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Exploiting agri-food residues for kombucha tea and bacterial cellulose production

Francesca Sabatini [a,b,1], Emanuela Maresca [c,1], Martina Aulitto [c], Veronica Termopoli [a,b], Arianna De Risi [b,c], Monica Correggia [c], Gabriella Fiorentino [c], Viviana Consonni [a], Fabio Gosetti [a,b], Marco Orlandi [a,b,*], Heiko Lange [a,b,d], Patrizia Contursi [b,c,**]

[a] University of Milano-Bicocca, Department of Earth and Environmental Sciences, Piazza della Scienza 1, 20126 Milan, Italy
[b] NBFC – National Biodiversity Future Center, 90133 Palermo, Italy
[c] University of Naples Federico II, Department of Biology, Naples, Italy
[d] Luleå University of Technology, Department of Civil, Environmental and Natural Resources Engineering, Luleå, Sweden

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ABSTRACT

Bio-valorization of agri-food wastes lies in their possible conversion into fermented foodstuffs/beverages and/or biodegradable polymers such as bacterial cellulose.

In this study, three different kombucha cultures were formulated using agri-food waste materials, citrus fruit residues and used coffee grounds, as alternative carbon and nitrogen sources, respectively. Over 21 days of fermentation, the kinetic profile was followed by monitoring cell density, pH variation, minerals, trace elements and production of bacterial cellulose. Moreover, the total phenolic and radical scavenging capacity was measured by spectrophotometric tests on the beverage and bacterial cellulose. Several classes of compounds were detected by gas chromatography coupled with mass spectrometry performing extractions on the headspace above fresh kombucha beverages and their lyophilized fractions, using solid phase micro extraction and liquid phase extraction, respectively. The obtained results allowed assessing molecular profiles of each kombucha beverages. A chemometric meta-analysis of the data revealed the individual impacts of the single ingredients and the effects of the fermentation process.

1. Introduction

Fruit and vegetable processing industries generate several million tons/year of valuable by-products [1] in form of agricultural and food residues (AFRs). Transitioning involves implementation of green valorization strategies for AFRs, repurposing them into high-quality foods that enhance human health and reduce food waste and losses [2–4].

Fermentation is a microorganism-based process that breaks down complex organic compounds/matrices to obtain energy through an anaerobic metabolism [5,6]. Interestingly, the degradative activity of the microbial hydrolytic repertoire releases not only peptides, monomeric amino acids, and monosaccharides sustaining microorganism growth, but also bioactive compounds present in the complex matrix of agrifood residues [7,8], *i.e.*, organic acids, exopolysaccharides, bioactive peptides, and phenolic compounds, often enhancing the flavor, and the safety [9,10]. One of the most ancient fermented beverages is kombucha, obtained by a symbiotic culture of bacteria and yeast (SCOBY) [11]. The community metabolism is based on yeast cells that hydrolyze sucrose into glucose and fructose, which are then metabolized in several organic acids and other substances [11]. During fermentation, glucose and fructose are also utilized to produce bacterial cellulose (BC), forming a thin film on the surface of fermented tea preparations. Fermentation parameters do not require sophisticated equipment, nor control of aerobiosis/anaerobiosis or temperature restrictions [11].

Kombucha derived from sugar tea is renowned for its health-promoting properties due to several organic compounds, including those with antioxidant and anti-microbial activities. Some of these molecules are also entangled in the film of bacterial cellulose [11]. Notably, such natural composite films on cellulose basis can be exploited, for example, in active food packaging applications and to produce

* Correspondence to: M. Orlandi, University of Milano-Bicocca, Department of Earth and Environmental Sciences, Piazza della Scienza 1, 20126 Milan, Italy.
** Correspondence to: P. Contursi, University of Naples Federico II, Department of Biology, Naples, Italy.
E-mail addresses: marco.orlandi@unimib.it (M. Orlandi), contursi@unina.it (P. Contursi).
[1] These authors contributed equally.

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fortified BC such as impregnation with active compounds [12]. While previous research on the use of BC for food packaging has focused on introducing functionalities based on the abundance of reactive groups within the BC structure [13], the non-covalent incorporation of bioactive molecules during cellulose film production has been relatively underexplored.

Interestingly, the use of alternative carbon and nitrogen sources that are naturally rich in antioxidant/antibacterial molecules can expand even further the repertoire of the metabolites produced upon kombucha fermentation. Some studies have explored the use of alternative raw materials, whereas only a few have investigated the potential of AFR as carbon and nitrogen sources for kombucha growth [14–16]. Huge amounts, *i.e.*, 10 millions of tons of citrus fruit processing wastes are annually generated worldwide, posing a significant ecological challenge [17]. Around 40% of citrus fruits cultivated worldwide are utilized by the juice processing industries, thus being a major source of citrus fruit waste (CFW) [13]. Likewise, large amounts of spent coffee grounds are produced every year (6–8 million tons/y, respectively) from domestic consumption, representing an ideal substrate for kombucha fermentation process as they contain approximately 24% lipids, 13% carbohydrates, and 11% proteins (m/m) [13]. Furthermore, a significant amount of bioactive molecules and their precursors remain in spent coffee grounds and pods since only a portion of these compounds are extracted during coffee preparation. Besides, the method of coffee preparation, *i.e.*, espresso *vs.* filter coffee, also influences the remaining main bioactive constituents [18,19]. On the other hand, the squeeze process that separates the liquid from the solid matrix of citrus for juice production utilizes only 34% of the total fruit composition, generating a significant portion of wastes made up of kernels, peels, pulp and pomaces [20]. Similarly to the edible parts, the non-edible components of CFW, including by-products or residues, contain significant quantities of potentially bioactive molecules like phytonutrients like flavonoids, phenolic acids, anthocyanins, carotenoids, vitamins such as A, C, and E, fatty acids, essential oils, and minerals. CFW has proven effective as carbon substrate in culture media for microbial fermentation [20] with various probiotics including *Lactobacillus*, *Limosilactobacillus*, *Lactiplantibacillus*, *Pediococcus* genera, yielding fermented animal feed of superior quality and/or precursors of biotechnologically relevant macromolecules such as lactic acidic, succinic acid, *etc.* [20].

Nevertheless, existing methods for valorizing CFW through fermentation often require physio-chemical pretreatments with notable environmental impact. For instance, steam explosion and/or extraction with organic solvents, have been used to remove antimicrobial compounds such as limonene as well as to increase the solubilization and the degradability of the complex CFW [20]. Recently, more attention is paid to the characterization of different types of kombucha preparations, with the aim of linking beneficial properties to molecular composition. Most of the studies are focused on the evaluation of total phenolic content, variation of the pH value before and after fermentation processes, antioxidant capacity, cytotoxicity, and enzymatic inhibition potential. Several of those studies provide an evaluation of the aroma by olfactory detection [21–24], the determination of ethanol content [25,26], and compositional profiling of volatile and/or more polar fractions of kombucha preparations by HS-SPME-GC–MS [21,22,24,27–30], GC–MS [26,31–34] and high performance liquid chromatography coupled with diode array detector and mass spectrometer (HPLC-DAD-MS) [23,30,34–36], often supported by chemometrics. The majority of the papers analyzed flavored commercial products [24,25] or in-house formulations prepared with black tea and sugar [24,27,30,31,34], in some cases with the addition/substitution of further vegetal and fruit species, *e.g.*, *Echium amoenum*, *Cichorium intybus* root, *Arbutus unedo* fruit, *Hibiscus rosa-sinensis,* turmeric or green tea leaves, in different ratios with traditional starting compounds [23,28,29,33,35,37,38]. In few cases formulations prepared with residual raw materials of possible nitrogen sources such as green and sweet potato tea leave, fermented tea broth [32,36,39] and carbon ones like banana peels or pineapple peels and cores [26,40] were analyzed. These existing studies relevant as background to this work are comparatively listed in Table 1.

To the best of our knowledge, the features and composition of kombucha beverages obtained with both waste starting materials have not yet been investigated.

In this study, we have integrated microbiological, biochemical and analytical chemistry techniques to unveil the differential chemical composition of kombucha beverages citrus waste and infusions of spent coffee ground and tea, as carbon and nitrogen sources, respectively, without any physico-chemical pre-treatment of the waste biomasses apart from shredding. As spent coffee ground and citrus fruit residues contain several antioxidants, the total phenolic and radical scavenging capacity was measured and traced back to the identification of volatile and volatizable compounds acquired by GC–MS performing head space solid phase micro (HS-SPME) and liquid phase (LPE) extractions.

2. Material and methods

2.1. Chemicals

The solvents and reagents used for HS-SPME-GC–MS and GC–MS procedure were dichloromethane (DCM; 99% purity, GC–MS grade, Merck KGaA, Germany), methanol (MeOH; LC-MS grade, Carlo Erba, Italy), ethyl acetate (AcOEt; HPLC grade, Carlo Erba, Italy), magnesium sulfate ($MgSO_4$; purity ≥97%, Merck KGaA, Germany) and sodium chloride (NaCl; ACS grade, Merck KGaA, Germany). For the GC–MS silanization reaction *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA; purity ≥97%, Merck KGaA, Germany) and pyridine (Py; 99.8% purity, Merck KGaA, Germany) were used. Cyclodecane (purity ≥90%, Merck KGaA, Germany) in AcOEt was used as internal standard for GC–MS procedures, while 1,2-dichlorobenzene (purity 99%, Merck KGaA, Germany) in methanol for the HS-SPME-GC–MS analysis. Ultra-pure milliQ water and sulfuric acid (95% Merck KGaA, Germany) were employed in the preparation steps.

