37 °C to reach an OD of 0.4 at 600 nm and eventually diluted (1:500) to get a final inoculum of ~5×10^5 CFU/mL. Ninety µL of the diluted cultures were pipetted into 96-well round-bottom microtiter plates prefilled with 10 µL of two-fold serial dilutions of the tested samples. The plates were incubated for 48 h at 37 °C. The MIC value was recorded as the lowest concentrations of the test samples able to inhibit visible bacterial growth. Content of the wells with no visible growth were spread on agar plates and incubated for 24 h at 37 °C. Plates with lowest concentration and no visible growth were scored as MBC.

2.5. Biofilm inhibition activity

Antibiofilm activity was assessed according to the protocol adopted by Saporito *et al.* (2018). Briefly, overnight cultures of PAO1 and MRSA were diluted 1:100 before inoculating 90 µL of bacterial suspension in a microtiter plate prefilled with 10 µl of SEC-F2 at concentrations equal to 1 × MIC, 1/10 × MIC and 1/100 × MIC. In the control wells, 10 µL of MQ-water were added instead of the sample. After incubation for 24 h at 37 °C, the supernatant fluids were removed and the wells were washed gently twice with 150 µL/well of phosphate buffered saline (PBS) to remove planktonic bacteria and cellular debris. The attached biofilms were stained by adding 125 µL/well of crystal violet (0.1% w/v in water) and incubating for 10 minutes at room temperature. The excess dye was removed by a washing step with PBS and the stained biofilm was dissolved by adding 200 µL/well of ethanol (96%) for 10 minutes. Eventually, 100 µL of each well was transferred to a clean flat bottom microtiter plate and the absorbance at 595 nm was recorded in a microplate reader (Synergy HT, BioTek).

The percent of biofilm inhibition was calculated by comparing the optical density values for the treated samples and the untreated control (Saporito *et al.*, 2018), as per the formula:

$$\text{Biofilm Inhibition (\%)} = \frac{OD_{595}\ \text{control} - OD_{595}\ \text{sample}}{OD_{595}\ \text{control}} \quad X\ 100$$

137

138

139 **2.6. Biofilm reduction assay**

140 Bacterial biofilm was formed as described in section 2.5. After 24 hours incubation the
141 biofilm was washed three times with PBS to remove any residual planktonic cells or cellular
142 debris from the plate wells. Next, a twofold dilutions series was prepared with SEC-F2 in Muller
143 Hinton broth and added to the wells. Mueller Hinton broth without SEC-F2 was added as a
144 positive biofilm control. The microtiter plates were incubated for 16 h at 37 °C, and then gently
145 washed, stained and measured at 595 nm as described in section 2.5. Biofilm reduction in % was
146 calculated as following:

$$\text{Biofilm Reduction (\%)} = \frac{OD_{595}\ \text{control} - OD_{595}\ \text{sample}}{OD_{595}\ \text{control}} \quad X\ 100$$

147

148 **2.7. Bacterial growth monitoring**

149 The bacterial growth was monitored using a microtiter plate assay (Godballe, Mojsoska, Nielsen,
150 Jenssen, 2016). In short, overnight cultures of PAO1 and MRSA were diluted with Mueller
151 Hinton broth to reach an optical density of 0.1 at 600 nm. Then, 90 µL/well of the diluted
152 cultures was inoculated into microtiter plates prefilled with 10 µL of SEC-F2 at concentrations
153 corresponding to 1 × MIC, 2 × MIC and 4 × MIC. The plates were incubated for 6 h at 37 °C
154 with periodical 5 minutes shaking prior to each reading and the OD_{600} was recorded by the
155 microplate reader every 30 min.

156 **2.8. Scanning Electron Microscopy (SEM)**

157 The ultrastructural and morphological changes in PAO1 and MRSA caused by SEC-F2 were
158 examined using the FEI Helios dual beam scanning electron microscope and in accordance with
159 standard protocols (Mojsoska, Carretero, Larsen, & Mateiu, 2017). Briefly, PAO1 and MRSA
160 were treated with 1 × or 4 × MIC concentrations of SEC-F2 for 2.5 h at 37 °C, then centrifuged

at 10 000 × g for 5 minutes. The bacterial pellets were fixed with 2% Glutaraldehyde in PBS, pH 7.3 at 4 °C for 16 h. The pellets were washed three times with distilled water and then post-fixed with 1% aqueous OsO_4, at 4 °C for 16 h. The pellets were rewashed three times with distilled water. The samples were then dehydrated in serial dilutions of ethanol (30%, 50%, 70%, 80%, 90%, 96% and 100 %) followed by serial dilutions of acetone (30%, 50% and 100%) at 25 °C for 10 minutes in each dilution. Samples were then dried to critical point in an Automated Critical Point Dryer (Leica EM CPD300, GmbH, Mannheim, Germany). Finally, samples were mounted on aluminum stub and platinum coated in a High Resolution Sputter Coater (Cressington 208HR, Cressington Scientific Instruments, UK) and examined by SEM at 2 KV. For the size analysis, FIJI (NIH public domain) was used (Schindelin et al., 2012).

