Waste and Biomass Valorization (2026) 17:3073–3093
https://doi.org/10.1007/s12649-025-03302-3

ORIGINAL PAPER

Circular Valorisation of Essential Oil Post-distillation by-products for Enhanced Xanthan Gum Bioproduction and Antimicrobial Treatments against *Staphylococcus aureus*

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Received: 17 July 2025 / Accepted: 30 August 2025 / Published online: 13 September 2025

Abstract

Essential oil (EO) post-distillation by-products, like hydrosols and wastewater, are sources of bioactive compounds. Aligned with the principles of the circular economy, these residues offer a cost-effective alternative for various applications. In this context, this study investigated xanthan gum (XG) biosynthesis by *Xanthomonas campestris* ATCC 33913 in a mineral salt medium supplemented with four hydrosols (*Cupressus leylandii* A.B. Jacks & Dallim, *Eucalyptus globulus* Labill., *Aloysia citrodora* Paláu, and *Melissa officinalis* L.), achieving a 15.8% increase in production (13.91 g/L) when 10% *C. leylandii* hydrosol was used, compared to the control (12.01 g/L). The combination of this XG sample with a 50:50 (*v/v*) *A. citrodora* hydrosol and wastewater mixture yielded a product with a strong antioxidant capacity (90.2% free radical scavenging) and bacteriostatic effects against *Staphylococcus aureus*. Moreover, its antimicrobial capacity increased over time, likely due to the bacteria's extended exposure to the bioactive compounds found in both by-products. These interactions may compromise the integrity of the cell membrane, thereby enhancing compounds' penetration, reducing *S. aureus* by 48.3% in clean conditions (low organic load) and 40.6% in dirty conditions (high organic load) after 45 min, both of which were significantly different from the control ($p < 0.0001$). The developed product demonstrated antimicrobial activity against *S. aureus* biofilms, reducing them by 35.9% ($p < 0.001$) after 45 min of treatment. This effect was increased (56.8%; $p < 0.0001$) by doubling the treatment time (90 min). Repurposing EO by-products as novel ingredients provides antioxidant and antimicrobial benefits for innovative products targeting *S. aureus* and its biofilms.

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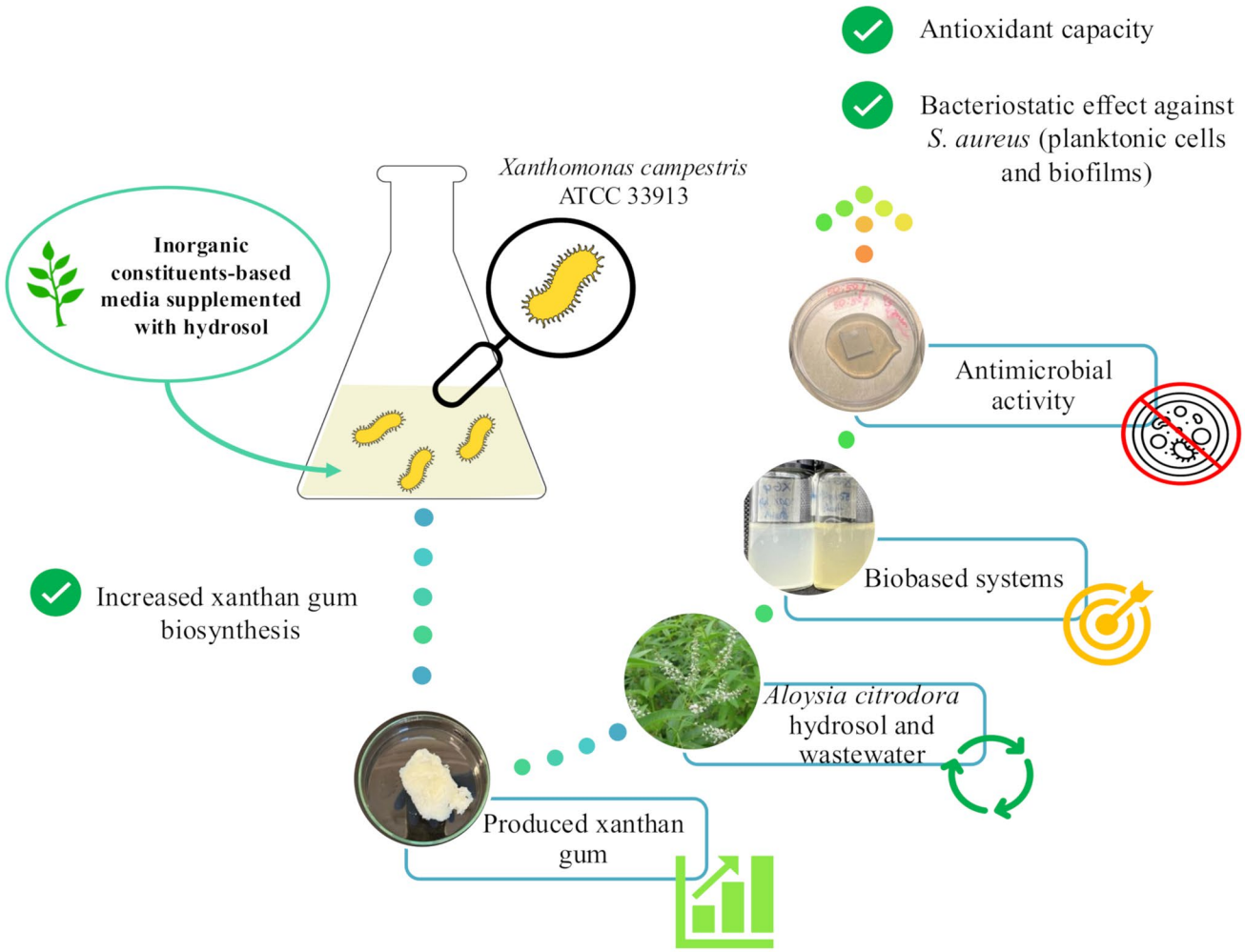
Graphical Abstract



Highlights

- Essential oil by-products provide novel antimicrobial and antioxidant ingredients;
- Supplementation with *C. leylandii* hydrosol uplifted xanthan gum (XG) biosynthesis;
- XG systems with *A. citrodora* hydrosol and wastewater led to innovative biobased systems;
- Biobased systems presented antimicrobial action against *S. aureus* and its biofilms;
- A sustainable, circular solution for the EO industry was revealed.

Keywords Xanthan gum · Essential oil by-products · Waste valorisation · Hydrosols · Antimicrobial activity · Biobased solutions

Introduction

Medicinal and aromatic plants (MAPs) are extensively studied for their bioactive compounds. Essential oils (EOs), a primary product obtained from MAPs, are widely used in cosmetics, food and beverages, pharmaceutical and healthcare, household products, and agriculture. However, EO extraction yields are relatively low compared to the substantial volume of generated by-products, requiring measures for their efficient management [1, 2]. Post-distillation by-products such as solid residues (residual biomass), aqueous condensates (hydrosols), and decoction water (wastewater) are discarded without further valorisation. Nevertheless, recent research indicates that these by-products are valuable sources of high-value products, offering environmental and economic benefits [3]. The solid residues and wastewater

are abundant in polyphenols like phenolic acids and flavonoids, and hydrosols mostly contain traces of EOs, which comprise a high content of volatile compounds (terpenoids and terpenes) [4]. Given the rising interest in sustainable resource utilisation, these bioactive compounds can be repurposed into innovative biomaterials with enhanced functional properties. In this context, hydrosols offer a new opportunity in biopolymer biosynthesis, allowing for cost-effective production, strengthening value chains, and supporting a shift toward zero-waste, eco-friendly processes.

Among biopolymers, polysaccharides have attracted considerable attention as sustainable alternatives due to their biodegradability, biocompatibility, and functional versatility. These characteristics make them ideal for use in the food, biotechnology, and pharmaceutical industries. Their rheological properties enable them to function effectively as thickeners, stabilizers, emulsifiers, bioadhesives, and gelling agents [5]. A particularly valuable example is xanthan gum (XG), a microbial exopolysaccharide produced by bacteria of the *Xanthomonas* genus. XG is notable for its non-toxic nature, water solubility, thermal stability, pH tolerance, and ability to impart high viscosity even at low concentrations [6]. Structurally, XG is an anionic pentasaccharide composed of D-glucose, D-mannose, and D-glucuronic acid in a 2:2:1 molar ratio. Its reactive hydroxyl and carboxyl groups enable chemical modification, allowing for tailored physicochemical properties and a broader application potential [7]. XG biosynthesis occurs via a three-step pathway: (i) uptake of simple sugars and conversion into nucleotide sugar precursors; (ii) sequential assembly of repeating pentasaccharide units with acetyl and pyruvate substitutions; and (iii) polymerization followed by secretion. This process is catalysed by enzymes encoded by the gum operon in *Xanthomonas campestris*, the primary species used in industrial production [8–10].

Exopolysaccharide secretion is a defensive response of microorganisms to adverse conditions such as shocks, heat, pH changes, salts, carbon-nitrogen limitations, and exposure to toxic compounds. These polysaccharides coat the cell surface, having biological roles such as preventing infections of bacteriophages, facilitating adhesion, and providing resistance to dehydration. Exposure to toxic compounds acts as a stressor, causing metabolic imbalances and cellular responses. However, a well-planned approach to effectively combining these conditions can significantly boost exopolysaccharide production [6, 11, 12]. In this scenario, combinations can be formulated to optimise the substrate and its composition to enhance XG secretion while achieving more cost-effective alternatives. Organic residues from agricultural, household, and industrial processes are rich in carbon and nitrogen sources and essential micronutrients supporting microbial growth. These residues often contain significant amounts of cellulose and hemicellulose, which can be enzymatically or chemically hydrolysed into fermentable simple sugars. Furthermore, specific plant-based residues may contain bioactive compounds that can create beneficial stress conditions, potentially enhancing microbial metabolic activity and increasing production. These residues offer a more sustainable alternative to traditional culture media for microbial growth and polymer production, significantly lowering process costs compared to traditional carbon sources like glucose and sucrose [12–14].

To ensure economically sustainable synthesis, several substrates have been previously evaluated, including the use of the solid residue from the distillation of *Melaleuca alternifolia* (EOs by-product) [15], potato crop residue [16], and cassava bagasse [17]. An example of the benefits of using these alternatives as substrates or medium supplements is demonstrated by the hydrolysate of lignocellulosic residues from cocoa husks and coconut shells. These substrates yielded xanthan gum productions of 3.57 g/L and 4.49 g/L, respectively, significantly outperforming the 1.31 g/L obtained using sucrose, a cheaper alternative substrate to conventional sugar sources [18]. Another approach was explored by Crugeira et al. [19], who investigated the use of moist olive pomace, a by-product of the olive oil industry rich in phenolic compounds, which induced bacterial stress and improved xanthan gum production by 50.9% compared to a control sample without this supplementation.

Antimicrobial resistance is a critical global health challenge arising from microorganisms' ability to survive and proliferate despite exposure to antimicrobial agents [20]. In this context, *Staphylococcus aureus*, a Gram-positive bacterium, is responsible for various infectious diseases, from skin and soft tissue infections to more severe and life-threatening conditions. It forms intricate extracellular polymeric biofilms, creating a protected environment for microcolony formation, sustenance, and recolonisation of sessile cells after dispersion [21, 22]. Rising concerns about the toxicity and environmental impact of synthetic preservatives and disinfectants have driven global interest in natural antimicrobial alternatives. Several plant-derived antimicrobial substances have been explored as safe disinfectants and sanitising solutions against this pathogenic bacterium, demonstrating higher efficacy against planktonic and biofilm cells of *Staphylococcus aureus* compared to conventional chemical agents, such as peracetic acid, sodium hypochlorite, and benzalkonium chloride, offering a promising natural and eco-friendly alternative suitable for industrial use [23–25].