2.2. Waste materials used for kombucha culture preparation

The citrus fruit (a mix of seeds, pulp, pith residues and peels) waste (CFWs), by-product of fruit juice manufacturing, was provided by Ortogel SpA (Sicily, Italy). Spent coffee ground (Caffè Borbone®) was taken from spent coffee pods, black tea was obtained from a local supermarket (Conad®) as well as sucrose.

Table 1
Recent works detailing non-commercial kombucha preparations using diverse ingredients other than black tea and sugar, compared to this work.

Entry	Carbon source	Nitrogen source	Additional ingredients	Ref
1	Sucrose	Black tea	Chicory	[23]
2	Sucrose	Black tea	Pineapple peel and core	[26]
3	Sucrose	–	Strawberry tree	[29]
4	Sucrose	Black tea waste	–	[32]
5	Sucrose	–	Turmeric rhizomes	[33]
6	Sucrose	–	Iranian Borage	[35]
7	Sucrose	–	Sweet potato leaves	[36]
8	Sucrose	Green tea	–	[37]
9	Sucrose	Fermented tea	Pineapple	[39]
10	Sucrose	–	Banana peel	[40]
11	Sucrose	–	Nettles	[40]
12	Sucrose	Black tea	–	[40]
13	Citrus fruit waste	Spent coffee or black tea	–	*THIS WORK*

2.3. Fermentation of kombucha cultures under standard and alternative conditions

Kombucha starter culture, *i.e.*, inoculum, was obtained by Prof. Donato Giovannelli from the Department of Biology, University of Naples, Italy. Standard growth conditions are based on commercial black tea (7 g/L) supplemented with sucrose (80 g/L). Briefly, the sugar dissolved in Ultra-pure milliQ water was autoclaved at 121 °C for 20 min and the tea was infused at 80 °C for 30 min. After cooling at room temperature, the growth medium was used to inoculate kombucha starter culture in a 1:5 ratio at 30 °C under static conditions. Then, the glass vessel was covered with a sterilized cloth to avoid contamination and fastened properly. For the replacement of the standard nitrogen and carbon sources, after 14 days the kombucha culture was collected by centrifugation at 2600 *x g* for 10 min at 4 °C and each pellet (*i.e.*, 30 mL) resuspended in the following different media (300 mL): (a) infusion of sucrose (80 g/L) and tea (7 g/L) as positive control; (b) infusion of citrus residues (13 g/L) and black tea (7 g/L); (c) infusion of sucrose (80 g/L) and spent coffee pods (22 g/L); (d) infusion of citrus fruit residues (13 g/L) and spent coffee pods (22 g/L).

Dried spent coffee pods and citrus residues were autoclaved before assembling them in the final medium. The pH of the solution was adjusted at pH 5.0 ± 0.1 with sulfuric acid (95 %) to even the acidity level among the different media. The methodological procedure is represented in Fig. 1.

The kombucha culture was monitored for three weeks and samples were collected at 7, 14, 21 days, by using the sterile Miracloth filters (Merck Millipore, Darmstadt, Germany) for pH and cell density analysis (see Section 2.4) and the remaining volume was freeze-dried for further studies (see Sections 2.8–2.9). The experiment was carried out in biological and technical replicates, the reported values are presented as mean values ± SD ($n = 3$). The resulting kombucha cultures analyzed in this study are listed in Table 2.

2.4. Determination of pH and cell density

For the evaluation of the physiological variation occurring during the growth on different nitrogen and carbon sources, cell density and pH values were monitored at different timepoints, *i.e.*, 7, 14, and 21 days. The cell density was measured by Bruker chambers and DM750 optical microscope PH 2 40× (Leica Microsystems, Wetzlar, Germany) for counting. Measurements were repeated at least ten times for each sample. The pH was monitored in triplicate using a digital pH-meter

Table 2
List of kombucha samples with relative controls used in this study.

Sample name	Carbon source	Nitrogen source	Kombucha culture
KST	Sucrose	Black tea	Yes
ST	Sucrose	Black tea	No
KCsT	Citrus	Black tea	Yes
CsT	Citrus	Black tea	No
KSC	Sucrose	Coffee pods	Yes
SC	Sucrose	Coffee pods	No
KCsC	Citrus	Coffee pods	Yes
CsC	Citrus	Coffee pods	No

S = sugar, Cs = citrus, T = tea, C = coffee, K = kombucha culture.

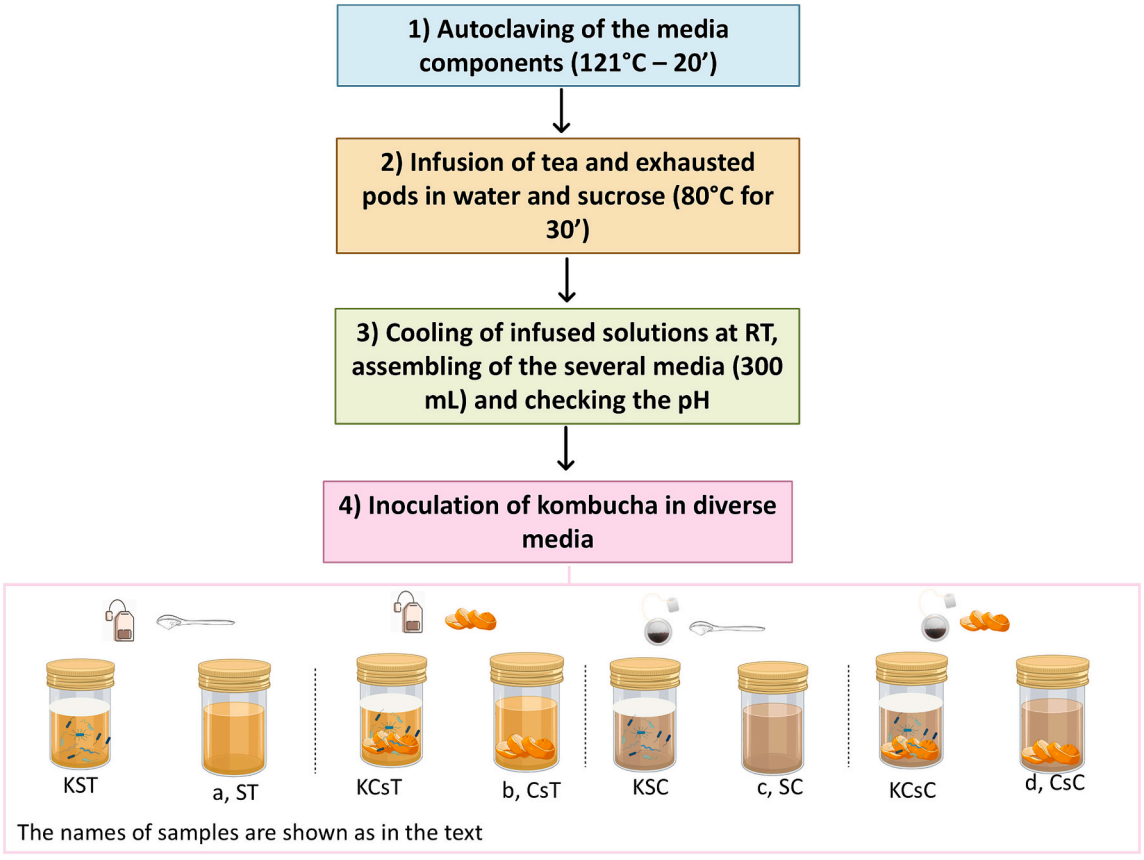

Fig. 1. Methodological workflow for preparing kombucha cultures under standard and alternative growth conditions. The different steps are shown: 1) autoclaving of the media components; 2) infusion of tea and exhausted coffee pods both in milliQ water and sucrose; 3) assembling of the final media; 4) inoculation of cells from kombucha in the followed media *i.e.* sucrose + infusion tea (**KST**), citrus waste + infusion tea (**KCsT**), citrus waste + infusion of coffee (**KCsC**), sucrose + infusion of coffee (**KSC**). The media used as negative control are named as follows: sucrose + infusion tea (**a, ST**), citrus waste + infusion of tea (**b, CsT),** citrus waste + infusion of coffee (**c, CsC**), sucrose + infusion of coffee **(d, SC).**

(Thermo Fisher Scientific, Waltham, MA, United States).

2.5. Determination of BC morphology by scanning electron microscopy (SEM)

The BC produced after 21 days was collected, dried through freeze-drying (Thermo Savant Modulyo Benchtop Freeze Dryer System, Savant Instruments Inc., Holbrook, NY, United States) and its weight was expressed as g/L of cultures. The morphology of the cellulose samples was investigated by Scanning Electron Microscope (SEM) using a FEI Quanta 200 FEG SEM (FEI, Eindhoven, The Netherlands). Before the SEM observations, the cellulose samples were sputter coated with a 10 nm-thick Au—Pd layer. All samples were observed at 10 kV acceleration voltage using a secondary electron detector.

2.6. Total phenol content (TPC) and antioxidants assays

KST, **KCsT**, **KSC** and **KCsC** samples collected at 21 days were assayed for total phenol content and antioxidant activity, after dissolving freeze-dried samples in water (10 mg/mL). Total phenol content (TPC) was determined with the Folin–Ciocalteu method by Kaashyap *et al.* adapted in 96-well microtiter plates (final volume 320 μL) [20]. An amount of 32 μg was mixed with 160 μL of 0.2 N Folin–Ciocalteau reagent for 5 min. Subsequently, 128 μL of 0.7 M sodium carbonate was added. The mixture was incubated at room temperature for 2 h. Absorbance at $\lambda = 760$ nm was measured against a water blank by UV–Vis spectrophotometer (BioTek Synergy HTX Multi-Mode Microplate Reader, Agilent Technologies Inc., Santa Clara, CA, United States). The TPC was assessed using gallic acid as a calibration standard (0–3.2 ppm) and results were expressed as the gallic acid equivalents (GAE) per liter of kombucha culture.

