Antimicrobial and antibiofilm activity of Cannabis sativa L. seeds extract against Staphylococcus aureus and growth effects on probiotic Lactobacillus spp

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Abstract: The growing concern on the antibiotic resistance spreading among bacteria has stimulated the search for valuable alternatives from plant sources.
This study dealt with the potential use of Cannabis sativa L. (cultivar Futura 75) seeds extract to inhibit the growth of selected pathogenic enterobacteria and the biofilm formation by Staphylococcus aureus, representing severe risks of food-borne illnesses. Effects on the probiotic bacteria growth were also examined. A double-staining viability/mortality assay was used to examine potential S. aureus membrane damage.
Our results highlighted a selective antimicrobial activity of C. sativa extract against pathogenic strains and no inhibitory effects on the growth of probiotic strains. For the best of our knowledge, this is the first work investigating the antibiofilm activity of C. sativa extract against the biofilm producer S. aureus. The inhibition of biofilm formation, at lower concentrations than the minimal inhibitory concentration (MIC), could be ascribed to the presence of high levels of caffeoyltyramine and cannabisin A, B, and C in cultivar Futura 75 seeds. The antibacterial action of C. sativa extract on S. aureus appeared to be only partially linked to a membrane damage mechanism.
Therefore, hemp seeds extracts represent a new exploitable and valuable antimicrobial and antibiofilm agent.

Highlights

Cannabis sativa L. seeds showed antimicrobial and antibiofilm activity.

C. sativa L. seeds selectively inhibit the growth of potentially pathogenic strains.

C. sativa L. seeds did not exert antimicrobial activity against probiotic bacteria.

C. sativa L. seeds inhibit the biofilm formation by *Staphylococcus aureus*.

C. sativa L. seeds have great potentiality use in food and nutraceutical industries.

Antimicrobial and antibiofilm activity of *Cannabis sativa* L. seeds extract against *Staphylococcus aureus* and growth effects on probiotic *Lactobacillus* spp.

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Abstract

The growing concern on the antibiotic resistance spreading among bacteria has stimulated the search for valuable alternatives from plant sources.

This study dealt with the potential use of *Cannabis sativa* L. ~~(cultivar Futura 75)~~ seeds extract to inhibit the growth of selected pathogenic enterobacteria and the biofilm formation by *Staphylococcus aureus*, representing severe risks of food-borne illnesses. Effects on the probiotic bacteria growth were also examined. A double-staining viability/mortality assay was used to examine potential *S. aureus* membrane damage.

Our results highlighted a selective antimicrobial activity of *C. sativa* extract against pathogenic strains and no inhibitory effects on the growth of probiotic strains. For the best of our knowledge, this is the first work investigating the antibiofilm activity of *C. sativa* extract against the biofilm producer *S. aureus*. The inhibition of biofilm formation, at lower concentrations than the minimal inhibitory concentration (MIC), could be ascribed to the presence of high levels of caffeoyltyramine and cannabisin A, B, and C in ~~cultivar Futura 75~~ seeds. The antibacterial action of *C. sativa* extract on *S. aureus* appeared to be only partially linked to a membrane damage mechanism.

Therefore, hemp seeds extracts represent a new exploitable and valuable antimicrobial and antibiofilm agent.

Keywords: *Cannabis sativa* L., pathogenic and probiotic bacteria, antimicrobial activity, *S. aureus*, biofilm inhibition**.**

1. ***Introduction***

Plants are rich in natural compounds with antimicrobial properties and, nowadays, there is a growing interest in natural antimicrobials, especially when deriving from plants. Plant extracts represent a promising and healthy solution to the rising antibiotic resistance and they may also provide better results than synthetic compounds in counteracting the bacterial growth (Hayek, Gyawali, & Ibrahim, 2013). Many studies pointed out the antibacterial activity of bioactive compounds from plants against e.g. Gram-positive and Gram-negative multidrug-resistant bacteria (Barbieri et al., 2017; Borges, Saavedra, & Simoes, 2015) as well methicillin-resistant *Staphylococcus aureus* (MRSA) (Gyawali, Hayek, & Ibrahim, 2015).

Several antimicrobial mechanisms of action have been suggested. Generally, phytochemicals can act harming the bacterial membrane, inhibiting the formation of bacterial biofilm or through the suppression of virulence factors such as enzymes and toxins (Barbieri et al., 2017).

