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ORIGINAL PAPER

Synthesis and characteristics of biocidal oil composites enhanced with thymol and salicylic acid

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Abstract

The research aimed to develop composite materials based on waste cooking oil containing thymol or salicylic acid and assess their antimicrobial properties against selected Gram-positive and Gram-negative bacteria strains. The study also investigated whether the use of a binder, such as catalyzed waste cooking oil, and the performing of annealing process would affect the antimicrobial effectiveness of the natural additive. The composite materials were characterized using FT–IR spectroscopy, thermal analysis, and scanning electron microscopy. Mechanical properties were also evaluated, along with abrasion resistance and soakability. Thymol-modified materials showed higher activity against *E. coli* strain; while, salicylic acid-modified materials were more effective against *P. aeruginosa*. The antibacterial activity against Gram-positive bacteria was generally lower than against Gram-negative bacteria. Flow cytometry and confocal microscopy were used to visualize and quantify bacterial death induced by the composite materials. The materials showed interactions with bacterial cells, leading to cell damage and inhibition of bacterial division. The most effective biocidal composite against the suspension of *P. aeruginosa* bacteria was 22 T (99% inactivation), containing 4% thymol. Against *E. coli*, composite 41 T containing 1% thymol caused a significant decrease in the viability of these bacteria by up to 45%. Similarly, on *S. aureus*, composites with the addition of thymol also exhibited strong effects, reaching up to 70% reduction, as observed in 43 T with 7% thymol. Composites containing salicylic acid also demonstrated biocidal properties, resulting in a 52% reduction in *E. coli* (33SA containing 7% salicylic acid); 99% reduction in *P. aeruginosa* (15SA containing 1% salicylic acid); 20% reduction in *S. aureus* (41SA containing 1% salicylic acid); and approximately 25% reduction in *S. epidermidis* (43SA with 7% salicylic acid). Furthermore, the composite materials demonstrated low cytotoxicity against human keratinocytes, indicating their potential safe use when contacted with human skin.

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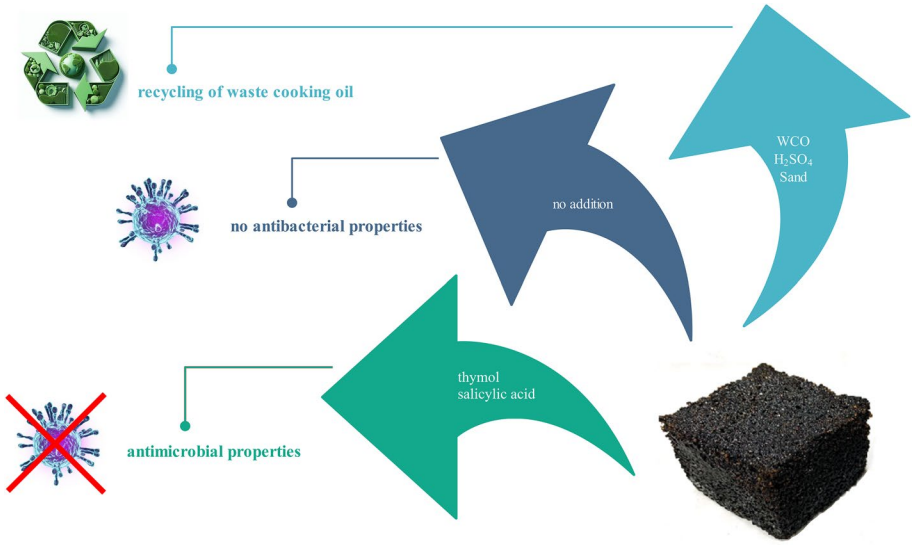
Graphical Abstract



Keywords Composite · Vegeblock · Oil block · Waste cooking oil · Functional materials · Microbiology · Salicylic acid

Introduction

Social development, urbanization, and increased travel contribute to the rapid spread of diseases (Connolly et al. 2020). The fast-paced lifestyle and associated stress make quick recovery crucial, leading to a high demand for public spaces for relaxation. However, crowded public places pose a significant risk of disease transmission due to varying hygiene levels and specific health conditions among users. Locations like swimming pools, saunas, and hot tubs, where direct skin contact with surfaces occurs, create favorable conditions for microbial growth. This increases the risk of bacterial, fungal, and viral infections among users, highlighting the need for effective hygiene measures in recreational facilities.

The most common bacteria in public recreation areas such as swimming pools, spas, hot tubs are *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Pseudomonas stutzeri*, *Pseudomonas mendocina*, *Staphylococcus epidermidis*, *Aeromonas bacteria*, *Aeromonas hydrophila/caviae* and *Lecburylcia adobacter*. Mostly, users of recreational facilities can pick up these pathogenic microorganisms on their hands or feet and transfer them to another location (AlTuraifi et al. 2019). Pathogenic strains are not only prevalent in SPA-type facilities but are also widespread in neighborhood chutes and parking lots. The high microbiological risk arises from the presence of organic waste, particularly food scraps, left unattended in areas not designated for disposal. Abundant literature confirms the substantial presence of pathogenic microorganisms in municipal waste collection sites (Bulski et al. 2016; Przybulewska et al. 2010). Bacteria most frequently multiplying in chutes and other municipal waste collection areas include both Gram-positive bacteria (*Staphylococcus spp.*, *Micrococcus spp.*, *Steptococcus spp.*, *Mycobacterium*, *Bacillus spp.*) and Gram-negative bacteria (*Pseudomonas spp.*, *Escherichia spp.*, *Enterobacter spp.* and others) (Traczewka et al. 2000; Łebkowska 2001; Hawrot-Paw and Domżał 2016). Enterotoxins produced by Gram-negative bacteria and found in their cell wall are responsible for various conditions such as allergic and toxic pneumonia, chronic bronchitis, acute respiratory obstruction, and skin problems (Deacon et al. 2009; Breza-Boruta 2012). The microorganisms multiplying among the waste, in addition to bacteria, include fungi, protozoa, viruses, intestinal parasites, as well as glucans, endotoxins, fungal metabolites, exotoxins and mycotoxins produced by microorganisms, with the degree of risk of infection by a biological agent varying (Kalwasińska et al. 2012; Nandhini et al. 2023).

In public places with a high microbiological risk, common methods involve surface cleaning with detergents or strong acidic formulations. However, there is currently no effective way to disinfect concrete surfaces while promoting proper health prophylaxis. Without preventive measures, pathogens can proliferate, leading to the potential spread of infectious diseases. Disease transmission involves a sequence of events known as the chain of infection, and

disrupting this chain is crucial for effective prevention. The use of flooring or paving materials with antimicrobial properties is considered advantageous in breaking this chain. Unfortunately, many agents used for this purpose are synthetic and toxic to human health (Connolly et al. 2020). For the sake of the environment and human safety, non-toxic antimicrobial agents are being sought.

Among substances of natural origin with antimicrobial properties, thymol (isopropyl methacresol, 2-isopropyl-5-methylphenol), considered by many researchers to be one of the most active antimicrobial substances among the constituents of essential oils, is a popular choice (Delgado et al. 2004). Thymol is found in plants such as *Thymus vulgaris, Ocimum gratissimum, Satureja thymbra, Thymus zygis, Carum copticum* and *Lippia multiflora* (Kowalczyk et al. 2020). It is a colorless, crystalline compound with an intense odor, dissolves in alcohol and other organic solvents, but is poorly soluble in water. Thymol is used in soft drinks and confectionery products. Thymol exhibits antibacterial activity against Gram-negative and Gram-positive bacteria (including *S. typhimurium*, *Escherichia coli*, *S. aureus*, *S. typhimurium*) also against verocytotoxic *E. coli* bacteria (Rivas et al. 2010; Mathela et al. 2010; Du et al. 2015) and also antifungal and against parasitic protozoa *Leishmania (Viannia) panamensis* (De Morais et al. 2014). In addition, it also shows antiviral activity (human rhinoviruses, herpes simplex virus type I and influenza viruses) (Li et al. 2017). Thymol contained in essential oils and esters show antiviral activity against influenza virus, HSV-1, HSV-2 and the results of studies on thymol activity against Sars-CoV-2 look promising (Walther and Schmidtke 2020; Kowalczyk et al. 2020).

Salicylic acid also has high antimicrobial efficacy. It occurs naturally in plants and regulates their flowering, growth and participates in the plant response to pathogens and abiotic stress (Balcke et al. 2012). Natural salicylates (salicylic acid and salicin) occur in large amounts in black poplar buds, willow bark, black poplar buds, elm herb and elm leaves (Bijttebier et al. 2016). Salicylic acid exhibits antiseptic and antibacterial properties, and is used as a food additive, in pharmaceuticals and cosmetics (Sedanova et al. 2023). Studies conducted on strains have confirmed antimicrobial efficacy against, among others, *Escherichia coli*, *Staphylococcus aureus, Bacillus subtilis*, and *Pseudomonas aeruginosa* (Polonio et al. 2001; Bandara et al. 2006; Fang et al. 2020). Moreover, in view of the fact that salicylic acid a phenolic compound considered safe for human health and the environment, that it can be an alternative to commonly used fungicides. Several studies have evaluated the antifungal activity of this acid against several pathogens (Wang et al. 2011; Panahirad et al. 2012; da Rocha Neto et al. 2015). Thus, it seems reasonable to conduct comprehensive research on new functional composite materials based on waste cooking oil for applications in places and public facilities with a higher risk of pathogens enriched with thymol or salicylic acid. Today, the annual consumption of vegetable oils for food use on a global scale exceeds 200 million tons (Shahbandeh 2023). Cooking oil is used not only in the food service sector, but also in households. It is common practice to dispose of frying oil residues directly into the sewage system, which generates undesirable effects such as unpleasant odors, clogged pipes, difficulties in the wastewater treatment process and environmental pollution. In addition, waste cooking oil (WCO) accumulated in illegal landfills can enter the sewage system, leading to ecosystem degradation. Due to the toxic properties of WCO, it is extremely important to avoid its delivery to the aquatic environment and soil (Hosseinzadeh-Bandbafha et al. 2022). Solid products based on used vegetable oils for use in the construction industry are becoming increasingly popular due to easy production technology, availability of raw material and attractive cost–benefit ratio (Noor et al. 2017; Staroń 2023; Staroń et al. 2023).

These materials would provide a means of preventing the spread of pathogenic pathogens. Such a solution can ensure sanitary safety in public places. Functional composite materials can be of any shape and size, e.g., paving blocks, bricks, paving slabs and edging, wall tiles, etc., so they can serve as flooring materials, wall elements or shelter edging, paving slabs, etc., which undoubtedly increases the spectrum of their applications. Incorporating substances of natural origin into the oil matrices will allow the active substances to be evenly released, and thus enable the antimicrobial properties of the substrate to be maintained. These materials will be used in the paving industry, which would allow to increase human safety and reduce the spread of pathogenic microorganisms.

The aim of the research was to obtain composite materials (so-called "vegeblocks") containing thymol or salicylic acid and to study their antimicrobial properties against selected Gram-positive and Gram-negative bacteria. This, in turn, will allow to answer the question of whether the binder such as catalyzed waste cooking oil and the performing of annealing process would affect negatively on the natural additive, disrupting its antimicrobial effectiveness.

Materials and methods

Production of functional composite materials based on waste cooking oil

Oil composites were produced by heating of WCO (rapeseed oil), which had undergone thermal food processing, together with sulfuric acid VI (pure for analysis), quartz sand with a fraction size of 0.5–1.4 mm, and a natural additive: either thymol or salicylic acid. Thymol and salicylic acid were a

commercial products (purified grade). The choice of quartz sand as a component of oil composites resulted from its high resistance to chemical and mechanical factors, which had a positive effect on the strength of the obtained oil composites (Wang et al. 2023). WCO came from a catering outlet where it was used for thermal processing of meat and vegetables (detailed characteristics of this raw material are presented in (Staroń et al. 2022)). The WCO was mixed with sulfuric acid, followed by the addition of sand. After homogenizing the mixture for 5 min in a planetary mixer, the natural additive was introduced and the entire mixture was re-homogenized. Subsequently, the mixture was transferred to aluminum molds and compacted on a vibrating table. The resulting samples were then heated at temperatures ranging from 190 to 210 °C for a duration of 12–20 h. The content of catalyzed oil and natural additive was counted in relation to the weight of sand.

Characteristics of composites

Thermal analysis of the composite materials, including thermogravimetry (TG), differential thermogravimetry (DTG) and differential thermal analysis (DTA), was performed using the SDT 650 apparatus from TA Instruments, within a temperature range of 25–1000 °C and a heating rate of 10 °C/min. Fourier-transform infrared spectroscopy was utilized to analyze the molecular structure of solid substances. This analysis was conducted using the Nicolet iS5 FT–IR spectrometer from Thermo Scientific, covering the wavelength range of 500–4000 cm^{-1}. For the observation of the surface structure of the oil blocks, the scanning electron microscope Hitachi TM-3000 was employed. This microscope was equipped with an energy-dispersive X-ray microanalyzer (EDS). The absorbability of the composites was tested by incubating them in distilled water (water mass/sample mass = 20:1) for 72 h. After this time, the samples were weighed again and their water absorption was calculated. To determine the abrasion resistance of composites, the authors used the test described in standard PN-EN 13892-3 (Bőhme method). Mechanical strength tests were carried out using the Zwick–Roell Z600 testing machine, applying an initial force of 25 N and a testing speed of 1 kN/min. A total of 100 materials each were obtained with both thymol and salicylic acid. Only those composites that met the strength requirements for pavers (tensile strength not less than 2.9 MPa, flexural strength at least 2.8 MPa) were selected for testing antimicrobial properties. The process parameters for these composites are shown in Table 1.