The radical scavenging activity of **KST**, **KCsT**, **KSC** and **KCsC** samples was determined against 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical content, following the method done by Chu and Chen [41]. An amount of 25 μg of kombucha supernatant was mixed with 225 μL of 200 μm DPPH solution in 96-well microtiter plates and incubated in the dark at RT for 30 min. Absorbance at $\lambda = 517$ nm was measured using UV–Vis spectrophotometer (BioTek Synergy HTX Multi-Mode Microplate Reader, Agilent Technologies Inc., Santa Clara, CA, United States). Methanol was used as a blank solution, while DPPH without kombucha culture was used as a control. The antioxidant activity of kombucha cultures was assessed by comparing the samples to standards of ascorbic acid (0 to 2.5 ppm) and expressed as micrograms of ascorbic acid equivalent (AAE) per mg/g infused.

The antioxidant activity released from the BC matrix was tested through the DPPH assay according to Bayer *et al.* with slight modifications [41]. The cellulose biofilms were first washed in sterilized MilliQ water (500 mL) under shaking in the dark for 16 h. The antioxidant test was performed on both washed and unwashed celluloses as control after freeze drying them. The lyophilized biofilms (10 mg) were resuspended in 10 mL of 200 μM DPPH solution. The vials were stored in dark and ambient conditions under gentle constant stirring for 30 min and the absorbance of the solution (250 μL) was measured using BioTek Synergy HTX Multi-Mode Microplate Reader, Agilent Technologies Inc., Santa Clara, CA, United States. DPPH radical scavenging activity was calculated as the following formula: DPPH scavenging activity $= [(A_{blank} - A_{sample})/A_{blank}] \times 100$ %, where A_{blank} is the absorbance of DPPH solution, A_{sample} is the absorbance of samples mixed with DPPH solution. Each analysis was carried out in triplicate.

2.7. Inductively coupled plasma mass spectroscopy (ICP-MS)

The concentration of metals was determined by an Inductively Coupled Plasma Mass Spectrometry (7900 ICP-MS) instrument (Agilent Technologies Inc., Santa Clara, CA, United States) by following Standard Operating Procedure published in Correggia *et al.* 2023 [42]. Briefly, all samples were filtered using filter 0.22 μm pore size and diluted with 1 % HNO_3, which was also used as blank. Subsequently, the prepared samples were introduced into the ICP-MS instrument. The methodical optimization of operational parameters, encompassing the argon gas flow rate, radiofrequency power, and ion lens configurations, was performed to ensure precision. Calibration curves were carried out using certified standards (Agilent Technologies Inc., Santa Clara, CA, United States). Data acquisition and analysis were carried out using MassHunter 4.6 software Version C.01.06.

2.8. Ion chromatography (IC)

The concentration of major cations and anions were measured using an Ion Chromatographic instrument (Eco IC, Metrohm, Herisau, Switzerland) equipped with a conductivity detector (Metrohm, Herisau, Switzerland) by following Standard Operating Procedure published in Correggia *et al.* 2024 [42]. Briefly, all samples were filtered using filter 0.22 μm pore size and diluted with type I water (18 MΩ/cm), which was also used as blank. Subsequently, the prepared samples were introduced into the IC instrument. Cations and anions were measured in two independent measurements, also anions separation was carried out in suppression mode. Calibration curves were carried out using certified CPA chem standards. Data acquisition and analysis were carried out using MagIC Net 3.3 software.

2.9. Head space-solid phase micro extraction (HS-SPME) coupled to GC–MS

All samples of the kombucha liquid fraction underwent analysis *via* HS-SPME-GC–MS to extract, analyze, and identify volatile compounds. The procedure was adapted on the basis of those reported in the literature [25,27]. A mixture of 8 mL of the sample, 200 μL of a standard solution of 1,2-dichlorobenzene at 50 ng/μL (Internal Standard, IS), and 1 g of NaCl were introduced into 20-mL headspace screw vials, equipped with polytetrafluorethylene-silicone septa. The solution was continuously stirred at 200 rpm with a magnetic bar and incubated for 10 min at 70 °C to establish temperature equilibrium. Subsequently, an SPME fiber (50/30 μm DVB/CAR/PDMS, 1 cm long; Supelco, Palo Alto, CA, United States) previously conditioned as recommended by the manufacturer, was inserted into the sample head space, and left to sample the volatile fraction for 10 min at same temperature of incubation. The fiber was then withdrawn from the vial and immediately introduced into the GC–MS injector for desorption step at 240 °C for 2 min. The analysis was performed with an Agilent 8860 N Gas Chromatograph coupled with 5977B single quadrupole mass spectrometer (Agilent Technologies Inc., Santa Clara, CA, United States). A DB-5 fused silica capillary column (stationary phase (5%-Phenyl)-methylpolysiloxane, 30 m × 0.25 mm i.d., 0.25 μm, J&W Columns, Agilent Technologies Inc., Santa Clara, CA, United States) was used for chromatographic separation. The injection volume was 1 μL, the injection port operated in splitless mode at 240 °C, and the purge flow to split vent was 7 mL/min at 2 min. The helium flow was kept constant at 1 mL/min (carrier gas, purity 99.995%) and the following chromatographic program was used: starting temperature 45 °C, then 10 °C/min to 60 °C, 15 °C/min to 200 °C, 20 °C/min to 230 °C, then hold for 6 min. The MS transfer line was kept at 280 °C while the ion source and quadrupole temperature were kept at 230 °C and 150 °C, respectively. The MS operated in electron ionization mode (EI) at 70 eV, in positive mode scanning in the range from 35 to 500 *m/z*.

2.10. Liquid phase extraction (LPE) coupled with GC–MS

All samples of the kombucha lyophilized fraction were subjected to GC–MS analysis. Starting from an amount of 10 mg of sample a combined procedure was performed to characterize separately the more apolar and more polar fraction. Thus, a first extraction with 750 μL of DCM:MeOH (2:1, v:v) in an ultrasonic bath (Super RK 512H Sonorex,

Bandelin electronic GmbH & Co. KG, Berlin, Germany) for 30 min was carried out. The supernatant (500 μL) was added with 5 μL of a standard solution of cyclodecane at 200 ng/μL (Internal Standard, IS) and injected in GC–MS, whereas the solid residue was subsequently extracted with 500 μL of acidic water (pH = 4) and 500 μL of AcOEt and vortexed for 5 min. The organic fraction was separated and $MgSO_4$ was added to remove residual water. The supernatant was collected (500 μL), added with 5 μL of IS, and subjected to silanizant derivatization using 15 μL of BSTFA and 15 μL of pyridine (RT for 30 min). Subsequently, the derivatized extract was injected into the GC–MS.

The same GC–MS instrument and data management of HS-SPME-GC–MS was adopted, and identical operative conditions were employed for both polar and less polar fractions. The injection volume was 1 μL, the injection port operated at 250 °C in splitless mode. The flow was kept constant at 1 mL/min (carrier gas He, purity 99.995%) and the following chromatographic program was used: initial temperature 50 °C for 1 min, 30 °C/min to 150 °C for 0.1 min, 20 °C/min to 250 °C for 3 min, 10 °C/min to 310 °C for 6 min. The interface temperature was kept at 280 °C while the MS transfer line, ion source and quadrupole temperature were kept at 310, 230 and 150 °C, respectively. The MS operated in electron ionization mode (EI) at 70 eV, in positive mode scanning in the range from 35 to 500 *m/z*.

Both LPE-GC–MS and HS-SPME-GC–MS raw data files were processed using a data analysis workflow through Agilent MassHunter Unknowns Analysis software version B.10.1 (Agilent Technologies Inc., Santa Clara, CA, United States). The data processing approach used for compound identification involved automatic peak detection, deconvolution and library searches. The NIST MS Search 2.4 (2020) mass spectral database was utilized for tentative identification of compounds. Only components that exhibited a match value >800 were included.

2.11. Statistical analysis

Statistical significance of difference between positive controls (**KST** and **KSC**) and the other microbiomes (Section 3.2) was determined by one-way ANOVA and Dunnett's multiple comparison test ($P < 0.05$).

MultiDimensional Scaling (MDS) and Maximally Regular Graph (MRG) are commonly used procedures for exploratory data analysis. MultiDimensional Scaling (MDS) projects samples in a low-dimensional space where the pairwise similarities are preserved with the aim to obtain a simple graphical visualization of the data structure. Each sample is represented by a point and the points are arranged in this space so that the distances between pairs of points have the maximal correlation to the similarities among the pairs of samples. Maximally Regular Graph (MRG) is a graph representation of the similarities between pairs of samples; it is generated through the optimization of the graph complexity by adding back to the original minimum spanning tree (MST), one by one, the missing connections previously skipped during the computation of the MST itself [43].

MDS and MRG were applied to disclose the mutual relationships among clusters of samples with similar chemical profiles. The 11 samples analyzed by GC–MS methods (Table 3) were described by 106-dimensional binary vectors, where 1 and 0 indicate the presence and the absence of a chemical compound, respectively. Pairwise sample similarity was quantified by Jaccard-Tanimoto coefficient [44], calculated from a contingency table as the following:

$$JT = \frac{a}{a+b+c} \quad (1)$$

where *a*, *b*, and *c* are frequency parameters related to the counts of common shared or absent chemicals; in particular, *a* is the number of common shared chemicals, while *b* and c count the chemicals that were detected in a sample but not in the other one.