2.9. Statistical analysis

Analysis of variance (ANOVA) was performed by Minitab® 18.1 (MINITAB Inc., Coventry, UK), using the general linear model (GLM) procedure and Tukey's test for pairwise comparison. All tests were performed in triplicate and the results were presented by the mean values ± standard deviation (SD).

3. Results and discussion

3.1. Antibacterial activity

The antibacterial activity of camel milk whey (CMW), camel milk whey hydrolysates (CMWH) and size exclusion fractions (SEC-F1 and SEC-F2) are presented in Table 1. No antibacterial activity of CMW at concentration of 10 mg/mL was observed against PAO1 and MRSA. Although, camel milk has showed antibacterial activity against various pathogenic and spoilage bacteria due to its higher content of lysozyme and lactoferrin (Alhaj et al., 2018), no activity was observed for CMW against PAO1 and MRSA in current work. In this context, Alhaj et al. (2018) reported that camel milk showed no antibacterial activity against *Bacillus cereus*, *Salmonella Typhimurium* and *S. aureus*, whereas Abdel-Hamid et al. (2016) reported that camel milk whey proteins exhibited antibacterial activity against *S. aureus* at concentration of 10 mg/mL. Additionally, camel milk proteins, camel colostrum proteins and whey proteins at concentration of 40, 20, 40 mg/mL, respectively, exhibited antibacterial activity against *E. coli and Listeria innocua* as reported by Jrad et al. (2014). These findings demonstrate that the antibacterial activity of camel milk is protein concentration and bacterial type dependent. As it

can be seen in Table 1, the hydrolysis of camel milk whey by papain for 4 h has shown a highly significant ($P < 0.01$) impact on the antibacterial activity against PAO1 and MRSA, while no inhibition zone was noticed for camel milk whey treatment (CMW). It is worth noting that the antibacterial activity of CMWH against PAO1 was significantly ($P < 0.01$) higher than that for MRSA. This may be attributed to the different membrane composition of PAO1 and MRSA. In this context, it should be noted that the antibacterial compounds must diffuse across the peptidoglycan and then act with the cytoplasmic membrane in order to inhibit the growth of Gram-positive rod shaped bacteria. Whereas, to kill the Gram-negative bacteria, the antibacterial peptides need to permeabilize the outer membranes (Li et al., 2017). The peptide resulted from camel milk whey hydrolysed by papain was able to permeabilize or disrupt the outer membrane of PAO1 (see SEM section 3.6). This may indicate that camel whey protein contains antibacterial peptide fragments which are released upon proteolysis. This is corroborated by the fact that camel milk whey mainly contains α-Lactalbumin, immunoglobulins, and lactoferrin (Al haj & Al Kanhal, 2010), the latter being a source of antimicrobial peptides like; LF1-11, lactoferrampin and lactoferricin (Sinha, Kaushik, Kaur, Sharma, & Singh, 2013). Our results are in agreement with those of Jrad et al. (2015) who reported that the antibacterial activity of camel milk casein increases via hydrolysis with pepsin or pancreatin. Furthermore, camel milk casein hydrolysed with Alcalase, α-chymotrypsin or papain exhibited antibacterial activity against *E. coli*, *B. cereus*, *S. aureus* and *Listeria monocytogenes* with inhibitory zone diameters ranged from 12.5 to 19.1 mm (Kumar et al., 2016). Compared with other milk types, buffalo whey proteins hydrolysed with papain at a concentration of 2 mg/mL showed antibacterial activity against *E. coli* and *S. aureus*, with an inhibition zone diameter of 14.5 and 15.4 mm, respectively (Meignanalakshmi & Vinoth Kumar, 2013). Tomita et al. (1991) found that low molecular weight peptides liberated during the hydrolysis of bovine lactoferrin by pepsin completely inhibited the growth of *E. coli* 0111. Goat whey hydrolysed with Alcalase demonstrated antibacterial activity against *E. coli*, *B. cereus*, *S. typhimurium*, and *S. aureus* with an inhibitory zones of 18.0, 13.3, 22.3 and 15.0 mm, respectively (Osman, Goda, Abdel-Hamid, Badran, & Otte, 2016). Overall, these results indicate that the antibacterial activity depends on the milk protein type, the enzyme type and the bacterial strain.

Size exclusion chromatography (SEC) fractionated the CMWH into fractions of proteins or peptides according to their molecular weight. SEC-F1 contains non-hydrolysed proteins and high