Table 1 Parameters of composite materials with natural additive production process

Composite no	Catalyzed oil (%)	H_2SO_4/catalyzed oil (g/g)	Temperature (°C)	Time (h)	Additive (%)
12	25	0.24	190	20	1
15	20	0.04	210	20	1
16	25	0.24	210	20	7
22	25	0.14	200	19	4
31	25	0.24	190	18	7
33	20	0.24	210	18	7
35	25	0.24	210	18	1
37	20	0.24	190	20	7
41	20	0.24	210	20	1
43	25	0.24	210	20	7
44	22.5	0.14	200	18	4
48	20	0.14	200	19	4
51	22.5	0.24	200	19	4
61	22.5	0.14	190	18	4
80	22.5	0.14	210	18	4
88	22.5	0.24	210	19	7
92	22.5	0.24	210	19	1
93	22.5	0.24	190	17	4
96	22.5	0.24	210	16	4
97	22.5	0.24	210	16	1
98	22.5	0.24	210	12	7
99	22.5	0.24	210	12	4

Bacteria culture

The Gram-positive bacteria (*S. aureus* and *S. epidermidis*) and Gram-negative bacteria (*E. coli* and *P. aeruginosa*) were used in the study. *S. aureus* (8325-4), *S. epidermidis* (ATCC12228), *E. coli* (K12) and *P. aeruginosa* (ATCC19660) cells were cultured in brain heart infusion (BHI) broth and LB broth, respectively, with aeration at 37 °C under shaking conditions in an orbital incubator (180 rpm). Cell growth was assessed with absorbance measurements at 600 nm (OD600) until the absorbance reached 0.5, which corresponded to approximately 10^7 CFU per mL.

Biofilm formation

S. aureus colonies (which have been grown on appropriate agar overnight), were suspended in media and the OD490 was adjusted to 0.65. The resulting bacterial suspension was then diluted 1:6 (1 mL bacterial suspension + 5 mL pre-warmed medium) and incubated at 37 °C with 5% CO_2 for approximately 3 h in order to reach mid-log phase. The mid-log growth suspension was diluted with pre-warmed media in a ratio of 1:2500 and 200 µL of dilution was placed into each well of an 8-well thin-layer agar-coated chamber slide. After approximately 16 h, medium from each chamber was aspirated and fresh medium was added. The biofilm was

incubated with selected composites for 24 h and then visualized with fluorescence microscopy.

Antibacterial activity of composite materials

The tested microorganisms were incubated with selected composites (0–100 mg/mL) of each in PBS for 24 h in the dark at room temperature. After this time, aliquots (10 ul) were transferred to 1 mL of fresh growth media in 12-well plate. Next, samples were mixed, serial-diluted in PBS, and plated (LB agar, BHI agar) to determine the number of CFUs. The viability was also monitored using the LIVE/DEAD BacLight Bacterial Viability Kit (Invitrogen; monitors membrane integrity) according to the manufacturer's instructions.

Flow cytometry

The antibacterial activity of composites was also determined using flow cytometry. For this purpose, bacteria were stained with propidium iodide (10 ug/mL) and the red fluorescence of dead bacteria was detected. For this analysis, untreated bacteria (control) and bacteria after treatment with composites (0.5×10^6 cells) were stained with propidium iodide, washed two times with HBSS and prepared for analysis. Bacteria were collected by centrifugation and then resuspended in 200 µL of PBS. Stained cells were then examined using BD c6 Accuri flow cytometer equipped with a 633 nm laser. The obtained data were analyzed using FlowJo software (Merck Millipore, Burlington, MA. USA) and Graphpad Prism 5.

Fluorescence imaging of bacteria

The antibacterial effect of materials was visualized with confocal imaging using a Zeiss LSM880 laser scanning microscope equipped with an argon-ion laser. The objective was oil immersion "dipping" lens (100×, Carl Zeiss Ltd., Jena, Germany) with a working distance of 1.46 mm. Accordingly, after incubation with selected composites bacteria were stained with Calcein AM (1 ug/mL) and propidium iodide (10 ug/mL). The living bacteria were imaged with green fluorescence and dead bacteria indicated red fluorescence. After washing, the bacteria samples were placed on the microscopic glass slides, and imaged with Zeiss880 confocal microscope. Registered images were analyzed with the Zeiss ZEN software.

Biofilm visualization

For biofilm imaging, the medium from each chamber was aspired and resident biofilm washed twice gently with sterile saline. Next, the dyes for staining were added (BacLight Live/Dead stain) to each well and incubated at room temperature for 15 min. During this time, the samples were protected from light. The stains were then aspirated, and biofilm was washed with sterile saline as before. The neutral buffered formalin was added to each well and incubated for 30 min at room temperature to fix the specimen. Biofilm was washed twice with saline and disposed of the wash fluids that contain formalin. The mounting medium was added, and a coverslip was put on the sample. The biofilms were imaged with Zeiss880 confocal microscope. Registered images were analyzed with the Zeiss ZEN software.

Cytotoxicity studies on human keratinocytes

A human keratinocytes cell line, HaCaT, was obtained from ATCC. The cells were cultured in DMEM medium with L-glutamine and $NaHCO_3$ supplemented with 5% heat-inactivated fetal bovine serum and penicillin (100 U/mL) at 37 °C in 5% CO_2-humidified atmosphere. When the cells reached 80% confluence, they were washed with phosphate-buffered saline and harvested with 2 mL of 0.25% trypsin-EDTA solution. Cells were then centrifuged and counted and plated at a density of 10,000/well in flat-bottom 96-well plates. On the following day, cells were incubated with selected composite materials at 100 mg/mL for 24 h. The medium was then replaced with fresh medium and cells were returned to the incubator. After overnight incubation the viability of cells was assessed using AlamarBlue assay and 3-(4.5-dimethylthiazol-2-yl)-2.5-diphenyltetrazolium bromide (MTT) assay. The cells were incubated for 4 h. At the end of incubation, the media was removed, and in the case of MTT, the cells were dissolved in dimethyl sulfoxide and absorbance from each well was measured at 570 nm using a microplate spectrophotometer. In the case of AlamarBlue assay, the fluorescence from each plate was measured at excitation at 560 nm, emission at 590 nm. Each experiment was repeated three times. Moreover, the bright-field microscopy was used for determining of HaCaT cells morphology after the treatment.

Statistical Analysis

The obtained data are presented as the mean and standard deviation (of the mean). Statistical significance was determined by one-way or two-way ANOVA with Bonferroni post hoc test using GraphPad Prism version 5.0.0 for Windows (GraphPad Software, San Diego, California USA).

Results

Oil composites characterization

Figure 1 shows the FT–IR spectrum of oil composites obtained at 210 °C but varying in the amount of catalyst, natural additive and annealing time. Bands with a wave number of about 1700 cm^{-1} are observed, which can be attributed to C = O stretching vibrations of the ester group, 1437 cm^{-1} C = C stretching vibrations of the alkenyl group, and 1080–1060 cm^{-1} coming from C–O stretching vibrations. C = H deformation vibrations are present for a wave number of 777–796 cm^{-1}. The peaks present for a wave number of 694 cm^{-1} originate from bending vibrations of C–H bonds of single-substituted aromatic compounds, while about 460 $cm^{-1}$are associated with torsional motion in organic molecules (Fečko et al. 2010; Taleb et al. 2020; Banchapattanasakda et al. 2023). In the abbreviations used, the number indicates the preparation of the composite according to Table 1, and the letter indicates the type of natural additive used: T—thymol and SA—salicylic acid.

Thermal analysis of the oil composites showed that the initial weight loss of the oil block occurred in the temperature range from 25 to 100 °C, which is due to the loss of

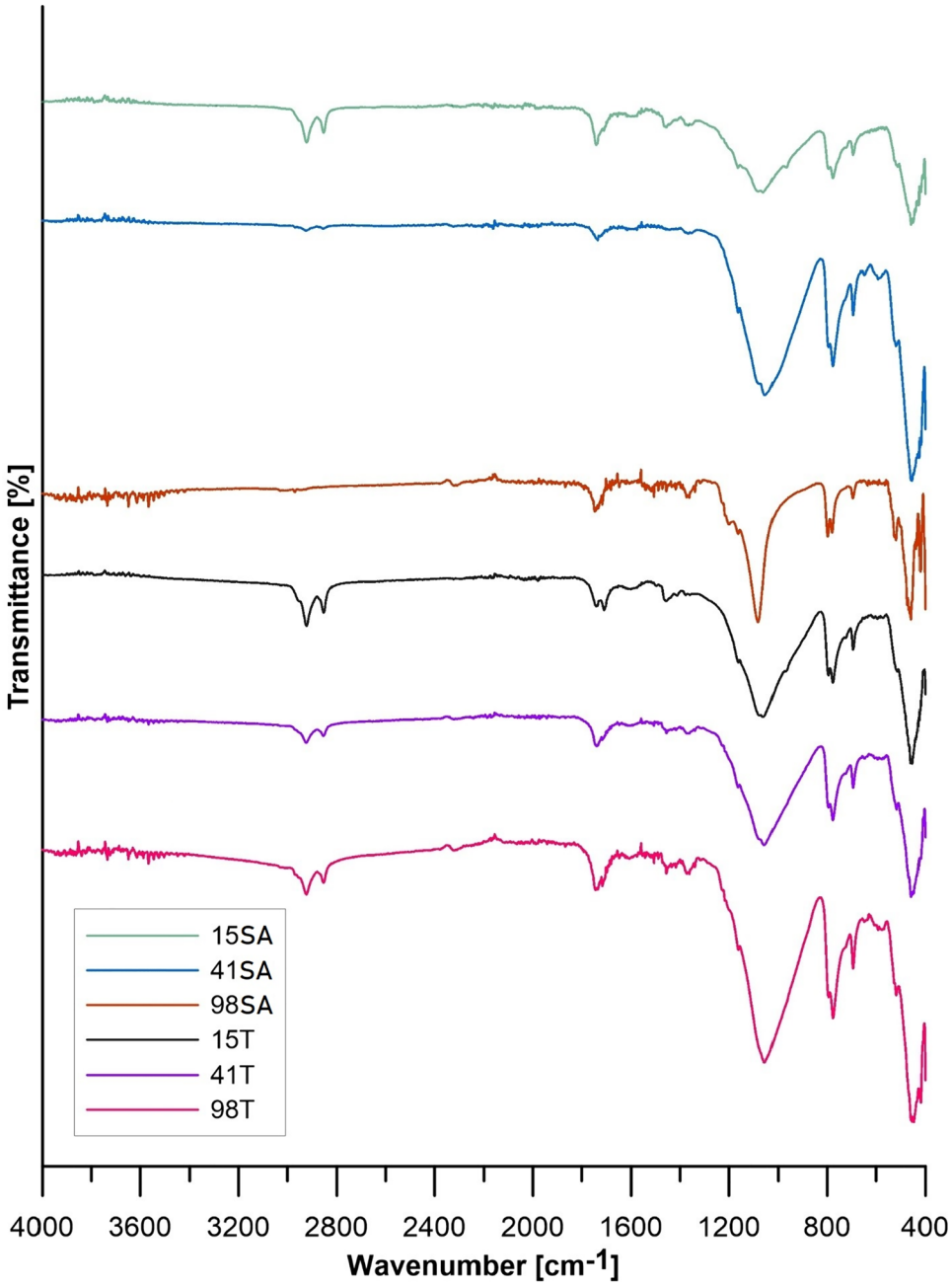


Fig. 1 FTIR spectra of composites No. 15SA, 41SA, 98SA, 15T, 41T and 98T

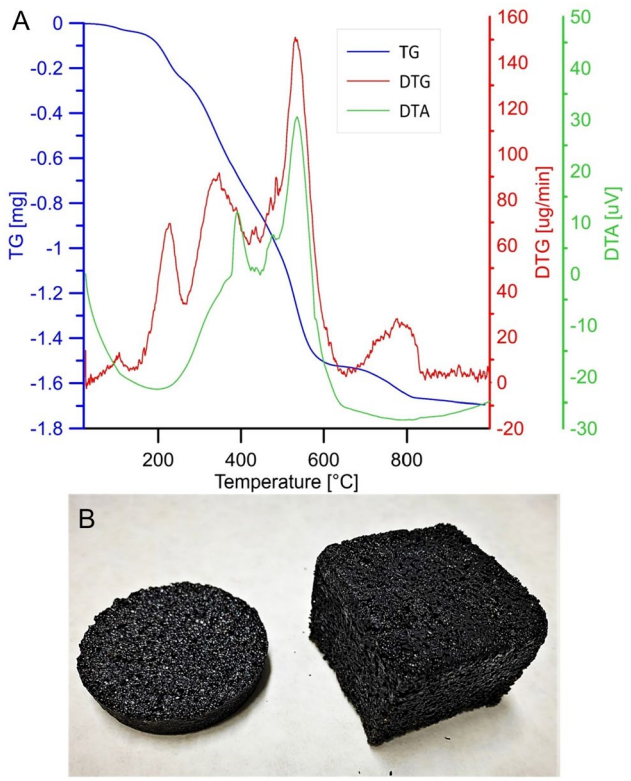


Fig. 2 Composite No. 98SA: **A** thermogravimetric/differential thermal analysis; **B** photograph

water absorbed by this material from the environment. The highest weight loss associated with the decomposition of the sample components is observed in the temperature range 250–600 °C, above this temperature the weight of the sample stabilizes. In the temperature range of 200–350 °C polyesters are degraded. Degradation at around 600 °C can be attributed to the oxidation reaction of double bonds in long chains of fatty acids (Ye et al. 2009; Mizera and Ryszkowska 2018; Alves et al. 2022). Figures 2A and B show the results of thermogravimetric/differential thermal analysis and a photograph of the 98SA composite.