The pairwise similarity matrix and MDS plot were computed by MATLAB software, using home-written scripts and appropriate packages. The software Pajek [45] was used to calculate the Maximally Regular Graph (MRG).

3. Results and discussion

3.1. Growth characteristics of kombucha cultures on different AFRs

The general purpose of this study is the valorization of agri-food residues (AFRs) that are abundant in Mediterranean countries through kombucha fermentation with special focus on the identification and characterization of bioactive and health-relevant molecules present in the tea beverages. Kombucha cultures are grown in sucrose-tea medium and complex metabolic interaction among bacteria and yeasts ensure the development of the full potential of carbon and nitrogen sources during fermentation. The standard nutrition components were replaced with untreated AFRs in form of citrus fruit waste (CFW) and spent coffee ground and combined them in different formulations as shown in Table 2. AFRs contain a significant fraction of bioactive compounds that can be exploited in food, pharmaceutical and cosmetic industries. Besides, they can be chemically modified upon microbial fermentation leading to the formation of derivatives that exhibit similar and/or additional bioactive profiles. Specifically, CFW is rich in vitamin C, folic acid, potassium, and pectin as well as health-promoting and therapeutic agents, especially with antioxidant but also with anti-inflammatory and anticancer properties [46,47]. Moreover, spent coffee grounds contain large amounts of organic compounds in form of fatty acids, amino acids, polyphenols, minerals and polysaccharides that support the growing interest in its valorization as potential source of bioactive molecules and/or functional ingredients for the food industry.

3.1.1. pH variation and cell density

The SCOBY is functional based on complex interactions among diverse microorganisms, each of them exhibiting a typical optimal pH-range that plays an important role in microbial growth. The initial pH-value of **KST**, **KCsT**, **KSC** and **KCsC** samples was set to 5.0 in agreement to the optimum growth pH of the main representatives of the consortium [48].

A drop of pH in kombucha beverages during the fermentation process has been generally observed across all samples, caused by the formation of various organic acids, such as oxalic, formic, acetic, lactic, succinic, malic, and citric acid, as reported in the literature [49]. Whereas a steep pH drop from 5.0 to 2.3 was observed for **KST** and **KSC** samples, only a slight pH variation from 5.0 to 4.0 occurred for **KCsC** and **KCsT** (Fig. 2A). This is probably due to the establishment of a citrate buffer system that maintains the pH in the observed range, made possible by the presence of the high amount of residual vitamin C in the citrus component. Citric acid-based buffer systems typically lie between 3.2 and 6.2 pH values, corresponding to range covered by the three acid functionalities present. Given the chemical mixture represented by the kombucha preparation (Table 2), it is expectable that the second deprotonation stage enters in equilibrium, with the produced carboxylic acids being less able to change the pH of the system. The difference in pH is noteworthy, and relevant for the fermentation effects.

In addition, the difference in terms of pH does not seem to affect the cell density among the different cultures as shown in Fig. 2B. Notably, citrus peels are rich in essential oils containing several antimicrobial compounds, such as limonene, pinolene, geraniol, γ-terpinene, neral, terpinene-4-ol, *etc.*, affecting micro-organisms growth and the fermentation performance. Terpenes, characteristic of citrus biomasses and identified in samples **KCsT**, **CsT**, **KCsC**, **CsC** by HS-SPME-GC-MS, are the following: D-limonene, linalool, terpinen-4-ol, α-terpineol and geraniol with the α-terpineol being the most abundant one. Both in the presence of coffee and tea as nitrogen sources, samples containing SCOBY highlight a decrease in the concentration of these terpenes by at least 50% with respect to control samples (Section 3.4) demonstrating that the community is able to deal with the presence of such antimicrobial

Table 3
List of the identified compounds in the GC–MS analysis performed on kombucha extracts, controls, and raw starting materials. The letters a, b, and c identify the DCM/MeOH, AcOEt, and HS-SPME extractions, respectively. The raw spent coffee grounds, tea, and citrus fruit waste are listed with the acronyms **C**, **T** and **Cs,** respectively. Compounds written in italics are those showing a potential antioxidant activity, while those reporting the term 'syn' in brackets are clearly synthetically derived compounds. Compounds identified (also) after derivatization of compounds *in situ* with *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) are indicated by (xTMS), where x indicates the number of TMS groups attached.

Code	Compounds / Class	**KST**	ST	**KCsT**	CsT	**KSC**	SC	**KCsC**	CsC	C	T	Cs
	Color code											
	Total number of identified compounds	35	31	56	48	39	24	60	54	13	14	30
	Alcohols											
1	1-Dodecanol	c	c									
2	2-Ethyl-1-hexanol	c	c			c						
3	2,5-Dimethyl-2,5-hexanediol		c			c	c	c	c			
4	Glycerin				a				a			
5	Perilla alcohol								c			
6	2-Phenylethanol	c		c		c		c				
7	Ethanol	c				c						
	Aliphatic & aromatic aldehydes											
8	Benzeneacetaldehyde		c				c				c	
9	3,5-di-*tert*-Butyl-4-hydroxybenzaldehyde (syn)		c			c	c					
10	4-Hydroxybenzaldehyde (TMS)											b
11	Benzaldehyde	c	c		c	c	c	c	c	c	c	
12	4-Methylbenzaldehyde	c	c	c	c	c	c	c	c			
13	Ethyl Vanillin							c				
14	*p*-Propylbenzaldehyde		c									
15	*Vanillin (TMS)*											b
	Aliphatic carboxylic acids											
16	Acetic acid			c				c				
17	*3-(4-Hydroxy-3-methoxyphenyl)propionic acid (2TMS)*							b	b			
18	3-Methylbutanoic acid (TMS)			b	b	b		b				
19	*4-Hydroxybenzeneacetic acid (2TMS)*			b	b			b	b			
20	*4-Hydroxyphenyllactic acid (3TMS)*			b				b	b			
21	Benzeneacetic acid (TMS)			b				b				
22	Glycolic acid (2TMS)			b								
23	Lactic Acid (2TMS)			b	b	b		b				
24	Pentanoic acid (TMS)							b	b			
	Alkaloids											
25	*Caffeine*	a,b,c	a,b,c	a,b,c	a,b,c	a,b,c	a,b,c	a,b,c	a,b,c	a,b,c	a,b,c	a,b,c
	Alkanes											
26	Hexadecane		a	a			a	a			a	a
27	Octadecane		a					a				
28	Tetradecane			a	a			a	a	a	a	
	Aromatic hydrocarbons											
29	5-*tert*-Butyl-*m*-cymene (syn)	c	c			c	c		c			
30	Methyl eugenol			c	c			c	c			
	Esters											
31	Ethyl phenylacetate					c						
32	Geranyl acetate											c
33	Isopropyl palmitate				c				c			c
34	Methyl linoleate									a	a	
35	Methyl stearate									a	a	a
36	Octanoic acid methyl ester					c						
37	Octyl caprylate					c		c				
	Fatty acids											
38	Adipic acid (2TMS)			b	b			b	b			
39	Azelaic acid (2TMS)			b	b			b	b			b
40	Decanoic acid	c	c		c	c						
41	Caproic acid	c				c		c				
42	Lauric acid (TMS)											b
43	*Linoleic acid (TMS)*	b						b	b			b
44	Myristic acid (TMS)	b		b	b	b		b	b			b
45	Nonanoic acid	c	c	c	c	c	c					
46	Octanoic acid	c	c		c	c						
47	*Oleic acid (TMS)*	b	b	b	b	b	b	b	b	b	b	b
48	Palmitic acid (TMS)	b	b	b	b	b		b	b	b	b	b
49	Stearic acid (TMS)	b		b	b	b		b	b	b	b	
	Ketones											
50	2-Hydroxy-2-cyclopenten-1-one	a	a			a	a					
51	4-Methyl-2-heptanone	c			c	c	c	c	c			
52	Acetophenone							c				
53	Mesityl methyl ketone	c					c					
	Phenols											
54	2,4-Di-*tert*-butyl-6-nitrophenol (syn)	c				c	c					
55	2,4-Di-*tert*-butylphenol (syn)	c	c	c	c	c	c	c	c	c		
56	*2-Methoxy-4-vinylphenol*			a				a	a	a		

(*continued on next page*)

Table 3 (*continued*)

