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ORIGINAL PAPER

Cell-free supernatant of *Streptococcus salivarius* M18 impairs the pathogenic properties of *Pseudomonas aeruginosa* and *Klebsiella pneumonia*

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Abstract
M18 strain of *Streptococcus salivarius* is a bacterial replacement probiotic that has been suggested for use in the oral cavity. Here, we have shown that *S. salivarius* M18 cell-free supernatant reduced the growth of the two most common human pathogens *Pseudomonas aeruginosa* and *Klebsiella pneumonia* and sensitized the pathogenic bacteria to antibiotic. Besides, the supernatant inhibited biofilm formation of *P. aeruginosa* drastically. For pinpointing the biomolecular changes that occurred in *P. aeruginosa* incubated with the probiotic supernatant, attenuated total reflectance-Fourier transform infrared (ATR-FTIR) spectroscopy was used. Unsupervised learning algorithms, principal component analysis (PCA) and hierarchical cluster analysis (HCA), and intensity analyses of individual spectral bands exhibited comprehensive alterations in the polysaccharide and lipid contents and compositions of *P. aeruginosa* cultivated with *S. salivarius* M18 cell-free supernatant. These results indicate that *S. salivarius* M18 has the potential for the prevention or alleviation of different pathogen-induced infections along with the infections of oral pathogens.

Keywords *Streptococcus salivarius* M18 · *Pseudomonas aeruginosa* · *Klebsiella pneumonia* · Biofilm · Polysaccharide · Lipid

Introduction

The World Health Organization describes probiotics as "Live microorganisms which, when administered in adequate amounts, confer a health benefit to the host." Probiotic microorganisms have been shown to help in the treatment of dental caries, pathologies of the gastrointestinal tract, acute respiratory tract infections as well as multiple autoimmune conditions and inflammatory disorders (Liu et al. 2018a, b; Hasslöf and Stecksén-Blicks 2019; Sengupta and Paramasivan 2019). Furthermore, in vivo studies and in vitro models suggested a supportive role of probiotics in the management of complicated burn injury, e.g., of burn wounds infected with *Pseudomonas aeruginosa* (Argenta et al. 2016; Satish et al. 2017; Besser et al. 2019). The observed antipathogenic effects of probiotic bacteria rely on a variety of competitive exclusion mechanisms that include reduction of luminal pH, the blocking attachment of other bacteria, sequestration of essential nutrients and metal ions, and production of inhibitory compounds, such as hydrogen peroxide, organic acids, and bioactive bacterial peptides or proteins (Dierksen et al. 2007; Barzegari et al. 2020).

Probiotics are also one of the strongest options to combat the pathogenic biofilm-residing microorganisms (Sengupta and Paramasivan 2019). Biofilms are the architecturally complex structures composed of microorganisms that are embedded in a self-produced matrix of extracellular polymeric substances (EPS) (López et al. 2010; Kostakioti

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et al. 2013). This three-dimensional matrix is mainly composed of exopolysaccharides along with other components such as carbohydrate-binding proteins, pili, flagella, and extracellular DNA (López et al. 2010; Kostakioti et al. 2013) in which exopolysaccharides function as scaffolds for other carbohydrates, nucleic acids, proteins, and lipids to adhere (Rabin et al. 2015). Biofilm structure allows for water, gasses, and substrates to get to all parts of the structure, distribution of metabolic products, removal of toxic end products, and it also protects the bacteria from detrimental effects of altered pH and osmolarity, radiation, antimicrobials, and other damaging agents together with shear forces and host immune system. In the clinic, biofilms are implicated in diverse pathologies such as wound infections, cystic fibrosis, otitis media, pneumonia, osteomyelitis, middle ear infections, and dental caries (Sharma et al. 2019). Besides, the recent reports emphasize that biofilm formation has a potential etiological role in the development of cancers attributable to chronic infection (Raskov et al. 2018; Li et al. 2019; Rizzato et al. 2019; Tomkovich et al. 2019). Biofilms can form on biotic or abiotic surfaces both in the environment and in the clinical setting (Sharma et al. 2019). Bacterial biofilms can also form on implanted devices, such as catheters, valves, stents, joint replacement, and shunts (Pletzer et al. 2016; Barzegari et al. 2020). It has been claimed that biofilms are involved in approximately 80% of all human bacterial infections (Fleming and Rumbaugh 2017) and the persistent presence of biofilms imposes huge healthcare and economic burdens (Chang 2018). Eradicating biofilms are extremely difficult and biofilm infections are highly recalcitrant (Fleming and Rumbaugh 2017). Antimicrobial drug-resistant or tolerant physiologies of established biofilms rely on the barrier effect of the matrix, spatial heterogeneity, the rise of hyper-resistance phenotypes, increased expression of antibiotic-degrading enzymes and efflux pumps, and activation of stress response signaling pathways (Singh et al. 2016). The key concern about biofilms is their contribution to the development of the ongoing emergence of antibiotic-resistant pathogens (Amaning Danquah et al. 2019). Biofilm can activate both innate and adaptive arms of the host–immune response, but neither of which can eliminate the biofilm pathogen; instead, the over activation of the immune system can damage the surrounding tissues (Moser et al. 2017). Therefore, the severe and prevalent clinical impacts of bacterial biofilms inspire many researchers to investigate new strategies to decrease bacterial pathogenicity and potentially enhance bacterial susceptibility to antimicrobial agents (Rémy et al. 2018). Because of their abilities to modulate the microbial ecology and the structure of biofilms, probiotics, and probiotic products have gained particular attention in the recent years to be used for biofilm eradication (Barzegari et al. 2020).

Streptococcus salivarius is a Gram-positive human commensal that colonizes within just a few hours of birth in the oral cavity and has been commercially available as an oral probiotic for more than a decade (Manning et al. 2016). It is also a common inhabitant of the gastrointestinal system, paranasal sinuses, and genitourinary tracts (Cebeci et al. 2015; Hegarty et al. 2016). *S. salivarius* strain M18 is a bacterial replacement probiotic specifically derived from the oral cavity and designed for use in the oral cavity. In 2019, US Food and Drug Administration (FDA) granted the *S. salivarius* M18 as Generally Recognized as Safe (GRAS; Notice No. 807) for addition to food, indicating that the strain can be used without any demonstrable harm to consumers (Santagati et al. 2012). The pathogen inhibitory effect of *S. salivarius* M18 has been mainly investigated on bacterial species putatively associated with the development of dental caries. Several numbers of nose, mouth, and throat pathogens were shown to be inhibited by the probiotic *S. salivarius* M18, including *Streptococcus mutans*, *Streptococcus sobrinus*, *Actinomyces naeslundii*, *Actinomyces viscosus*, *Streptococcus pneumoniae*, *Streptococcus agalactiae*, *Haemophilus influenzae*, *Enterococcus faecalis*, *Listeria monocytogenes*, *Staphylococcus cohnii*, and *Staphylococcus saprophyticus* (Wescombe et al. 2012; Burton et al. 2013a; Di Pierro et al. 2015; Poorni et al. 2019). The present work aimed to investigate whether the cell-free supernatant of the probiotic *S. salivarius* M18 strain provides inhibitory activity against the pathogens *P. aeruginosa* and *Klebsiella pneumoniae*. *P. aeruginosa* is an opportunist bacteria and can cause a wide range of severe human infections, including pneumonia (associated with chronic obstructive pulmonary disease and cystic fibrosis), urinary tract infections (commonly caused by the use of urinary catheter), diabetic foot, otitis externa, bacteremia, keratitis, otitis media folliculitis (hot-tub rash), and soft tissue infections (high risks for burns and open wounds) (Lyczak et al. 2000). Infections of *P. aeruginosa* often exhibit resistance to antibiotics and antimicrobial therapies and are characterized by an intense neutrophilic response resulting in significant damage to host tissues that leads to high morbidity and mortality (Gellatly and Hancock 2013). *K. pneumonia*, another Gram-negative pathogen, is also associated with a broad range of human infections, such as pneumonia, bacteremia, urinary tract infections, bloodstream infection, infection of burns, intra-abdominal infection, meningitis, and pyogenic liver abscess (Caneiras et al. 2019). Both *P. aeruginosa* and *K. pneumoniae* are among the most common causes of nosocomial infections (Azimi et al. 2019). Besides, they are known as important food-borne pathogens that can be frequently found in raw vegetables, milk and milk products, fish, meat, and street foods (Correa et al. 1991; Arslan et al. 2011; Zhang et al. 2018).