molecular weight peptides, whereas, SEC-F2 contains low molecular weight peptides. The largest proteins/peptides in SEC-F1 exhibited no antibacterial activity against PAO1 and MRSA. In contrast, SEC-F1 in our previous study showed antibacterial activity against *S. aureus* and had no activity against *B. cereus*, *E. coli* and *S. typhimurium* (Abdel-Hamid et al., 2016). Nevertheless, SEC-F2 demonstrated a significantly ($P < 0.01$) higher antibacterial activity against PAO1 and MRSA compared to CMWH and positive (ampicillin) control. These results indicating that through the SEC technique, the potential antibacterial peptides were eluted and concentrated in SEC-F2. In agreement with this finding, Salami et al. (2010) reported that the fraction < 3 kDa of camel whey protein hydrolysates showed the highest inhibition of growth of *E. coli* compared to the total hydrolysates and their fractions of <5 kDa and <10 kDa. Furthermore, size SEC-2 of camel milk whey hydrolysed by papain exhibited the highest antibacterial activity against *E. coli*, *B. cereus*, *S. aureus* and *S. typhimurium* (Abdel-Hamid et al., 2016). Additionally, Cheng, Tang, Wang, & Mao (2013) reported that the second fraction of yak κ-casein hydrolysates fractionated by sephdex G-25 column exhibited the highest antibacterial activity against *E. coli*.

Considering the obtained highest antibacterial activity of SEC-2 among all experimental treatments, it has been selected for further analysis including minimum inhibitory concentration, minimum bactericidal concentration, monitoring of bacterial growth rate, the antibiofilm activity and mode of action using scanning electron microscopy.

3.2. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

MIC and MBC of SEC-F2 was evaluated using micro-dilution method and results are given in Table 2. The concentration of SEC-F2 (mg/mL) required to inhibit the visual growth of MRSA was almost twice the concentration needed to inhibit PAO1 growth. Furthermore, the MBC values of each microbe were twice the MIC values (Table 2). This finding goes in parallel with the antibacterial activity of SEC-F2 (Table 1) and confirming that MRSA is less sensitive to SEC-F2 peptides than PAO1. Similar results were observed by Dosler & Karaaslan, (2014) who reported MIC around 0.128 mg/mL of cationic antimicrobial peptides (LL-37, CAMA, Melittin, Defensin, Magainin II) against *P. aeruginosa* ATCC 27853. Furthermore, the same authors found that the MBC value was twice the MIC value. It is worth noting that Abdel-Hamid et al.

(2016) reported lower MIC values for SEC-F2 of papain camel whey hydrolysate against *B. cereus, S. aureus* and *S. Typhimurium* (0.09, 0.09 and 0.01 mg/mL, respectively) compared to the MIC values obtained here. Nevertheless, a higher MIC value (62.5 mg/mL) of bovine milk casein hydrolysed by latex *Jacaratia corumbensis* protease was recorded against *P. aeruginosa* ATCC 27853 (Arruda et al., 2012). Additionally, bovine lactoferrin hydrolysed with pepsin showed antibacterial activity against *P. aeruginosa* MMI-603 with an MIC value of 0.63 mg/mL (Tomita et al., 1991).

3.3. Bacterial growth rate of PAO1 and MRSA exposed to SEC-F2.

PAO1 and MRSA were treated with SEC-F2 at different concentrations (1 ×, 2 × and 4 × MIC) for 5 h at 37 °C. The optical density (OD_{600} nm) was recorded in order to evaluate the bacteriostatic and bactericidal mode of action of SEC-F2. SEC-F2 at 1 × MIC concentration delayed the growth of PAO1, while at 2 × MIC and 4 × MIC concentrations growth was almost completely inhibited for PAO1 (Fig. 1A). These results indicate that SEC-F2 exhibited bactericidal effect against PAO1 and the peptides in SEC-F2 able to disrupt the outer and cytoplasmic membranes. With respect to MRSA, 1 × and 2 × MIC of SEC-F2 showed lower growth inhibition activity compared to the control MRSA treatment. However, at 4 × MIC concentration of SEC-F2 the growth of MRSA was also completely inhibited (Fig. 1B), which evidences the bacteriostatic effect of SEC-F2 against MRSA at this concentration (4 × MIC). It should be noted that SEC-F2 showed a lower antibacterial effect in the growth curve experiment than in the MIC assay, which is most probably attributed to the higher initial bacterial count in the growth assay (~10^7 CFU/mL) compared to the initial bacterial count in MIC test (~10^5 CFU/mL) (Godballe et al., 2016).

3.4. Antibiofilm activity of SEC-F2

The ability of SEC-F2 to prevent biofilm formation of PAO1 and MRSA was evaluated, and results are given in Tables 3. SEC-F2 significantly ($P < 0.01$) inhibited the biofilm formation of both PAO1 and MRSA in a concentration-dependent manner. It is worth noting that the inhibitory effect was more pronounced in MRSA than in PAO1, whereas at sub-MIC concentrations (1/10 × MIC) the effect was similar for both strains (Table 3). The potential antibiofilm activity of SEC-F2 most probably attributed to the peptide derived from camel milk α-lactalbumin and lactoferrin by papain, results corroborated by Kamiya et al. (2012) reporting

inhibition of *P. aeruginosa* biofilm formation by bovine lactoferrin. A similar trend of results was reported for lactoferrin derived peptides against biofilm formation of *C. parapsilosis, K. pneumonia and P. aeruginosa* (Fais et al., 2017; Morici et al., 2017; Xu et al., 2010). In contrast to the previous results on the ability of hydrolysis to enhance the antibiofilm activity, Rogan et al. (2004) demonstrated that the hydrolysis of lactoferrin by cathepsin resulted in loss of antibiofilm activity against *P. aeruginosa*.