Biofilms are formed by bacterial cells communities enclosed in a self-produced polymeric matrix able to adhere to inert or living surfaces (Costerton, 1999). Bacterial biofilms are mostly diffused in the clinical environment and in many industrial activities. Biofilm-related infections caused over 75% of human microbial infections (Davies, 2003) and are of medical attention. For instance, in the dairy industry or other food processing industries, including drinking water distribution systems, the biofilm formation is a potential source of contamination and may be responsible for severe hygiene problems as well as economic loss (Satpathy, Sen, Pattanaik, & Raut, 2016). Bacteria within biofilms are more resistant to antibiotics and chemical agents than planktonic cells and their increased tolerance towards antimicrobial agents can impair the treatment of infections by biofilm (Nostro et al., 2016; Satpathy et al., 2016). As described by Beoletto, Oliva, Marioli, Carezzano and Demo (2016) the bacterial cells can become 10-1000 times more resistant to antimicrobial agents when in a biofilm form. *Staphylococcus aureus* is a well known pathogen living inside biofilms in a wide variety of environment. *S. aureus* biofilms represent a severe risk of food contamination; they

have been frequently found on surfaces of food processing plants and are responsible for infections linked to fresh and processed foods consumption (Doulgeraki, Di Ciccio, Ianieri, & Nychas, 2017).

Cannabis sativa L. is an annual herbaceous plant, belonging to the Cannabaceae family, originated in Central Asia where it played a historically important role in medical treatments, over as food and fiber sources. Two main types of *Cannabis* are distinguished: the drug type, *C. sativa* subsp. *indica*, can contain up to 20% of the psychoactive compound D9-tetrahydrocannabinol (THC), while the non drug type, *C. sativa* subsp. *sativa*, is industrial hemp and is characterized by a low content of THC. Industrial hemp can be found as fiber or seed oil (Rodrıguez Leyva, & Pierce, 2010). In most European countries, the current upper legal limit for cultivation of hemp for fiber and seeds production is 0.2% THC on dry basis (Petrovic´, Debeljak, Kezic´, & Dzidara, 2015; Russo & Reggiani, 2015). To date, hemp seeds are mainly used as animals feed; however, their products including oil, meal, flour, and protein powder, are achieving growing popularity as excellent sources of essential amino acids and fatty acids for human nutrition (Callaway, 2004; Russo & Reggiani, 2015; Andre, Hausman, & Guerriero, 2016). The antibacterial activity of cannabinoids from *C. sativa* subsp. *indica* against MRSA strains has been described (Appendino et al., 2008) whereas the antimicrobial activity of *C. sativa* low-THC varieties isolated compounds has been poorly investigated. An inhibitory effect on both Gram-positive and Gram-negative bacteria growth by three industrial hemp essential oils have been described by Nissen et al. (2010). To date, data on the antibacterial activity of hemp seeds are lacking.

Biofilm inhibition by plant extracts rich in polyphenols has been reported against *S. aureus*, including MRSA strains, *E. coli* and *Pseudomonas aeruginosa* (Nostro et al., 2016). Moreover, an inhibitory effect of seeds extracts rich in flavonoids against the biofilm formation by *Candida albicans*, *S. aureus*, and *Pseudomonas aeruginosa* has been described (Onsare & Arona, 2014). No data are at present available on the effect of hemp seeds extracts on biofilm formation.

In a recent paper, we showed that *C. sativa* L. seeds are rich in polyphenols, with caffeoyltyramine and cannabisin A, B, C being the main components of the polyphenolic fraction (Frassinetti et al., 2018). Moreover, seeds extract showed a good ~~antioxidant activity (measured by DPPH and ORAC assays),~~ antioxidant effects on human erythrocytes, ~~as well as~~ anti-mutagenic activity against *Saccharomyces cerevisiae*.

The goal of our study was to evaluate, for the first time, the inhibitory activity of *C. sativa L*. seeds extract, cultivar Futura 75, on the biofilm formation by *S. aureus*. A double-staining viability/mortality assay was used to examine potential *S. aureus* membrane damage. Furthermore, the effects on the growth of gut potential pathogens and beneficial bacteria belonging to the *Lactobacillus* genus were investigated.

2. Materials and Methods

2.1. Chemicals and reagents

Ethanol, gentamycin, vancomycin, fluorescein diacetate (FD) and propidium iodide solution (PI) were purchased from Fluka-Sigma-Aldrich, Inc. (St. Louis, MO). Phosphate buffer saline (PBS) was purchased from VWR (Radnor, PA).

2.2. Plant material and extraction

Cannabis sativa L. cultivar Futura 75 was ~~kindly given by~~ ASSOCANAPA (Carmagnola, Turin, Italy). This cultivar was chosen because it is the most widespread and cultivated in Italy in the last five years and it is well characterized ~~from the biochemical and nutritional point of view~~ (Russo & Reggiani, 2015; Lesma et al., 2014). *C. sativa* seeds ~~extraction was performed as~~ described by Frassinetti et al. (2018). Following centrifuge, supernatants in 80% ethanol were filtered (0.2µm) and kept in the dark at 4°C until use.