In this manuscript, we demonstrated that the cell-free supernatant of the probiotic *S. salivarius* M18 can reduce the growth of the pathogenic bacteria *P. aeruginosa* and *K. pneumonia* dramatically and enhance their susceptibility to antibiotic treatment. Besides, the results of this study showed that the supernatant has an antibiofilm effect on *P. aeruginosa*. The cell-free supernatant of *S. salivarius* M18 was found to decrease cellular polysaccharide content and enhance lipid saturation in *P. aeruginosa* which can be related to the observed antipathogenic properties of the supernatant, mechanistically.

Materials and methods

Bacterial strains, culture conditions and collection of cell-free supernatants

Bacterial strains used in this study are listed in Table 1. *S. salivarius* M18, *P. aeruginosa*, and *Escherichia coli* were kindly provided by Dr. Rafig Gurbanov (Bilecik Şeyh Edebali University, Department of Molecular Biology and Genetics) and *K. pneumonia* was kindly provided by Dr. Ülküye Dudu Gül (Bilecik Şeyh Edebali University, Vocational School of Health Services). All the strains were cultured at 37 °C with orbital shaking in tryptic soy broth (TSB) medium composed of peptone from casein 17.0 g/l, peptone from soymeal 3.0 g/l, D(+)-Glucose monohydrate 2.5 g/l, NaCl 5.0 g/l and K_2HPO_4 2.5 g/l or on agar plates containing 1.5% (w/v) agar in TSB.

For supernatant collection, optical densities (OD) of overnight cultures at 600 nm were adjusted to 0.1 and the bacteria were grown for 24 h at 37 °C using a shaking incubator (160 rpm). The bacterial growth medium, TSB, which has not been inoculated with bacteria, but incubated under the same conditions, was used as "only medium (OM) control" in the experiments. At the end of 24 h incubation, the cultures were centrifuged at 4 °C, 5000 rpm (1844 *xg*) for 10 min to collect supernatants before filtration using 0.22-µM PES filters. The cell-free supernatants were used immediately or aliquoted and stored at − 80 °C for later use. Where stated, supernatants of *S. salivarius* M18 were collected after 14 h incubation.

Determination *S. salivarius* M18 growth kinetics

OD_{600} of *S. salivarius* M18 overnight culture was adjusted to 0.1 in the growth medium and the bacteria were cultivated in a shaking incubator (160 rpm) at 37 °C. Starting from zero hour (h), the OD_{600} value of 1.0 ml culture was measured at an interval of 2 h using an ultraviolet–visible light spectrophotometer (Agilespec, New Jersey, USA). The bacterial growth curve was plotted as the optical density OD_{600} vs. the culture time (h). The uninoculated growth medium was used as blank.

Growth inhibition test

Overnight culture of *P. aeruginosa* or *K. pneumoniae* (10^6 colony-forming units-CFU/ml) were incubated with 100 µl of *S. salivarius* M18 cell-free supernatant (SsM18 sup.) or uninoculated TSB medium (only medium; OM) in 96-well flat-bottom polystyrene cell culture plates (Nest Scientific, Maryland, USA) without shaking at 37 °C for 8 h or 24 h. To investigate the effect of the environmental pH on the pathogen inhibitory action of the supernatant, a sterile solution of 1.0 N NaOH was added into *S. salivarius* M18 cell-free supernatant or a sterile solution of 1.0 N HCl was added into the control medium (OM) to adjust the pH of the media to pH 7.0 or pH 5.0, respectively, before incubation with *P. aeruginosa* or *K. pneumonia*, as previously described (Furtado et al. 2014). An equal volume of sterile dH_2O (2.0% v/v) was added to the media in which pH was not adjusted.

To evaluate the antibiotic sensitivity, *P. aeruginosa* (10^6 CFU/ml) or *K. pneumonia* (10^6 CFU/ml) incubated with *S. salivarius* M18 cell-free supernatant containing the indicated amount of Cefoperazone sodium salt (CPZ; Cat no: C4292, Sigma-Aldrich, Missouri, USA) for 24 h. At the end of incubation, absorbances were measured at OD_{600} using a multi-well plate reader (Multiskan FC, Thermo Scientific, Massachusetts, USA). The optical density of the uninoculated bacterial growth medium at 600 nm was used as blank.

For spot plate assays, *P. aeruginosa* or *K. pneumonia* was grown in *S. salivarius* M18 cell-free supernatant (or in control growth medium; OM) in the presence or absence of CPZ in 96-well plates and subjected serial dilutions in the growth medium TSB. 3.0 µl of each dilution was spotted on

Table 1 Bacterial strains used in the study

Strain	Source
Streptococcus salivarius M18 (BLIS M18™)	Blis Technologies (New Zealand)
Pseudomonas aeruginosa (ATCC 27853)	American Type Culture Collection-ATCC (Manassas, Virginia, USA)
Klebsiella pneumoniae (ATCC 700603)	ATCC
Escherichia coli (ATCC 8739)	ATCC

agar plates, and the plates were incubated overnight at 37 °C. The spots were photographed using G:BOX imaging system, equipped with GeneSys image capture software (Syngene, England).

Biofilm formation assay

Static biofilms formed in the wells of the 96-well microtiter plates were assessed using crystal violet (CV) staining assay, as described previously (Merritt et al. 2011). Briefly, *P. aeruginosa* (10^6 CFU/ml) was inoculated in 100 µl of cell-free supernatants obtained from 14 h or 24 h cultured *S. salivarius* M18 in 96-well flat-bottom polystyrene cell culture plates (Nest Scientific). The plates were incubated at 37 °C for 24 h without shaking. At the end of incubation, the medium was removed and the wells were washed in dH_2O for at least three times to remove planktonic bacteria from each well. 150 µl of methanol was added to the wells and the plates were incubated for 15 min at room temperature for fixation. Methanol was removed and the plates were air-dried. 130 µl of a 0.1% (w/v, in dH_2O) solution of CV (Sigma-Aldrich) was added to each well of the microtiter plate and the plates were incubated at room temperature for 15 min. The stains were discarded and the plates were washed at least three times by submerging the plates in trays containing dH_2O to remove any CV that was not specifically stained the adherent bacteria. After air drying, 130 µl of 30% acetic acid (v/v, in dH_2O) was added to each well to solubilize the CV. OD of each stained wells was measured using a multi-well plate reader (Multiskan FC, Thermo Scientific) at 550 nm. The wells containing uninoculated plain culture medium were used as blank controls.

Congo red assay

To observe the changes in exopolysaccharide production of *P. aeruginosa* incubated with the *S. salivarius* M18 supernatant, Congo red assay was used as previously described (Friedman and Kolter 2004; Fu et al. 2017). In brief, Congo red agar plates were prepared with tryptone (10 g/l), 1.0% (w/v) agar, Congo red (40 µg/ml) and Coomassie brilliant blue G250 (20 µg/ml). 200 µl of *S. salivarius* M18 cell-free supernatant (or control growth medium) spread on the Congo red plates and allowed to dry onto the plates at room temperature. 3.0 µl of *P. aeruginosa* (4×10^8 CFU/ml) was spotted onto the agar surfaces and the plates were incubated at 37 °C for 24 h. The spots were observed and imaged by stereomicroscope (LEICA DM750, Wetzlar, Germany). For the quantification of Congo red binding, ImageJ (NIH, USA; https://rsbweb.nih.gov/ij/) color threshold tool, considering the spot area, was used as previously described (Tian et al. 2019). The color threshold of the image was adjusted using the following parameters: hue = 0–255, saturation = 0–255, brightness = 130–255, threshold color = red, background = dark, and color space = HSB.