Therefore, this work investigated two sustainable approaches to contribute to the integral valorisation of the EOs productive cycle. Firstly, the biosynthesis of XG by *Xanthomonas campestris* was studied by analysing the effect of adding 10 and 20% (*v/v*) of four different hydrosols

(*Cupressus leylandii* A.B. Jacks & Dallim, *Eucalyptus globulus* Labill., *Aloysia citrodora* Paláu and *Melissa officinalis* L.) supplemented to the production medium. The objective was to induce bacterial stress and boost the exopolysaccharide excretion, triggering a response mechanism to counteract the adverse conditions caused by the antimicrobial bioactive components present in hydrosols. The produced XG was characterised (chemical structural analysis and thermal behaviour) and compared to a commercial counterpart. Secondly, the biosynthesised XG was combined with prepared biobased systems comprising a mixture of two *A. citrodora* post-distillation by-products (hydrosol and wastewater). The XG-based products' antioxidant (free radicals scavenging) and antimicrobial (against *Staphylococcus aureus*) activities were investigated. This approach emphasises the utilisation of different by-products of the EOs extraction industry through various strategies to develop novel biobased antimicrobial systems.

Materials and Methods

Materials

Fresh plant (*Cupressus leylandii* A.B. Jacks & Dallim, *Aloysia citrodora* Paláu, *Eucalyptus globulus* Labill., and *Melissa officinalis* L.) supplied by Deifil Technology Lda (Póvoa de Lanhoso, Portugal), were received, cut, frozen and stored at -20 °C (Hotpoint-Ariston, Italy). HPLC grade n-hexane (CarloErba Reagents, France) and anhydrous sodium sulphate (Sigma-Aldrich, Merck, Germany) were used in sample preparation for the chemical analysis. Commercial xanthan gum (viscosity of 1.45 to 2.00 mPa.s for 1% solution at 20 °C) was acquired from TCI chemicals (Switzerland). For the phenolic and flavonoid content assays, Folin-Ciocalteu reagent (PanReac AppliChem, ITW Reagents, Spain), sodium carbonate (Scharlau, Scharlab, Spain), aluminium chloride (VWR Reagents, Belgium), sodium nitrite (Riedel-de-Haën, Honeywell, Germany), sodium hydroxide (Sigma-aldrich, Merck, Germany), gallic acid and catechin (Merck, USA). ABTS (2,2'-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid), bovine serum albumin, polysorbate 80, lecithin and sodium chloride were acquired from Sigma-Aldrich (Saint Louis, MO, USA) were used. The microbial cultures used in this study were *Staphylococcus aureus* ATCC 6538 (Mistracon, Sharlab, Spain) and *Xanthomonas campestris* ATCC 33913 (DSMZ, Leibniz Institute, Germany). The culture media brain-heart infusion (BHI) broth, nutrient agar, yeast extract, malt extract, peptone, glucose, and sucrose were purchased from Liofilchem (Italy). Saponin was bought from PanReac Applichem (ITW Reagents, Spain). The used acetone (99.8%) and ethanol (99.8%) were purchased from CarloErba Reagents (France) and Honeywell (Germany), respectively. Commercial benchmark product (Unilever) was purchased in a local supermarket (ingredients: aqua, coco-glucoside, benzalkonium chloride, sodium carbonate, perfume, dimethyl cocamine, trisodium citrate). The used water was distilled water.

Essential Oil by-products and Characterisation

This work considers the by-products of two EOs' productive processes: hydro-distillation and steam-distillation. Hydrosols obtained by hydro-distillation of four selected plants (*C. leylandii*, *A. citrodora*, *E. globulus*, and *M. officinalis*) were studied and characterised in a previous work of the group [26], were used in the XG biosynthesis. The hydrosol and wastewater obtained from steam-distillation of the most promising plant selected based on the antimicrobial activity of the previous study [26] (*A. citrodora*), were combined in different ratios (*v/v*) and added with the produced XG to produce a biobased antimicrobial system prototype (see Sect. Development of the Biobased Antimicrobial Systems).

Hydro-distillation Hydrosols

The hydrosols obtained by hydro-distillation presented the following characteristics: *C. leylandii* hydrosol (pH of 2.9, with terpinen-4-ol (36.2%) and oplopanonyl acetate (12.8%) as major compounds), *A. citrodora* hydrosol (pH of 4.1, with geranial (38.9%) and neral (39.0%) as major compounds), *E. globulus* hydrosol (pH of 3.2, with 1,8-cineole (90.1%) as major compound), and *M. officinalis* hydrosol (pH of 3.2, with geranial (50.1%) and neral (42.0%) as major compounds). According to Almeida et al. [26], the antimicrobial activity of the hydrosols was ranked as: *A. citrodora* > *M. officinalis* > *E. globulus* > *C. leylandii*.

Steam-distillation Hydrosol and Wastewater

Steam-distillation was performed using a steam-distillation apparatus (Fig. 1) following an adapted procedure [27]. Briefly, 100 g of plant material and 1500 mL of distilled water were used. The process lasted 90 min, and the produced EO and hydrosol were separated and stored under refrigeration. Wastewater corresponding to the condensed water dripping from the plant material was also collected and stored. The pH of all samples was measured with a pH meter (InoLab, WTW Series pH 720, Weilheim, Germany).

Hydrosol chemical characterisation was conducted using gas chromatography-mass spectrometry (GC–MS, Shimadzu, Japan) as described elsewhere [26]. Briefly, 15 mL of hydrosol underwent liquid-liquid extraction with 5 mL of

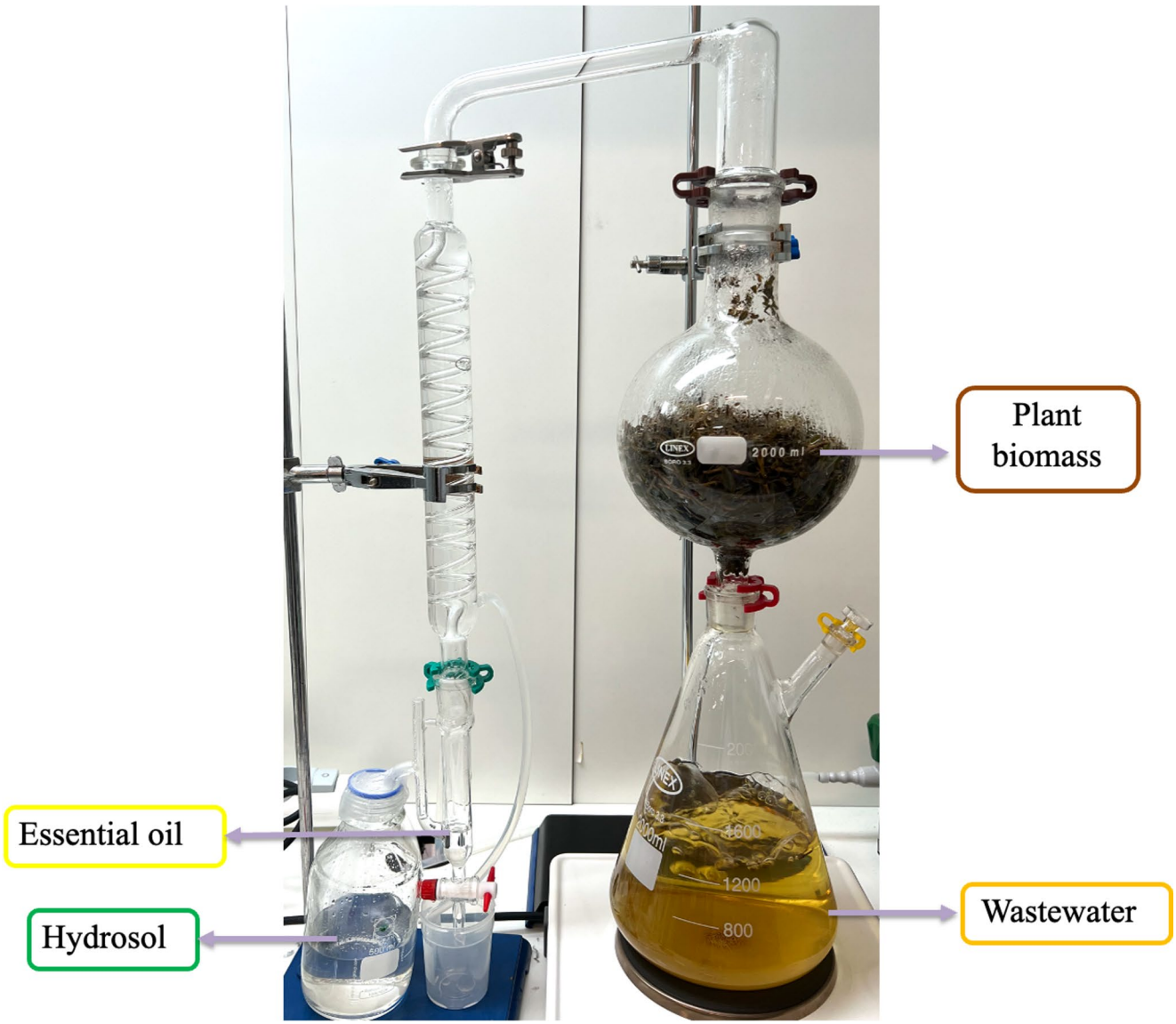


Fig. 1 Steam-distillation system and its respective essential oil and by-products

n-hexane, followed by phase separation, drying with anhydrous sodium sulfate, and filtration. Compounds were analysed on an SH-RXi-5 ms column system (30 m × 0.25 mm × 0.25 µm) using a 1:10 split ratio and helium as the carrier gas. Identification relied on linear retention indices (LRI) and mass spectra matched to the NIST17 library (similarity > 90%), with LRI values calculated using n-alkanes (C_8–C_{40}) as references. Quantification was based on relative peak area percentages from total ion current (TIC) readings.

The phenolic composition was analysed using ultra-performance liquid chromatography (Dionex Ultimate 3000 UPLC, Thermo Scientific) with an electrospray ionisation mass spectrometry detector (LQT XL, Thermo Scientific) and a diode array detector (HPLC-DAD-ESI/MS). Separation was performed on a Waters Spherisorb S3 ODS-2 C18 column (3 µm, 150 × 4.6 mm) at 35 °C with a mobile phase of 0.1% formic acid (A) and acetonitrile (B) using a gradient flow (0.5 mL/min). Mass spectra were acquired in negative ion mode (100–1500 *m/z*) with a spray voltage of 5 kV at 325 °C. Compound identification was based on standards, UV spectra, retention times, MS-MS data, and literature. Results are expressed in µg/mL.

Xanthan Gum Biosynthesis and Characterisation

Media Preparation

The biosynthesis of xanthan gum comprised the inoculum activation and biopolymer production. For the activation, the yeast malt broth (YM) medium was prepared (*w/v*) using

0.3% yeast extract, 0.3% malt extract, 0.5% peptone, and 1.0% glucose, added to 1 L of distilled water. The control production medium was an inorganic constituents-based mineral salt medium (MSM) supplemented with organics like sucrose (2.5%) and yeast extract (0.05%) [19], added with 1 L of distilled water. The medium added with the hydrosols was prepared similarly to the control medium by substituting part of the water with the corresponding hydrosol volume (10 and 20% (*v/v*)). All the prepared media were subjected to pH correction to 7 with 5 M sodium hydroxide, then submitted to a pasteurisation process (70 °C for 3 h).