On the surface of the samples, silicon and aluminum were identified as sand components, sulfur from the acid catalyst, carbon component of the WCO and gold and palladium as a result of sample sputtering. Figures 3 and 4 show photography and SEM micrograph of composites No. 16T and 88SA.

The materials were characterized by high porosity and sharp edges but varied in mechanical strength due to the different size and number of pores inside the composites. The lowest splitting tensile strength of about 1.3 MPa was characterized by composite No. 51SA, which was obtained at 200 °C for 19 h with 4% salicylic acid. On the other hand, the highest among composites with thymol was material No. 98T (4.4 MPa), and with salicylic acid was 33SA and 88SA (4.2 MPa). These composites were obtained by annealing at 210 °C. The strength properties of the obtained composites can be compared with those of the layered composite obtained from polyester resin and kenaf fiber [45]. In the case of flexural strength, a similar relationship was observed—16SA and 98 T composites with the highest flexural strengths of 7.3 and 5.7 MPa, respectively, were obtained at 210 °C and the mass ratio of acid to catalyzed WCO was 0.24. 80SA and 80 T composites with the lowest strengths were also annealed at the highest temperature but the mass ratio of acid to catalyzed WCO was 0.14 (Fig. 5). The oil blocks with the highest mechanical strength had the highest mass ratio of acid catalyst to catalyzed oil, which was expected to result in faster polymerization of the samples and their curing.

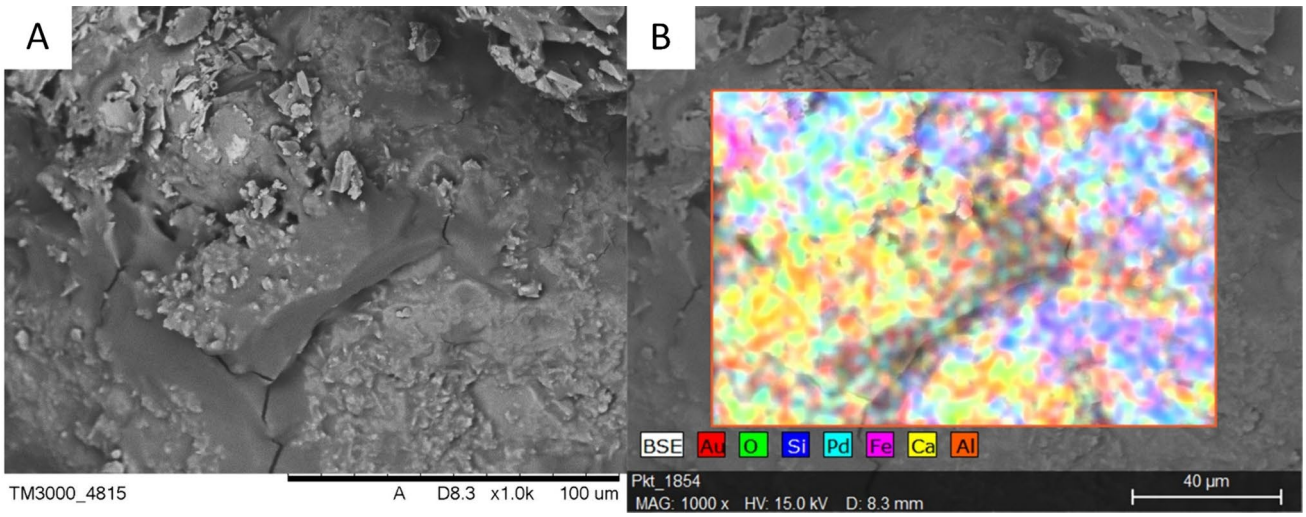


Fig. 3 Photography (**A**) and SEM micrograph (**B**) of composites No. 16T

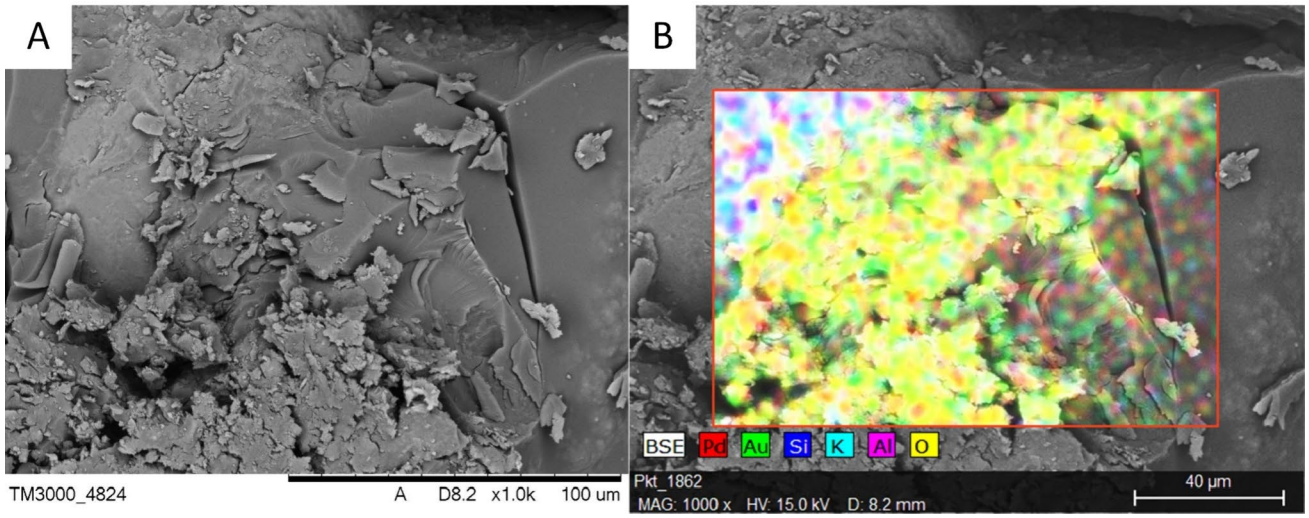


Fig. 4 Photography (**A**) and SEM micrograph (**B**) of composites No. 88SA

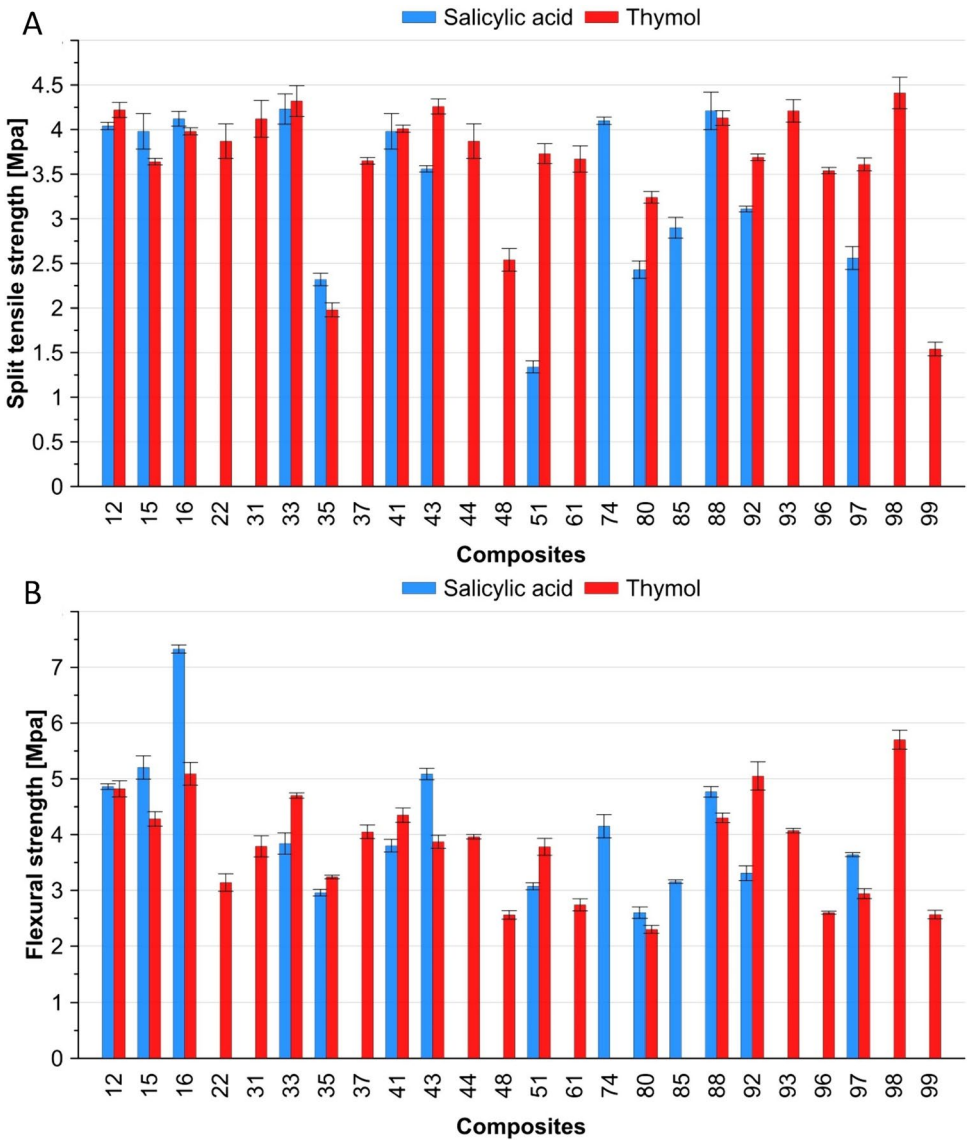


Fig. 5 Split tensile (**A**) and flexural strengths (**B**) of oil composites

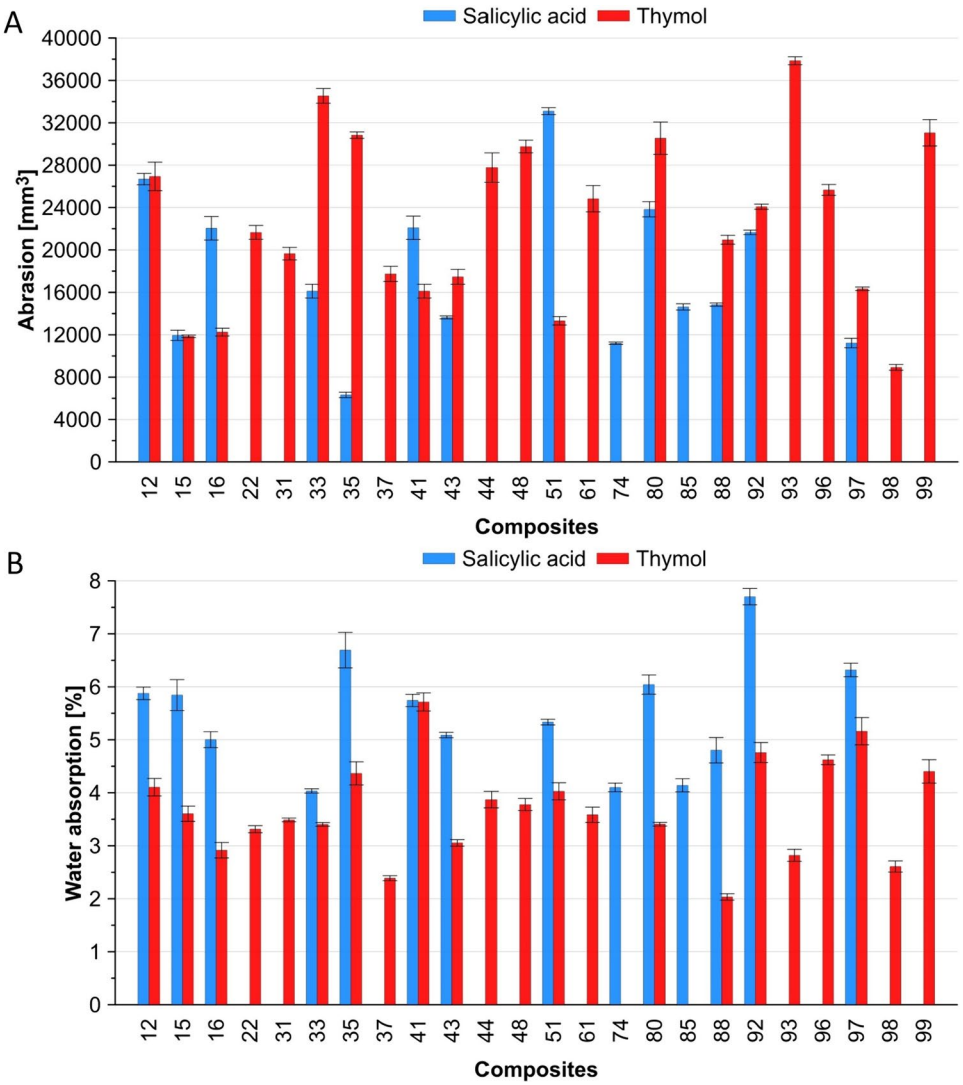


Fig. 6 Abrasion resistance (**A**) and soakability (**B**) of oil composites

The composites also showed variations in abrasion resistance and soakability (Fig. 6A and B). For example, the 35SA composite had a low abrasion resistance of about 6300 mm^3 but a high soaking capacity of 6.7%. In contrast, the abrasion resistance of the 93 T thymol-added composite was almost 38,000 mm^3 and the soakability was only 2.8%.