DAPI staining

Surface-associated nucleic acid was investigated using DAPI (4′,6-diamidino-2-phenylindole) staining as performed before (Tran et al. 2018). Pre-sterilized coverslips were placed in the wells of a 6-well plate and 1.5 ml of *P. aeruginosa* (10^6 CFU/ml) in cell-free supernatant of *S. salivarius* M18 (or control growth medium; OM) were added into the wells. Followed by 24 h incubation at 37 °C without shaking, the planktonic cells were discarded and the wells were washed three times with PBS (phosphate buffered saline). The coverslips incubated with 5 µM of DAPI for 30 min at room temperature. Upon washing with PBS three times, the coverslips were mounted on glass slides, and then sealed with nail polish. The samples were observed under a fluorescent microscope (Olympus BX53, Shinjuku, Tokyo, Japan) using DAPI filter U-FUNA (excitation: 358 nm, emission: 461 nm; Olympus) and imaged. Images of eight random fields of DAPI stained samples were taken at 100X magnification and analyzed using ImageJ (NIH).

ATR-FTIR spectroscopy

Overnight culture of *P. aeruginosa* was inoculated in cell-free supernatant of *S. salivarius* M18, or control growth medium (only medium; OM) as 10^6 CFU/ml and 200 µl of each inoculum was spread on agar plates. The plates were incubated at 37 °C for 24 h. The raw spectra of single colonies were collected using Frontier FTIR Spectrometer (PerkinElmer, Massachusetts, USA) equipped with a universal ATR Miracle accessory. The spectrum of air was used as a reference. Single colonies were collected from the solid media and placed on a ZnSe crystal plate (PerkinElmer). Two biological replicates with two technical replicates were analyzed. The samples were scanned in the spectral range between 4000 and 650 cm^{-1} at room temperature with a resolution of 4 cm^{-1} and the spectra were collected as an average of 32 scans. OPUS 5.5 software (Bruker, USA) was used to quantify the absolute intensities of particular spectral bands in the second derivative and vector-normalized IR spectra, as previously described (Gurbanov et al. 2018).

Principal component analysis

Principal component analysis (PCA) was applied to the mean-centered and single transformed (unit-vector normalized) absorbance spectra in the particular polysaccharide region (1100–900 cm^{-1}) of fingerprint infrared region via The Unscrambler X 10.4 software (Camo, Norway). Full-cross validation method, singular value decomposition

(SVD) algorithm, and Hotelling's T2 statistics were used in the model, and the results were presented as scores and loadings plots (Gurbanov et al. 2019).

Hierarchical cluster analysis

Hierarchical cluster analysis (HCA) was applied to the single transformed (unit-vector normalized) absorbance spectra in the particular polysaccharide region (1100–900 cm^{-1}) via The Unscrambler X 10.4 software (Camo). Hierarchical complete-linkage method using squared Euclidean distance was used in the model. Clustering was based on either the magnitude of similarities or distance between the spectra. The results were presented as a dendrogram (Gurbanov et al. 2018).

Statistical data analysis

The results were expressed as mean ± standard error of the mean (SEM). *t* test was applied for comparisons using Prism 6.01 (GraphPad, La Jolla, CA, USA). The degree of significance was shown as $*p \leq 0.05$, $**p \leq 0.01$, $***p \leq 0.001$, $****p \leq 0.0001$.

Results

Streptococcus salivarius M18 cell-free supernatant showed antibacterial activity on *P. aeruginosa* and *K. pneumonia*

The turbidity of *P. aeruginosa*, inoculated on 96-well plates in *S. salivarius* M18 cell-free supernatant (SsM18 sup.) was determined by measuring the optical density at 600 followed by 8 h and 24 h incubation. As shown in Fig. 1a and b, the supernatant inhibited the growth of *P. aeruginosa* significantly compared with *P. aeruginosa* inoculated into only medium-OM control. In addition, the antibacterial activity was evaluated by spot plate assay by performing serial dilutions of *P. aeruginosa* incubated with *S. salivarius* M18 supernatant (8 h and 24 h) or with the control medium (Fig. 1a and b; right panels). The growth inhibitory effect of *S. salivarius* M18 supernatant was also investigated in *K. pneumonia* (Supplemental Fig. 1a and b).

The influence of *S. salivarius* M18 cell-free supernatant on the antibiotic susceptibility was examined on *P. aeruginosa* and *K. pneumonia* incubated with the antibiotic cefoperazone (CPZ). Incubation with the *S. salivarius* M18 supernatant was found to enhance the susceptibility to CPZ dramatically in both *P. aeruginosa* (Fig. 1c) and *K. pneumonia* (Supplemental Fig. 1c). The growth inhibitions in the presence of CPZ were also shown by spot plate assays (Fig. 1c and Supplemental Fig. 1c; right panels). On the other hand, cell-free supernatant collected from the commensal *E. coli* (Monk et al. 2013) did not affect the growth of *P. aeruginosa* the way *S. salivarius* M18 supernatant did (Supplemental Fig. 2) indicating that the observed antibacterial activity caused specifically by the probiotic *S. salivarius* M18.

Streptococcus salivarius is a species of lactic acid bacteria. Growth of lactic acid bacteria generates acidic end products that create unfavorable conditions for many other organisms, including pathogens by reducing the pH of the environment (Barbour and Philip 2014; Vieco-Saiz et al. 2019). To investigate if the observed pathogen inhibitory effect of the supernatant was related to pH, we first measured the pH of the *S. salivarius* M18 cell-free supernatant at the end of 24 h incubation at 37 °C, as described in materials and method section. The pH of the growth medium (control medium; OM) which has not been inoculated with the bacteria but incubated under the same conditions with *S. salivarius* M18 culture was measured as 7.1 ± 0.015 (belongs to measurements of three independent biological replicates) and the pH of the *S. salivarius* M18 cell-free supernatant was measured as 5.02 ± 0.08 (belongs to measurements of three independent biological replicates). We, therefore, adjusted the pH of the control medium (only medium; OM) to pH 5.0. Besides, the pH of the *S. salivarius* M18 cell-free supernatant was adjusted to 7.0 to investigate if the acidified growth medium was the only reason behind the pathogen inhibitory effect of the supernatant. We found that in the early phase of the growth (8 h), the growth of *P. aeruginosa* incubated with pH 5.0 control medium (OM) reduced compared with *P. aeruginosa* incubated with pH 7.0 control medium (OM); but still, the growth inhibitory action of the *S. salivarius* M18 supernatant (pH 5.0) was significantly higher than the inhibitory action of the acidified (ph 5.0) control medium, OM (Fig. 1d). After 24 h incubation, the probiotic supernatant was found to inhibit the growth of *P. aeruginosa* by about 70% (Fig. 1e). On the other hand, in comparison to the growth kinetics of 8 h incubated *P. aeruginosa,* the pathogenic bacteria were found to gain resistance to the devastating effect of the acidified control medium (pH 5.0 adjusted OM) at the end of 24 h incubation (Fig. 1e). Appearing at the early stages of the growth, the adaptation of *P. aeruginosa* to the acidic environment has been already shown before (Aleanizy et al. 2018). *P. aeruginosa* is well known for its successful adaptation to environmental conditions including highly variable pH values. For instance, under conditions of cystic fibrosis, the airway surface liquid of the lung was found acidified (pH < 6.5) because of the defective bicarbonate ion transport, and *P. aeruginosa* can easily colonize under this acidic conditions. However, similar adaptation behavior was not observed in *K. pneumonia* (Supplemental Fig. 1d and e) which was previously shown as intolerant to acidic conditions (Ajayasree and Borkar 2018).