Code	Compounds / Class	**KST**	ST	**KCsT**	CsT	**KSC**	SC	**KCsC**	CsC	C	T	Cs
57	4-Ethylresorcinol					c	c					
58	*Catechol* (TMS)			b				b	b			
59	Eugenol			c	c			c	c			
60	*Hydroquinone* (2TMS)			c				c	c			
61	4-Ethyl-phenol	c		c		c		c				
62	*4-Ethyl-2-methoxy-phenol*	c		a,b,c	b,c	a,c	b,c	c	c			
63	Thymol			c	c			c	c			
	Phenolic acids											
64	*3-Hydroxybenzoic acid (2TMS)*			b				b	b			b
65	*4-Hydroxybenzoic acid (2TMS)*			b	b	b		b	b	b	b	
66	Benzoic acid (TMS)			b	b			b	b			b
67	*Gallic acid (4TMS)*	a	a	b	b	b		b	b	b	b	b
68	*Protocatechoic acid (3TMS)*			b	b			b	b			b
69	*Syringic acid (2TMS)*			b		b						
70	*Vanillic acid (2TMS)*			b	b			b	b			b
71	*3,4-Dihydroxyhydrocinnamic acid (3TMS)*							b	b			
72	*4-Coumaric acid (2TMS)*				b			b	b			b
73	*Isoferulic acid (2TMS)*			b	b			b	b			b
74	*Sinapinic acid (2TMS)*											b
	Sugar derivatives											
75	5-Hydroxymaltol (2TMS)			b	b			b	b			
76	γ-Decalactone			c				c				
77	γ-Nonalactone			c								
78	2,4-Dihydroxy-2,5-dimethyl-3(2*H*)-furan-3-one	a	a			a	a					
79	2*H*-Pyran-2,6(3*H*)-dione	a				a						
80	3-Deoxy-D-glucuronic acid	a					a					
81	3-Deoxy-D-glucuronic acid lactone		a									
82	2,3-Dihydro-3,5-dihydroxy-6-methyl-4*H*-pyran-4-one	a	a			a	a					
83	5-Hydroxymethyl-2-furoic acid (2TMS)			b	b			b	b			
84	5-Hydroxymethylfurfural	b	b	b	a,b,c	b	b	b	a,b			a,b,c
85	2,3-Dihydrobenzofuran				a				a			
86	Furandimethanol (2TMS)			a,b	a			a	a			a,b
87	Furfural		c		c		c			c		
88	Furfuryl methyl ether		c				c					
89	Levoglucosan	a				a						
	Tannins											
90	*Catechin (5TMS)*			b								
91	*Epigallocatechin (6TMS)*			b								
	Terpenes											
92	Carveol			c	c			c	c			
93	Carvone			c	c			c	c			c
94	Citronellal			c	c				c			
95	Cubenol			c								
96	*D-Limonene*			a,c	a,c			a,c	a,c		c	a,c
97	2,6-Di-*tert*-butylquinone (syn)	c	c	c		c	c	c	c			
98	Fenchol				c				c			
99	Geraniol		c		c				c			a
100	Linalool	c	c	c	c			c	c		c	c
101	Nootkatone			c	c			c	c			c
102	*α-Terpineol*	b,c	b,c	b	b,c	b,c		b,c	b,c			b,c
103	β-Damascenone			c								
104	Terpinen-4-ol			c	c			c				
105	ß-Myrcene	c	c	c	c			c	c			
106	α-Pinene											c

compounds by degrading them during the fermentation [50].

3.1.2. BC production

BC production was monitored and expressed in g/L of culture produced at the end of the fermentation time. The formation of the BC, that often occurs in multiple layers and increases in thickness with prolonged fermentation time, peaks at 14–21 days of cultivation [50]. As expected, the higher production of BC was observed when sucrose was used as carbon source (**KST** and **KSC**, Fig. 2C). This is in agreement with the BC biosynthesis pathway that requires glucose monomers as substrate and in form of UDP-glucose to initiate the polymerization process [51]. Whilst **KCsC** and **KCsT** were grown on CFWs (1.3%) containing about 50% of complex polysaccharides, *i.e.*, cellulose and hemicellulose, [52] **KST** and **KSC** were cultivated in the presence of 8% of sucrose, which can be promptly converted into glucose and fructose by yeast invertase activity [52]. Therefore, the physiological growth as well as BC productions of **KCsC** and **KCsT** is strictly dependent on the degradative ability of the SCOBY culture to convert complex polysaccharides into simple sugars (Fig. 2B), thus accounting for the minor production of BC compared to **KST** and **KSC** cultures. Moreover, the decrease in BC production of **KSC** compared to the **KST** culture is caused by the replacement of the standard nitrogen source tea with coffee, which compared to the non-roasted tea leaves carries less readily available additional sugar sources. Nevertheless, the ultrastructure of BC produced when sucrose was replaced by the complex CFW carbon source (Fig. 3A) was comparable to that obtained under standard conditions (Fig. 3B). Both samples show a cellulose network constituted by well separated and randomly oriented cellulose fibrils, with diameters lower than 100 nm.

These results highlight the metabolic flexibility of SCOBY in exploiting alternative nutrient sources to sustain the fermentation process as well its physiological adaptability in tolerating not only antimicrobial compounds but also a quite wide range of pH values.

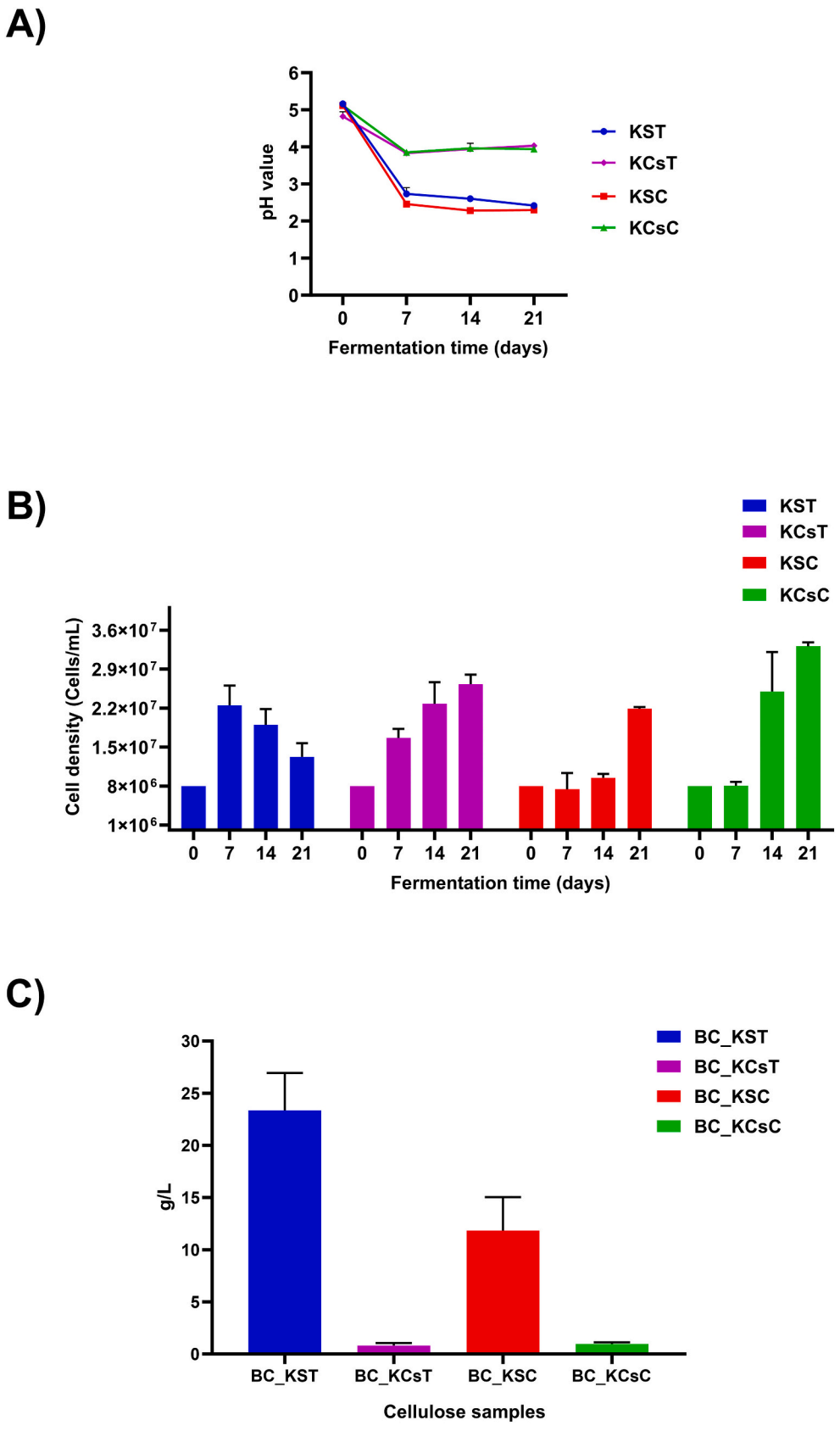

Fig. 2. Evaluation of growth parameters: **A)** pH measurements, **B)** cell density of Kombucha culture and **C)** cellulose production over 21 fermentation days.

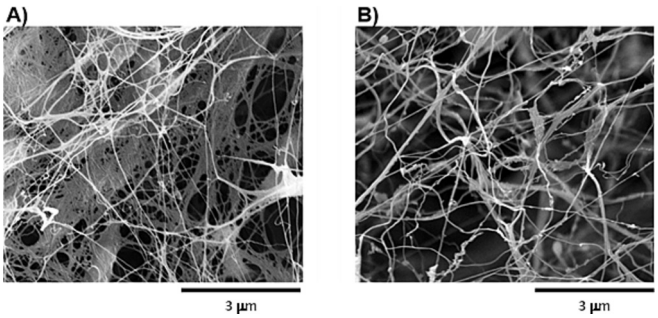

Fig. 3. SEM images of BC produced upon replacement of sucrose with citrus residues (A) and under standard condition (sucrose + infusion of tea) (B). Cellulose fibrils have a diameter lower than 100 nm.

3.2. Analysis of the total phenol and antioxidant content of kombucha

Naturally occurring antioxidants in food consist of phenols, mainly in form of tannins, lignans, and flavonoids and alkenes present in unsaturated fatty acids, carotenes, tocotrienes, *i.e.*, vitamin E subcomponents, and squalene, and in form of other multifunctional smaller organic compounds like citric acid, *i.e.*, vitamin C. Noteworthy, the antioxidant content in food products is affected by processing techniques, with industrially produced food being significantly less rich in these compounds due to the oxidative stress caused by the industrial preparations [48].