It has been reported that the minimum bactericidal concentration for bacteria in the biofilm state are 4 to 10× higher than those reported for the planktonic cells (Marques et al., 2015; Wang, Wu, Ciofu, Song, & Høibya, 2012). Accordingly, obtaining a noticeable reduction in biofilm biomass at the lowest concentration of MIC (1/100 × MIC), reflects the potential activity of SEC-F2 as an antibiofilm and/or antibacterial agent.

3.5. Biofilm reduction by SEC-F2

The activities of two-fold serial dilutions of SEC-F2 (10 to 0.31 mg/mL concentrations) on biofilm reduction of PAO1 and MRSA were tested on 24 h mature biofilms. For both PAO1 and MRSA strains, the highest tested concentration (10 mg/mL) exhibited the highest significant ($P < 0.01$) reduction in the amount of biofilm biomass (Table 4). The biofilm reduction activity showed a significant ($P < 0.01$) peptide concentration-dependence in both strains, with a more pronounced impact in PAO1. By decreasing the concentration of SEC-F2 the reduction activity was progressively reduced to be eventually lost at lowest concentration tested (0.31 mg/mL) in PAO1 (Table 4). Whereas, the MRSA biofilm was significantly ($P < 0.01$) reduced by all the applied SEC-F2 concentrations even at the lowest SEC-F2 concentration, which resulted in more than 60% reduction of the biofilm. As discussed above for the MIC data (section 3.2), the significant ($P < 0.01$) difference in biofilm reduction obtained between PAO1 and MRSA could be imputed to the different nature of their bacterial membranes. Moreover, *P. aeruginosa* is considered as a potent biofilm former compared to MRSA (Yadav, Chae, Go, Im, & Song, 2017). Additionally, the biofilm composition, architecture, and quorum sensing mechanisms may explain and/or contribute to these differences in biofilm reduction between PAO1 and MRSA. In this context, Lebeaux, Ghigo and Beloin (2014) suggested that the iron chelating properties of lactoferrin is the key function that explains the lactoferrin antibiofilm activity, which may contribute to explain our obtained differences between PAO1 and MRSA. It has been reported

312 that iron is required for normal biofilm development in *P. aeruginosa* (Banin, Vasil, &
313 Greenberg, 2005), whereas iron deprivation promotes biofilm production in *S. aureus* (Johnson,
314 Cockayne, Williams, & Morrissey, 2005). It is worth noting that further work is needed to
315 elucidate the nature and chemical features of SEC-F2 to address its mode of action on PAO1 and
316 MRSA more thoroughly

317 **3.6 Changes in bacterial membrane morphology**

318 The impacts of the size exclusion chromatography fraction 2 (SEC-F2) of camel milk
319 whey protein hydrolysates on the ultrastructural and morphological changes in PAO1 and MRSA
320 are shown in Fig. 2 and 3, respectively. It has been reported that small cationic peptides with
321 balanced charge and hydrophobicity as key structural elements of bovine lactoferrin, exhibited
322 the ability to interact with bacterial membranes and caused membrane damage through various
323 forms of pore formation (Jenssen & Hancock, 2009; Mojsoska & Jenssen, 2015). The key
324 structural elements aid initial electrostatic interaction, followed by hydrophobic interactions and
325 other bio-events that govern the fate of the bacteria. The manifested ultrastructure clearly reveals
326 a higher degree of damaged bacteria in presence of SEC-F2 (Fig. 2 AI-VI, 3B and 3C) compared
327 to both control samples PAO1 and MRSA (Fig. 2A I and 3A). We have previously investigated
328 the mode of action of SEC-F2 using several bacterial models and transmission electron
329 microscopy (Abdel-Hamid et al., 2016). These authors concluded that 2 × MIC concentrations of
330 SEC-F2 caused substantial cell distortion and cell lysis in both Gram-negative and Gram-positive
331 bacteria. In corroboration to this, the current SEM micrograph clearly show that the cell
332 membrane damage of PAO1 and MRSA is more pronounced at the highest tested concentration 4
333 × MIC of SEC-F2 (Fig. 2A IV-VI and 3C).

334 A closer observation of the PAO1 micrograph details revealed that a noticeable
335 filamentation occurred in the bacterial cells resulted from SEC-F2 treatments (Fig. 2A II).
336 Furthermore, an obvious leakage of cytoplasmic content that further intensified by increasing the
337 MIC concentration (Fig. 2A III-VI). These findings were confirmed by images analysis and size
338 measurements, which showed that the PAO1 bacterial cells at both tested concentrations (1 × and
339 4 × MIC) (Fig. 2B) were noticeably longer than that of control PAO1 (Fig. 2A I). In this context,
340 Vega, Martínez, Chalá, Vargas, & Rosas, (2018) have demonstrated the antimicrobial activities
341 of the peptides of bovine lactoferrin and bovine lactoferricin fractions in a similar trend of SEC-

F2 results. These authors reported that small amphiphilic peptides of bovine lactoferricin caused morphological alteration in *P. aeruginosa* such as surface shrinkage, wrinkling formation of protrusions and leakage of cellular contents.