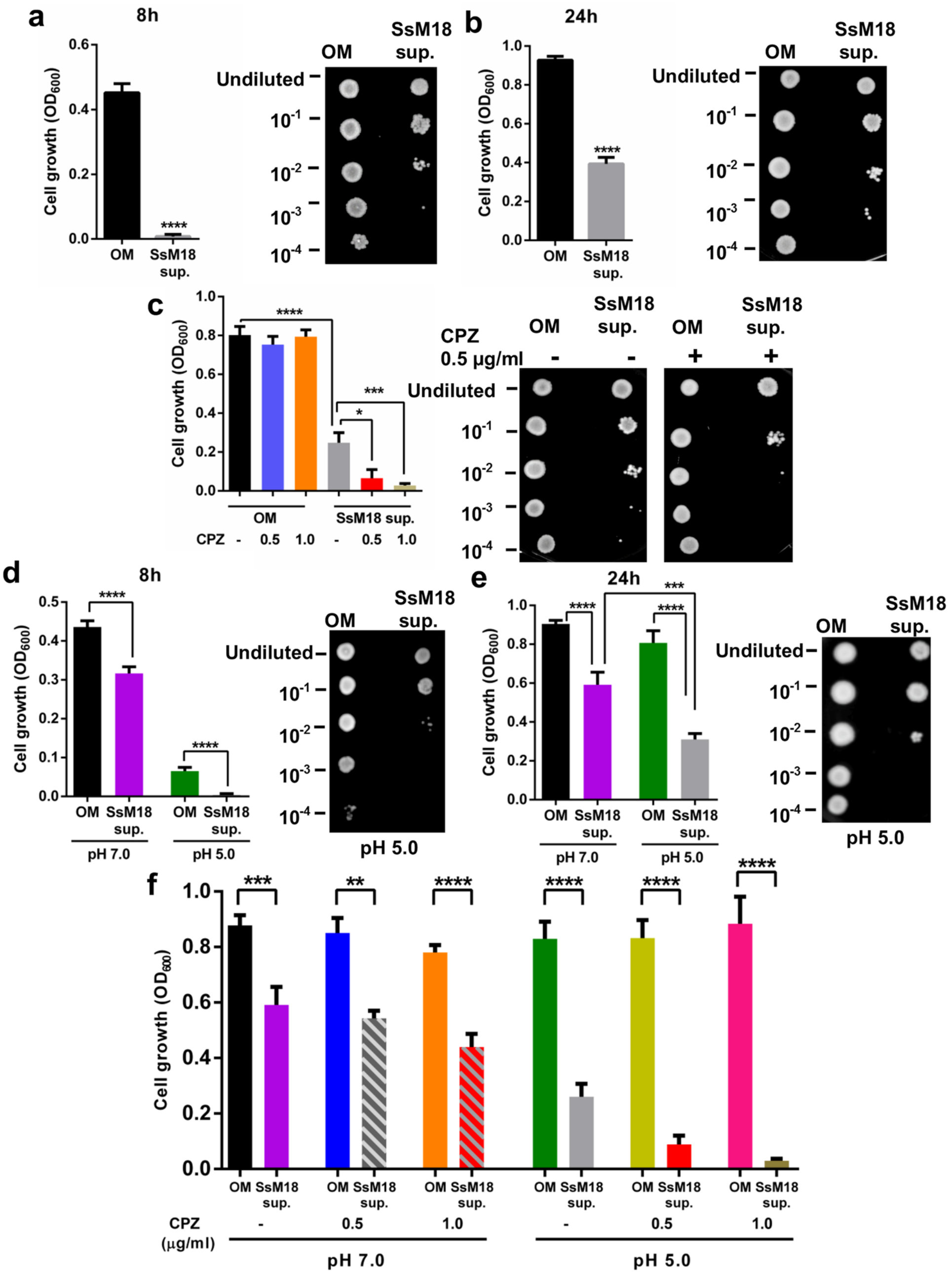

Fig. 1 *Streptococcus salivarius* M18 cell-free supernatant inhibited the growth of *P. aeruginosa* and sensitized the pathogenic bacteria to antibiotic. *P. aeruginosa* was incubated with *S. salivarius* M18 supernatant (SsM18 sup.) or with control medium (only medium; OM), which has not been inoculated with *S. salivarius* M18 but incubated under the same conditions, for **a** 8 h and **b** 24 h. The bacterial growth was determined at OD_{600}. **c** *P. aeruginosa* incubated with *S. salivarius* M18 cell-free supernatant containing 0.5 µg/ml or 1.0 µg/ml cefoperazone (CPZ) for 24 h. Change in the antibiotic susceptibility of *P. aeruginosa* was determined by measuring OD_{600}. **d** The pH of the *S. salivarius* M18 cell-free supernatant (SsM18 sup.) was adjusted to pH 7.0 and the pH of the control medium (only medium; OM) was adjusted to pH 5.0. The effect of the environmental pH on the growth of *P. aeruginosa* was investigated by measuring OD_{600} after 8 h and **e** 24 h incubation to evaluate the pH responsiveness of antibacterial substances present in the *S. salivarius* M18 cell-free supernatant. **f** Effect of pH adjusted *S. salivarius* M18 cell-free supernatant (SsM18 sup.) and control medium (only medium; OM) on the growth of *P. aeruginosa* in the presence of Cefoperazone (CPZ; 0.5 µg/ml or 1.0 µg/ml) was determined at OD_{600}. Spot plate assays were used for visualizing cell growth followed by the incubation with the cell-free supernatants or with the control medium. The experiments were repeated three times with eight technical replicates of each and the results were presented as mean ± SEM. *t* test was used to analyze the results ($^{**}p \leq 0.01$, $^{***}p \leq 0.001$, $^{****}p \leq 0.0001$)

Nevertheless, adjustment of the pH of *S. salivarius* M18 cell-free supernatant to 7.0 diminished its growth inhibitory effect indicating that to a considerable extent, the supernatant requires a slightly acidic environment to show the antipathogenic activity and neutralization of the environmental pH results in a decrease in the pathogen inhibitory effect of the probiotic supernatant (Fig. 1d and e). This acidic condition was also found to be critical for the sensitivity to the antibiotic CPZ (Fig. 1f).

Streptococcus salivarius M18 cell-free supernatant inhibited the biofilm formation in *P. aeruginosa*

The effect of *S. salivarius* M18 supernatant on biofilm formation was evaluated on *P. aeruginosa* grown in 96-well polystyrene plates using crystal violet (CV) staining. The results of the previous studies about one of the most studied and extensively used probiotic bacteria *Lactobacillus plantarum* showed that the concentration of the antimicrobial substances of *L. plantarum* reaches a maximum at the onset of the stationary phase of the growth (Messi et al. 2001; De Giani et al. 2019). Depending on this observation, we also aimed to evaluate the antipathogenic potential of *S. salivarius* M18 culture supernatant collected at the beginning of the stationary phase of the growth. Based on the *S. salivarius* M18 growth curve (Fig. 2a), the growth inhibitory (Fig. 2b) and antibiofilm (Fig. 2c) effects of *S. salivarius* M18 cell-free supernatant collected at the beginning of the stationary phase of the growth (14 h after inoculation) and collected at the end of 24 h were investigated on *P. aeruginosa*. Treatment with *S. salivarius* M18 culture supernatant (SsM18 sup.), collected after 24 h followed by inoculation, tremendously inhibited biofilm formation of *P. aeruginosa* (about 90%). 14 h supernatant also showed growth inhibitory and antibiofilm effects, but to a lesser extent when compared with 24 h culture supernatant indicating that the antipathogenic compounds were also present and active during the entry into the stationary phase, but the types of these compounds were modulated or their amounts/activities reached highest levels during the stationary phase of growth. Of note, the pH of the 14 h cell-free supernatant was measured as 5.00 ± 0.03 (belongs to two independent biological replicates).