Production and Isolation

The cryopreserved culture of *Xanthomonas campestris* pv. *campestris* ATCC 33913, stored in an ultra-freezer (ThermoFisher, STP, AS) at -70 °C, was inoculated in 18 mL of YM medium in a 100 mL Erlenmeyer flask, incubated for 14 h at 28 °C under 150 rpm in a shaker-incubator (ES-20/80, BioSan, Latvia). Subsequently, 10% (*v/v*) of the pre-inoculum at a mid-exponential phase culture was added to the YM medium. After 8 h of incubation using the above-mentioned conditions, the bacterial culture was ready for the biopolymer production assays.

Xanthan gum production trials were carried out in triplicate using 250 mL Erlenmeyer flasks containing 45 mL of the MSM medium and 5 mL of the inoculum (10% (*v/v*), final volume basis). The flasks were subjected to 200 rpm orbital shaking at 30 °C (ES-20/80, BioSan, Latvia) for 76 h. This time was predefined after preliminary studies (48, 76 and 96 h) to achieve the highest productive levels. After the production, XG was recovered as previously described by [19]; briefly, samples were centrifuged at 10,000 g for 30 min at 4 °C (centrifuge 5810 R, Eppendorf, Hamburg, Germany) to separate the biomass from impurities. The resulting supernatant was mixed with ethanol (99.8%) at a 1:3 (*v/v*, supernatant/ethanol) ratio and kept at 4 °C for 18 h for XG precipitation. The precipitated biopolymer was recovered by filtration (Whatman filter n°4) and dried in an oven at 30 °C (ED115, Binder, Tuttlingen, Germany) until it reached a constant weight. The production yield quantification was determined gravimetrically using an analytical balance (AS 60/220.R2 Plus, Radwag, Radom, Poland).

Characterisation

The produced XG was characterised by Fourier transform infrared spectroscopy (FTIR) and thermogravimetric analysis (TGA) and compared with a commercial product.

FTIR analysis was conducted using an MB3000 FTIR spectrometer (ABB, Zurich, Switzerland) in attenuated total reflectance (ATR) mode equipped with a diamond crystal cell. The instrument was operated under the following settings: 32 scans/min, a spectral resolution of 4 $cm^{-1,}$ and a scan range from 4000 to 550 cm^{-1}. Spectral data were acquired and processed with Horizon MB version 3.4 software. For measurement, the sample was applied directly onto the crystal surface.

TGA analysis was performed on Netzsch equipment (TG 209F3 Tarsus, Selb, Germany). Approximately 7 mg of each sample was placed in alumina crucibles and heated from 30 to 800 °C at a constant heating rate of 10 °C/min under a nitrogen flow rate (40 mL/min). Thermograms were processed using Netzsch Proteus thermal analysis software (version 5.2.1).

Biobased Antimicrobial Systems and Characterisation

Development of the Biobased Antimicrobial Systems

The biobased antimicrobial systems were prepared by dissolving the most promising produced xanthan gum (1% (*w/v*)), acting as a thickener, in an aqueous medium combining the two *A. citrodora* EO steam-distillation by-products, hydrosol and wastewater. The selected hydrosol-to-wastewater ratios emphasize the hydrosol as the primary focus, with wastewater included as a supplement for added effects. Three different ratios (*v/v*) were applied: 70:30, 50:50, and 100:0, and the corresponding samples were named XG_70:30, XG_50:50, and XG_100:0, respectively. Three control samples were prepared using only distilled water: one comprising the most promising produced XG (XG_MP), another the produced control XG (XG_CT), and the latter the commercial XG (XG_CM). These samples were designated as C-XG_MP, C-XG_CT, and C-XG_CM, respectively. The developed biobased antimicrobial systems are shown in Fig. 2.

Phenolic and Flavonoid Content

The biobased antimicrobial systems total phenolic and flavonoid content was determined using colorimetric methods. Phenolic content was measured by the Folin-Ciocalteu method, with absorbance at 765 nm as described by Duarte et al. [28], and results were expressed as milligrams of gallic acid equivalents (GAE) per gram. Flavonoid content was determined using the aluminium chloride assay, with absorbance at 510 nm described by Barros et al. [29], and results were expressed as milligrams of catechin equivalents (CE) per gram. Distilled water was used as a blank control, and the absorbances were read in a microplate reader (Epoch, BioTek Instruments, USA) for both methodologies.

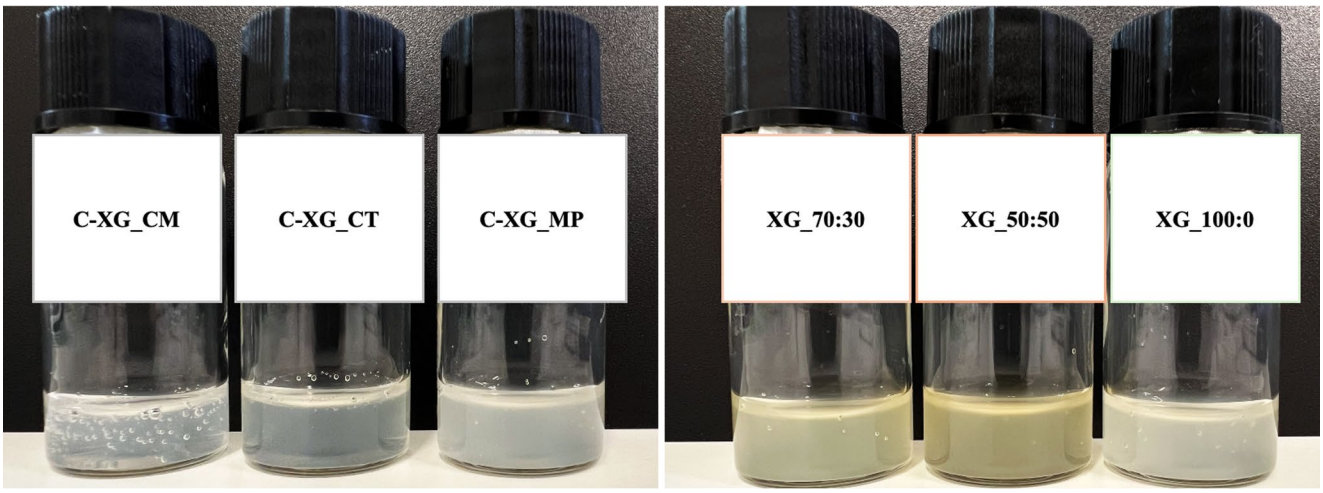


Fig. 2 Developed biobased antimicrobial systems, including the control systems and the systems incorporating *A. citrodora* steam-distillation by-products (hydrosol and wastewater) in different ratios

Calibration curves for gallic acid and catechin showed R^2 values of 0.9989 and 0.9993, respectively.

Antioxidant Capacity

The antioxidant capacity was evaluated through the discolouration and deactivation of the free radical $ABTS^{\cdot+}$ (2,2'-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid) [30]. A stock solution of $ABTS^{\cdot+}$ was prepared and left in the darkness for 16 h before use, then diluted to an absorbance of 0.70 ± 0.04 at 734 nm (V-730 UV–visible, Jasco, Madrid, Spain). Aliquots of 150 µL of each sample were mixed with 4.85 mL of the $ABTS^{\cdot+}$ solution and incubated in the dark for 20 min at room temperature. Distilled water was used as the blank for control. The absorbance was measured at 734 nm, and the scavenging capacity percentage (SC) was calculated using the following Eq. (1):

$$SC\ (\%) = \frac{Ac - As}{Ac}\ x\ 100 \quad (1)$$

where A_C is the absorbance of the blank, and A_S is the absorbance of the sample.

Antimicrobial Activity

The bacterial culture of *Staphylococcus aureus* ATCC 6538, preserved at -70 °C in an ultra-freezer (ThermoFisher, STP, AS), was reactivated in BHI broth and incubated at 37 °C for 24 h in a bacteriological oven (Raypa, Incutterm, Barcelona, Spain). Following incubation, the inoculum was prepared by adjusting the bacterial suspension in BHI broth to the desired cell density using a densitometer (DEN-1 McFarland densitometer, Grant-bio, UK) at a wavelength of 550 nm.

The antimicrobial capacity of the biobased antimicrobial system was evaluated using the quantitative suspension test, according to the standard procedure EN 1276:2009 [31] described by Campana and co-workers [32] with adaptations. In brief, 1 mL of the sample was added to sterile tubes containing 8 mL of sterile distilled water. Then, the influence of organic matter presence in the system was also investigated, with the addition of bovine serum albumin to simulate dirty (0.3% (*w/v*)) and clean (0.03% (*w/v*)) conditions. At this point, 1 mL of the bacterial culture prepared according to the conditions previously described, adjusted to a final cell density of about 1.5×10^6 cells/mL, was added and left in contact with the mixture in the tubes for 1, 15 and 45 min at room temperature. A control was prepared by replacing the sample with sterile distilled water. At each time point, 1 mL from each tube was transferred to another series of tubes containing 9 mL of neutralising buffer (polysorbate 80 (3% (*v/v*)), saponin (3% (*w/v*)), and lecithin (0.3% (*w/v*)) for 2 min. Then, 1 mL aliquot from each tube was diluted in saline solution (0,85% (*w/v*)), plated in triplicate onto nutrient agar (using the pour plate method) and incubated at 37 °C for 24 h. After the incubation, the colony-forming units (CFU) were enumerated. The results of CFU counting were converted to CFU/mL and used to calculate the bacteria reduction in percentage (%). Previous studies were conducted to ensure that the neutralising buffer solution did not affect microbial strains while neutralising the antimicrobial action of each sanitiser (data not shown).

Anti-biofilm Efficacy

To evaluate the anti-biofilm efficacy of the biobased antimicrobial product, stainless steel coupons were prepared, treated, and inoculated with *S. aureus* as a biofilm-forming bacterial, followed by a quantitative assessment of bacterial adhesion and survival based on CFU counts, according to the procedure proposed by Campana et al. [32] with modifications. Briefly, stainless steel coupons (2 × 2 × 0.8 cm) were washed with acetone to remove grease, then treated with HCl 5 N for 15 min and cleansed with ethanol 70% (*v/v*). Finally, the coupons were doubly rinsed with distilled water, air-dried, and autoclaved (121 °C for 15 min). The optical density of *S. aureus* (under the previously described growth conditions) was adjusted to approximately 1.5×10^6 cells/mL and used to assemble biofilms on stainless steel coupons. Briefly, sterilised coupons were placed in sterile Petri dishes containing the adjusted bacterial suspension and BHI broth, then incubated at 37 °C for 24 h. After incubation, the coupons were aseptically removed and washed with sterile PBS to remove unbound bacteria. Adherent bacteria were swabbed off the coupons with a sterile cotton swab, transferred into saline solution (0.85%, *w/v*), and vigorously shaken. The bacterial suspensions were serially diluted and plated on nutrient agar. Plates were incubated at 37 °C for 48 h, and CFU counted.

After forming the biofilms, the test included two groups of coupons, experimental and control, comprising two subgroups reflecting the contact duration of the biobased antimicrobial product or sterile distilled water, 45 and 90 min (time selection based on the quantitative suspension test). The coupons with the *S. aureus* biofilms were gently washed with 5 mL of sterile PBS before being dipped into new dishes containing 5 mL of the biobased antimicrobial system (or sterile distilled water) at the best concentration effective in the quantitative suspension test. After 45 and 90 min of contact, the coupons were transferred for new dishes with 5 mL of neutralising solution (2 min). The adhering bacteria were collected from each coupon using sterile swabs, plated on nutrient agar, and counted.