With alteration of size in respect to MRSA, it can be seen from Fig. 3A that the MRSA control sample was abundant in cells that adhere in a big cluster. Whereas, MRSA treated with both 1 × and 4 × MIC concentrations showed different levels of bacterial membrane damage (Fig. 3B and 3C). In this context, Hartmann et al., (2010) have demonstrated *S. aureus* bacterial cell membrane damage and lysis caused by short peptides at supra-MIC concentrations. It is worth noting that we have demonstrated in our previous study using a transmission electron microscopy (TEM) technique that SEC-F2 exhibited bacteriostatic action on *S. aureus*, however, no significant damage on the bacterial cell membrane was observed (Abdel-Hamid et al., 2016). Minor morphological changes on MRSA surface roughness and impaired cell division at 1 × and 4 × MIC concentrations were observed, respectively (Fig. 3B and 3C), which is in agreement with the TEM findings reported by Abdel-Hamid et al. (2016). The size measurement analysis showed that in presence of SEC-F2 the bacteria exhibit one directional elongation at 1 × MIC (Fig. 3D), whereas at 4 × MIC the cell size expansion is smaller than 1 × MIC, but it happens in both directions (Fig. 3A-D). Overall, the PAO1 and MRSA ultrastructure micrographs findings are in support of the results of antibacterial activity, MIC and growth rate assay (sections 3.1, 3.2 and 3.3).

4. Conclusion

In the present study camel milk whey protein was evaluated as a source for potential bioactive peptides. The antibacterial and antibiofilm activities of the camel milk whey protein hydrolysate (CMWH) and its obtained fractions from size exclusion chromatography (SEC-F1 and SEC-F2) were assessed against *P. aeruginosa* PAO1 and Methicillin-Resistant *S. aureus* (MRSA). CMWH showed significant antibacterial activity against PAO1 and MRSA. It is worth noting that SEC-F2 exhibited higher antibacterial activity against PAO1 and MRSA compared to control and CMWH treatments. Moreover, SEC-F2 has significantly inhibited the biofilm formation, as well as leading to a reduction of preformed biofilms of both pathogen strains in a peptide concentration-dependent manner. In addition, the growth rate profile and scanning electron microscopy analyses revealed that SEC-F2 exhibited bacteriostatic effect toward MRSA

and PAO1. The obtained data clearly demonstrates the robust antibacterial and antibiofilm activities of SEC-F2 against the both tested Gram-negative and Gram-positive species, which may provide a basis for the dairy industry to develop innovative products and to optimize the processing conditions. Nevertheless, further studies on SEC-F2 isolation, purification and structural identification, along with synthesis opportunities *in vitro* will expand our knowledge and understandings of the relationship between the chemical structure and the bioactivity profile of this crucial fraction.

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Conflicts of interest

The authors declare no conflict of interest.

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Figure captions

Figure 1. Bacterial growth curve under exposure of 1 × MIC, 2 × MIC and 4 × MIC of SEC-F2 against (A) *P. aeruginosa* PAO1 and (B) Methicillin-Resistant *S. aureus* (MRSA).

Figure 2. Scanning electron micrographs of A) (I) untreated (control) and treated P. aeruginosa PAO1 with 1 × (II-III) and 4 × MIC (IV-VI) of size exclusion chromatography fraction 2 (SEC-F2). B) Cell length of untreated and SEC-F2 treated PAO1 is shown. Scale bars are 1 and 2 µm.

Figure 3. Scanning electron micrographs of A) (I) untreated (control) and B-C) (II-III) treated Methicillin-resistant S. aureus (MRSA) with 1× and 4 × MIC, respectively, of size exclusion chromatography fraction 2 (SEC-F2), D) Size measurements for untreated and treated bacteria. Scale bars are 1 and 500 µm.

Table 1. Antibacterial activity of camel milk whey, camel milk whey hydrolysate and size exclusion chromatography fractions 1 and 2 (SEC- F1 and SEC-F2)

Samples	Inhibition zone diameter (mm)	
	PAO1	MRSA
Positive control*	$18.3 \pm 2.1^{Ca**}$	12.3 ± 0.6^{Cb}
Camel milk whey	NI[¤]	NI
Camel milk whey hydrolysate	22.3 ± 2.1^{Ba}	19 ± 1^{Bb}
SEC -F1	NI	NI
SEC -F2	27.9 ± 0.7^{Aa}	22.3 ± 1.5^{Ab}

Data are mean of triplicate measurements ± SD.

* Positive control was ampicillin 10 mg/ml.

** Capital letters indicate the pairwise comparison between whey treatments (same column); lower case letters indicate the pairwise comparison between microbes (same row).

NI= No inhibition zone was observed.

PAO1, *P. aeruginosa* PAO1– MRSA, Methicillin-Resistant *S. aureus*

Table 2. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of size exclusion chromatography fraction 2 (SEC-F2)

Strains	mg/mL	
	MIC	MBC
PAO1	0.16	0.31
MRSA	0.31	0.63

The MIC and MBC values are mean of three biological replicates.