Microorganisms can form sessile, sedentary communities that enable single-cell organisms to adopt a temporary multicellular lifestyle which assists survival in adverse environments. Formation of biofilm comprises distinct stages: (i) "initial adhesion" where bacteria attach to a surface, (ii) "microcolony formation" where bacteria begin to divide and produce EPS that embeds the cells and enhances adhesion, (iii) "biofilm maturation" where EPS matrix forms a three-dimensional structure providing a multi-functional and protective scaffold, (iv) "detachment" (also termed dispersal) where bacteria re-enter the planktonic phase, leave the biofilm, and may re-colonize new areas (Crouzet et al. 2014; Koo et al. 2017). CV is a basic dye that can bind to negatively charged molecules and can be used to stain and quantify total biofilm biomass comprising bacteria and EPS (Stiefel et al. 2016). In addition to microtitre-plate tests using the dyes CV or safranin, quantification of biofilm formation can also be carried out by staining with DAPI which binds to double-stranded DNA of both viable and dead cells (Hannig et al. 2010). After 24 h incubation of *P. aeruginosa* with control medium (OM) or supernatant collected from *S. salivarius* M18 (SsM18 sup.), nucleic acid content on coverslip surfaces was determined through the quantification of fluorescence intensity of DAPI, as described previously (Raymond et al. 2018; Lei et al. 2019b). The nucleic acid content of the *S. salivarius* M18 supernatant incubated *P. aeruginosa* biofilm developed on coverslips was found to be significantly lower (90%) than the corresponding control ones (Fig. 2d).

Streptococcus salivarius M18 cell-free supernatant altered the content and composition of polysaccharides and lipids in *P. aeruginosa*

Infrared (IR) absorption spectra of different kinds of samples over a wide spectral range can be obtained using Fourier transform infrared (FTIR) spectroscopy coupled with the attenuated total reflectance (ATR) technique. The method provides information about the content and structure of cellular macromolecules with high accuracy, high sensitivity, and reliability. The simplicity of the sample preparation steps, fast detection, and low cost are the main advantages of ATR-FTIR (Gurbanov et al. 2015). In this study, ATR-FTIR was used to monitor biomolecular changes (Tunçer et al. 2018) that occurred in *P. aeruginosa* which was inoculated in cell-free supernatant of *S. salivarius* M18 and spread on agar plates. After 24 h incubation at 37˚C, the raw spectra of single *P. aeruginosa* colonies were collected and analyzed. The experimental set-up was shown in Fig. 3a.

To investigate if *P. aeruginosa* colonies grown in the presence of *S. salivarius* M18 products can be discriminated from their counterpart controls depending on different polysaccharide constituents, unsupervised learning algorithms PCA and HCA were applied to IR spectra in 1100–900 cm^{-1} region which has been assigned to C–OH stretching and C–O–C and C–O ring vibrations in polysaccharides, and C-P-O stretching associated with polysaccharide species (Mader et al. 2016; Soler-Arango et al. 2019). The PCAs of the IR spectral data were displayed in a two-dimensional plot using the first two principal components which together accounted for 84% and 15% (99% total) of the total variation; indicating that the groups (OM vs. SsM18 sup. incubated *P. aeruginosa*) were clearly

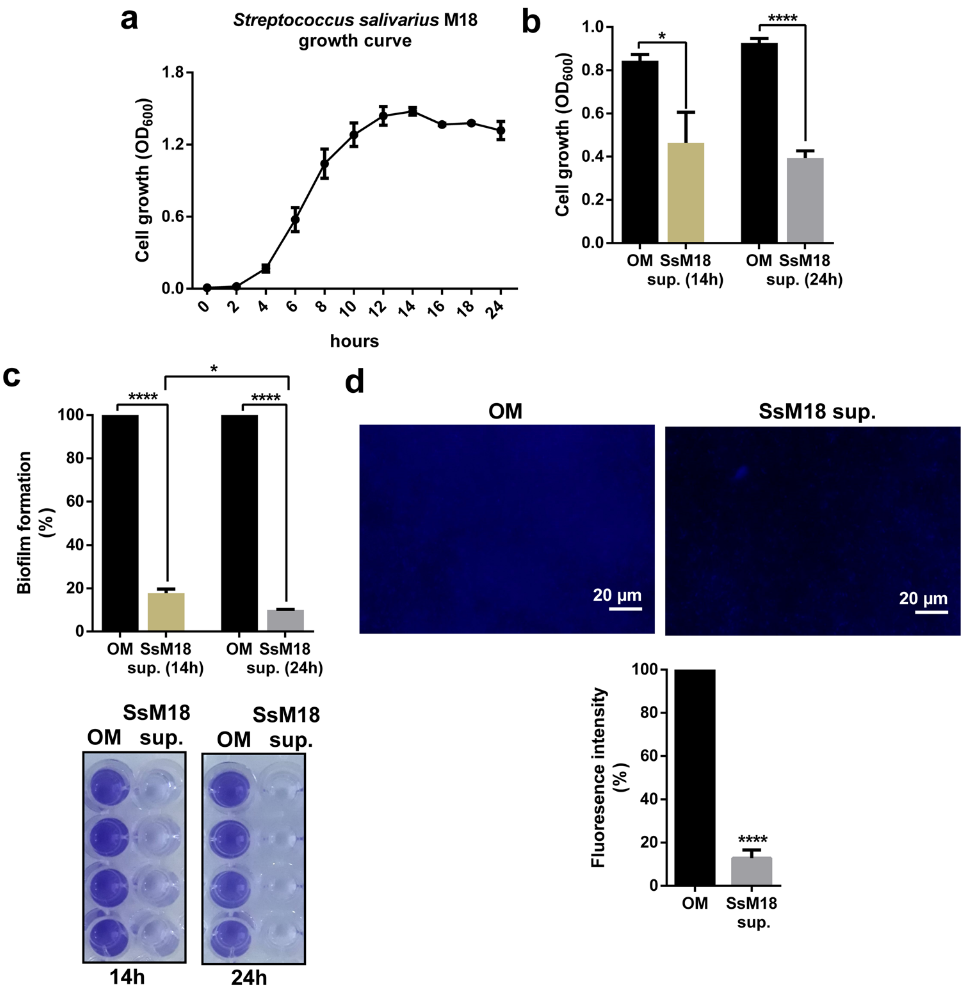

Fig. 2 *Streptococcus salivarius* M18 cell-free supernatant suppressed *P. aeruginosa* biofilm formation. **a** The OD_{600} values as a function of time were plotted to obtain the growth curve of *S. salivarius* M18. **b** Growth of *P. aeruginosa* incubated with the supernatant obtained from 14 h or 24 h cultured *S. salivarius* M18 (SsM18 sup.) measured by optical density (OD_{600}). **c** Change in the biofilm formation property of *P. aeruginosa* incubated with the supernatant obtained from 14 h or 24 h cultured *S. salivarius* M18 (SsM18 sup.) was determined using crystal violet (CV) staining. The quantitative results were represented as percent (%) respect to the only medium (OM) control biofilms. Images showing CV stained wells (biofilm growth) were given on the bottom. **d** The nucleic acid content of coverslip attached biomass of *P. aeruginosa*, incubated with the supernatant of 24 h cultured *S. salivarius* M18 (SsM18 sup.) or incubated with only medium (OM), was observed using a fluorescence microscope. Representative fields of the DAPI stained biomass were shown in the upper panels. Change in the fluorescence intensity was given as percent (%) respect to mean fluorescence of OM incubated control cells (lower panel). The results belong to at least three biological replicates and represented as mean ± SEM. *t* test was used to compare the results ($^{*}p \leq 0.05$, $^{****}p \leq 0.0001$)

differentiated from each other (Fig. 3b). This segregation can be explained from the loading plots of PC-1 (Fig. 3c). HCA, based on calculations of the pairwise distances of the observations in multidimensional data space, allows the determination of the number of clusters and also shows that which sample belongs to which cluster: in other words, the HCA algorithm groups data into clusters with similar attributes and the dendrogram is a visual presentation of the clustering (Mossoba et al. 2007). In the study, the groups, which were *P. aeruginosa* incubated in the presence of *S. salivarius* M18 supernatant products (SsM18 sup.) or with control growth medium (OM), were successfully differentiated in 1100–900 cm^{-1} region. As shown in Fig. 3d, the samples belong to the same groups were separated by relatively small distances (left side of the dendrogram), while the samples with diverse content and structures were separated by relatively large distances (right side of the dendrogram). In the 1100–900 cm^{-1} region, the bands with the significant spectral differences observed between the groups in the average second derivative spectra were shown in Fig. 3e. The intensity of an IR band corresponds to the concentration of the specific molecule (Tunçer et al. 2018). Intensities of the bands located at 1053 cm^{-1} (C–C, C–O, C–O–C, and C–O–P