Fermentation is a rather gentle process, involving a series of enzymatic reactions that modify the chemical components of the substrate. Therefore, the nutritional value, the content of bioactive compounds as well as the functional properties of food can be improved by bioconversions, including the 'activation' of antioxidants using the enzymes produced by microorganisms. For instance, bacterial degradation of tannins, mainly *via* bacterial tannases [53,54], can overcome the well-known and widely applied inhibitory effects of tannins *versus* bacteria. SCOBY has, however, not yet been reported to contain gene sequences that code for tannase-like enzymes.

Other bacterial processes include the production of unsaturated fatty acids as uncontrolled secondary metabolites [55,56]. Also in this case, however, SCOBY has not been shown to exhibit this activity. It is hence more likely that the general conditions of the kombucha mixtures, especially the acidic milieu, favor a gradual hydrolysis and (subsequent) extraction of phenolics, present most likely, in the starting tea and CFW materials, in form of hydrolysable tannins and complex tannins (*i.e.*, in form of gallate esters, such as epigallocatechin gallate, a common and well known ingredient in tea) [48]. The same can be assumed for the unsaturated fatty acids and the phenolic acids detected.

The choice of AFR wastes in form of CFWs exploited in this study has been thus motivated by the exceptional abundance of antioxidants and their potential precursors in citrus fruit residues [48]. In order to highlight the contribution of this waste in enhancing the antioxidant power of the kombucha tea beverages, a commercial low-cost black tea and spent coffee ground were used as nitrogen source for medium preparation.

The presence of phenols and other organic antioxidants was analyzed through the Folin-Ciocalteau test for total phenol content (TPC) and the 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical-based antioxidant assay, respectively (Fig. 4A and B) over a 3-week time range, at regular time intervals (see Section 2.5 for the procedures). Since no relevant differences were observed among the samples over the time, only results relative to the end of fermentation time (21 days) are shown. Accordingly, most literature indicates that the production of antioxidants peaks after 7 days of fermentation and keeps constant up to 21 days [57,58].

The basal amount of TPC diverges significantly in diverse black tea/coffee sources [59]. In the applied experimental setting, the TPC value in samples grown in the absence of citrus, *i.e.*, **KST** and **KSC** samples, was quite low, with 16.65 ± 2.55 mgGAE/L and 1.86 ± 0.43 mgGAE/L, respectively (Fig. 4A), and close to the minimal values found in the literature for other black tea/coffee [60,61]. Conversely, when CFW was used as carbon source, the TPC content of **KCsT** and **KCsC** samples rose up to 62.91 ± 9.21 and 29.08 ± 3.31 mgGAE/L, respectively. These values are significantly higher, *i.e.*, 3.77 and 1.75-fold, than the control **ST** and **SC** cultures grown in the presence of sucrose. Furthermore, a synergistic effect derived from the infusion of fresh black tea leaves and citrus residues was observed in the **CsT** sample. Interestingly, the highest antioxidant activity was revealed in the **KCsT** sample as supported by DPPH assay, demonstrating the significant contribution of CFW, in raising the antioxidant power of the tea beverage (Fig. 4B).

When the antioxidant properties of the bacterial celluloses (BCs) produced under the aforementioned growth conditions were tested in the DPPH assay, all the BCs exhibited free-radical scavenging activity (Fig. 4C). Assuming that the cellulose itself exhibits very limiting reductive character by virtue of its aldehydic end-groups, this indicates that antioxidant molecules present in the kombucha preparation in the presence of SCOBY were incorporated and accumulated in the cellulose matrix. The only significant difference in antioxidant activity was observed between the **BC_KSC** (Fig. 4C) and **KSC** (Fig. 4B) samples, as only **BC_KSC** showed clear antioxidant activity (74 ± 2 %). Therefore, it is conceivable that antioxidant molecules are entangled within the BC matrix, and/or have been better protected against oxidative environment that could consume those antioxidants present in solution. Overall, these results demonstrate that polymers with antioxidant properties can be biosynthesized from agri-food wastes, paving the way for the production of active food packaging films.

3.3. Analysis of trace metals and inorganic ion content

So far, few studies have been addressed to assess the amount of minerals and trace elements in kombucha tea beverages [62,63]. Recommended intakes of nutrients vary by age and sex and are known as Recommended Dietary Allowances (RDAs) and Adequate Intakes (AIs) according to Food and Drug Administration. The amount of minerals in kombucha drinks is affected by the tea infusion, the preparation method and the initial mineral content of the water used to prepare the growth medium [60]. In this study, deionized water was utilized to highlight the contribution of the different carbon and nitrogen substrates in the increase/decrease of the basal concentration of ions (determined by IC) and trace elements (ICP-MS) upon kombucha fermentation. Results are reported in Fig. 5.

Noteworthy, the presence of CFW in **KCsT** modifies substantially the content of chloride, phosphate, potassium, magnesium, calcium, and iron, among others (see Fig. 5) compared to the **KST** sample [59], and contribute to their general impact on a healthy diet. Also iron and copper concentration are higher in kombucha preparations based on CFWs, *i.e.*, **KCsC** and **KCsT**. Zinc content shows an interesting trend, with the two samples just mentioned being less concentrated than the **KST** sample, and more concentrated than **KSC**. This, in light of the amounts observed for chromium, nickel and lead, points to an important aspect that comes into play using waste materials. Indeed, some industrial processing steps contact metal surfaces and metal-based coatings thus leaving traces in the products and the waste materials, with the latter especially expectable in case the waste materials were not meant for being further valorized in food preparations. Since, the sugar has been industrially produced and packed, and the same can be stated for the tea, it is not surprising that these products contain higher amounts of nickel and chromium [64,65].

Chloride, especially in combination with potassium, is commonly found in nuts and kernels [66,67], such as the latters coming into the kombucha preparation *via* the CFWs, and can be readily extracted in medium acidic aqueous solutions, such as the kombucha suspension under study itself. As expected, nitrite is found in higher concentrations in the samples containing the spent coffee ground, *i.e.*, a waste material

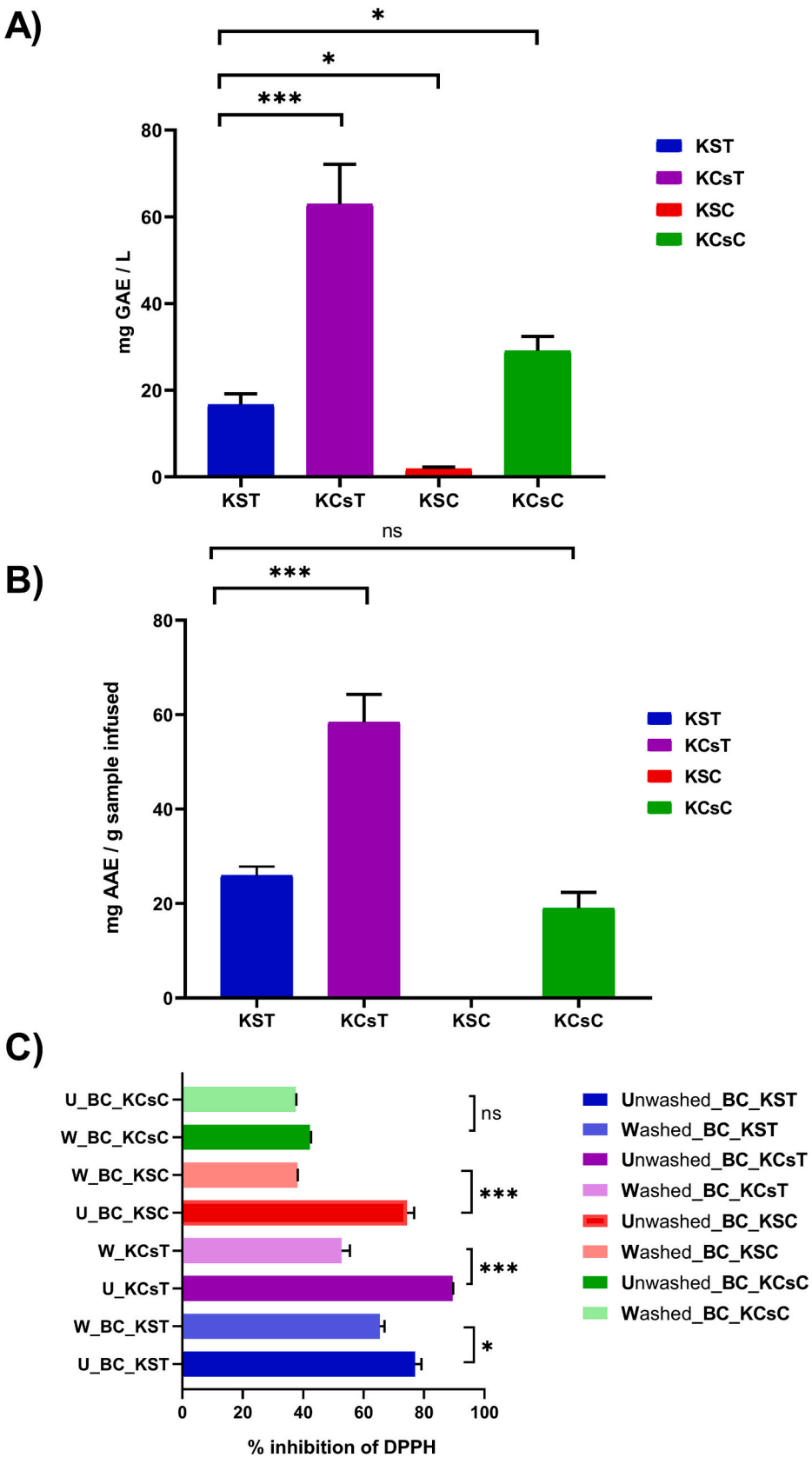

Fig. 4. Determination of antioxidant activity *in vitro*: **A)** TPC, **B)** and **C)** DPPH assay of kombucha beverage at 21 fermentation days.