PAO1, *P. aeruginosa* PAO1– MRSA, Methicillin-Resistant *S. aureus*

Table 3. Antibiofilm activity of size exclusion chromatography fraction 2 (SEC-F2)

Concentration	Biofilm Inhibition %	
	PAO1	MRSA
MIC	60.5 ± 1.5^{A}	85.5 ± 1.0^{A}
1/10 MIC	43.5 ± 1.8^{B}	41.0 ± 2.9^{B}
1/100 MIC	20.9 ± 1.8^{C}	36.2 ± 0.8^{C}

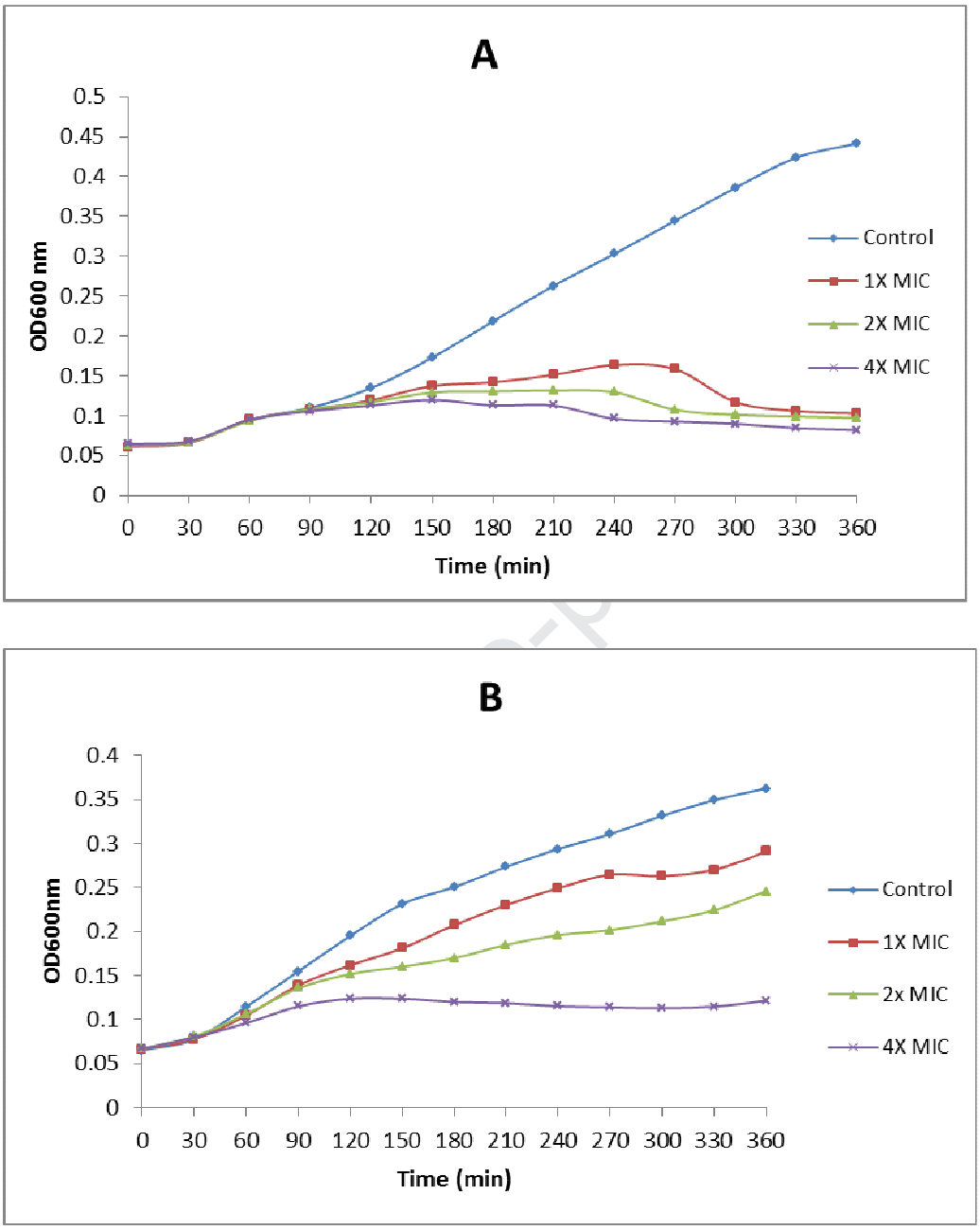
Data are mean of triplicate measurements ± SD.
Values in the same column with different superscript capital letters are significantly different ($P < 0.01$).
PAO1, *P. aeruginosa* PAO1– MRSA, Methicillin-Resistant *S. aureus*

Table 4. Minimum biofilm reduction concentration of size exclusion chromatography fraction 2 (SEC-F2)