2.4. Antimicrobial activity

2.4.1. Bacterial media

Mueller Hinton Broth (MHB), de Man Rogosa Sharpe (MRS) medium, Mc Farland standard 0.5 were purchased from Oxoid (Basingstone, UK). Tryptone, Phytone, and Yeast extract (TPY) broth was prepared homemade as follows: 10.0 g/L tryptone, 5.0 g/L soy peptone, 10.0 g/L glucose, 2.5 g/L yeast extract, 1.5 g/L K_2HPO_4, 0.5 g/L $MgCl_2.6H_2O$, 0.5 g/L Cystein-HCl, and 0.5 g/L (pH 6.5) Tween 80.

2.4.2. ~~Bacterial~~ strains and growth conditions

Pathogenic bacterial strains were purchased by the American Type Culture Collection (ATCC) and grown overnight under aerobic conditions on MHB at 37°C. The antibacterial activity of *C. sativa* extracts was evaluated on *Enterobacter aerogenes* ATCC, *Salmonella enterica* ser. *Typhimurium* ATCC 14028 and *Escherichia coli* ATCC 25922, three Gram-negative bacteria, and on *Staphylococcus aureus* ATCC 25923 and *Enterococcus faecalis* ATCC 29212, two Gram-positive bacteria. Seven strains of human origin (four lactic acid bacteria and three bifidobacteria) were also used: *Lactobacillus paracasei* MB395, *Lactobacillus reuteri DSM 20016, Lactobacillus brevis* ATCC 14869, *Lactobacillus plantarum* MB91, *Bifidobacterium bifidum* B2009, *Bifidobacterium longum* Re11 and *Bifidobacterium breve* B632. Apart from strains deriving from international culture collections, all the other bacteria were from the Bologna University Scardovi Collection of Bifidobacteria available at the Department of Agricultural and Food Science (University of Bologna). *Lactobacillus* strains were grown for 24 hours on MRS medium at 37°C under anaerobic conditions created in a capped jar using an anaerobic atmosphere generation system (Anaerocult A, Merck, Darmstadt, Germany). *Bifidobacteriun* strains were cultivated in TPY broth and incubated anaerobically at 37°C for 24 hours.

2.4.3. The minimum inhibitory concentration (MIC) assay

136 The minimum inhibitory concentration (MIC) of the *C. sativa* seeds extract against the selected
137 bacteria was established as described by Blando, Russo, Negro, De Bellis and Frassinetti (2019). *C.*
138 *sativa* seeds extract was diluted at different concentrations in sterile water. The pathogenic bacterial
139 strains were cultured for 16 hours on MHB at 37°C and cultures were diluted to obtain the 0.5
140 McFarland standard turbidity. A working suspension corresponding to about 1-5 x 10^5 CFU/ml was
141 prepared in sterile MHB. An aliquot of bacterial suspensions (50 µl) and hemp seeds extract
142 dilutions (100 µl) were added to a transparent and sterile 96-well plate containing 100 µl of MHB.
143 A control (containing only bacterial inoculum) was included on each microplate. Gentamycin and
144 vancomycin were used as positive control (1 mg/ml in sterile physiological solution, corresponding
145 to 0.05 mg/ml in the well). Then, the plate was incubated for 24 hours at 37°C under aerobic
146 conditions. The optical density (O.D.) at 600 nm was recorded and the MIC was established as the
147 lowest concentration of hemp seeds extract capable to suppress the microorganisms' growth.

148 The MIC value was also determined for the *Bifidobacterium* and *Lactobacillus* strains using the
149 same procedure except for the growing media conditions. Furthermore, plates were incubated for 24
150 hours at 37°C under anaerobic conditions. O.D. at 600 nm was measured as described above.

151 ***2.4.4. S. aureus viability by a double-staining fluorescence assay***

152 The *S. aureus* viability in the presence of hemp seeds extract was detected using a live/dead double
153 staining method by which cells with the damaged membrane can be stained by propidium iodide
154 (PI) while intact cells are stained by fluorescein diacetate (FD). Briefly, 1 ml of *S. aureus* cells were
155 cultured at 37°C with or without 0.5 and 1 mg/ml of hemp seeds extract without shaking.
156 Vancomycin (0.01 mg/ml) was used as positive control. After 1 hour, bacteria mixtures were
157 centrifuged for 5 min at 3000 x g. The supernatants were discarded and the pellets were washed
158 with PBS buffer pH 7.4. The collected pellets were finally resuspended in 1 ml of PBS and 4 µl of 1
159 mg/ml FD and 8 µl of 1 mg/ml PI were added to the microbial cell suspension, gently mixed and
160 incubated for 10 min in the dark at room temperature. Microbial suspensions were centrifuged for 5

min at 3000 x g. Supernatants were discarded and pellets washed to remove excess of PI and FD dyes. Pellets were resuspended in PBS (1 ml) and an aliquot of each microbial suspension (200 µl) was transferred in a 96-well blackened fluorescence plate. FD ($485_{ex}/535_{em}$ nm) and PI ($485_{ex}/616_{em}$ nm) fluorescence was recorded using a Victor™ X3 Multilabel Plate Reader (Waltham, MA). All data were measured in quadruplicate.