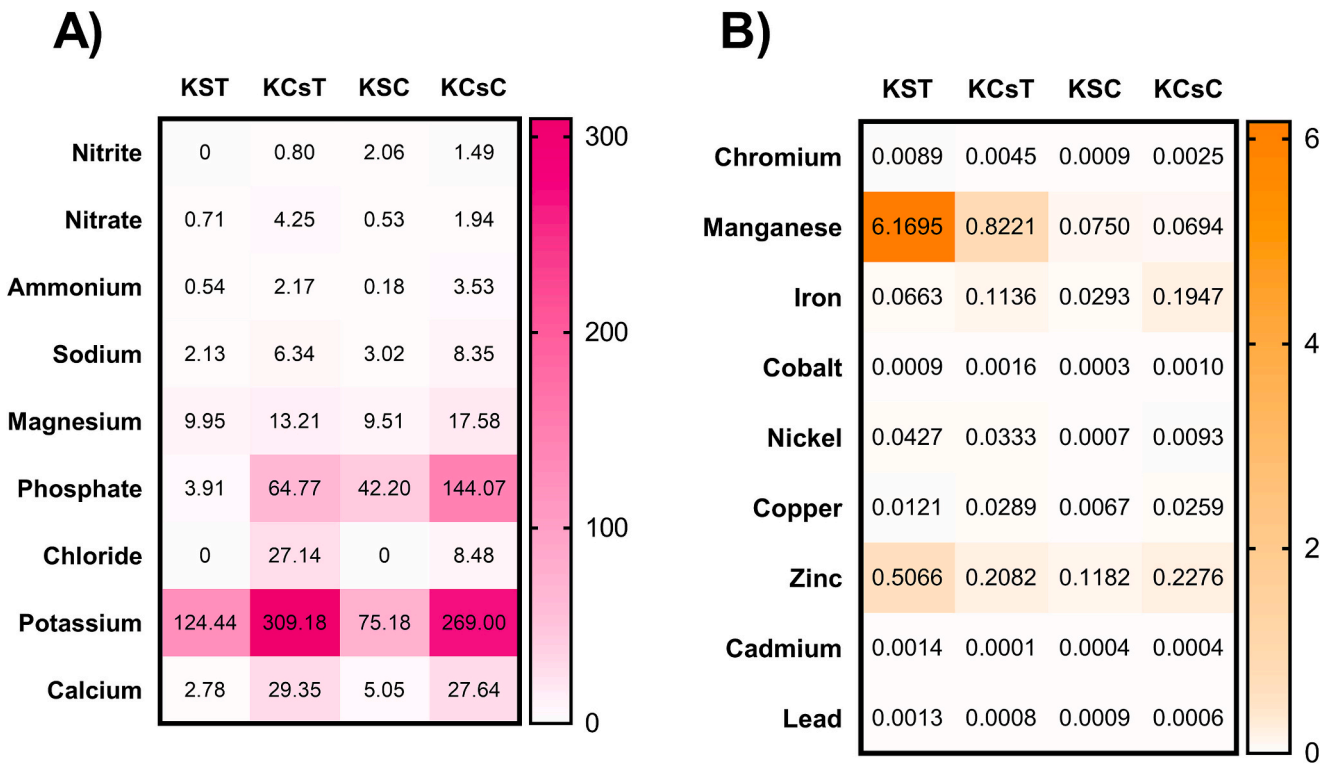

Fig. 5. Concentration of A) most abundant ions and B) trace elements in kombucha cultures determined by IC and ICP-MS, respectively. All the data are expressed as ppm and presented as mean values ± SD ($n = 3$).

stemming from a roasted organic matter [68]. The fact that higher nitrate contents are found in the **KCsC** and **KCsT** samples is interesting, hinting at the fact that the CFW must bear a higher amount of nitrates, that add to those contributed by the black tea component. Since the data suggest a more-than-additive increment of the nitrate content in the **KCsT** sample, it can be further postulated that nitrates are eventually additionally produced by the SCOBY. Also, the presence of the phosphates can, in contrast, be better explained by the metabolic activity of the SCOBY, transforming originally organic phosphates and phosphorous from such AFR waste into phosphates. The concentrations of the most abundant ions and the trace elements are consistent with a safe use of the beverage [68]. Finally, the supplementation of several minerals as observed for CFWs, increases the added value of the kombucha beverage by enriching it with nutrients essential for general human metabolic functions.

3.4. Analysis of the volatilizable and volatile compounds in kombucha samples by LPE-GC–MS and HS-SPME-GC–MS

All the sample extracts were processed by GC–MS to perform their comprehensive qualitative analysis and to evaluate as their bioactive profile can be modified using different sources, exploring the potential benefits of CFWs and spent coffee grounds as alternative substrates [61].

The analysis of kombucha samples were not aimed at delineating a kinetic profile but carried out after the 21st day of fermentation to characterize the final AFR-valorizing product. Consequently, and generally in line with what has been observed in available kombucha characterizations [26,32], the presence of expectable compounds produced in different steps of sugar fermentation, such as volatile ethanol (code **7,** Table 3) acetic acid (code **16,** Table 3) or, were detected only in few kombucha samples, such as those containing citrus or sugar as carbon source, respectively. This trend can be attributed to the different raw materials used and fermentation conditions selected [69]. These compounds, being as such of poor specificity, have not been considered any further in this study.

Three different extraction procedures were applied to explore the complete volatile chemical profile of both lyophilized and fresh kombucha samples. Furthermore, the contributions of the three interesting raw materials citrus fruit waste (CFW), spent coffee ground, and black tea were evaluated. A total of 106 individual compounds were tentatively identified and listed in Table 3.

The chromatograms relative to **KCsT** extracts, chosen for their rich and representative profiles, are reported in Fig. 6.

To obtain a wider range of non-volatile compounds from the lyophilized samples, a preliminary extraction was conducted using a polar mixed aprotic and protic solvent system, *i.e.*, DCM-MeOH. This allowed to extract, however, only a small number of compounds, mainly sugar derivatives and caffeine (**25**), with the latter being the dominant peak in the collected chromatograms, as reported in Fig. 6A. The solid residues were further extracted to promote the detection of more non-volatile compounds with medium polarity and higher affinities to ethyl acetate as aprotic organic solvent that is known to better host more heterogeneous polarities. These extracts were characterized by the presence of several acids, such as aliphatic carboxylic acids, fatty acids, and benzoic and phenolic acids, as well as simple phenols like 4-ethyl-2-methoxy-phenol (**62**), detected in several other fermented beverages, *e.g.*, beer, cider, and wine, in concentrations depending on the microbial compositions and fermentation conditions [70], and sugar derivatives, as shown in Fig. 6B. All the samples, including reference starting materials, were characterized by the presence of gallic acid (**67**).

The headspaces above fresh kombucha liquid fractions were analyzed by the use of HS-SPME, to identify the most volatile components. The main classes of identified molecules are olfactory terpenes stemming from citrus, such as citronellal (**94**), D-limonene (**96**) and fenchol (**98**), all contributing to the characteristic citrusy odor of fresh oranges and lemons, as reported in Fig. 6C [71]. These compounds are considered principal responsible for the pleasant sensory profile improving the flavor of the kombucha beverage in case of CFWs starting material. Other categories of detected compounds were esters, such as octyl caprylate (**37**), and benzaldehyde derivatives, such as vanillin