Statistical Analysis

For all assays, results were reported as the average ± standard deviations of replicates. When applied, the results were analysed using an ANOVA statistical test with Tukey's multiple comparison post-test using the GraphPad Prism® 8.0 software (San Diego, CA, USA).

Results and Discussion

Steam-distillation Hydrosol and Wastewater

Understanding the chemical composition and biological effects of EO hydrosols and wastewater is becoming increasingly important as these by-products are gaining popularity. Despite the absence of quality standards in pharmacopoeias for these products, chemical analysis is essential for their standardisation, quality assurance, and safety, facilitating their application across various industries [2]. The *A. citrodora* hydrosol and wastewater obtained by steam-distillation, presented acidic characteristics (pH of 5.96 and 5.42, respectively). The complete chemical composition of hydrosol volatile compounds is presented in Table 1. Briefly, with 98.4% of the total compounds identified, the citral isomers were the main components, which account for 31.2% of neral and 36.7% of geranial, a pretty similar chemical profile to the one of the hydro-distillation hydrosols [26]. Minor variances can be attributed to the different times used with both processes, 90 min and 10 min, for steam-distillation and hydro-distillation, respectively. This is an advantage, as steam systems are favoured at an industrial scale for their ability to process large volumes of raw materials efficiently, resulting in lower investment costs and minimising chemical degradation caused by hydrolysis [1]. The complete chemical characterisation of the hydrosol and wastewater in terms of phenolic components is shown in Table 2. The results revealed a higher total phenolics content in the wastewater (120 µg/mL, including a total of 59.4 and 58.0 µg/mL of phenylpropanoid and flavonoid compounds) than the hydrosol (20.5 µg/mL). The flavonoid luteolin-7-diglucuronide was the main component of *A. citrodora* wastewater (39.7 µg/mL wastewater), followed by martynoside (11.5 µg/mL wastewater) and forsythoside H (13.8 µg/mL wastewater). Essential oil (EO) by-products, including wastewater and solid residues, are valuable sources of phenolic compounds. Following distillation, the non-volatile components of aromatic plants are retained in these by-products [33]. These findings highlight both by-products as promising sources of bioactive compounds.

Xanthan Gum Biosynthesis and Characterisation

The results of the xanthan gum biosynthesis obtained with the inorganic constituents-based mineral salt medium supplemented with the hydrosols (*Cupressus leylandii* A.B. Jacks & Dallim, *Aloysia citrodora* Paláu, *Eucalyptus globulus* Labill., and *Melissa officinalis* L.), 10 or 20% (*v/v*), is represented in Fig. 3. It was verified that the higher production level was achieved with 10% (*v/v*) *C. leylandii* hydrosol, resulting in 13.91 g/L, corresponding to an increase of

Table 1 Chemical composition of volatile compounds presents in the hydrosols extracted from *A. citrodora* by steam-distillation (mean ± SD, $n = 3$)

Compound	RT	LRI[a]	LRI[b]	*A. citrodora*
				Relative %
1-Octen-3-ol	16.16	976	974	0.77 ± 0.04
Sulcatone	16.52	983	981	1.15 ± 0.03
3-Octanol	17.01	993	988	0.19 ± 0.01
o-Cymene	18.42	1020	1022	0.054 ± 0.001
1,8-cineole	18.72	1026	1026	9.59 ± 0.45
cis-sabinene hydrate	20.61	1062	1065	0.71 ± 0.03
Linalool	22.33	1096	1095	1.08 ± 0.02
cis-Verbenol	22.46	1098	1137	0.33 ± 0.01
Camphor	24.49	1139	1141	0.15 ± 0.01
exo-Isocitral	24.60	1141	1140	0.11 ± 0.02
Citronellal	25.00	1150	1148	0.07 ± 0.01
(Z)-Isocitral	25.56	1161	1160	0.87 ± 0.02
δ-Terpineol	25.65	1163	1162	0.23 ± 0.02
Rosefuran epoxide	26.09	1171	1173	0.46 ± 0.04
Terpinen-4-ol	26.16	1173	1174	0.23 ± 0.02
(E)-Isocitral	26.44	1179	1177	1.42 ± 0.07
α-Terpineol	26.82	1186	1186	2.34 ± 0.14
Oxiranecarboxaldehyde, 3-methyl-3-(4-methyl-3-pentenyl)-	28.39	1219	1215	0.1292 ± 0.0001
Nerol	28.64	1224	1227	2.88 ± 0.05
Isogeraniol	28.88	1230	1222	0.38 ± 0.04
Neral	29.25	1237	1235	**31.21 ± 0.86**
Geraniol	29.87	1251	1249	3.14 ± 0.15
Geranial	30.64	1267	1264	**36.69 ± 0.28**
Bornyl acetate	31.38	1283	1284	0.15 ± 0.01
Thymol	31.70	1290	1289	0.10 ± 0.01
Carvacrol	32.13	1299	1298	0.20 ± 0.01
Geranyl acetate	35.68	1380	1379	0.27 ± 0.01
β-caryophyllene	37.31	1418	1417	0.16 ± 0.02
Germacrene D	39.88	1480	1484	0.38 ± 0.02
Bicyclogermacrene	40.52	1495	1500	0.23 ± 0.01
Cubebol	41.27	1514	1514	0.21 ± 0.02
(E)-Nerolidol	43.10	1560	1561	0.14 ± 0.01
Spathulenol	43.74	1576	1577	1.33 ± 0.04
Caryophyllene oxide	43.96	1582	1582	0.43 ± 0.05
β-Spathulenol	46.05	1637	1619	0.183 ± 0.002
epi-α-Cadinol	46.14	1639	1638	0.29 ± 0.01
α-Cadinol	46.65	1653	1652	0.14 ± 0.01
Total identified				98.42 ± 0.02
Monoterpene hydrocarbons				0.054 ± 0.001
Oxygen-containing monoterpenes				92.75 ± 0.20
Sesquiterpenes hydrocarbons				0.77 ± 0.05
Oxygen-containing sesquiterpenes				2.72 ± 0.12
Others				2.12 ± 0.06

RT = retention time, LRI[a] = linear retention index determined on a SH-RXi-5 ms fused silica column relative to a series of n-alkanes (C_8-C_{40}); LRI[b] = linear retention index reported in the literature; Relative % is given as mean ± SD, $n = 3$

15.81% ($p < 0.001$) compared to the control (12.01 g/L). This production was even higher than that observed for 20% *C. leylandii* hydrosol. These results can be attributed to the effect of the main components of *C. leylandii* hydrosol, namely terpinen-4-ol and oplopanonyl acetate, which, at 10% concentration, did not reveal toxicity to the bacteria. Instead, it activated the bacteria's protective mechanism against adverse conditions, improving exopolysaccharide production. These findings highlight the potential of *C. leylandii* hydrosol, which exhibited the lowest antimicrobial capacity among the studied hydrosols but induced sufficient stress to the microorganism, offering an alternative use for this by-product.

Adding *A. citrodora*, *M. officinalis*, and *E. globulus* hydrosols (at both concentrations, 10 or 20% (*v/v*)) reduced biosynthesis levels compared to the control, which is not an ideal or attractive outcome from an economic point of view. Overall, it was observed that increasing the hydrosol concentration from 10 to 20% resulted in an XG production decrease of 30–50%, compared to the control. In conclusion, hydrosol bioactive compounds influence *Xanthomonas campestris* growth and XG production. Increasing their concentration increases toxicity, creating an adverse environment due to their antimicrobial properties, thus decreasing XG production [26, 34, 35]. From an industrial perspective, the observed concentration-dependent effects highlight the importance of carefully balancing microbial strain selection, stress induction, process parameters (pH, temperature, stirring, aeration and dissolved oxygen), and nutrient medium composition to improve microbial performance and biopolymer production [36]. Given that hydrosols are complex mixtures, conducting assays to evaluate the antimicrobial activity of hydrosols on *Xanthomonas campestris* and further characterising the resulting biopolymer are important steps to ensure biosafety and industrial-grade product consistency. Moreover, this approach offers economic and environmental advantages, positioning EO by-products as a promising resource for sustainable biopolymer production.

Aligned with this work, several studies have also explored cheap by-product alternatives for sustainable XG production. Regarding cost-effective carbon sources, studies carried out by Li and collaborators [15] have investigated XG production from an EO industrial by-product, the solid residue of *Melaleuca alternifolia.* This lignocellulosic biomass underwent acid hydrolysis, yielding 5.0 g/L XG after 48 h at 30 °C and 200 rpm, with 44.2% sugar consumption, demonstrating potential for large-scale use. Other studies using low-cost substrates reported yields of 4.98 g/L (crude glycerol) [37], 11.73 g/L (kitchen waste) [38], 8.90 g/L (broomcorn stem) [39], 7.10 g/L (acid-treated tapioca pulp) [40], and 5.23 g/L (sugarcane molasses) [41]. Kang et al. [42] found that low concentrations of furfural,

Table 2 Phenolic compounds composition of *A. citrodora* hydrosol and wastewater obtained by steam-distillation (phenolic compound quantification = µg/mL)

Peak	Rt (min)	λmax (nm)	[M-H] m/z	MS^2 (m/z)	Identification	*A. citrodora* hydrosol	*A. citrodora* wastewater
1	5.53	311	389	371(100),345(22),209(18),179(7)	Theveside	nd	0.7 ± 0.01
2	6.71	327	487	323(100),251(35),179(15)	Cistanoside isomer I	nd	1.24 ± 0.03
3	7.58	327	487	323(100),251(62),179(10)	Cistanoside isomer II	nd	0.75 ± 0.02
4	7.88	328	487	323(100),251(52),179(15)	Cistanoside isomer III	nd	0.664 ± 0.005
5	8.26	321	769	675(46),519(100),425(6),331(3)	Allysonoside isomer I	0.98 ± 0.01	nd
6	9.48	327	755	593(100),575(21),623(16)0.461(8)	Forsythoside B	0.47 ± 0.01	1.35 ± 0.02
7	10.22	323	535	441(100),347(21)	Hydroxypionresinol-*O*-glucoside	nd	nd
8	10.66	321	769	675(46),519(100),425(6),331(3)	Allysonoside isomer II	0.1841 ± 0.0004	nd
9	10.95	321	785	691(100),597(6),521(10),425(7)	Echinacoside isomer I	0.188 ± 0.001	nd
10	11.42	321	769	675(32),519(100),425(12),331(5)	Allysonoside isomer III	0.25 ± 0.002	nd
11	11.62	330	639	621(100),461(21),459(70),179(11)	β-hydroxy-verbascoside	0.213 ± 0.002	3.9 ± 0.1
12	12.06	330	639	621(100),461(16),459(70),179(8)	β-hydroxy-isoverbascoside	0.1952 ± 0.0001	3.9 ± 0.1
13	13.17	347	637	351(100),285(68)	Luteolin-7-diglucuronide	nd	**39.7 ± 0.8**
14	14.49	327	639	621(100),461(63),441(33),245(31),179(13)	Plantamajoside	nd	4.4 ± 0.1
15	14.76	327	639	621(100),459(100),441(7),323(19),251(25), 179(12)	Isoplantamajoside	0.542 ± 0.002	5.3 ± 0.1
16	15.19	324	639	621(100),459(68),323(15),245(22),179(9)	Forsythoside A	0.18 ± 0.001	1.6 ± 0.03
17	16.06	335	621	351(100),269(54)	Apigenin-7-diglucuronide	4.3337 ± 0.001	13.1 ± 0.2
18	16.71	330	623	461(100)	Verbascoside	0.185 ± 0.0004	5.5 ± 0.2
19	16.88	330	623	461(100)	Isoverbascoside	nd	2.29 ± 0.03
20	18.11	330	651	351(100),193(65),175(16)	Martynoside	0.1869 ± 0.0003	11.5 ± 0.2
21	18.96	330	651	351(100)	Isomartynoside	2.04 ± 0.04	0.813 ± 0.004
22	19.37	327	623	461(100),315(64)	Forsythoside H	nd	13.8 ± 0.1
23	21.68	321	769	675(32),519(100),425(12),331(5)	Allysonoside isomer IV	0.443 ± 0.001	nd
24	22.54	327	653	621(100),459(80)0.323(31),287(26),179(15)	Campneoside I	0.298 ± 0.004	0.72 ± 0.01
25	24.91	327	635	351(100),193(68),175(11)	Acacetin-7-diglucuronide	4.466 ± 0.002	5.372 ± 0.001
26	25.12	327	637	461(100),315(88)	Plantainoside C	nd	0.925 ± 0.004
27	26.77	321	769	675(32),519(100),425(12),331(5)	Allysonoside isomer V	0.54 ± 0.001	nd
28	29.77	321	785	691(100),597(6),521(10),425(7)	Echinacoside isomer II	0.2733 ± 0.0002	nd
29	31.94	331	677	659(100),547(3),451(26),225(18)	4-*O*-methylhypolaetin-7-*O*-acetyl-allosyl-O-acetylglucoside	4.3732 ± 0.0004	nd
30	33.61	277	363	295(5),269(100),193(3)	Diterpene derivative I	nd	nq
31	34.13	277	363	295(5),269(100),193(3)	Diterpene derivative II	nd	nq
32	35.28	294	499	363(100)	Diterpene derivative III	nd	nq
					Total Phenylpropanoid	7.2 ± 0.1	59.4 ± 1
					Total Flavonoids	6.41 ± 0.04	58 ± 1
					Total Phenolic compounds	20.5 ± 0.1	120 ± 2