2.5. Biofilm Inhibition

2.5.1. Biofilm production and inhibition assay

The biofilm production and inhibition was determined as previously described by Blando et al. (2019) using two *Staphylococcus* reference strains, specifically the biofilm producer *Staphylococcus aureus* ATCC 35556 and the *Staphylococcus epidermidis* ATCC 12228, used as negative control. The percentage of biofilm inhibition was calculated using the formula of Bazargani and Rohloff (2016):

$$\text{Biofilm reduction \%} = \frac{\text{O.D. control} - \text{O.D. sample}}{\text{O.D. control}} \times 100$$

2.5.2. Microscopic visualization of biofilm in the presence/absence of hemp extract

The inhibition of biofilm was also evaluated by microscopic technique as described by Bazargani and Rohloff (2016) with some modifications. Briefly, *S. aureus* cells were inoculated on round cover glass slides (diameter 1 cm) placed in 24-well polystyrene plate (Greiner Bio-One Gmbh, Austria) and cultured with or without hemp seeds extract as described above. After 24 hours of incubation the cultures were stained with Giemsa 1/20 solution (v/v) for 20 min at room temperature. Stained glass pieces were placed on the slides. Biofilm was evaluated by microscopy at 100x magnification.

2.6. Statistical analysis

Assays were carried out in triplicate and results were expressed as mean values ± standard deviation (SD). Data were analyzed by one-way analysis of variance (ANOVA) with Dunnett's multiple comparison test (GraphPad Prism software, version 6.00 for Windows San Diego, CA). A *p*-value lower than 0.05 was considered statistically significant.

3. Results and discussion

In this work the beneficial role and potential use of hemp seeds extract as antimicrobial and antibiofilm agent have been studied.

3.1. Cannabis sativa L. extract antimicrobial activity against pathogenic and beneficial bacteria

The antimicrobial activity against selected enteric bacterial strains was measured evaluating the strain growth in the presence of increasing doses of hemp seeds extract (Table 1). Growth in the presence of standard antibiotics, specifically gentamycin and vancomycin, was used as a positive control. The most sensitive Gram-negative microorganisms were *E. coli* ATCC 25922 and *S. typhimurium* ATCC 14028 showing MIC values of 1 mg/ml, while *E. aerogenes* ATCC 13048 was inhibited by 2.5 mg/ml hemp seeds extract. For both Gram-positive bacteria (*S. aureus* ATCC 25923 and *E. faecalis* ATCC 29212) MIC values of 1 mg/ml were found. Therefore, the inhibitory effect was similar for Gram-positive and Gram-negative strains. The MIC against planktonic cells of the biofilm producer *S. aureus* ATCC 35556 was 1 mg/ml. The antimicrobial activity of hemp seeds extract may be related to its high content of polyphenols, mainly caffeoyltyramine and cannabisin. Indeed, as reported by Patnaik, Dey and Gouda (2008) the caffeoyltyramine exhibited antibacterial activity against *S. aureus* and *E. coli* and this is probably linked to the presence of phenolic hydroxyl groups forming hydrogen bonds with the active sites of target enzymes. Besides, Nissen et al. (2010) reported that essential oils from inflorescences of industrial hemp, mainly the Futura variety, showed good antimicrobial activity against Gram-positive opportunistic/pathogenic bacteria. For the first time this study highlighted the antimicrobial effect of *C. sativa* seeds, which have the benefit to be stored more easily with respect to inflorescence and have greater industrial potentials. Differently from the action on pathogens, *C. sativa* seeds extract did not exert any marked antimicrobial activity against the tested *Bifidobacterium* and *Lactobacillus* spp. strains, as shown in Table 2. Selective inhibition against potential pathogenic strains is a crucial matter since the maintenance of a balanced intestinal microbiota is necessary for the health of the host (Di Gioia,

Aloisio, Mazzola, & Biavati, 2014). Besides, the maintenance of a proper amount of beneficial bacteria, such as lactobacilli and bifidobacteria, in the gut is essential not only for the host health status, through production of bioactive molecules and detoxification of harmful compounds (Di Gioia, Gaggia, Baffoni, & Stenico, 2015), but also for the protection against incoming pathogens (Montier et al., 2012; Symonds et al., 2012). In fact, beneficial bacteria are capable to compete for nutrients with enteric pathogens, to stimulate the development of both humoral and cellular mucosal immune system, and to strongly adhere to the intestinal mucosa, thus preventing pathogen adhesion (Tremaroli & Bäckhed, 2012). The selectivity of the action is therefore of outmost importance for the maintenance of a healthy gut microbiota.