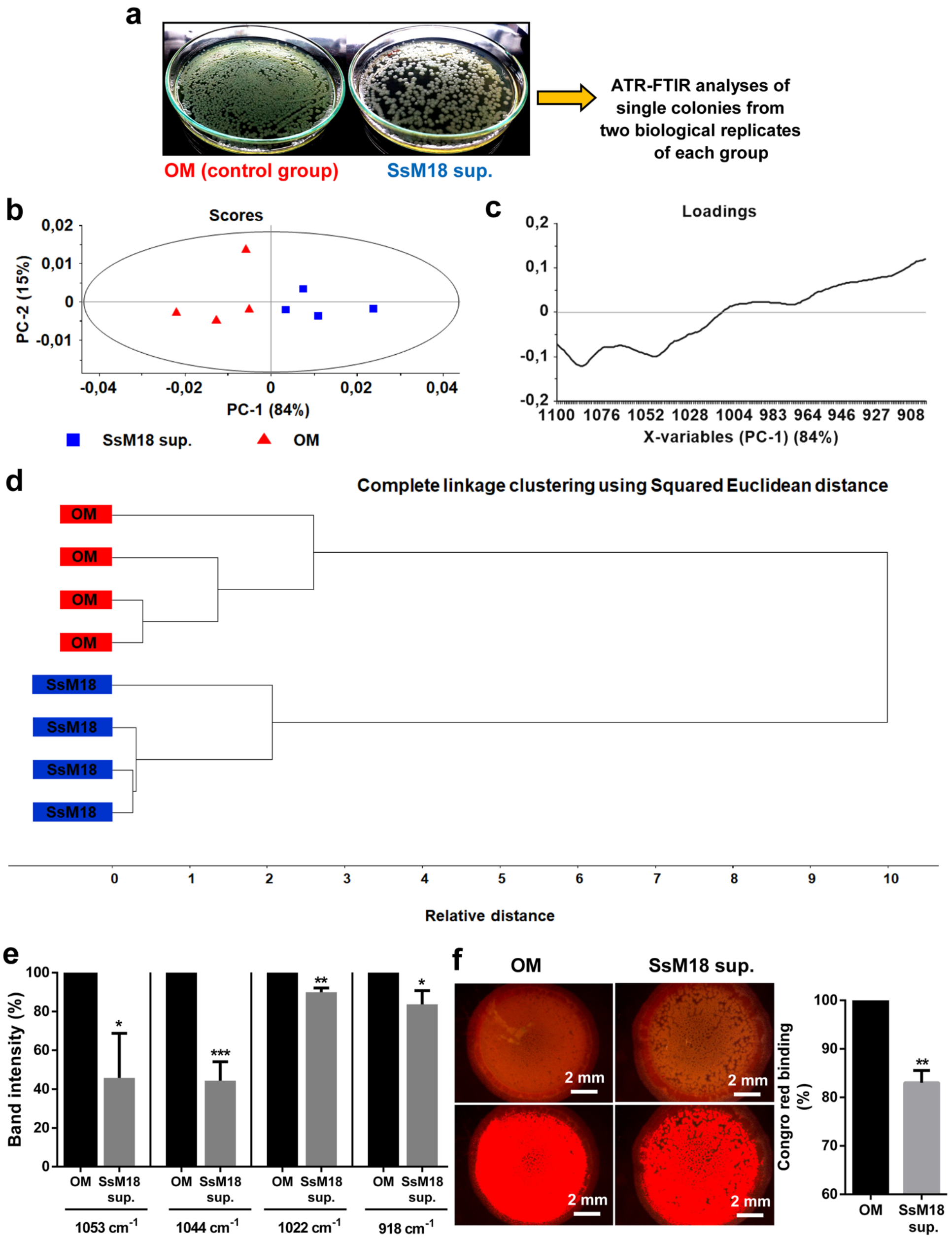

Fig. 3 The polysaccharide content of *P. aeruginosa* decreased by incubation with *S. salivarius* M18 cell-free supernatant. **a** *P. aeruginosa* was inoculated in *S. salivarius* M18 supernatant (SsM18 sup.) or control medium, (only medium; OM), and spread on agar plates. The plates were incubated at 37 °C for 24 h and the single colonies were collected to be used in ATR-FTIR spectroscopic analyses. **b** In the particular polysaccharide region (1100–900 cm^{-1}), PCAs were applied to the mean-centered and single transformed (unit-vector normalized) absorbance spectra obtained from *P. aeruginosa* colonies grown in the presence of *S. salivarius* M18 supernatant (SsM18 sup.) or incubated only with control medium (OM). **c** The corresponding PCA loading plot is shown for the PC-1. **c** HCA dendrogram illustrates the successful differentiation of the *S. salivarius* M18 supernatant (SsM18 sup.) or control medium (OM) incubated *P. aeruginosa*. An index at the bottom is an arbitrary scale which was used to compare similarities at different distances. **d** In the 1100–900 cm^{-1} region, the intensities of IR bands with significant spectral differences were presented as percent change respect to the control group (OM). **e** Congo red binding pattern of *P. aeruginosa* grown in the presence of *S. salivarius* M18 supernatant (SsM18 sup.) or control medium (OM) was shown. The intensity of the binding was quantified using ImageJ (NIH, USA; https://rsbweb.nih.gov/ij/) color threshold plugin (lower panel). The change in the Congo red binding as an indicator for exopolysaccharide production was represented as percent change respect to control (OM) (right panel). The experiments were repeated two times each with two technical replicates. The results represented as mean ± SEM and the statistical significances were analyzed using *t* test (*$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$)

of saccharides) (D'Souza et al. 2008), 1044 cm^{-1} (C–O stretching of polysaccharides; attributed to β-glucans) (Liu et al. 2006; Venkataramana et al. 2010; Synytsya and Novak 2014), 1022 cm^{-1} (C–O stretching of polysaccharides; assigned to β-1,4 glucans of polysaccharides) (Ramamurthy and Kannan 2007; Pebotuwa et al. 2020), and 918 cm^{-1} (C–O, COH, and C–C ring vibrations in carbohydrates; probably corresponding to α-glycosidic linkage) (Ushasree and Ahmad 2017; Li et al. 2018a) were found to be decreased significantly in *P. aeruginosa* grown in the presence of the molecules present in the *S. salivarius* M18 cell-free supernatant (SsM18 sup.). Collectively, PCA and HCA results together with the band intensity analyses showed that (i) polysaccharide constitutes of the *P. aeruginosa* grown in the presence of *S. salivarius* M18 extracellular compounds were different from the *P. aeruginosa* grown in the presence of regular growth environment and (ii) secreted molecules of *S. salivarius* M18 decreased the polysaccharide content of *P. aeruginosa*. Depending on the results obtained from the spectral data, we used Congo red binding assay to observe the changes in the polysaccharide production (Calderón et al. 2019). Quantitative analysis of the Congo red coverage of *P. aeruginosa* was achieved using ImageJ. The decrease in the Congo red binding, indicating decreased polysaccharide content, in the spots of *P. aeruginosa* grown in the presence of extracellular compounds of *S. salivarius* M18 was shown in Fig. 3f.

The effects of *S. salivarius* M18 cell-free supernatant on *P. aeruginosa* cellular lipid content and structure were also investigated from the spectral data. The band 1740 cm^{-1} is designated to C=O stretches of ester functional groups from lipid triglycerides and fatty acids and therefore assigned for total lipids in the cell (Leskovjan et al. 2010; Shapaval et al. 2014). The absorption bands in the ranges between 3050 and 3000 cm^{-1} belong to the –CH and between 2950 and 2800 cm^{-1} belong to the $–CH_2$ groups of unsaturated and saturated aliphatic chains of lipids, respectively. The absorption ratio of these two ranges can be used to determine the changes of the unsaturated to saturated lipid ratio as a marker for lipid oxidation (Mecozzi and Sturchio 2017). As shown in Fig. 4a, a significant decrease found in the cellular total lipid content of *P. aeruginosa* grown in the presence of *S. salivarius* M18 extracellular secreted compounds. Besides, the ratio of unsaturated lipids to saturated lipids was significantly higher in these bacteria compared with the control group (OM) (Fig. 4b).