Rt = retention time; λmax = maximum absorption wavelengths; MS^2 = mass spectral data; calibration curves used: verbascoside (y = 124,233x − 18,873, R2 = 1; LOD = 0.70 µg/mL and LOQ = 2.13 µg/mL) and apigenin 7-O-glucoside (y = 10683x − 45794; R2 = 0.999; LOD = 0.10 µg/mL; LOQ = 0.53 µg/mL); nd = not detected.; nq = not quantified: compounds could not be quantified due to the absence of similar standards available

a by-product of the acid-catalysed dehydration of lignocellulosic biomass (as noted in most studies above) maintained XG production (13 g/L) and enhanced cell proliferation. In a different approach, Crugeira and co-workers [19] reported promising results in XG production using moist olive pomace (15%) as a substrate supplement, achieving 21.64 g/L, a 50.91% increase over the control (14.34 g/L), attributed to the presence of phenolic compounds inducing bacterial stress (28 °C, 250 rpm, 76 h). Faced with the cited works, the yields reached in this study can be considered relevant, outperforming similar substrates and biosynthesis times. Adding *C. leylandii* hydrosol enhanced XG production by inducing bacterial stress, including when compared to other EO industrial by-products, requiring only pH correction, potentiating it as cost-effective for industrial applications.

As previously discussed, XG biosynthesis is influenced by various factors, beginning in the early growth phase and continuing beyond cell proliferation. During fermentation,

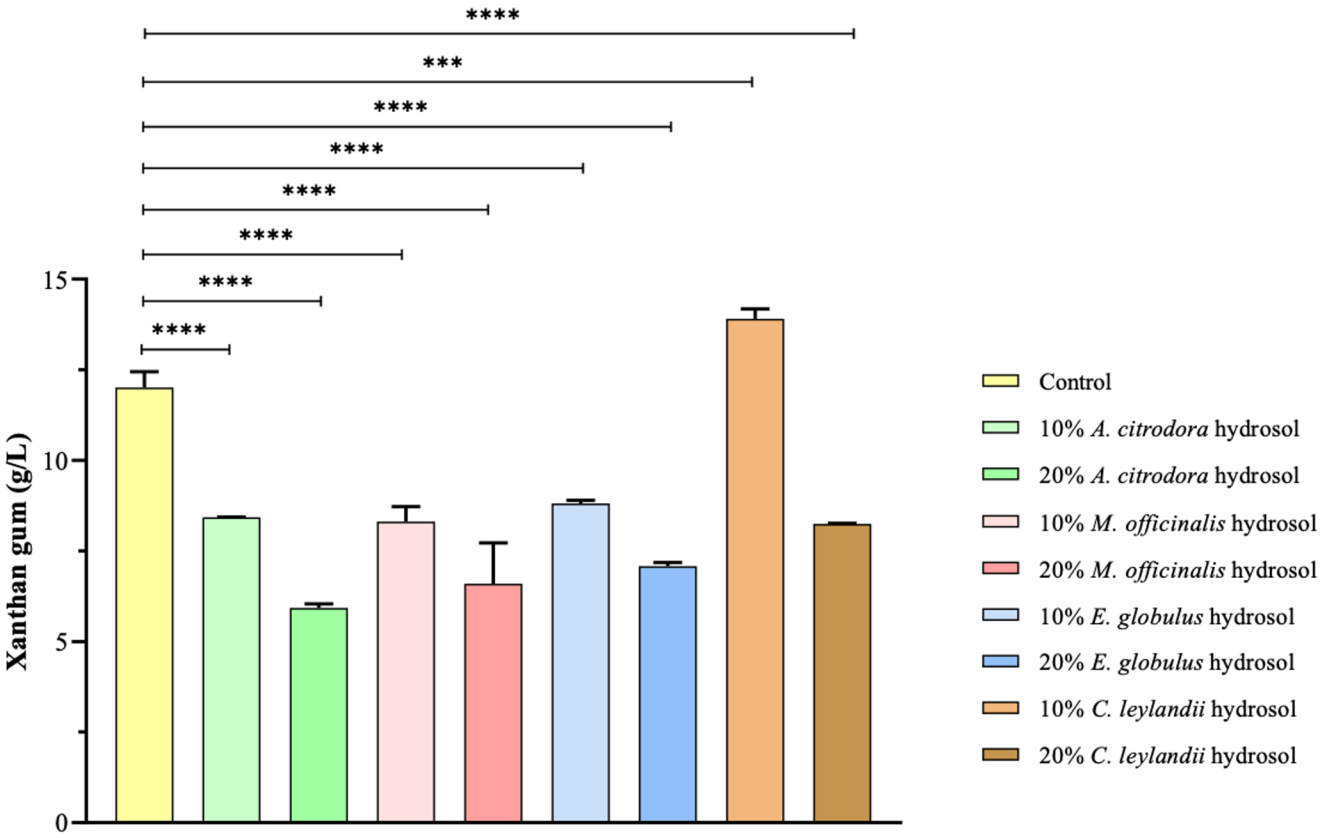


Fig. 3 Xanthan gum biosynthesis using 10% and 20% (*v/v*) of each studied hydrosol as supplement to the production medium. ****$p<0.0001$, ***$p<0.001$

XG secretion significantly increases the viscosity of the culture broth, which complicates immediate cell quenching and downstream separation processes [43, 44]. To address this challenge, a solvent in which XG is insoluble, such as ethanol, was employed, facilitating both the precipitation and separation of the polymer, thereby indicating successful XG formation. Although several *Xanthomonas* species, including *X. malvacearum*, *X. phaseoli*, and *X. carotae*, are known to produce XG, *Xanthomonas campestris* is consistently highlighted as the most efficient one. Its superior performance is attributed to its ability to metabolize a wide variety of carbon sources and to produce XG with desirable rheological properties, making it well-suited for cost-effective production using low-cost substrates [45]. This is further supported by numerous studies reporting xanthan gum biosynthesis using the same bacterial strain employed in the present study, *X. campestris* ATCC 33913 [9, 19, 46, 47].

Given that the structure and functional group composition of XG can vary depending on the microbial strain and substrate used, characterisation of the produced biopolymer is essential to confirm its identity and assess its properties. To this end, the most promising sample (XG_MP), produced with the addition of 10% (*v/v*) *C. leylandii* hydrosol, was compared to a control sample produced without hydrosol (XG_CT) and a commercial xanthan gum sample (XG_CM). Fourier-transform infrared spectroscopy (FTIR) and thermogravimetric analysis (TGA) confirmed the presence of characteristic functional groups and thermal stability consistent with xanthan gum, thereby validating its formation under the tested conditions. Concerning FTIR (Fig. 4), comparable absorption peaks among samples (XG_CT, XG_MP, and XG_CM) indicated that their chemical structures were similar. All the samples presented the bands at 3250–3350 cm^{-1} characteristic of the O-H stretching vibrations, the peaks in the range 2800–2950 cm^{-1} related to the absorption of symmetrical and asymmetrical C-H stretching (CH_2 and CH_3 groups) [48, 49], the signal at 1700–1750 cm^{-1} corresponding to C=O stretching attributed to the esterified residues of the symmetric stretching of the pyruvate group and the asymmetric stretching of the carboxylate group in glucuronic acid [50]. The 1525–1650 cm^{-1} vibration corresponds to axial deformation of the enol C=O, driven by the asymmetric stretching of the acetonate carbonyl group. The vibration in the range 1420–1430 cm^{-1} is attributed to the C-H bending, with a -CH_2 bending mode around 1250 cm^{-1}. The 1000–1150 cm^{-1} vibration is associated with acetal groups, involving the stretching of the C-C and C-O bonds [48, 51, 52]. The presence of this band