3.2 Cannabis sativa L. extract ~~effect~~ on S. aureus viability

The effect of hemp seeds extract on the biofilm producer *S. aureus* ATCC 35556 was evaluated as percentage of live or dead bacterial cells using a FD/PI double-staining assay. Regarding Gram-positive bacteria, antibacterial compounds may impair the bacterial cell wall causing leakage of the cytoplasm and its coagulation (Tian et al., 2018).The use of PI as a membrane-impermeable fluorescent dye, which is totally excluded by live cells, is useful to understand if hemp seeds extract is able to damage the *S. aureus* membrane.

A clear reduction in FD staining was observed after all treatments compared to control (untreated cells), which showed the maximum FD fluorescence intensity and the minimum PI signal. As shown in Figure 1 (panel A), at both tested concentrations, hemp seeds extract was able to reduce in a dose-dependent manner the *S. aureus* viability (52% and 70% of fluorescence reduction at 0.5 and 1 mg/ml, ***$p<0.001$ vs CNT vitality for both). Our results showed a 5-fold increase in dead cells following the exposure to 1 mg/ml hemp seeds extract with respect to the lowest tested concentration. A similar pattern was observed after vancomycin exposure, which targets bacterial protein synthesis, with a decrease (~43%, ***$p<0.001$ vs CNT vitality) in viable stained cells and increase (~15%, #$p<0.05$ vs CNT mortality) in dead stained cells lesser than the effect caused by

239 the highest concentration of hemp seeds extract. In panel B we reported the live/dead cells ratio
240 showing a percentage of mortality higher than 50% for all treatments with the greatest effect
241 (~78%) following 1 mg/ml hemp seeds extract exposure.

242 These results suggest a strong antibacterial effect of 1 mg/ml hemp seeds extract on biofilm
243 producer *S. aureus* that seems to be in part linked to a membrane damage mechanisms. In our case,
244 although the increased signal in dead-stained cells, we observed a much higher decrement in viable
245 cells suggesting, besides membrane damages, multiple antibacterial activity mechanisms. Indeed,
246 among others, hemp seed compounds could be involved in different bacterial biosynthetic pathways
247 by acting eg. as inhibitors of the cell wall, DNA, lipid and/or protein synthesis. Further analyses
248 will be necessary to clarify this aspect.

249 ***3.3. Microscopic visualization of biofilm and biofilm inhibition (crystal violet assay)***

250 The effect of hemp seeds extract on the adhesion ability of *S. aureus* was checked via a microtiter
251 plate method. The bacteria were cultured in the presence of sub-MIC concentration of extract,
252 ranging from 0.1 to 1 mg/ml. Bacterial biofilm was stained with crystal violet. *Staphylococcus*
253 *epidermidis* ATCC 12228, used as negative control, was not able to produce biofilm. As shown in
254 Figure 2, *C. sativa* seeds extract at a concentration lower than 0.5 mg/ml, reduce (*$p<0.05$ and
255 **$p<0.01$ vs *S. aureus* at 0.1 and 0.25 mg/ml, respectively) but not completely inhibit the biofilm
256 formation. Conversely, 0.5 and 1 mg/ml *C. sativa* seeds extract totally blocked the biofilm
257 formation with an inhibition rate (calculated as described in 2.5 section) of about 80% (***$p<0.001$
258 vs *S. aureus* for both concentrations).

259 These results showed that the biofilm inhibition occurs at lower concentration than the MIC (1
260 mg/ml). Our results are in accordance with results of Silva et al. (2015) on extracts from *Vaccinium*
261 *corymbosum*, showing that *S. aureus* biofilm is inhibited at half the concentration that inhibits
262 planktonic cells. Several studies demonstrated that sub-MIC concentrations of antimicrobial
263 compounds can hinder the formation of bacterial biofilm (Dong et al., 2012; Magesh et al., 2013;

Silva et al., 2015; Ding et al., 2017). The mechanisms of this activity remain unclear, however the down regulation of two-component signal transduction systems (TCSs), important cell-to-cell communication systems known to play a key role in biofilm formation (Rasamiravaka, Labtani, Duez, & El Jaziri, 2015), has been suggested. As demonstrated using water extracts from rhubarb, a TCS down-regulation by sub-MIC concentrations can impair the correct transfer of information within the cell during the biofilm formation and inhibit quorum sensing mechanisms (Ding et al., 2017).

Finally, the effect of 0.5 mg/ml *C. sativa* L. seeds extract on the biofilm formation by *Staphylococcus aureus* ATCC 35556, which has been shown to form strong biofilms (Thiran et al., 2018), has been also investigated. The biofilm formation was evaluated by microscopic visualization (Fig. 3) and the antibiofilm activity of hemp seeds extract is shown in Figure 3B. The inhibition pattern of biofilm formation by vancomycin (positive control) was similar to that of hemp seeds (Fig. 3C). In our study, the inhibition of biofilm can be ascribed to the high content of phenolic compounds, particularly caffeoyltyramine and cannabisin A, B, C, in hemp seeds extract. It has been recently reported by Beoletto et al. (2016) that terpenes and flavonoids inhibited the biofilm formation.