Antibacterial activity of investigated composite materials

The antibacterial activity of 20 composite materials modified with thymol and 12 composites modified with salicylic acid was determined against Gram-positive (*S. aureus*, *S. epidermidis*) and Gram-negative (*E. coli*, *P. aeruginosa*) bacteria. For each material the maximal concentration at 100 mg/mL was used. The bacteria were grown in both planktonic and biofilm. In each case, bacteria were incubated with the materials for 24 h and then the viability of bacteria was assessed. Figures 7, 8, 9 and 10 show the antibacterial activity of materials against all tested strains. Moreover, recognizing that bacteria in biofilms can be 10–1000 times more resistant to antibacterial agents than planktonic bacteria, we investigated the activity of composite materials on bacterial biofilms. Biofilms were obtained after incubation of the bacteria in 12-flat-bottom microplates coated with thin layer of agar for 24 h. After the establishment of the biofilms, they were incubated for 24 h with selected composite materials at 100 mg/mL concentration (bottom panels on Figs. 7, 8, 9 and 10). The potency of antimicrobial agents can be reported in minimum inhibitory concentrations (MIC). Thus, for the most active materials the MIC values were also determined (Table 2 and Figure S1).

It is important to note that the specific antibacterial activity of waste cooking oil composites can vary depending on the formulation and the intended application. Experimental studies and testing are necessary to determine the precise antibacterial efficacy of a particular composite system based on waste cooking oil. Based on the presented data, it can be

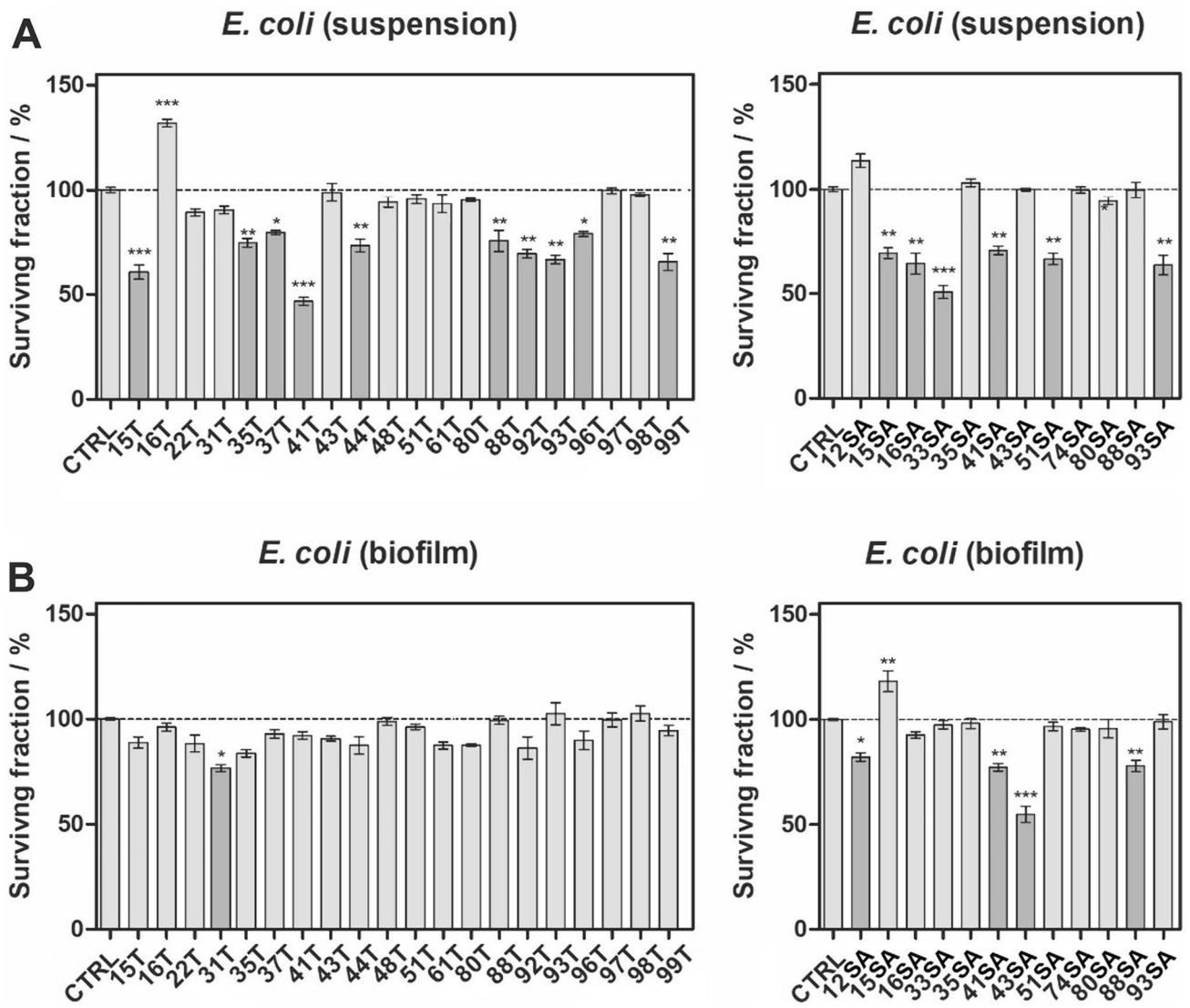


Fig. 7 Determination of antibacterial activity of investigated composite materials against *E. coli* in **A** planktonic culture, **B** biofilm. *E. coli* cells were exposed to each material in concentration of 100 mg/mL for 24 h. Bacteria viability is expressed as a percent of the viability of control (non-treated bacteria). Data are presented as mean ± SEM. The asterisks denote p values < *0.05. **0.01. ***0.001 compared to the control

concluded that the increased percentage of modifier content (both T and SA) do not increase the antimicrobial potency of the material. For instance, 15 T reduced bacteria viability about ca. 45%, contrary to 16 T that can increase the bacteria activity. Thus, it suggests that the antimicrobial activity is closely related to other physicochemical properties of composite material and derives from synergistic effect between the action of base material and modifier.

In the case of *E. coli*. 10 thymol-modified materials caused inactivation of the bacteria. Among these, 41 T was the most active, causing a decrease in viability of up to 45%. The 15 T material also caused inactivation of the bacteria by about 40%. Other materials: 35 T, 37 T, 44 T, 88 T, 92 T, 93 T, 96 T and 99 T caused a decrease in bacterial viability of 20–30%. As for the materials with the second modifier used—salicylic acid, 6 out of 12 samples showed an antibacterial effect against *E. coli*. The most active was 33SA, which caused inactivation of bacteria by 52%. The other active materials 15SA, 16SA, 41SA, 51SA and 93SA caused a decrease in viability by about 30%. It is worth mentioning that both groups of materials were less active against biofilm-growing bacteria. Of the thymol-modified materials, only one 31 T caused biofilm inactivation of 20%. In the case of salicylic acid-modified materials, 43SA showed a bland antibacterial effect (about 50% decrease in viability). The 41SA, 88SA and 12SA materials showed biofilm inactivation at 15–20%. The MIC value was determined for

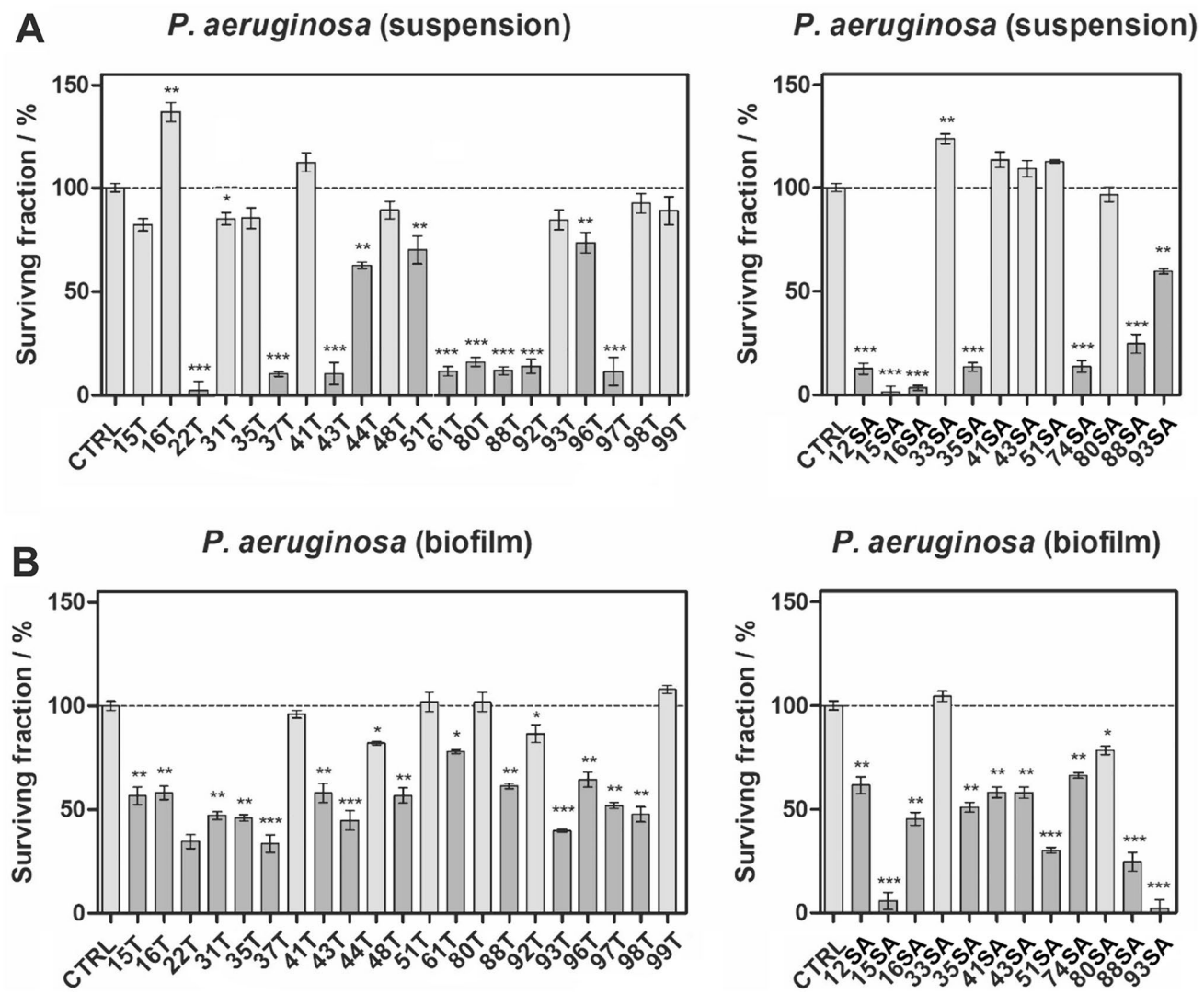


Fig. 8 Determination of antibacterial activity of investigated composite materials against *P. aeruginosa* in **A** planktonic culture. **B** biofilm. *P. aeruginosa* cells were exposed to each material in concentration 100 mg/mL for 24 h. Bacteria viability is expressed as a percent of the viability of control (non-treated bacteria). Data are presented as mean ± SEM. The asterisks denote p values < *0.05, **0.01, ***0.001 compared to the control

the two materials 41 T and 33SA and was 91.43 mg/mL and 100.56 mg/mL, respectively.

The strain most susceptible to the tested materials is *P. aeruginosa*. As many as 8 materials caused inactivation of bacteria growing in suspension which was higher than 90% (22 T(98%) > 37 T(90%) > 43 T(90%) > 97 T(89%) > 88 T(89%) > 80 T(89%) > 92 T(86%) > 61 T(84%)). The most active material was 22 T (99% inactivation). Materials with moderate activity also included 44 T, 51 T and 96 T. The antibacterial effect observed for them was 30–40% inactivation. The SA group materials were also the most active against *P. aeruginosa*. The 15SA material caused almost complete inactivation of the bacteria (inactivation level of 99%). The 16SA material also caused significant inactivation of 95%. A 90% decrease in viability was observed for the 12SA, 35SA and 74SA materials. A large antibacterial effect observed for the 88SA material (70% decrease in lifetime); while, moderate and 93SA showed a moderate antibacterial effect of 45%.