In summary, FTIR spectral analyses revealed that significant changes in the content and composition of polysaccharides and lipids occur in the pathogenic bacteria *P. aeruginosa* grown in the presence of extracellular compounds of *S. salivarius* M18. Therefore, one can suggest that these biomolecular alterations have reflected the changes in the cellular activities of the pathogenic bacteria, such as growth and biofilm formation capabilities.

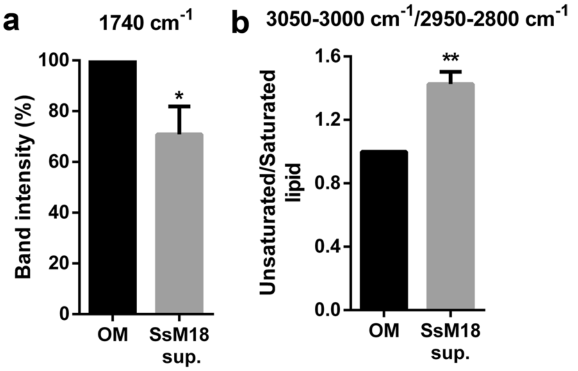

Fig. 4 Incubation with S. *salivarius* M18 cell-free supernatant altered cellular lipid content and status of *P. aeruginosa*. **a** Change in the intensity of the 1740 cm^{-1} band was given as percent (%) respect to the band intensity belong to the control medium (OM) incubated *P. aeruginosa*. **b** Change in the unsaturated (3050–3000 cm^{-1}) to saturated lipid (2950–2800 cm^{-1}) ratio was displayed as fold change. Mean ± SEM was used to report variability in the experiments repeated as two biological replicates with two technical replicates each. *t* test was used for comparisons ($^{*}p \leq 0.05$, $^{**}p \leq 0.01$)

Discussion

Oral microbiota appears as an important concept since a variety of health conditions, including but not limited to pneumonia, hepatic or brain abscesses, dementia, cardiovascular diseases, cystic fibrosis, and digestive cancers were reported to be linked either directly or indirectly to the composition of oral microbiota (Offenbacher et al. 2009; Hale et al. 2012; Burton et al. 2013b; Koliarakis et al. 2019). Recently, dysbiosis of colonic microbiota was also suggested to be affected by oral microbiota (Koliarakis et al. 2019) pointing out that oral–colon interaction should be studied in detail, especially for the evaluation of oral bacteria-mediated systemic inflammatory responses. Therefore, the development of functional probiotics and probiotic products capable of fostering a healthy oral microbiota is a special area of interest not only for treating periodontal diseases but also for treating systemic diseases (Wescombe et al. 2012; Kumar 2013).

Streptococcus salivarius M18 is a Gram-positive oral probiotic which can inhibit several numbers of nose, mouth, and throat pathogens. The unusually broad spectrum of inhibition suggests that *S. salivarius* M18 may also have additional benefits for the host in helping to limit the growth of a variety of other common bacterial pathogens to prevent different types of infections in addition to the infections of the oral cavity. In this study, we analyzed the antipathogenic properties of the cell-free supernatant of *S. salivarius* M18 on the pathogens *P. aeruginosa* and *K. pneumonia*. The supernatant was found to inhibit the growth of *P. aeruginosa* and *K. pneumonia* and enhance their susceptibility to antibiotic treatment. Besides, surface attachment ability and biofilm formation capacity of *P. aeruginosa*, a commonly used bacteria for the biofilm development model (Ryder et al. 2007), was inhibited dramatically in the presence of *S. salivarius* M18 extracellular molecules. Analyses of ATR-FTIR spectra allowed us to pinpoint some of the biomolecular changes, which can potentially affect the cellular activities, occurred in *P. aeruginosa* incubated with the *S. salivarius* M18 cell-free supernatant products.

Pathogen inhibitory activity of probiotic lactic acid bacteria is mostly caused by the metabolites, including organic acids, diacetyl, hydrogen peroxide, bacteriocins, and bacteriocin-like inhibitory substances (BLISs) (Kormin et al. 2001; Dierksen et al. 2007; Barzegari et al. 2020). Living organisms, including bacteria, fungi, animals, and plants, produce low molecular weight host defense peptides referred to as antimicrobial proteins (AMPs) (Chikindas et al. 2018; Lei et al. 2019a). Bacterial AMPs can be classified as non-ribosomally synthesized AMPs, with no structural genes coding for these AMPs and ribosomally synthesized AMPs namely bacteriocins (Chikindas et al. 2018), a huge family of secreted peptides that display antimicrobial activity (Perez et al. 2014; Hols et al. 2019). Bacteriocins are produced by different genera of lactic acid bacteria, including *S. salivarius*, and can inhibit or kill other taxonomically close or unrelated bacteria from both Gram-negative and Gram-positive groups (Silva et al. 2018; Kumariya et al. 2019). They can be easily degraded by proteolytic enzymes and the degradation fragments are not long-lived in the human body or the environment; therefore, the opportunity of target strains to interact with the degraded antibiotic fragments, which is one of the common reasons behind the development of antibiotic resistance, can be minimized. Accordingly, bacteriocins are suggested to be used as a replacement for antibiotics to which pathogenic bacteria have become resistant (Perez et al. 2014). In addition to their physical stability, the above-mentioned properties of bacteriocins make them attractive for various applications expanding from human health to food industry, such as treatment of infections and diseases caused by antibiotic-resistant bacteria, selective killing of cancer cells, and inhibition of foodborne pathogens (Chikindas et al. 2018; Silva et al. 2018). Different classes of bacteriocins have been shown to exert their antimicrobial action mainly through disrupting the integrity of the cell wall via generating ion-selective pores which cause dissipation of the proton motive force and depletion of intracellular ATP (adenosine triphosphate), leakage of intracellular substrates, and eventual death (Cleveland et al. 2001; Kumariya et al. 2019) or through inhibiting the protein or nucleic acid synthesis of the target cell (Ahmad et al. 2017). Bacteriocins that contain the modified amino acid lanthionine are referred to as lantibiotics (Barbour et al. 2013). Lantibiotics are the antimicrobial peptides that are active against Gram-positive bacteria but they show relatively poor activity against most Gram-negative bacteria (Li et al. 2018b). Bacteriocins of *S. salivarius* are referred to salivaricins (Grazia et al. 2017). The data obtained from the *S. salivarius* M18 genome sequencing revealed that the chromosome contains *slm* locus encoding the lantibiotic salivaricin M. The strain also harbors an about 183 kilobase pairs long megaplasmid, namely pSsal-M18, with 172 protein-coding sequences in which 16 of them belong to the known bacteriocin-encoding loci for the lantibiotics salivaricin A2 (encoded by *salA*), salivaricin 9 (encoded by *sivA*), and salivaricin MPS (Wescombe et al. 2010; Heng et al. 2011; Barbour et al. 2020). Furthermore, recent research revealed that although *S. salivarius* lantibiotics are usually known to be encoded by megaplasmids, *S. salivarius* typically has an incomplete chromosomal *blp* (bacteriocin-like peptide) locus followed by several secondary loci, comprising the putative bacteriocin genes *slvW*, *slvY*, and *slvZ* for the strain M18, that potentially code for class II salivaricins with an N-terminal double-glycine maturation site. It has been also shown that since the human commensal *S. salivarius* does not contain a functional BlpRH pair; the ComRS-regulated ComA, which is homologous to BlpA, is responsible for bacteriocin secretion, maturation and so, for competence commitment (Mignolet et al. 2018; Hols et al. 2019). Like most bacteriocins, salivaricins can disrupt cell membranes via pore formation through electrostatic interactions (Eijsink et al. 2002), but salivaricin B was shown to interfere with cell-wall biosynthesis in the target cells by inhibiting peptidoglycan formation through the accumulation of the final soluble cell-wall precursor UDP-*N*-acetylmuramyl-pentapeptide (UDP-MurNAc-pentapeptide) (Barbour et al. 2020). In this study, we have shown that *S. salivarius* M18 supernatant was potent to inhibit the pathogenicity of the two Gram-negative species *P. aeruginosa* and *K. pneumonia*. Noteworthy, lantibiotics are considered to predominantly if not exclusively, act on Gram-positive bacterial targets (Wescombe et al. 2009), and depending on the present evidence, the inhibitory activity of the defined lantibiotic salivaricins is against solely on other Gram-positive pathogens (Wescombe et al. 2011; Barbour et al. 2020). However, some bacteriocins and BLISs, yet uncharacterized compounds that appear to have "bacteriocin-like" inhibitory effects (Rahmdel et al. 2019), have been reported to exhibit much broader antimicrobial activity; in other words, some of them can be active against both Gram-positive and certain Gram-negative bacteria. Besides, some bacteriocin-like compounds have been claimed as more active at acid pH than at neutral or alkaline pH (Li et al. 2015). In addition to the AMPs, lactic acid bacteria produce acidic end products which reduce the pH of the environment and create unfavorable conditions for the growth of many pathogenic bacteria (Barbour and Philip 2014; Vieco-Saiz et al. 2019). The undissociated form of the organic acids enters the bacterial cell and dissociates inside its cytoplasm and reduces the intracellular pH or the intracellular accumulation of the ionized form of the organic acid can lead to the death of the pathogen (Bermudez-Brito et al. 2012). Thus, we investigated the effect of pH modulation on the pathogen inhibitory action of the *S. salivarius* M18 cell-free supernatant. Because *S. salivarius* M18 has been found to lower the pH of the growth medium to 5.0, we adjusted the pH of the uninoculated control medium (only medium; OM) to 5.0 and determined the growth inhibitory properties of the cell-free supernatant of the probiotic bacteria and the uninoculated control medium (pH adjusted to 5.0). We found that at the end of 24 h incubation, acidified control medium did not show any growth inhibitory effect on *P. aeruginosa* while incubation with the *S. salivarius* M18 cell-free supernatant resulted in almost 70% inhibition in the growth of *P. aeruginosa*. On the other hand, when the pH of *S. salivarius* M18 supernatant was neutralized (pH adjusted to 7.0), almost 50% of the *P. aeruginosa* growth inhibitory action of the supernatant was lost. Accordingly, these results suggest that in a slightly acidic environment (pH 5.0), the antipathogenic activity of the *S. salivarius* M18 cell-free supernatant was potentially caused by the combined effects of the products of *S. salivarius* M18 which can be proteinaceous in nature and more active at pH 5.0 and the organic acids produced by *S. salivarius* M18 (Makras and De Vuyst 2006) in which the activities have been disappeared by neutralizing the environmental pH.