Conclusions

The emergence of multiple drug-resistant strains in the last decade due to the large and often indiscriminate use of antibiotics has stimulated the research of alternatives, the use of plant extracts being one of them. The present study showed that *C. sativa* L. seeds extract have a selective inhibitory action against pathogenic strains as well as a potential role as a new antibiofilm agent. To the best of our knowledge, this is the first time that the antibiofilm activity of *C. sativa* L. seeds has been reported against biofilm producer *S. aureus.* Therefore, the use of hemp seeds extracts in controlling microbial growth, especially opportunistic and pathogenic contaminants in the food and

nutraceutical industry, has been described as possible alternatives to antibiotics/antibacterial compounds. This approach is particularly valuable also considering the lack of toxicity and positive effects on human health of these extracts that have been previously demonstrated.

Declaration of Interest Statement

The authors declare that have no conflicts of interest.

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Table 1

Table 1. Growth of selected pathogen strains in the presence of different amounts of hemp seeds extract and target antibiotics (0.05 mg/ml) as negative control.

Strains	Hemp seeds extract concentrations (mg/ml)				
	Control	0.5	1	2.5	*Gentamycin*
Escherichia coli ATCC 25922	1.12 ± 0.2	1.24 ± 0.05	0.18 ± 0.06	0.12 ± 0.01	0.05 ± 0.00
Salmonella typhimurium ATCC 14028	0.82 ± 0.03	0.80 ± 0.02	0.12 ± 0.01	0.16± 0.05	0.049 ± 0.01
Enterobacter erogene ATCC 13048	1.07 ± 0.03	1.3 ± 0.04	1.3 ± 0.01	0.3 ± 0.02	0.05 ± 0.01
					Vancomycin
Enterococcus faecalis ATCC 29212	1.1 ± 0.09	1 ± 0.02	0.37 ± 0.02	0.3 ± 0.05	0.045 ± 0.00
Staphylococcus aureus ATCC 25923	1.3 ± 0.07	1.08 ± 0.07	0.27 ± 0.07	0.26 ± 0.02	0.04 ± 0.01
Staphylococcus aureus ATCC 35556	0.9 ± 0.07	0.88 ± 0.06	0.25 ± 0.05	0.24 ± 0.02	0.04 ± 0.01

Bacterial growth (O.D. 600 nm).

Table 2

Table 2. Growth of the *Lactobacillus* and *Bifidobacterium* strains tested in the presence of different amounts of hemp seeds extract

Strains	Hemp seeds extract concentrations (mg/ml)			
	Control	0.5	1	2.5
Lactobacillus paracasei MB395	0.42 ± 0.08	0.52 ± 0.01	0.42 ± 0.01	0.42 ± 0.01
Lactobacillus reuteri DSM 20016	0.78 ± 0.06	0.72 ± 0.04	0.71 ± 0.01	0.70 ± 0.31
Lactobacillus brevis ATCC 14869	0.80 ± 0.02	0.74± 0.05	0.77 ± 0.06	0.86 ± 0.01
Lactobacillus plantarum MB91	0.77 ± 0.03	0.85 ± 0.05	0.83± 0.01	0.85± 0.02
Bifidobacterium bifidum B2009	0.75 ± 0.05	0.74 ± 0.06	0.76± 0.05	0.77 ± 0.03
Bifidobacterium longum Re11	0.80 ± 0.05	0.91 ± 0.01	0.93± 0.03	0.86 ± 0.06
Bifidobacterium breve B632	0.76 ± 0.03	0.78 ± 0.02	0.79 ± 0.03	0.81 ± 0.05

Bacterial growth (O.D. 600 nm).The evaluation was done after 24 h for *Lactobacillus* spp. strains and after 30 h for *Bifidobacterium.*