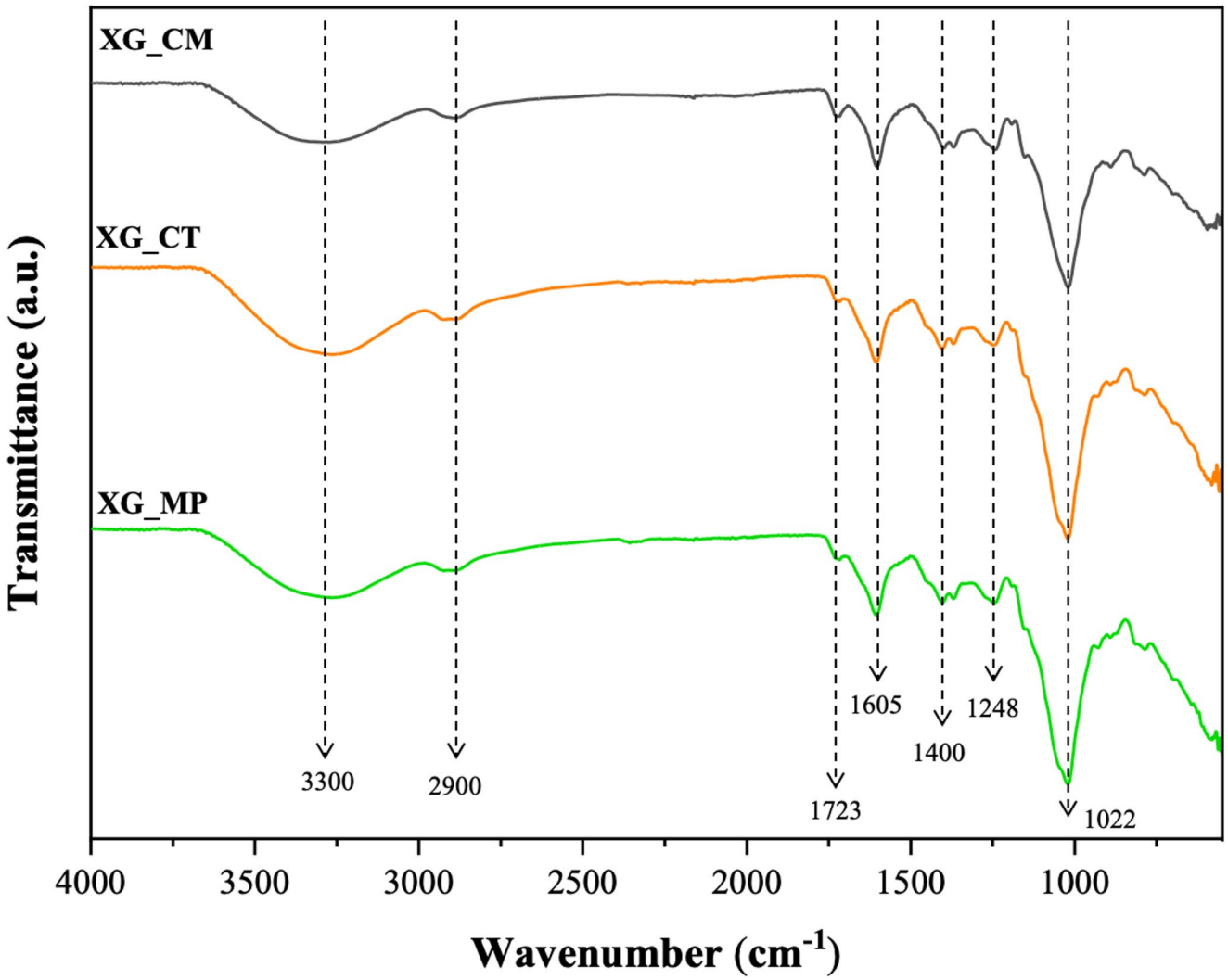


Fig. 4 FTIR spectra of the commercial xanthan gum (XG_CM), produced xanthan gum control (XG_CT) and the most promising produced xanthan gum (XG_MP, produced with 10% (*v/v*) of *C. leylandii* hydrosol as a supplement)

can be associated with a higher solubility of xanthan gum, attributed to acetal groups, which is beneficial for industrial uses in food, pharmaceuticals, and cosmetics [51].

The TGA and derivative thermogravimetry (DTG) curves for the XG_CT, XG_MP, and XG_CM samples are presented in Fig. 5. The produced biopolymers (XG_CT, XG_MP) decompose in four thermal events, presenting only slight differences from the commercial (XG_CM), which presented only two thermal events. The initial mass loss event found for all samples corresponds to the polysaccharide's dehydration (27–177 °C), associated with the easy water absorption of XG [18, 52]. The second mass loss, attributed to polysaccharide backbone degradation (side and main chains), varied between samples. For commercial XG (XG_CM), it occurred in one stage at 179–457 °C (61.08% mass loss). For the produced XG (XG_CT, XG_MP), this loss is divided into two (157–234 °C (9% mass loss; side chains) and 234–377 °C (40% mass loss; main chain). These differences are likely attributable to variations in pyruvate and acetate content or differences in polymer conformation, as suggested by [18]. Similarly, Cancella et al. [53] reported a comparable DTG profile for xanthan gum produced by *Xanthomonas campestris* using dairy by-products, attributing the observed thermal behaviour differences to residual substrate retention and structural variations arising from the production and purification processes. T_{max}, the temperature at which the maximum mass loss rate occurs, was observed at 273.0 (XG_CM) and increased to 282.4 (XG_CT) and 281.5 °C (XG_MP), indicating a higher thermal resistance for the synthesised XGs. In addition, the produced xanthan gums showed a fourth mass loss of around 379–537 °C (circa 9%), which could be related to a highly resistant polysaccharide fraction [18, 19]. The residual masses were 26.3, 32.0 and 30.0, respectively, for XG_CM, XG_CT, and

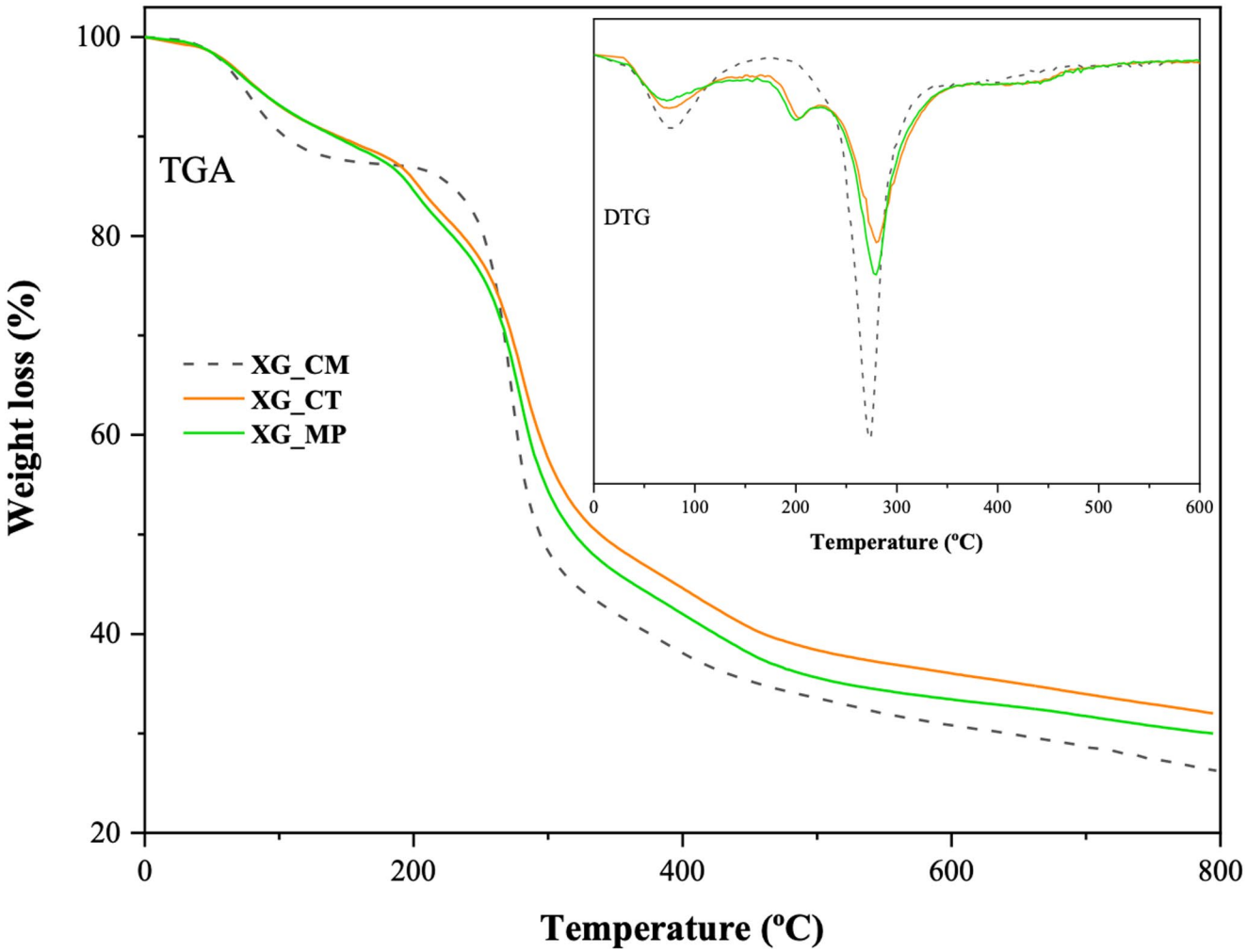


Fig. 5 Thermal degradation mechanism: weight loss and DTG curve of commercial xanthan gum (XG_CM), produced xanthan gum control (XG_CT) and the most promising produced xanthan gum (XG_MP, produced with 10% (*v/v*) *C. leylandii* hydrosol as a supplement)

XG_MP. Summarising, the two synthesised XGs exhibited similar degradation profiles, indicating that the hydrosol did not interfere with the produced XG structure. The observed differences from the commercial are likely due to variations in the synthetic route (type of bacteria used, fermentation conditions, presence of additives), leading to biopolymers with different molecular structures, e.g., polysaccharide chain conformation, side groups (like pyruvate and acetate), or the overall polymer structure.

Biobased Antimicrobial Systems

Antioxidant Capacity

Regarding antioxidant activity (Fig. 6), free radical scavenging capacity increased with the rising concentration of *A. citrodora* wastewater. The XG_50:50 solution presented 90.2% scavenging capacity, resulting in the most promising system, followed by the XG_70:30 with 67.7%. The system containing only the hydrosol (XG_100:0) presented a lower scavenging potential (18.3%). All the biobased systems presented significant differences ($p<0.0001$) compared to the control C-XG_MP. This antioxidant potential is related to the high phenolic content in *A. citrodora* wastewater, as can be perceived by the results shown in Table 3. This might be associated with the main compound in *A. citrodora* wastewater (luteolin-7-diglucuronide), which is reported to have strong antioxidant potential [54, 55]. The control systems (C-XG_CM, C-XG_CT, and C-XG_MP) showed expected antioxidant capacity, which can be associated with XG and be dependent on its source and structure, namely the presence of hydroxyl, ester, and carboxylic groups, as well as pyruvate [56]. The C-XG_MP system presented a higher scavenging capacity (28.3%) in comparison to C-XG_CT (17.4%) and C-XG_CM (10.3%) (both $p<0.0001$), which may be related to the anchoring of some *C. leylandii* hydrosol bioactive component during XG biosynthesis. This characteristic highlights another positive impact of adding

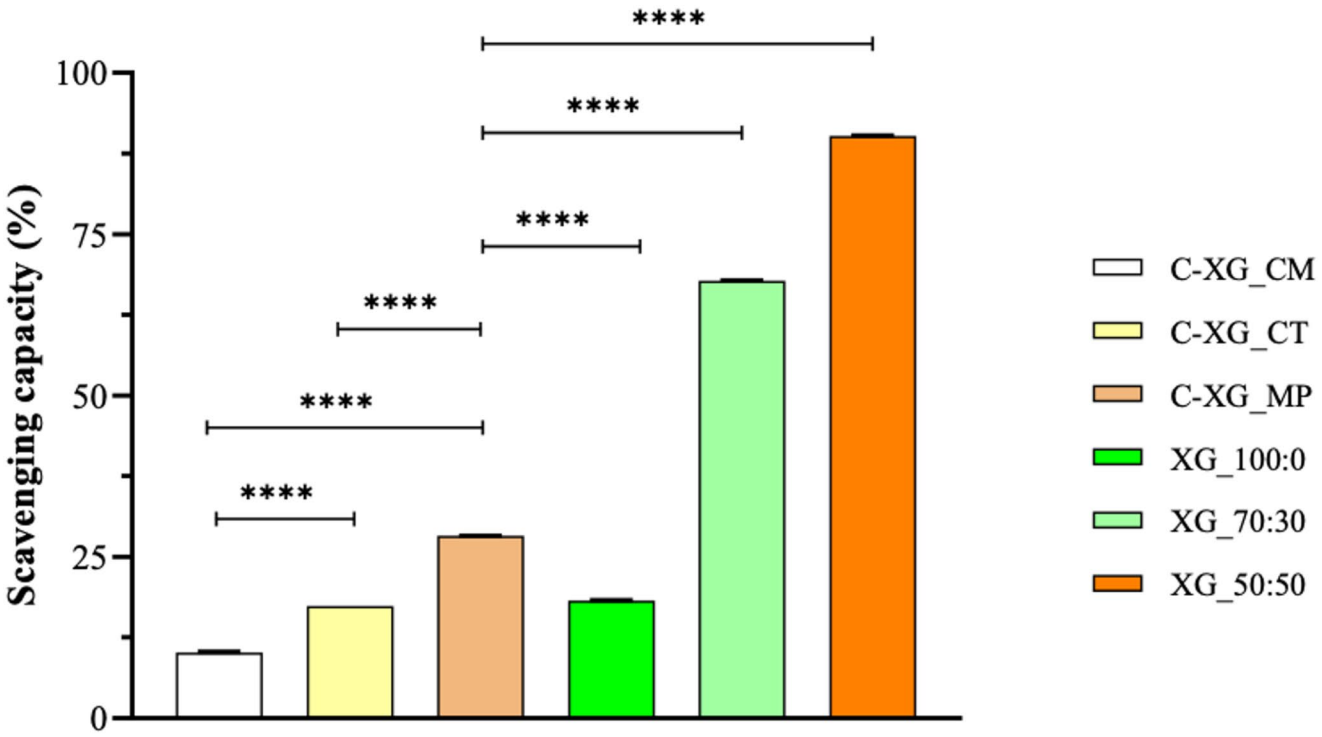


Fig. 6 Scavenging effect of XG control and biobased systems on $ABTS^{\cdot+}$ free radicals. ****$p<0.0001$