The results of this study also represent that incubation with *S. salivarius* M18 cell-free supernatant decreases the cellular polysaccharide content of *P. aeruginosa*. Bacterial polysaccharides can be classified depending on their morphological localizations: (i) intracellular polysaccharides, such as peptidoglycan, periplasmic glucans, lipopolysaccharide (LPS), lipooligosaccharide (LOS), are found inside or as part of the cytoplasmic membrane or cell wall, (ii) capsular polysaccharides form a structural part of the cell wall, and (iii) extracellular polysaccharides (exopolysaccharides), consisting of branched, repeating units of sugars or sugar derivatives, are secreted by microorganisms into their environment (Chapot-Chartier and Kulakauskas 2014; Misra et al. 2015). Many bacteria produce polysaccharides to be secreted out of the cell. Some of these secreted polysaccharides show only limited association with the cell surface, and these are often referred to as extracellular polysaccharides or slime polysaccharides. On the other hand, some of the secreted polysaccharides can form a discrete surface layer (capsule) that is intimately associated with the cell surface. Extracellular polysaccharides and capsular polysaccharides play important roles in the biology of microorganisms, including biofilm formation, and they have been known to be essential virulence determinants in pathogens of humans, plants, and livestock (Cuthbertson et al. 2009). Moreover, surface-associated polysaccharides are involved in a wide range of bacterial properties and functions, including surface adhesion which is an important step in biofilm formation. They also participate in interactions with other microorganisms and host cells (Chapot-Chartier and Kulakauskas 2014). More specifically, one of the major aspects of the cystic fibrosis–*P. aeruginosa* interaction is the production of high levels of an exopolysaccharide (Pier 2000). Accordingly, inhibiting the production of polysaccharides has been suggested as an alternative approach to reduce biofilm formation (Huang and Stewart 1999). Besides, bacterial cell wall polysaccharides are required for normal cell morphology and play a role in cell division (Chapot-Chartier and Kulakauskas 2014). Polysaccharides and glycoconjugates of the bacterial cell wall are also important for cell viability by protecting the cell protoplast from damage. In Gram-negative bacteria, the outer membrane mainly contains LPSs that cause powerful immune reactions in humans such as fever and septic shock and even multiple organ failure and mortality (Pier 2000; Misra et al. 2015). In addition to the alterations in the polysaccharides, another finding of this study is the modification of the cellular lipid content and composition of the pathogen *P. aeruginosa* by the cell-free supernatant of *S. salivarius* M18. Cell size and cell shape are among important determinants for surface adhesion and biofilm formation in bacterial cells (Alim et al. 2018) and wall/membrane lipid composition has been claimed to affect the size and shape of a bacterial cell (Huang et al. 2006; Alim et al. 2018). The bacterial cell wall is the most important site for the attack by antibiotics (Misra et al. 2015; Liu and Breukink 2016) and the bacterial lipid composition arises as an important factor in determining the sensitivity of bacteria to antimicrobial agents (Epand et al. 2007). Our results showed that *S. salivarius* M18 supernatant increases the lipid unsaturation index of *P. aeruginosa*. This result is notable since the percentage of saturated fatty acids are shown to be increased in cells of biofilm compared with the cells of planktonic state (Dubois-Brissonnet et al. 2016). In another study, sessile *P. aeruginosa* was shown to have higher lipid stability in the membrane bilayer and lower membrane fluidity (an increase of membrane saturation) as compared with planktonic counterparts (Benamara et al. 2014). As a summary, it can be proposed that through decreasing polysaccharide production and secretion, decreasing surface attachment, along with modifying cellular lipid composition, the products of *S. salivarius* M18 present in the cell-free supernatant can reduce propagation, and inhibit biofilm formation of the pathogenic bacteria.

By producing bacteriocins that target the important cariogenic species and through the production of the enzymes dextranase and ureas that help reduce dental plaque accumulation and acidification, respectively, the probiotic *S. salivarius* M18 has been suggested to be used in preventing cavities and breaking-up dental plaques (Burton et al. 2013a). First time in the literature, we showed that *S. salivarius* M18 has an inhibitory activity also on the two Gram-negative pathogens *P. aeruginosa* and *K. pneumonia*, both of them are associated with a broad range of human infections and known as important food-borne pathogens. In the global medicine market, more than one-third of the medicines based on the natural resources, including microorganisms, and bioactive compounds obtained from natural resources are extremely important for the pharmaceutical industry for drug development (Butler 2004). The results presented in this manuscript indicate that *S. salivarius* M18 has a huge potential for the prevention or alleviation of several types of pathogens along with the ones responsible for oral infections. The probiotic potential of *S. salivarius* M18 should be investigated in more detail for further characterization of known inhibitory agents as well as for the investigation of possible novel inhibitor compounds produced by this species.

Availability of data and material

All the data generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Author contributions Both authors contributed to the conception and design of the study. The final manuscript has been read and approved by both authors. ST designed the experiments, supervised the study, analyzed the results, and wrote the manuscript. SK carried out the experiments with help from ST. This study is a part of the Master of Science project of SK under the supervision of ST.

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Compliance with ethical standards

Conflict of interest The authors declare they have no conflict of interest relevant to this article.

Ethics approval This study does not require ethical approval.

Informed consent For this type of study, consent is not required.

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