Figure 1

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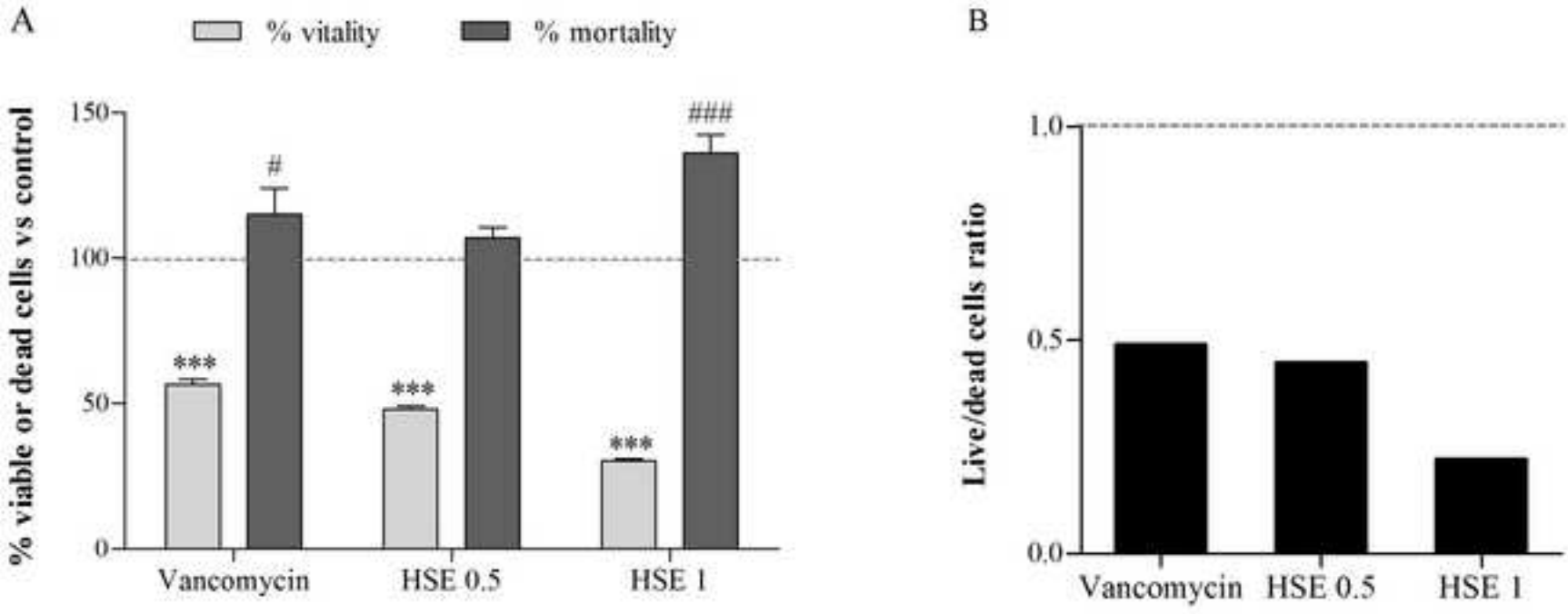

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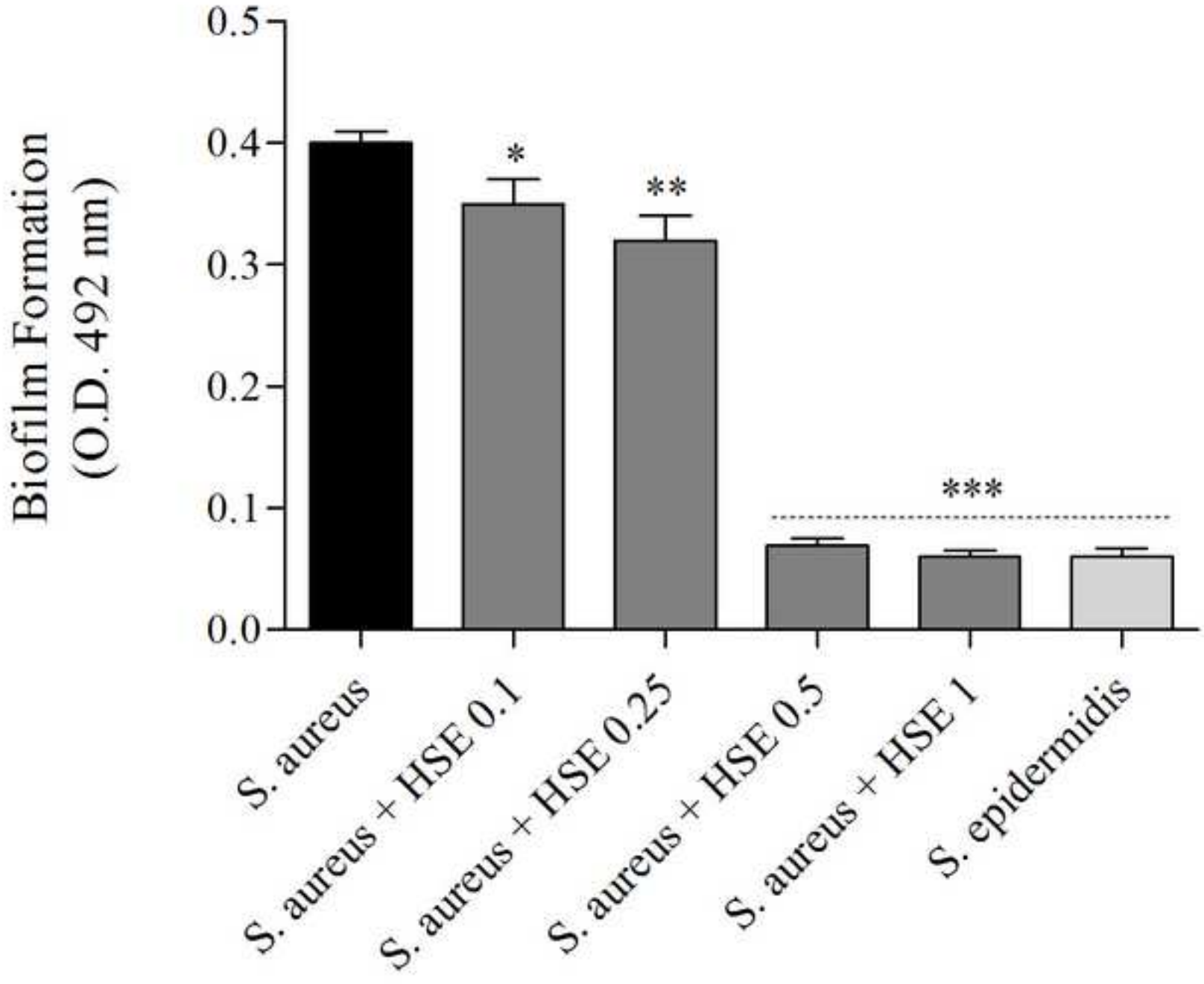