SEC-F2 Concentration (mg/mL)	Biofilm reduction (%)	
	PAO1	MRSA
10	$89.0 \pm 1.6^{Ab*}$	92.6 ± 0.5^{Aa}
5	80.4 ± 4.8^{Bb}	85.7 ± 1.2^{ABa}
2.5	64.9 ± 1.0^{Cb}	80.7 ± 1.8^{Ba}
1.25	51.0 ± 4.3^{Db}	71.1 ± 3.2^{Ca}
0.62	20.2 ± 2.2^{Eb}	65.5 ± 4.6^{CDa}
0.31	-7.7 ± 1.9^{Fb}	61.5 ± 2.1^{Da}

Data are mean of triplicate measurements ± SD.
A-F Different uppercase letters within a column indicate significant differences ($P < 0.01$) in the pairwise comparison between peptide concentrations
a-b different lowercase letters within a row indicate significant differences ($P < 0.01$) in the pairwise comparison between bacteria.
PAO1, *P. aeruginosa* PAO1– MRSA, Methicillin-Resistant *S. aureus*

Figures



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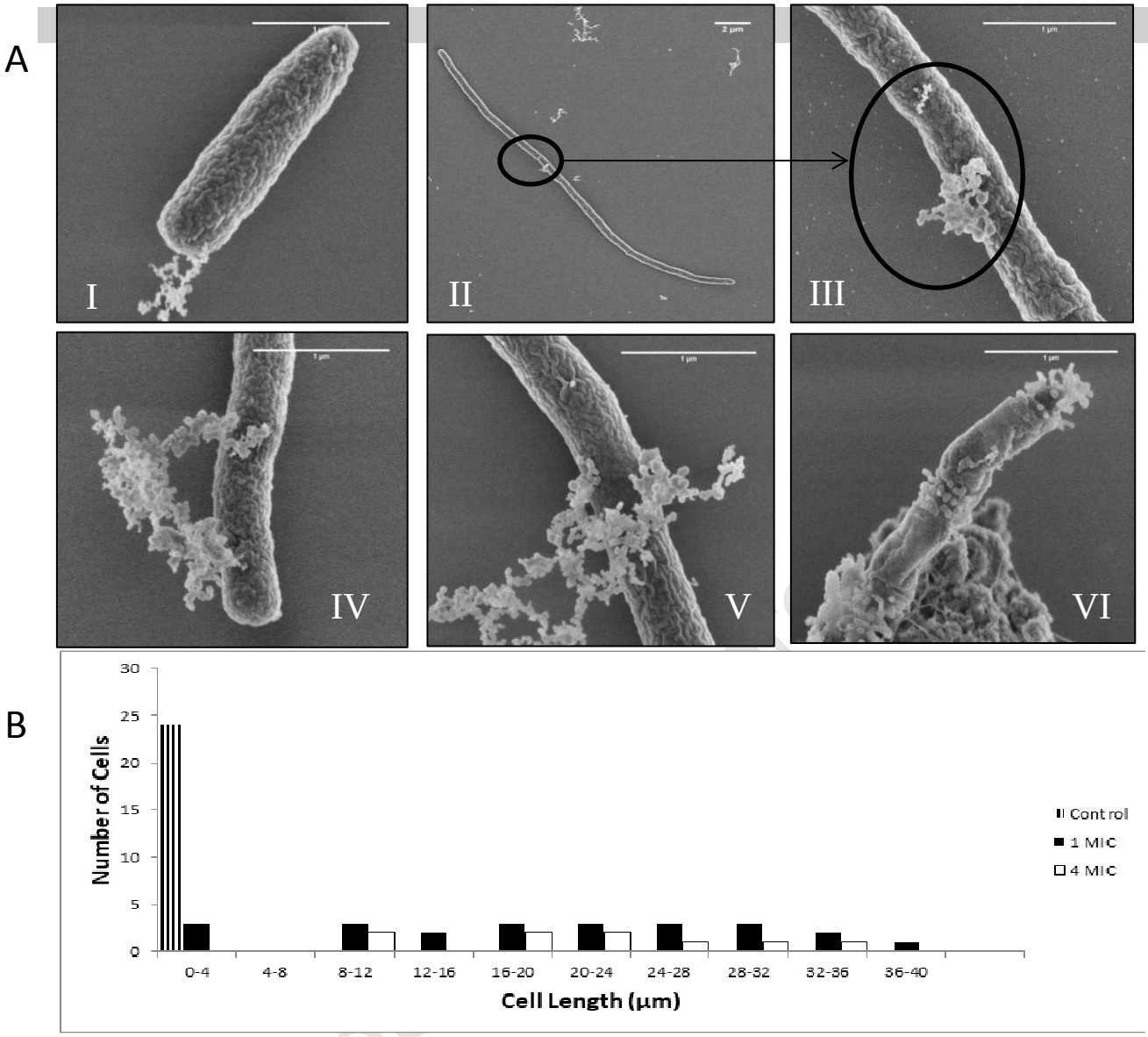