Interestingly, the tested materials appeared to be active against *P. aeruginosa* biofilm. Most thymol-modified materials caused a significant decrease in biofilm viability. The effect was varied and the recorded decrease in viability was in the range of 25–70%. The activity of the materials in descending order can be rearranged as follows: 41 T(77%) > 22 T(66%) > 97 T(60%) > 48 T(55%) > 37 T(54%) > 35 T(53%) > 31 T(52%) > 99 T(48%) > 61 T(43%) > 15 T(43%) > 44 T(42%) > 16 T(42%) > 98 T(36%) > 88 T(22%).

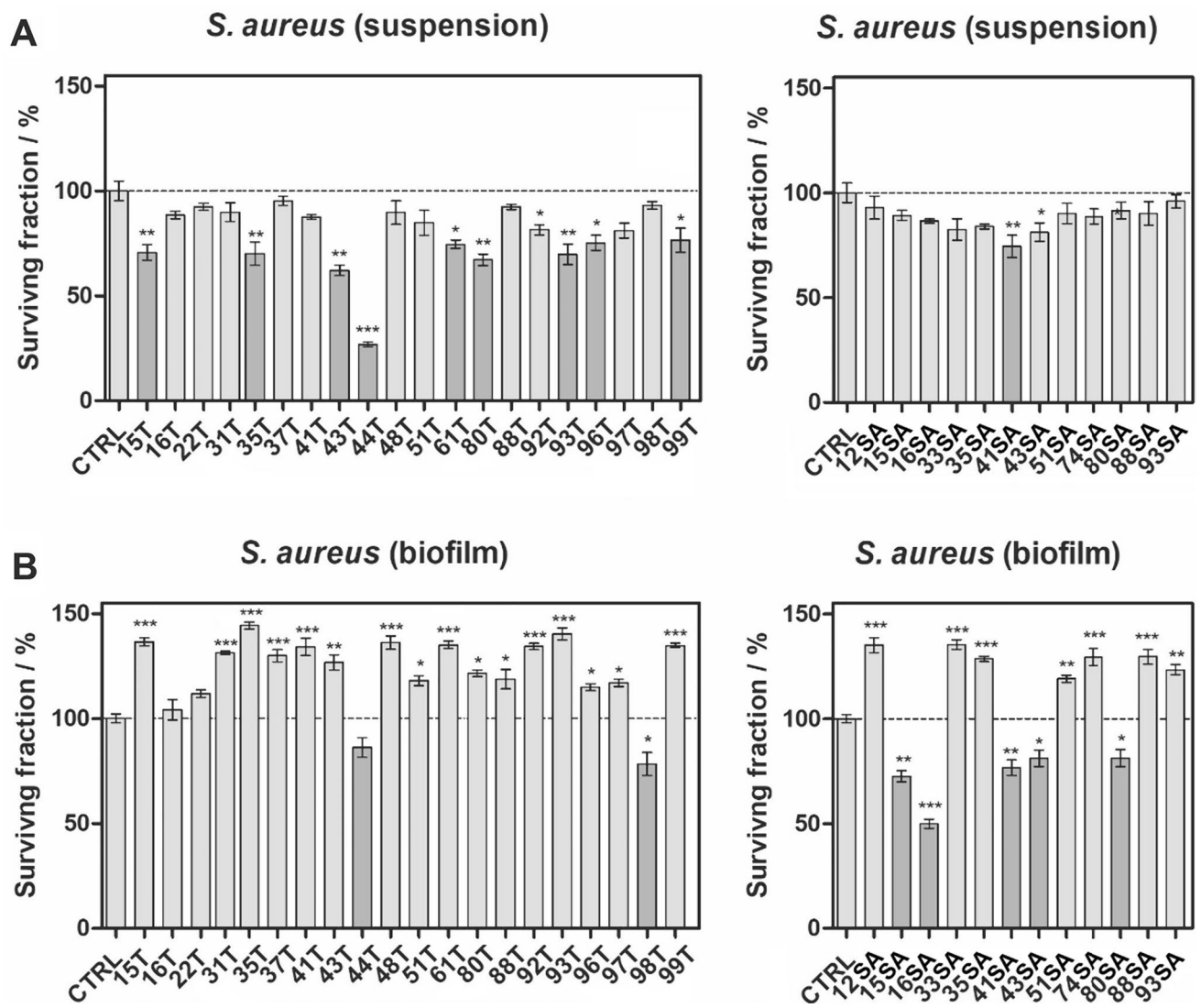


Fig. 9 Determination of antibacterial activity of investigated composite materials against *S. aureus* in **A** planktonic culture, **B** biofilm. *S. aureus* cells were exposed to each material in concentration 100 mg/mL for 24 h. Bacteria viability is expressed as a percent of the viability of control (non-treated bacteria). Data are presented as mean ± SEM. The asterisks denote p values < *0.05, **0.01, ***0.001 compared to the control

In addition, almost all salicylic acid-modified materials (10 of 12) were highly active. A nearly complete bactericidal effect (99%) was noted for the 93SA material. A decrease in viability of 90% was observed for 15SA, 88SA and 51SA caused inactivation of 70% of the biofilm, while 16SA, 35SA, 41SA, 43SA, 12SA and 74SA by 30–40%. For 13 compounds, MIC value was obtained, for: 22 T—65.28 mg/mL, 37 T—78.25 mg/mL, 43 T—69.36 mg/mL, 61 T—65.09 mg/mL, 88 T—47.57 mg/mL, 92 T—77.18 mg/mL, 97 T—62.63 mg/mL, 80 T—81.24 mg/mL, 12SA—64.38 mg/mL, 15SA—48.68 mg/mL, 16SA—55.23 mg/mL, 35SA—63.10 mg/mL, 88SA—49.59 mg/mL.

The susceptibility of gram-negative and gram-positive bacteria to composite materials based on waste cooking oil can vary due to inherent differences in their cell wall structure and composition. That is why the antimicrobial activity of materials was tested also against Gram-positive species.

For *S. aureus*, 9 of the materials tested showed antibacterial properties. However, only one compound showed significant activity resulting in 70% bacterial inactivation. The others (15 T, 35 T, 43 T, 61 T, 93 T, 96 T, 99 T, 80 T) caused mortality of 30% of the bacterial population. In the case of materials with salicylic acid, their activity was insignificant. Only 41SA inactivated 20% of *S. aureus* bacteria. In the case of bacteria growing as a biofilm for many materials, an increase in viability of up to 50% was recorded. Only two of the materials tested—44 T and 98 T—resulted in a biofilm reduction of about 20–25%. Interestingly, of the salicylic

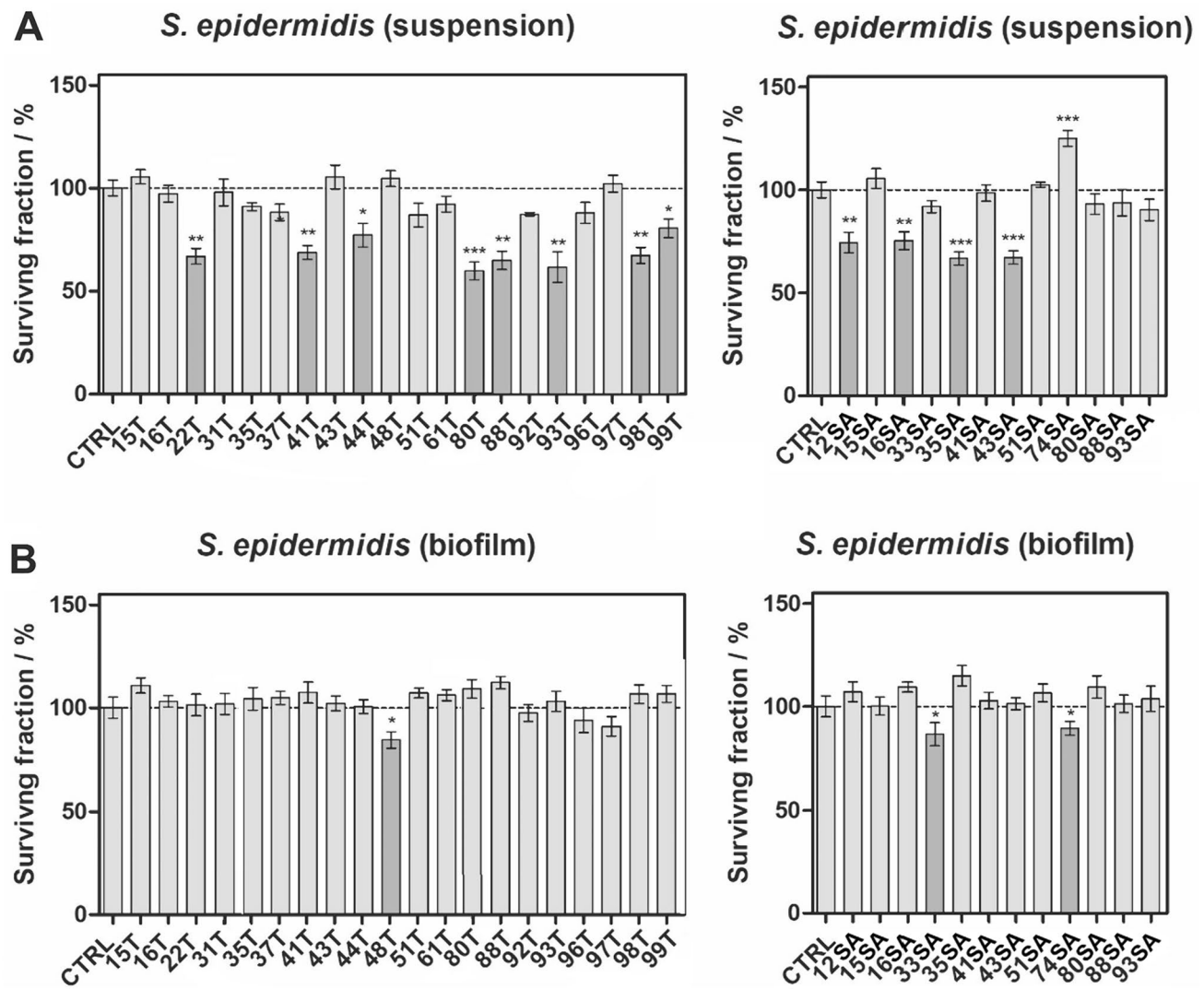


Fig. 10 Determination of antibacterial activity of investigated composite materials against *S. epidermidis* in **A** planktonic culture. **B** biofilm. *S. epidermidis* cells were exposed to each material in concentration 100 mg/mL for 24 h. Bacteria viability is expressed as a percent of the viability of control (non-treated bacteria). Data are presented as mean ± SEM. The asterisks denote p values < *0.05, **0.01, ***0.001 compared to the control

acid-modified materials, a 50% decrease in viability was observed for one of them—16SA, relative to the control. For 12SA, 41SA, 43SA, 80SA materials, the decrease was 20–30%. As with thymol-modified materials, the opposite effect and an increase in biofilm viability were shown for the others. For the 44 T material, it was possible to determine the MIC value, which was 76.60 mg/mL.

In the case of *S. epidermidis* generated in a suspension culture of 8 materials. An antibacterial effect was demonstrated. Materials 22 T, 41 T, 44 T, 88 T, 93 T, 98 T, 99 T, 80 T effect reduction by 20–30%. In the case of biofilm, only the 48 T material exhibited negligible biological activity and released biofilm by approximately 15%. As for the salicylic acid materials, 4 of them: 12SA, 16SA, 35SA, 43SA had 25–35% activity against *S. epidermidis* in suspension. One of the 74SA materials later in turn increased efficiency by about 25%. In the case of biofilm, a slight effect on the microbiological activity of the materials was observed. For the 33SA and 74SA materials, the way of looking at biofilm effects by about 10% was noted.

The determined minimum inhibitory concentrations (MIC) values obtained for best materials were summarized in Table 2 (and Figure S1).

Table 2 Average MIC values and standard deviation (SD) determined for the most active materials against all tested bacterial strains

Strain	Material	MIC (mg/mL) ± SD
E.coli	41 T	91.43 ± 3.87
E.coli	33SA	100.56 ± 2.44
P.aeruginosa	22 T	65.28 ± 1.37
P.aeruginosa	37 T	78.25 ± 3.46
P.aeruginosa	43 T	69.36 ± 1.67
P.aeruginosa	61 T	65.09 ± 2.05
P.aeruginosa	88 T	47.57 ± 1.35
P.aeruginosa	92 T	77.18 ± 2.53
P.aeruginosa	97 T	62.63 ± 2.71
P.aeruginosa	80 T	81.24 ± 3.65
P.aeruginosa	12SA	64.38 ± 2.92
P.aeruginosa	15SA	48.68 ± 2.41
P.aeruginosa	16SA	55.23 ± 3.63
P.aeruginosa	35SA	63.10 ± 4.11
P.aeruginosa	88SA	49.59 ± 3.83
S.aureus	44 T	76.60 ± 4.24

Antibacterial activity of investigated materials—flow cytometry analysis

In the course of further antimicrobial activity studies, we investigated the viability of bacteria after treatment with selected composite materials using flow cytometry (Fig. 11). For this reason, we selected the most representative materials due to its most prominent inactivation in each studied microorganism. After the incubation with material, the bacteria were collected and stained with propidium iodide for dead cells visualization.