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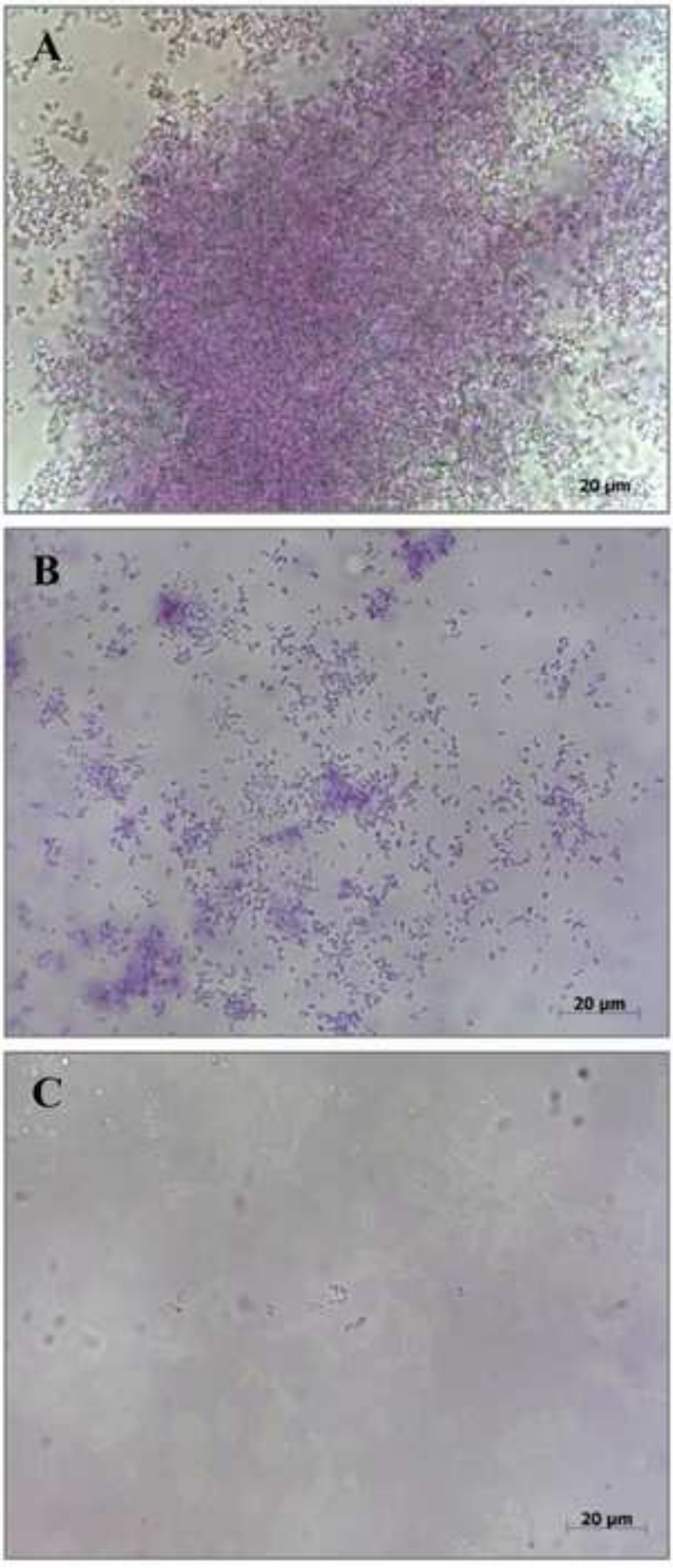

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