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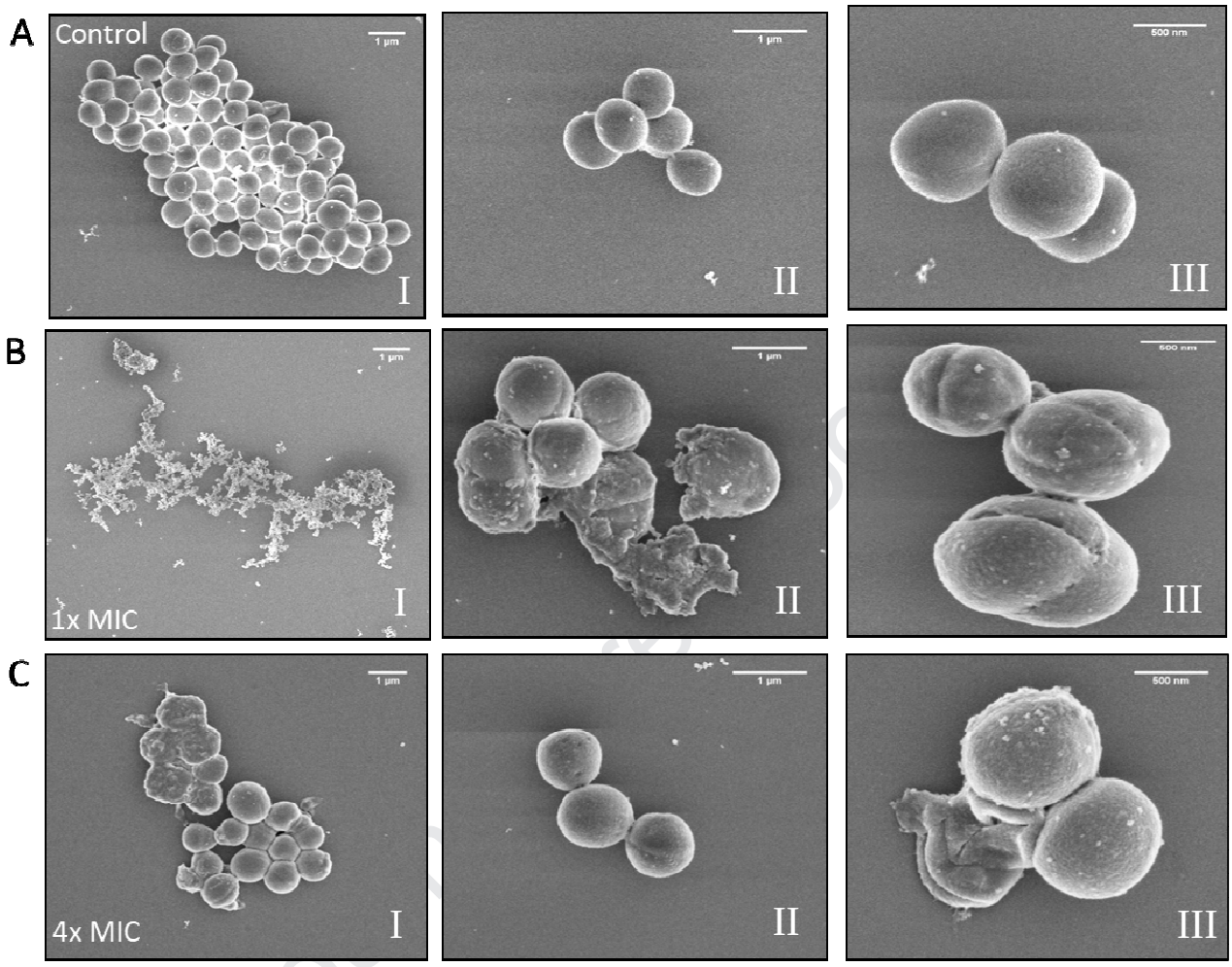

D

Sample	Diameter (µm) x-axis	Diameter (µm) y-axis
Control	0.8 ± 0.1	0.7 ± 0.1
MRSA 1x MIC	1.1 ± 0.2	0.9 ± 0.2
MRSA 4 x MIC	0.9 ± 0.1	0.9 ± 0.1

Figure 3. Scanning electron micrographs of A) (I) untreated (control) and B-C) (II-III) treated Methicillin-resistant *S. aureus* (MRSA) with 1× and 4 × MIC, respectively, of size exclusion chromatography fraction 2 (SEC-F2), D) Size measurements for untreated and treated bacteria. Scale bars are 1 and 500 µm.

Highlights

- Hydrolysis of camel milk whey by papain enhanced the antibacterial activity against PAO1 and MRSA
- Size exclusion chromatography fraction 2 (SEC-F2) exhibited the highest antibacterial activity.
- SEC-F2 inhibited the formation of the biofilm by PAO1 and MRSA.
- SEC-F2 eradicated the biofilm formed by PAO1 and MRSA.

Conflict of Interest Form

The authors declare no conflict of interest

Best Regards

Mahmoud Abdel-Hamid

Resource	Source	Identifier
Chemical		
acetone		
aluminum		
Ampicillin		
crystal violet		
ethanol		
Glutaraldehyde		
OsO4		
PBS		
phosphate buffered saline		
platinum		