Propidium iodide is impermeable to live bacteria with intact cell membranes but can enter bacteria with compromised or damaged membranes. It intercalates with the bacterial DNA, resulting in a red fluorescence signal. Thus, in this experiment the fluorescence emitted by propidium iodide-stained bacteria after treatment with selected composite materials can be captured and analyzed by flow cytometry. In general, the fluorescence intensity in the red channel corresponds to the propidium iodide staining, indicating dead or permeabilized bacteria. Based on the results, the different populations observed in the flow cytometry plot, such as live bacteria (PI-negative) and dead bacteria (PI-positive). The more red-shifted fluorescence is the more dead cells are. The most toxic compound was found to be compound 15SA for the bacteria *P. aeruginosa*.

Composite material-treated bacteria imaging with confocal microscopy

To further visualize the induced damages and the differences in bacterial death mechanisms, we performed confocal imaging after the treatments with selected composites. The representative confocal images of most sensitive to treatment bacteria—*E. coli*, *P. aeruginosa*, *S. aureus* are shown in Figs. 12, 13, and 14. In these studies, the bacteria were stained with Calcein AM and propidium iodide (PI) for their viability examination after treatment with selected composite materials. Calcein AM is a green fluorescent dye that is often used to label live cells. When live bacteria are stained with Calcein AM, they emit a bright green fluorescence under excitation. This indicates their viability and that the bacteria are metabolically active and have intact cell membranes, allowing Calcein AM to enter and produce a fluorescence signal. Contrastingly, propidium iodide is a red fluorescent dye that selectively stains dead or permeabilized cells. When dead bacteria are stained with propidium iodide, they exhibit a red fluorescence. PI cannot enter live bacteria with intact cell membranes but can penetrate compromised or damaged membranes of dead bacteria. As may be observed, the performed treatment with composites indicated a disruption of bacterial cells and may inhibit the bacteria division without visible membrane disruption. Nevertheless, it is believed that these bacteria were dying or losing cell functions with the intact cell walls (see confocal pictures below, Figs. 6, 7 and 8).

In order to obtain further insight into the interaction of selected composite materials with biofilm, we used confocal microscopy to observe *S. aureus* biofilms incubated with tested materials—31 T, 43SA and 88SA. Figure 15 shows representative images of biofilm after 24 h incubation with these materials. In the next step, the biofilm was stained with propidium iodide (PI, Red, dead cells). Calcein AM (green, live cells) and Hoechst dye (nucleic acids). Imaging was performed using laser scanning confocal microscopy.

The presence of biofilm can be observed as island-like structures. The red fluorescence from dead bacteria seems to be inside the biofilm, which may demonstrate that material activity can be attributed to its surface interaction and possible penetration in the biofilm structure.

The cytotoxicity of investigated composite materials toward human keratinocytes

The selectivity of selected for microbial cells over host mammalian cells was demonstrated using the human keratinocytes cell line HaCaT. The viability of HaCaT cells and their morphology after treatment with composite materials after 24 h incubation is shown in Fig. 16 (viability) and

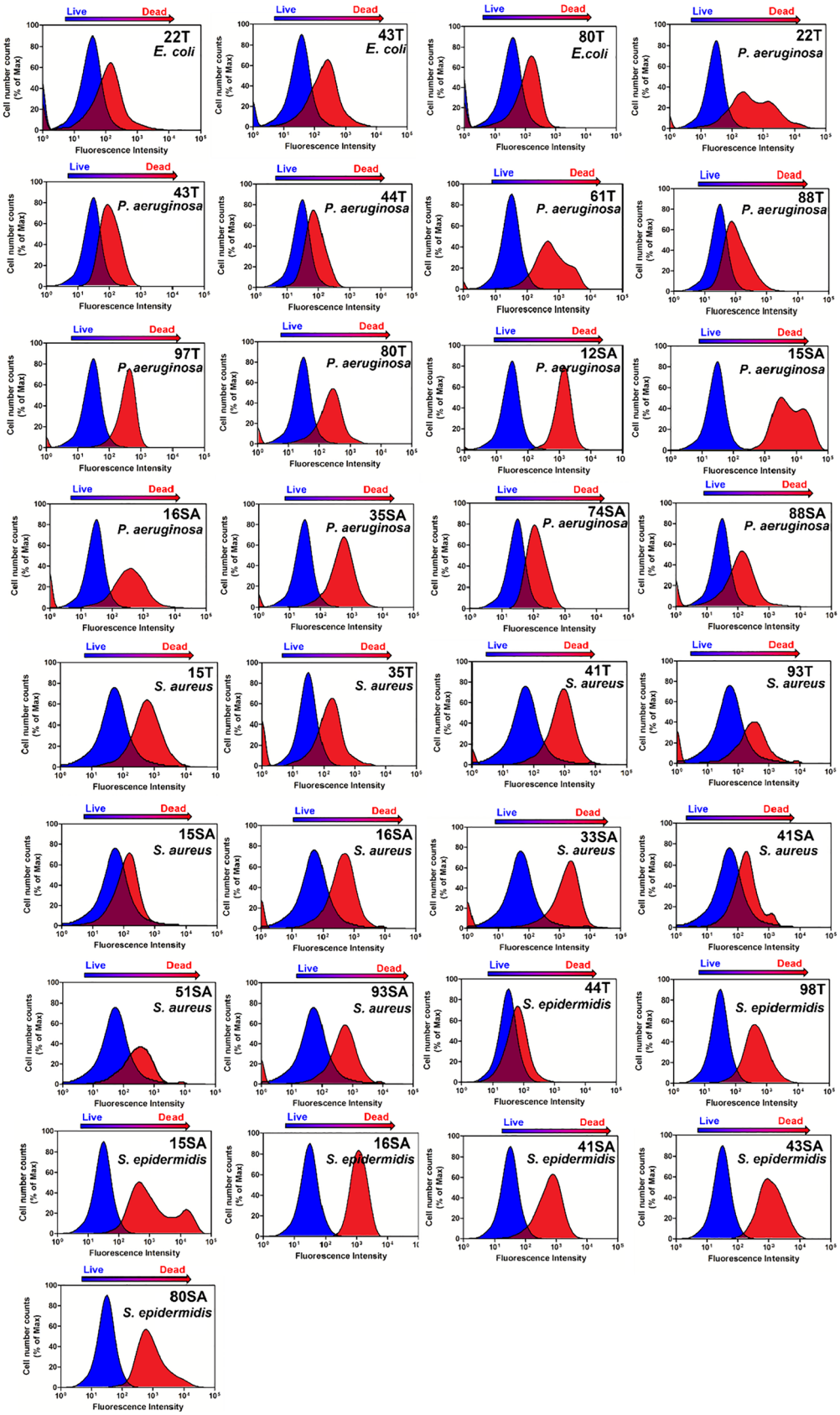


Fig. 11 Flow cytometry analysis of bacteria after 24 h treatment with investigated composite materials. For this purpose, after treatment bacteria were stained with propidium iodide (10 ug/mL) and the red fluorescence of dead bacteria was detected

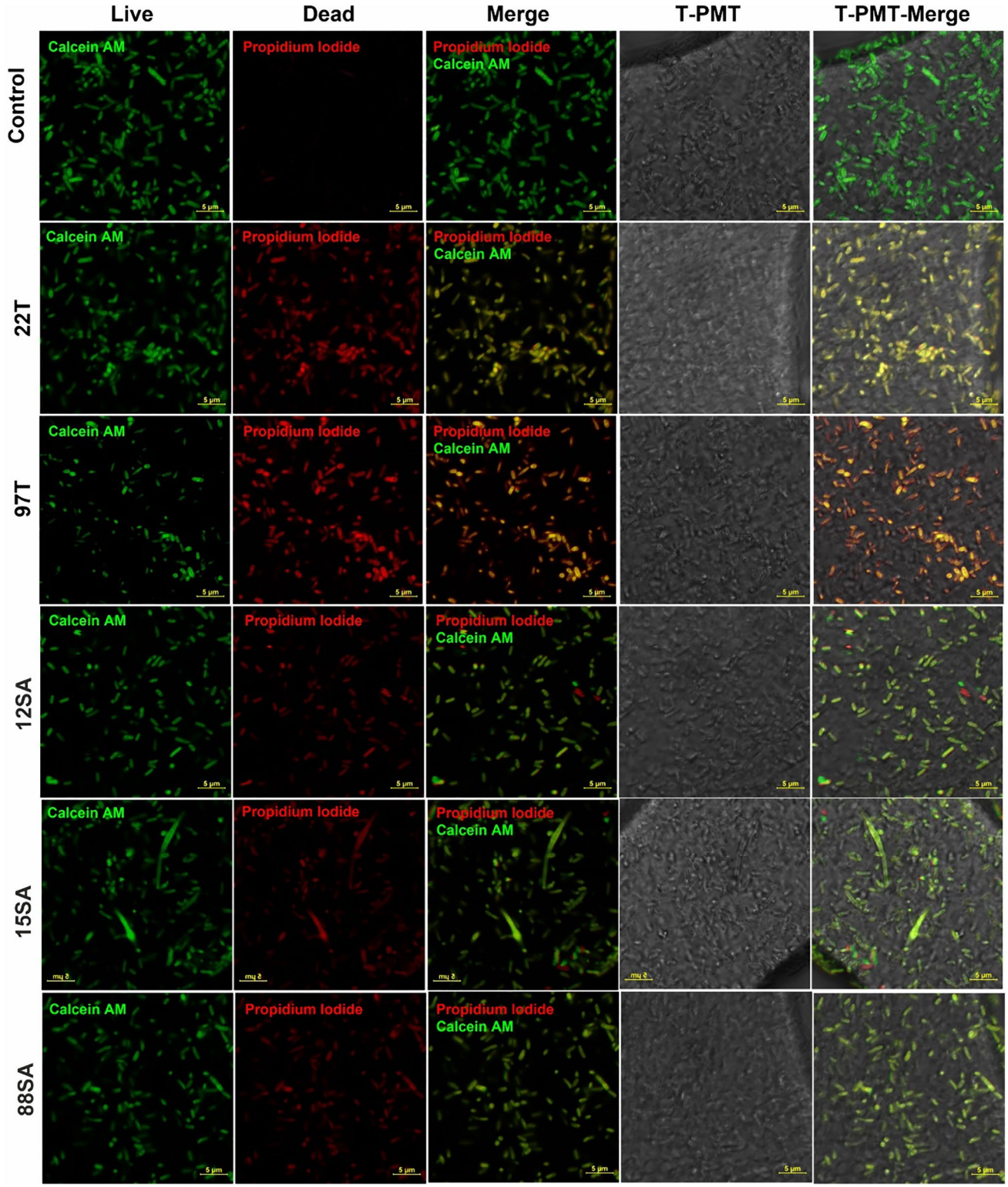


Fig. 12 Laser scanning confocal microscopy images of the non-treated (upper panel) and selected composite material-treated (bottom panels) *E. coli* stained with Calcein AM and propidium iodide with bright-field (T-PMT) contrast

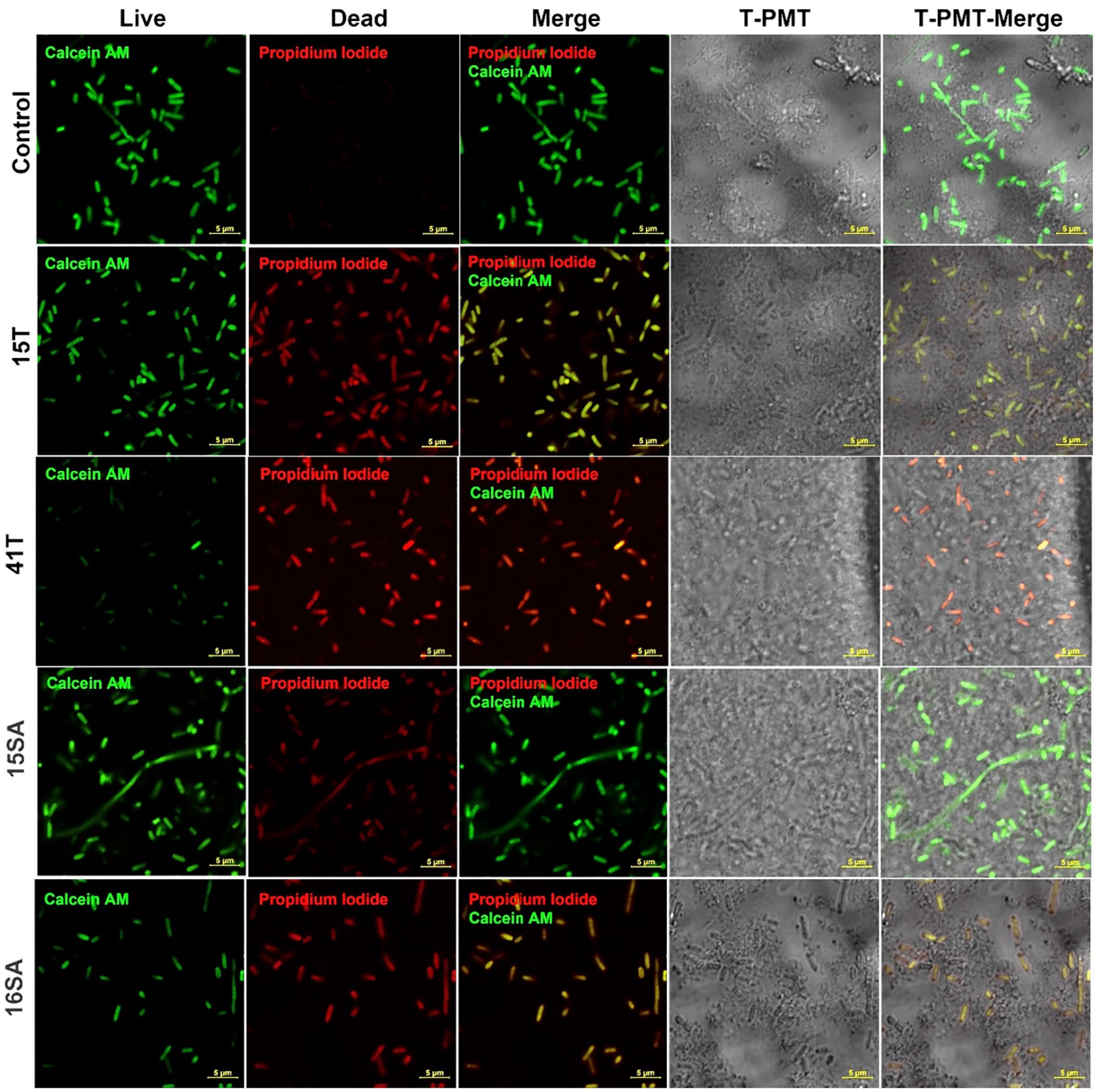


Fig. 13 Laser scanning confocal microscopy images of the non-treated (upper panel) and selected composite material-treated (bottom panels) *P. aeruginosa* stained with Calcein AM and propidium iodide with bright-field (T-PMT) contrast