Table 3 Total phenolic and flavonoid content in each developed biobased system

Biobased system Codification	Phenolic content	Flavonoid content
	mg gallic acid equivalent/ g solution	mg catechin equivalent/g solution
XG_70:30	24.953 ± 0.009	16.549 ± 0.002
XG_50:50	35.877 ± 0.004	25.513 ± 0.004
XG_100:0	8.709 ± 0.006	5.438 ± 0.001
C-XG_CT	6.674 ± 0.001	0.415 ± 0.002
C-XG_MP	7.763 ± 0.001	2.089 ± 0.002
C-XG_CM	2.176 ± 0.001	2.249 ± 0.004

hydrosol as a medium supplement, i.e., producing an antioxidant XG.

Antimicrobial Activity

The antimicrobial assay to evaluate the effectiveness of the biobased systems on *S. aureus* is represented in Figs. 7 and 8. Microbial reduction was observed over time in clean and dirty conditions. After 45 min of contact, the XG_50:50 system led to the highest antimicrobial activity, presenting a reduction of 48.3% ($p<0.0001$) in clean conditions (Fig. 7) and 40.6% ($p<0.0001$) in dirty conditions (Fig. 8) for *S. aureus* culture, compared to the control (without biobased systems addition, *S. aureus* control). The solution containing only hydrosol (XG_100:0) presented circa half of this reduction, demonstrating the advantage of combining the two bioactive residues. This was also corroborated by the results of the XG control solutions (C-XG_CT, C-XG_MP and C-XG_CM), which presented the lowest microbial reductions (<20%) compared to a control (without biobased systems addition, *S. aureus* control). According to the literature, the major components identified in *A. citrodora* hydrosol, neral and geranial (citral isomers), also found in their respective EOs, are linked with antimicrobial properties [57, 58]. Dai and collaborators [59] studied the influence of citral concentration on the antibacterial efficacy against *S. aureus*. Their findings revealed a significant cell membrane disruption at a concentration of 0.5 mg/mL, resulting in the leakage of intracellular macromolecules and rapid cell death. The citral mode of action operates similarly to other aldehydes, which can crosslink amino groups to cell walls and cytoplasm. When it reaches significant quantities in the cytoplasm, citral can cause coagulation and precipitation of cytoplasmic components [60]. Gkalpinos and co-workers [61] reported the minimal inhibition concentration of *A. citrodora* aqueous extract (rich in phenolic compounds) of 6.25 mg/dry matter/mL against *S. aureus*. Another study [62] attributed the antibacterial effect against *S. aureus*, using a natural extract to luteolin-7-diglururonide (the main phenolic compound identified in *A. citrodora* wastewater). Plant phenolic compounds have been shown to have many mechanisms of action against bacteria, with the most prevalent being membrane disruption and permeability. Hydroxyl

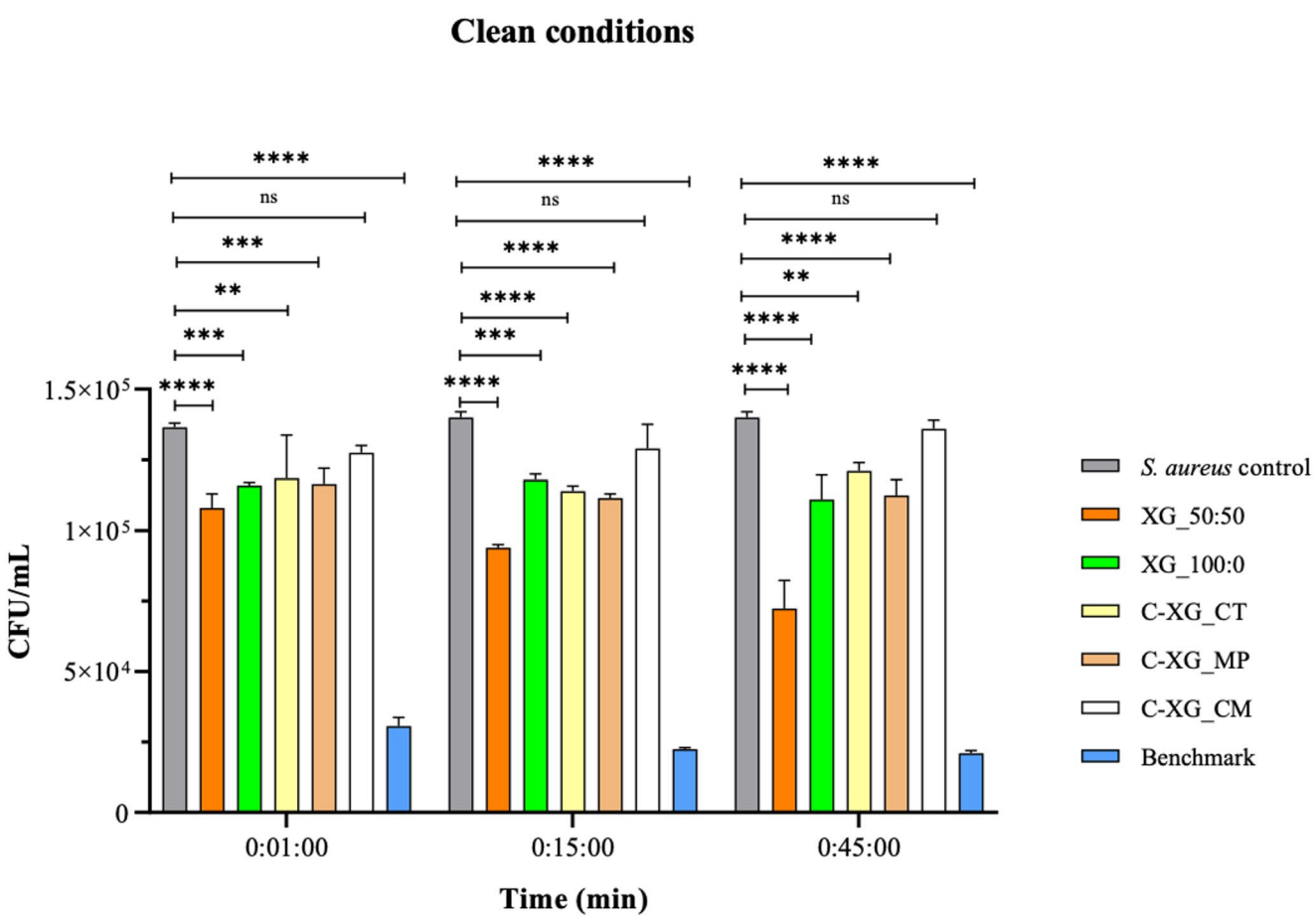


Fig. 7 Efficacy of the XG control and biobased antimicrobial systems on planktonic *S. aureus* culture assessed by quantitative suspension test with clean conditions (0.03% w/v organic matter concentration). ****$p<0.0001$; ***$p<0.001$; **$p<0.01$; ns means not significant

groups in the phenolic structure increase the interaction by hydrogen bonding with the microbial cells [63].

Statistical analysis revealed significant differences in the antimicrobial activity between clean and dirty conditions, particularly for 1 and 15 min. The XG_50:50 solution exhibited higher reduction potential under clean conditions, demonstrating enhanced antimicrobial activity at both 1 min ($p<0.001$) and 15 min ($p<0.05$) compared to dirty conditions. However, at 45 min, no significant differences were observed between clean and dirty conditions. The used commercial benchmark product presented the highest microbial reduction from the initial contact time of 1 min onwards, showing consistent effectiveness regardless of the level of organic matter. After 45 min, it achieved a reduction of 84.6% ($p<0.0001$) in clean conditions and 86.9% ($p<0.0001$) in dirty conditions, compared to the control (*S. aureus* control). This product comprises naturally derived cleaning ingredients, including natural oils, non-anionic and cationic surfactants, and benzalkonium chloride, an active ingredient commonly found in commercial disinfectants. Cationic surfactants have a strong affinity for microbial surfaces because of their opposite charge, allowing the penetration of cell walls [64]. The benzalkonium chloride mode of action involves the disturbance and disruption of the membrane bilayers by the alkyl chains in its composition and the alteration of the membrane's charge distribution [65]. This combination of ingredients likely contributes to its potent antimicrobial properties.

Sanitiser efficacy is assessed by its ability to achieve a logarithmic reduction of 5 logs against a target microorganism within 1 to 60-minute intervals (EN 1276, 2009) [31]. However, in this work, all logarithmic reductions were less than 1 log, with even the benchmark product also failing to meet the specific reduction requirement. As a result, neither the biobased systems nor the benchmark could be considered effective sanitising agents. Despite this, the developed prototype (XG_50:50), composed entirely of natural compounds without synthetic additives like the benchmark sample, showed promising bacteriostatic results. Consequently, this biobased system was selected to evaluate the anti-biofilm efficacy against *S. aureus* on stainless steel surfaces.