Fig. 17 (bright-field microscopy imaging). For evaluation of the cytotoxic effect of investigated composite materials toward human keratinocytes, the cell viability was determined with two assays MTT and Alamar Blue. The MTT assay measures cell viability based on the reduction of a yellow tetrazolium salt by metabolically active cells. In this case, cells with intact mitochondrial function convert MTT into a purple formazan product, which intensity of the purple formazan product is directly proportional to the number of viable cells. Therefore, a higher intensity indicates higher cell viability. The Alamar Blue assay is another commonly used assay for assessing cell viability and metabolic activity. It utilizes a redox indicator that changes color based on the metabolic activity of the cells. Viable cells convert the non-fluorescent, oxidized Alamar Blue into a highly fluorescent reduced form. The fluorescence intensity is measured and used as an indicator of cell viability.

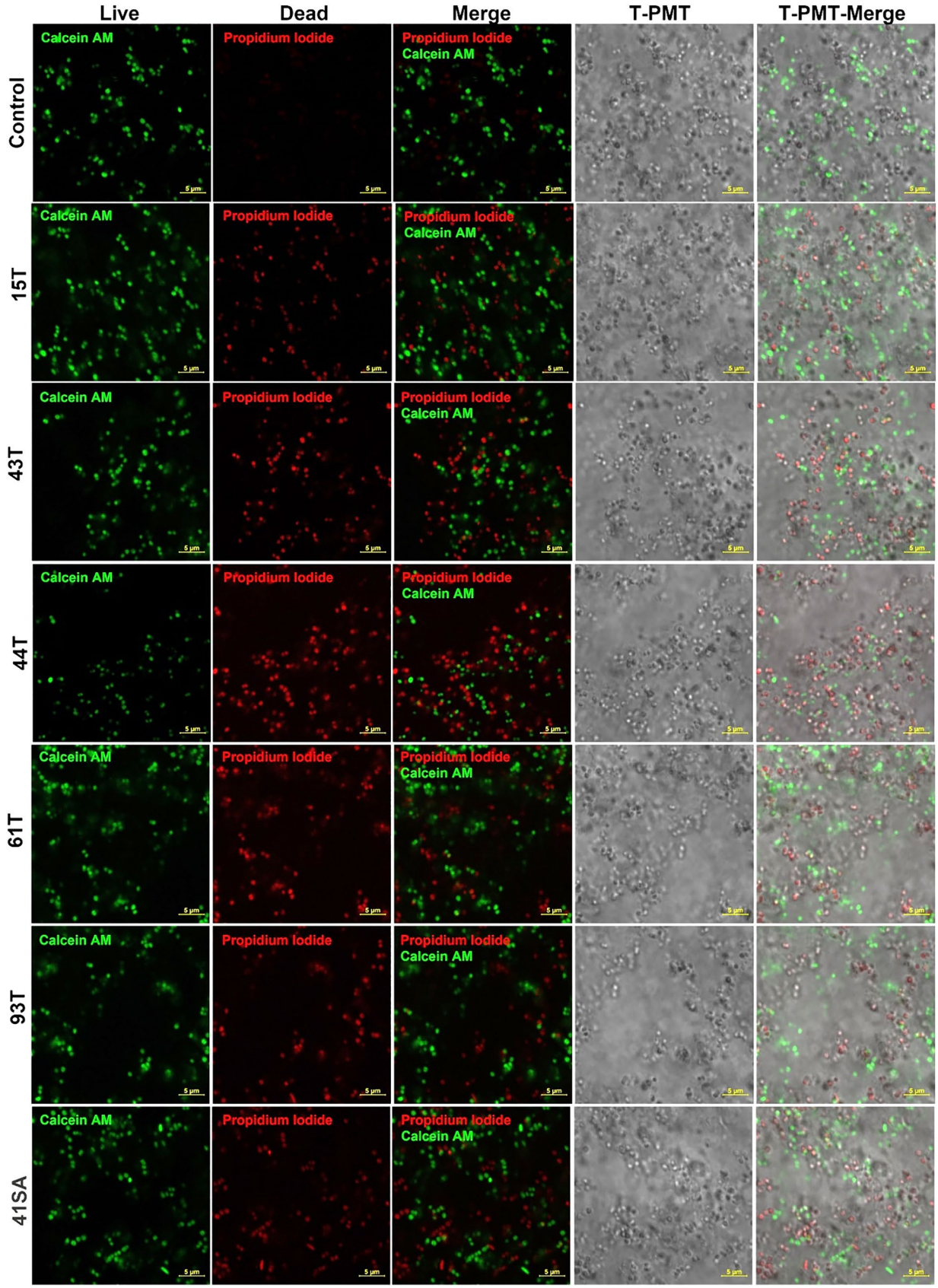


Fig. 14 Laser scanning confocal microscopy images of the non-treated (upper panel) and selected composite material-treated (bottom panels) *S. aureus* stained with Calcein AM and propidium iodide with bright-field (T-PMT) contrast

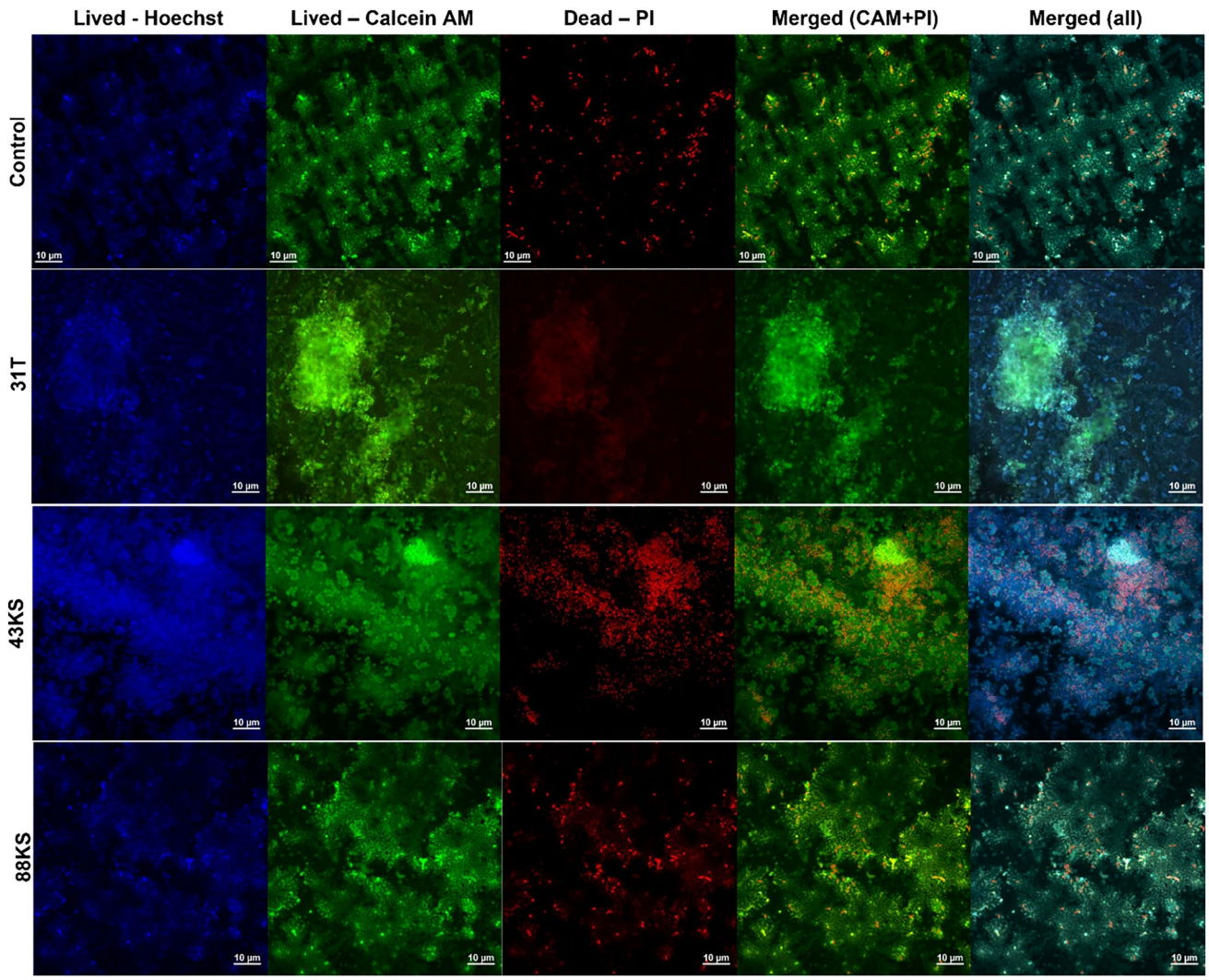


Fig. 15 Laser scanning confocal microscopy images of the non-treated (upper panel) and selected composite material-treated (bottom panels) *S. aureus* biofilms stained with Hoechst33342, Calcein AM and propidium iodide

Based on the obtained results, it may be noted, that the tested materials did not induce significant cell death or cell damage in human keratinocytes (HaCaT cells). The data from cytotoxicity assays and microscopic observations indicate that after incubation with selected composited that are active against bacteria HaCaT cells remain viable and continue to carry out their normal cellular processes. It may mean that tested materials do not inhibit the ability of human cells to proliferate and divide. Moreover, the materials do not induce the release of toxic substances or trigger toxic responses in human keratinocytes, including cellular stress, oxidative damage, or DNA/RNA damage.

In summary, it can be concluded that tested materials may be considered compatible with human keratinocytes, suggesting that it is not likely to cause adverse effects when in contact with human skin or other keratinocyte-rich tissues. It also suggests that the material may be safe for use or exposure in contexts involving these cells such as in the development of skincare products or biomaterials intended for contact with skin.

Discussion

The obtained composites showed the mechanical strength required for paving blocks (Hein and Eng 2016). This was due to the structure and hardness of the composites. Materials with higher porosity absorbed more water, which could fill the interior of the pores. At the same time, such material was more abrasive compared to non-porous material. In addition, abrasion was influenced by the hardness of the material resulting from the degree of polymerization of the WCO during the annealing process.