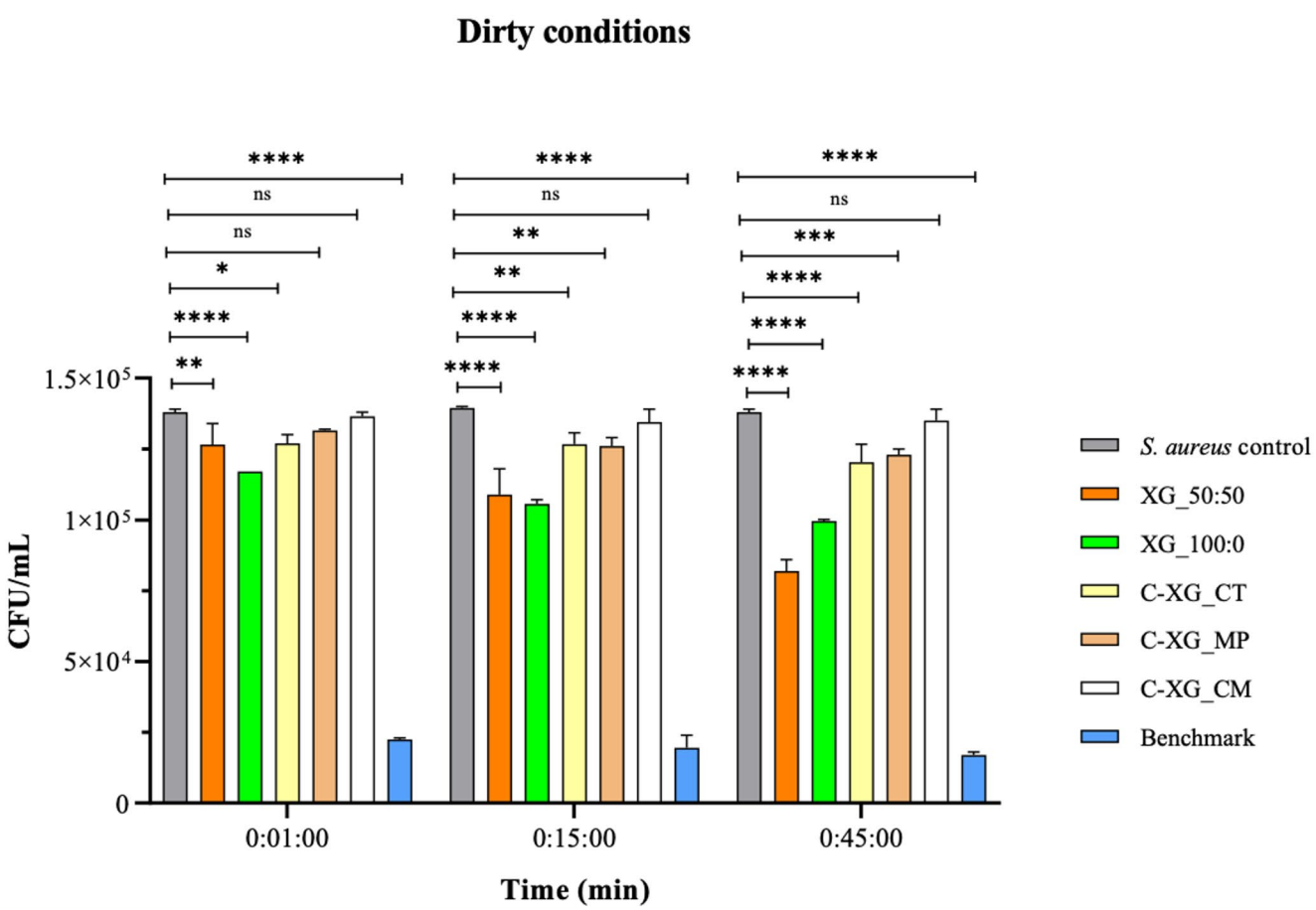


Fig. 8 Efficacy of the XG control and biobased antimicrobial systems on planktonic *S. aureus* culture assessed by quantitative suspension test with dirty conditions (0.3% w/v organic matter concentration). ****$p<0.0001$; ***$p<0.001$; **$p<0.01$; *$p<0.05$; ns means not significant

Anti-biofilm Efficacy

The *S. aureus* formed a biofilm on stainless steel surfaces after 24 h, with viable colony counts of 7.5 log CFU/mL (6.3 CFU/cm^2), comparable to reported values in the literature of 8.2 log CFU/cm^2 [66] and 7.6 log CFU/cm^2 [67]. The susceptibility of the *S. aureus* biofilms to biobased antimicrobial systems is represented in Fig. 9. Unlike the antimicrobial efficacy test on planktonic cells, there is no established threshold logarithmic reduction for biofilms. According to the protocol established by [68], a decrease of 3 logs indicates antimicrobial activity.

One of the key properties of biofilms is their strong resistance to antimicrobial agents, enduring concentrations up to 100–1000 times higher than for planktonic cells. Previous research has shown that *S. aureus* biofilms are more tolerant to antibiotics and disinfectants than stationary-phase planktonic cells, likely due to the higher presence of persistent cells [69, 70]. This was corroborated by the results for 45-minute treatments, where the antimicrobial capacity of the XG_50:50 was lower for the biofilm than for planktonic cells, 35.9% reduction ($p<0.001$) compared to the control (without biobased antimicrobial prototype treatment). By doubling the contact time (90-minute treatment), a reduction of 56.8% ($p<0.0001$) compared to the control. The sanitising controls presented no significant differences in biofilm reduction. Gao and collaborators [71] reported that citral reduced the biomass and cell viability of 24-hour dual-species (*S. aureus* and *Candida albicans*) biofilms. Citral disrupted the biofilm matrix by targeting nucleic acids, proteins, and carbohydrates and suppressed genes involved in quorum sensing, peptidoglycan, and fatty acid biosynthesis in *S. aureus*. Many studies presented evidence suggesting that plant extracts and phenolic compounds can disrupt bacterial membranes, hindering biofilm formation, decrease exoproteome secretion, and disperse the cell wall's biosynthetic machinery [72]. When combined with citral, these mechanisms may explain the anti-biofilm potential of phenolic compounds in wastewater, leading to enhanced effectiveness.

The benchmark sample reduced 3 logs ($p<0.0001$) after both treatment times, which agrees with the 99.9% reduction described by the manufacturer. When comparing the effects of the active ingredient (benzalkonium chloride) on

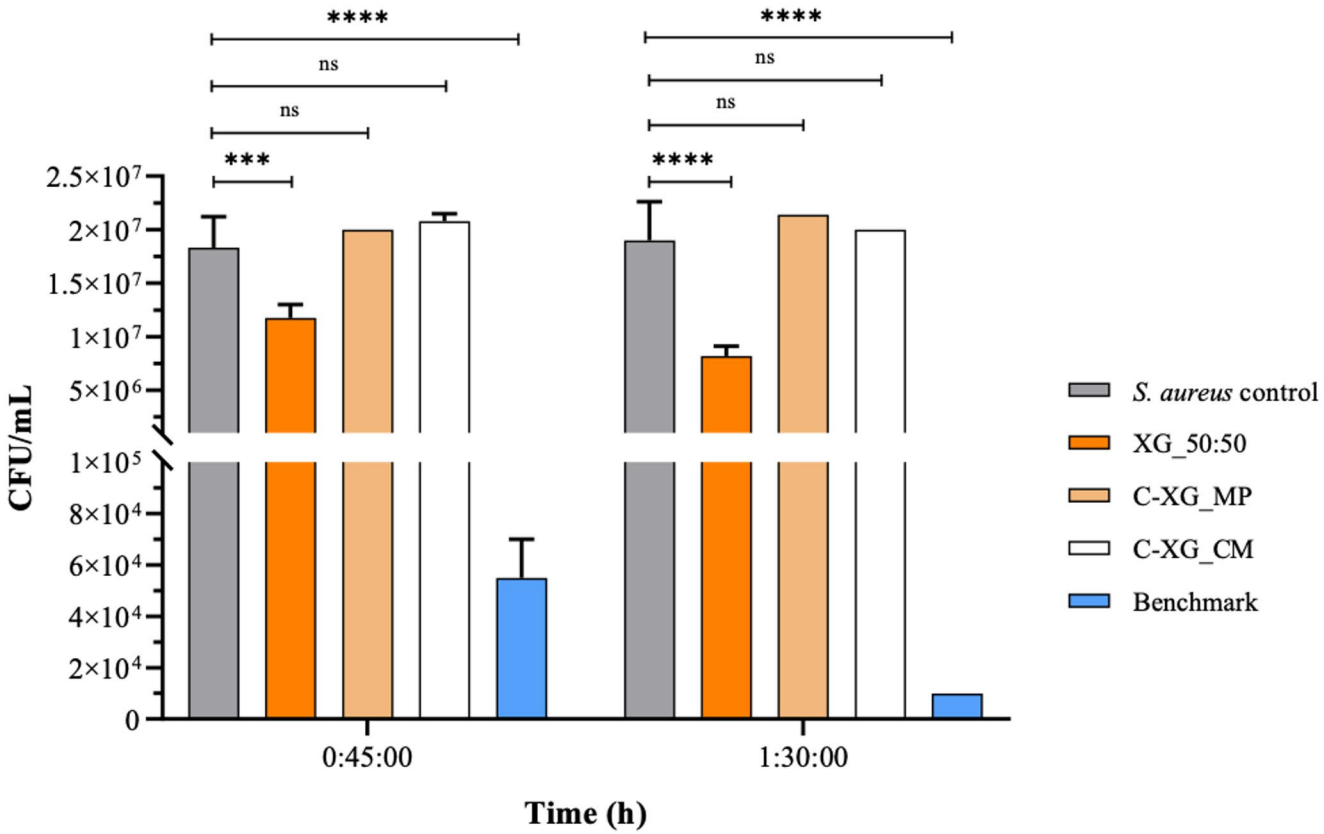


Fig. 9 Efficacy of the XG control and biobased antimicrobial systems on *S. aureus* biofilm developed for 24 h on stainless steel coupons at 37 °C. ****$p < 0.0001$; ***$p < 0.001$; ns means not significant

planktonic cells and biofilms, a more significant reduction was observed for the biofilm. This unusual behaviour is likely due to the absence of interfering components, such as the used organic matter (bovine serum albumin) added to the previous quantitative suspension tests. External factors can impact the antibacterial activity of sanitising agents, as chemical or ionic interactions between organic materials and antimicrobial treatments may reduce their effectiveness [73].

Conclusions

In summary, the biosynthesis of XG in the presence of hydrosols, aimed at reducing the costs associated with biopolymer industrial production, was successfully achieved. However, scaling up to industrial production may present challenges, including process optimization, cost management, and maintaining consistent quality. The produced XG exhibited the characteristic functional groups and higher thermal resistance, which can be due to differences in polymer molecular structure compared to its commercial counterpart. The biobased systems developed using the produced XG, along with a mixture of *A. citrodora* hydrosol and wastewater, presented effective results regarding their antioxidant and antimicrobial potential. The combination of EO by-products increased both biological activities due to their rich bioactive composition. These findings also support further research on the systems containing only wastewater in their composition, as the inclusion of this component significantly improved the results. Overall, the findings presented in this work demonstrate the promising properties of a sustainable system that closes the productive cycle of EO production, supporting waste reduction and a circular economy. In addition, future life cycle assessments could help quantify the environmental benefits of using EO by-products These bio-based systems, either independently or in combination with other natural polymers, have the potential to serve as natural antimicrobial agents for further applications in the food, cosmetics, and pharmaceutical industries.

Acknowledgements Financial support through national funds FCT/MCTES (PIDDAC) to CIMO UIDB/00690/2020 (https://doi.org/10.54499/UIDB/00690/2020) and UIDP/00690/2020 (https://doi.org/10.54499/UIDP/00690/2020), and SusTEC LA/P/0007/2021 (https://doi.org/10.54499/LA/P/0007/2020), LSRE-LCM UIDB/50020/2020 (https://doi.org/10.54499/UIDB/50020/2020) and UIDP/00690/2020 (https://doi.org/10.54499/UIDP/50020/2020), ALiCE LA/P/0045/2020 (https://doi.org/10.54499/LA/P/0045/2020); and national funding by FCT, P.I., through the institutional scientific employment program

contract for L. Barros (CEEC-INST, https://doi.org/10.54499/CEECINST/00107/2021/CP2793/CT0002). Pedro Crugeira thanks Ole aChain (NORTE-06-3559-FSE14 000188) for his research contract. National funding by FCT, through the institutional program contract for scientific employment of A. Santamaria-Echart, and the PhD grant of Heloísa Helena Scorsato de Almeida (https://doi.org/10.54499/SFRH/BD/148124/2019). Deifil Technology Lda (www.deifil.pt) for suppl ying the studied plants.

Author Contributions HHSA: Conceptualization, Methodology, Investigation, Writing – original draft preparation. PJLC: Methodology, Writing – review and editing. ASE: Methodology, Writing – review and editing. JSA: Methodology, Writing – review and editing. TCF: Investigation, Writing – review and editing. LB: Supervision, Writing – review and editing. AER: Supervision, Writing – review and editing. MFB: Conceptualization, Supervision, Resources, Writing – review and editing.

Funding Open access funding provided by FCT|FCCN (b-on).

Data Availability Not applicable.

Declarations

Competing Interest The authors declare no conflict of interest.

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