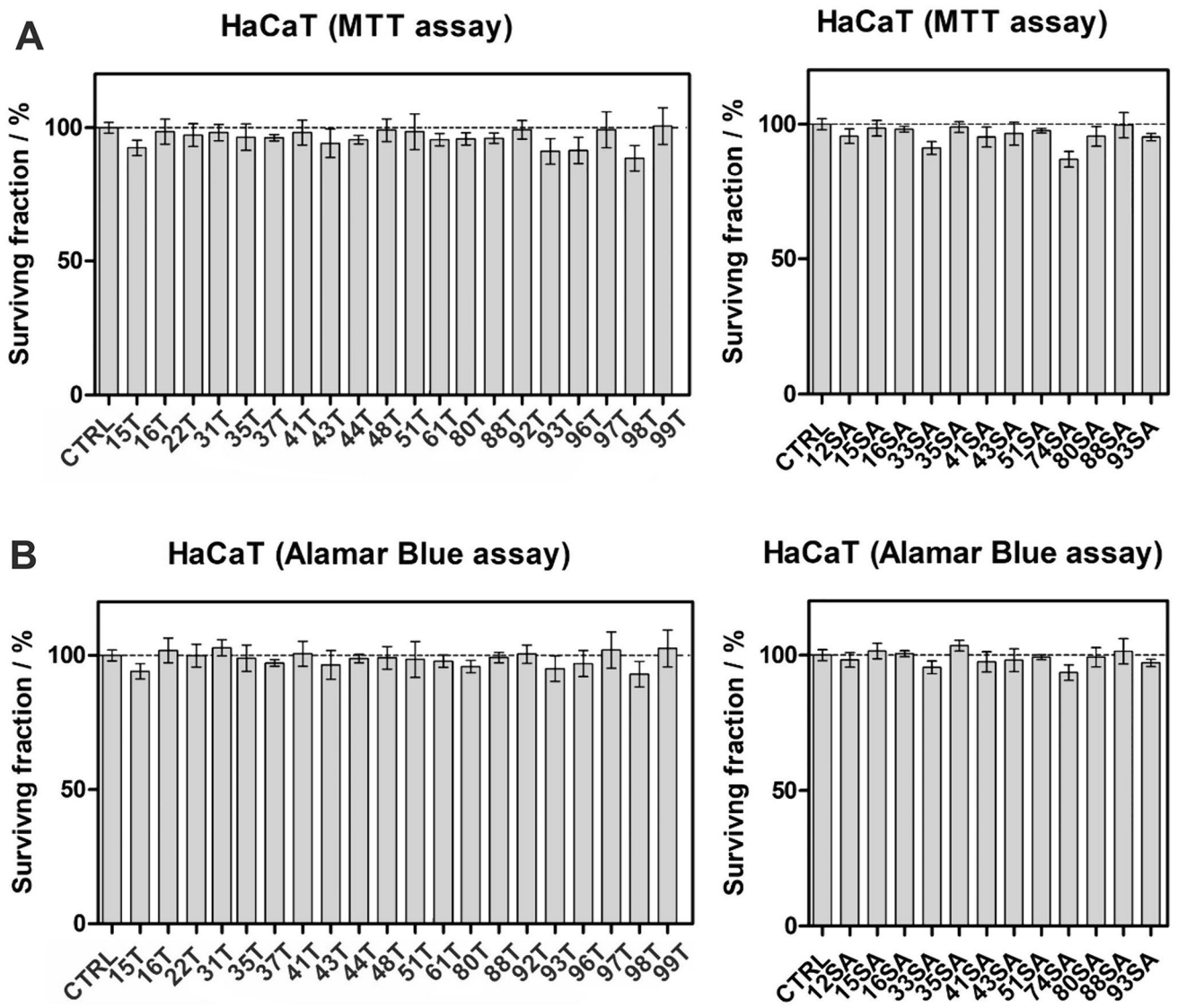


Fig. 16 Cytotoxicity of tested materials—thymol-modified (left) and salicylic acid (right) against human keratinocytes (HaCaT cells) assessed by **A** MTT assay and **B** Alamar Blue assay

The porosity of materials used in construction can lead to a variety of problems, such as moisture, fungal colonization and moss growth. These factors can negatively affect the condition of a building, leading to its deterioration. A common practice is to apply coatings to the surface of materials to prevent moisture from entering these materials (Herrera et al. 2004; Korkanç and Savran 2015). At the same time, such a surface loses its roughness and becomes more slippery. In addition, it is a temporary procedure because the coating becomes abraded over time (Shirtcliffe et al. 2011; Khammas and Koivuluoto 2022).

Waste cooking oil composites can inhibit the growth of bacteria by interfering with their cellular processes. Fatty acids can disrupt the bacterial cell membrane, causing leakage of cellular contents and cell death. The presence of waste cooking oil may create an unfavorable environment for bacterial growth, such as by reducing nutrient availability or altering pH levels (Marchetti et al. 2020; Bustamante-Torres et al. 2022). Moreover, due to the addition of modifiers like thymol or salicylic acid. Synergistic effects can be observed. For instance. incorporating waste cooking oil into a composite alongside essential oils or plant extracts known for their antibacterial properties can enhance the overall antibacterial activity of the material (Zhang et al. 2017). In addition, the antibacterial activity of WCO composites may primarily be observed on the surface of the material. When in contact with bacteria, the released fatty acids or other antimicrobial components can directly interact with and inhibit bacterial growth (Rodhi et al. 2022; Ustadi et al. 2022).

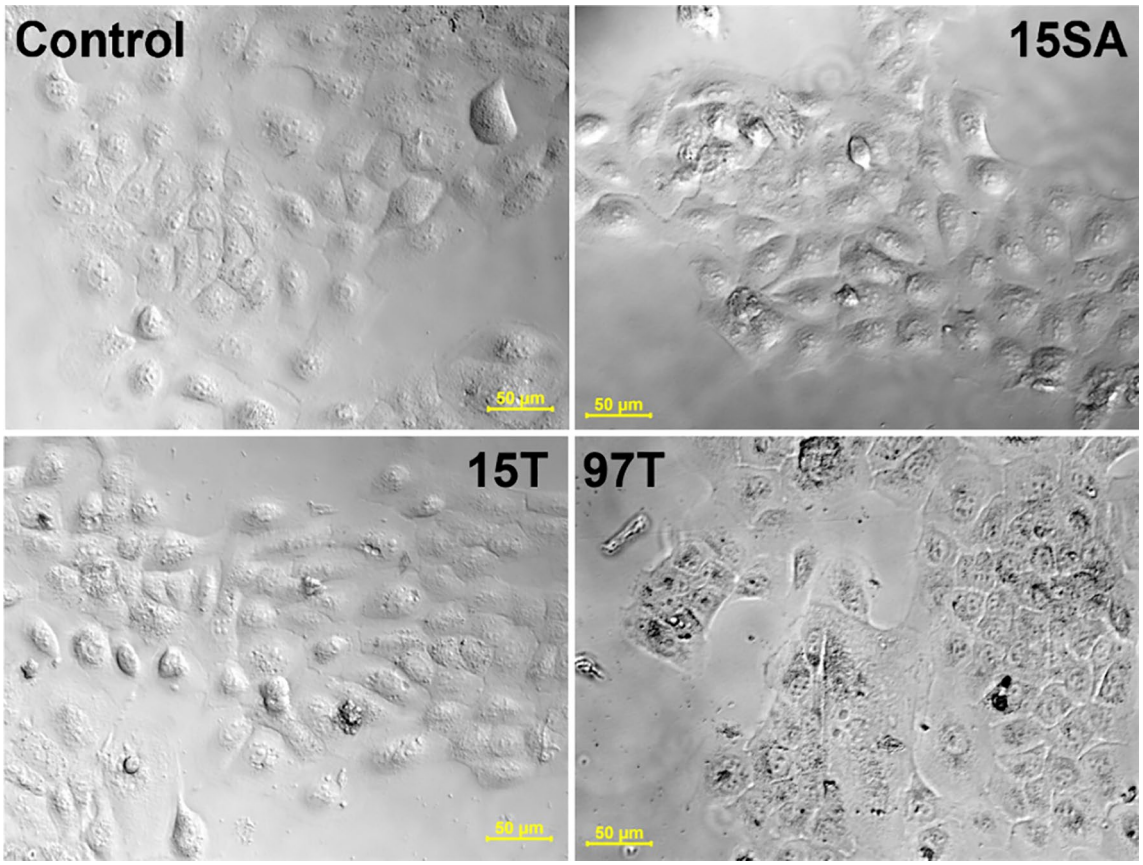


Fig. 17 Morphology of HaCaT cells treated with selected composite materials visualized with bright-field microscopy

In our studies, we used two modifiers thymol (T) and salicylic acid (SA) to enhance the antimicrobial activity of composite materials. Thymol is a natural compound found in thyme oil and is known for its antimicrobial properties. When incorporated into composites, thymol can enhance the antibacterial activity of the material. Thymol possesses broad-spectrum antimicrobial activity, which means it can inhibit the growth of various types of bacteria (Falcone et al. 2007). It has been shown to be effective against both Gram-positive and Gram-negative bacteria. Thymol can disrupt the cell membrane of bacteria, leading to leakage of cellular contents and cell death. It can also interfere with essential bacterial enzymes, Proteins, and DNA, inhibiting bacterial growth and replication. By incorporating thymol into composites, the release of the compound can be controlled, allowing for a sustained and prolonged antimicrobial effect. This controlled release mechanism ensures that the composite remains effective over an extended period (Michalska-Sionkowska et al. 2017; Cometa et al. 2022; Turki et al. 2023). Thymol has been also reported to inhibit the formation of bacterial biofilms. Biofilms are communities of bacteria that adhere to surfaces and can be highly resistant to antibiotics. By preventing biofilm formation, composites modified with thymol can help reduce the risk of infections associated with biofilms (Marchese et al. 2016; Michalska-Sionkowska et al. 2017; Piri-Gharaghie et al. 2022).

The second applied modifier, salicylic acid (SA) is a compound commonly used in skincare products for its anti-inflammatory and exfoliating properties. While it has some antimicrobial effects, its antibacterial activity is not as potent as other compounds like thymol. Salicylic acid has moderate activity against certain bacteria, particularly *Propionibacterium acnes*, which is associated with acne. It can help reduce the population of bacteria on the skin and inhibit their growth to some extent (Kong et al. 2021).

Salicylic acid's primary mechanism of action is through its anti-inflammatory properties. By reducing inflammation, it indirectly contributes to the management of bacterial infections, as inflammation can provide a favorable environment for bacterial growth (Zhang et al. 2023). Salicylic acid is generally effective against Gram-positive bacteria, including *Propionibacterium acnes*, but its activity against Gram-negative bacteria may be limited. Gram-negative bacteria have an outer membrane that acts as a barrier, making them less susceptible to the effects of salicylic acid. Composites

modified with salicylic acid can be used in skincare products such as creams, gels, cleansers. They are commonly used for the treatment of acne and other skin conditions where bacterial overgrowth plays a role (Zhang et al. 2023). As indicated with our results, salicylic acid may also be combined with other antimicrobial agents or material to enhance its antibacterial activity. The combination of salicylic acid with other ingredients, such as benzoyl peroxide or antibiotics, can have a synergistic effect and improve the overall antimicrobial efficacy of the composite (Tanghetti and Popp 2009; Jayakumar et al. 2023).

While composite materials can offer certain antimicrobial properties in planktonic bacteria, their effectiveness against biofilms may be limited due to the factors like (i) protective matrix (EPS) which can limit the effectiveness of composite materials in direct contact with the biofilm, and (ii) composite materials may have limitations in their ability to penetrate and diffuse through the biofilm matrix, limiting their efficacy against the microorganisms residing within (Mah and O'Toole 2001). Moreover, biofilms contain a subpopulation of persisted cells that are highly tolerant to antimicrobial agents and heterogeneity within biofilms. While composite materials may be effective against some biofilm cells, others may remain resistant, leading to incomplete eradication of the biofilm (Davies 2003; Ono et al. 2007).

Composites based on a waste cooking oil with antibacterial activity have potential applications in various fields. They can be used in food packaging materials, where they can help inhibit bacterial growth and extend the shelf life of food products. Additionally, they may find applications in the development of antibacterial coatings, Films, or surfaces for use in healthcare settings or other environments where microbial contamination is a concern. Composites modified with thymol have found applications, including medicine, dentistry, and food packaging. They can be used in the development of antibacterial coatings, films, dental materials, wound dressings, and other biomedical devices.

Based on our research, it can be mentioned that antibacterial materials dedicated to public places like swimming pools, several important factors should be considered: (i) the antibacterial material should be effective against a wide range of bacteria, including both Gram-positive and Gram-negative bacteria. This ensures that it can target and inhibit the growth of various bacterial strains commonly found in swimming pools; (ii) the material should have long-lasting antibacterial properties to withstand the harsh conditions typically present in swimming pools, such as exposure to chlorine, UV radiation, and fluctuating pH levels. It should be resistant to degradation or leaching over time; (iii) the antibacterial material should be safe for human contact and environmentally friendly. Additionally, it should not leach harmful substances into the pool water or the surrounding environment; (iv) the antibacterial material should be compatible with the surfaces commonly found in swimming pools, such as tiles, Concrete, or plastic. It should be capable of adhering or integrating with these surfaces without compromising their structural integrity. Moreover, the promising materials should be effective in biofilm inactivation. Biofilms are notoriously difficult to remove and can harbor harmful bacteria. Thus, the antibacterial material should have properties that prevent biofilm formation, making it easier to maintain a clean and hygienic public places environment.

By considering these factors, chemists and engineers can create antibacterial materials specifically tailored to public places like swimming pools, promoting a cleaner and safer environment and reducing the risk of bacterial infections.

Conclusion

The article presents promising applications of oil-based composites with antibacterial properties. The research focused on obtaining composites based on waste cooking oil, containing additives such as thymol and salicylic acid, and evaluating their antimicrobial activity against selected Gram-positive and Gram-negative bacteria.

The obtained composites exhibited satisfactory mechanical strength, allowing for their potential use in the construction industry. By controlling the process parameters of oil composite production, it is possible to modify their functional properties. The utilization of waste cooking oil in the composites disrupts bacterial cellular processes, particularly by affecting their cell membranes, leading to cell death. Additionally, the modification of composites through the addition of substances like thymol or salicylic acid showed synergistic effects, enhancing the overall antimicrobial activity of the materials.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s10098-024-02812-3.

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Author contributions AS designed the experiment, organized the experimental process, participated in the experiments and wrote the paper, BP, AB and JPP participated in the experiments. All authors have read and approved the final manuscript.

Data and code availability Not Applicable.

Declarations

Competing interests The authors declare no competing interests.

Ethical approval Not Applicable.

Supplementary information Figure S1 shows the potency of antimicrobial agents as a minimum inhibitory concentrations (MIC) for the most active materials.

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