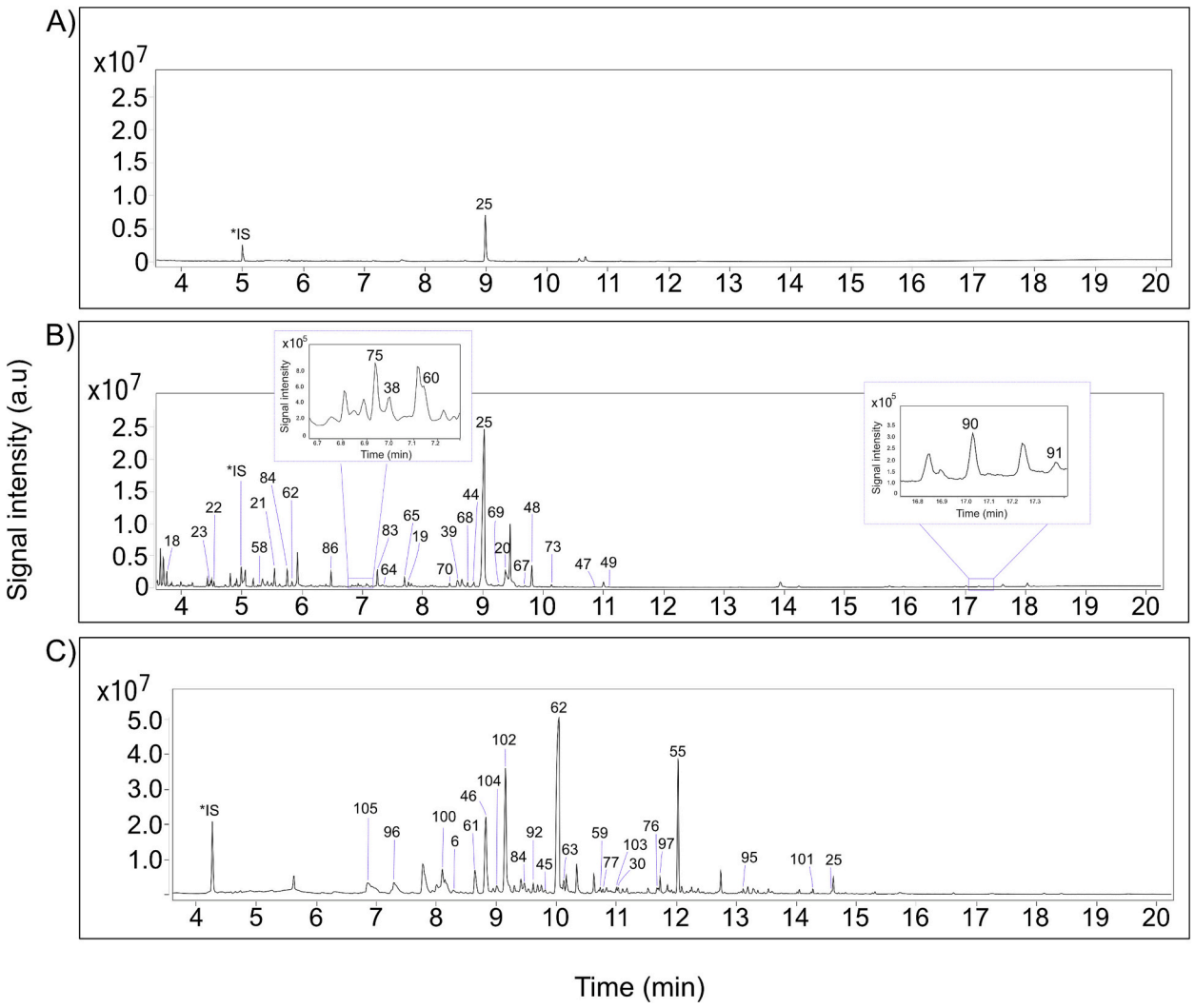

Fig. 6. GC/MS Total Ion Chromatograms (TIC) of representative **CsT** extracts: **A)** LPE in DCM/MeOH (70/30); **B)** LPE- in EtOAc; **C)** HS-SPME-GC/MS.

(**15**). A high content of esters and benzaldehyde derivatives impart a flavor to the kombucha preparation that is characteristic also for the fresh fruits used, contributing to improve the overall quality and style of the product [72–74]. While some esters and benzaldehyde derivatives are of natural origin and already present in the starting materials, as suggested in literature, the identified octyl caprylate, on the other hand, could be related to SCOBY-based fermentation; it has been detected in black tea-based kombucha preparations before [75]. Another volatile product providing floral and fruity aromas and possibly generated by fermenting yeast is the underlying caproic acid (**41**), detected only the fermented kombucha preparations, and not in the controls [76,77]. These two compounds can be seen as exemplary for the general findings in this study. Both compounds have been reported before as indicators of the fermentation processes leading to better flavor and smell of the kombucha preparations. In addition, these two substances can have very different origins: octyl caprylate is commonly used in the production of plastics, and is a general industrial food additive for improving olfactory characteristics [78]. Caproic acid, causing the opposite olfactory result, *i.e.*, an unpleasant smell, is clearly the hydrolysis product of the aforementioned octyl caprylate, and it can be also in this case discussed whether its presence is due to a fermentation process involving esterases, or due to a chemical hydrolysis in acidic medium [79]. Other substances found and listed in Table 3, the di-*tert*-butylphenols (**54** and **55**), as well as the derivative **97**) (2,6-di-*tert*-butylquinone), have been found in kombucha preparations before and claimed to result from fermentation [33]. Moreover, no biosynthesis of these compounds is proven, reports so far suggest their origin from bacterial action. Unfortunately, it is much more likely that these substances, with the tert-butyl group being a structural feature completely unknown in biochemistry, are of human, *i.e.*, purely synthetic origin, and that their presence hints simply at the fact that the ingredients used have been handled in plastic containers. Other aromatic derivatives, such as phthalic acid derivatives, are well known as contaminants in samples, and are commonly simply ignored. In light of the results obtained in this study, and the findings in related kombucha studies, it seems that the list of substances to exclude from this kind of analysis due to their ubiquity and manifold origin.

Another interesting finding is the presence of myristic acid (**44**) in the CFW-based kombucha preparations. Myristic acid is naturally not to be expected in the ingredients used, but is industrially used to protect fresh citrus fruits [80]. It can thus not be excluded that this compound is an example for organic substances that are brought into the preparations as a reminiscent of the history of the starting ingredients (see Section 3.3).

3.5. *Chemometric meta-analysis relative to volatilizable and volatile compound data*

To evaluate the differences among the chemical profiles of the extracts, a multidimensional analysis was conducted, using the single

compounds and their detected presences for the analyzed samples. The pairwise sample similarities were analyzed by MultiDimensional Scaling (MDS) and Maximally Regular Graph (MRG), as shown in Fig. 7. In the MDS plot (Fig. 7A), each point represents a sample; two similar samples, that is, two samples with similar chemical profiles, are represented by two points that are close together, and two dissimilar samples are represented by two points that are far apart. In the MRG plot (Fig. 7B), the samples are represented by the graph vertices and their relationships by the graph edges. The degree of each vertex, *i.e.*, the number of incident edges, serves as a measure of the overall similarity of the sample represented by that vertex to all other samples in the graph. When a group of vertices is fully connected, it indicates that the samples they represent are highly similar to one another.

Both the MDS and the MRG show the presence of two clusters of samples with similar chemical profiles; one cluster includes the samples with citrus as the carbon source, *i.e.*, **KCsC**, **KCsT**, **CsC**, and **CsT**, and the other cluster includes the samples with sugar, *i.e.*, **KSC**, **KST**, **SC**, **ST**. It is noteworthy that the two clusters of similar samples are quite far apart, indicating some relevant differences in their common chemical profiles. By comparing the chemical profiles of the two clusters it is apparent that their separation, that is, their dissimilarity, is mainly due to a set of chemicals that were identified only in the samples derived from CFWs. Most of these compounds generally occurring in citrus matrices, such as azelaic acid (**39**), vanillic acid (**70**), carvone (**93**), D-limonene (**96**) or while others, not present in the raw material, have been identified in kombucha samples and respective controls, *i.e.*, 4-hydroxybenzeneacetic acid (**19**), methyl eugenol (**30**), adipic acid (**38**), eugenol (**59**), thymol (**63**), 5-hydroxymaltol (**75**), 5-hydroxymethyl-2-furoic acid (**83**) and carveol (**92**). Moreover, these samples exhibited phenolic cinnamic acid derivatives such as 4-coumaric (**72**) and isoferulic acid (**73**). These compounds are indicative and characteristic for the lignocellulosic nature of parts of the CFWs starting material, *e.g.*, in form of the contained kernels [81]. Furthermore, the sample **KCsT** was found to contain condensed tannins like catechin (**90**) and epigallocatechin (**91**). To corroborate the choice for CFWs as starting material with potential antioxidant properties, it is important to note that only **KCsT** and **KCsC** samples and their controls exhibit the presence of well-known antioxidants, as reported in *italics* in Table 3, such as 4-hydroxybenzeneacetic acid (**19**), 4-hydroxyphenyllactic acid (**20**), hydroquinone (**60**), 3-hydroxybenzoic acid (**64**), and protocatechoic acid (**68**).

In order to get an idea of how much the composition in terms of blunt composition changes upon incubation of the mixtures with SCOBY, a comparison of the numerosity of the different classes identified in Table 3 has been made. The graphical outcomes are displayed in Figs. 8 and 9.

The analyses essentially confirms the findings outlined before on the basis of the statistical analysis, underlying the three main insights regarding the importance of the starting materials and the real effect of the SCOBY activity on the various mixtures of starting ingredients: i) the high amount of components in the CFWs material compared to tea and spent coffee ground is important for arriving at a kombucha preparation rich in compounds considered important; ii) the fermentation is only slightly changing the composition to what is obtained by simply summing up the compounds of the single starting ingredients (dotted data points in Fig. 9); iii) the fermentation seems mainly to target more ester hydrolysis than ester formation, and fatty acid production. Assuming a very optimistic error of ±1 in the detection of compounds per class, fermentation effects are rather constraint with respect to all compounds and compound classes identified in the discussed analyses. For example, the hydrolysis of esters is nearly complete in all samples, *i.e.*, also in the control samples not incubated with SCOBY.

SCOBY-incubated preparations differ significantly more from untreated preparations in case of sugar-based preparations looking at the simple numerousness of interesting compounds in the preparations (Fig. 8B and D *vs.* 8A and 8C, respectively). Here, the amount of ingredients is much higher than that of small molecules brought in by the coffee or the tea alone. It seems that in these cases, the SCOBY is more active in general, producing metabolites that effectively enrich the kombucha preparation.

Direct comparative analysis displayed in Fig. 9 between the effects of sugar (**S**) *vs.* CFWs (**Cs**) thus always underlines that more important than an eventual fermentation is the choice of starting material. In this respect, the CFWs hold a great potential to determine essentially alone the quality of the kombucha preparation with respect to the quantity of bioactive compounds relevant for human health, whereas the use of simple sugars lead to kombucha preparations of much less beneficial characteristics in terms of bioactive ingredients (Fig. 9 *vs.* Fig. 8).

It is unlikely that a different picture would emerge from an analysis based on the aqueous phases avoiding an extraction: expected tannins were found in the analyses, as well as polyhydroxylated compounds. Only *water-soluble* molecules of higher molecular weight, *i.e.*, not the film-forming bacterial cellulose formed upon SCOBY activity, and/or very polar molecules could not be targeted by these analyses. Nevertheless, these structures, with hemicelluloses, pectines, *etc.*, being prominent examples in case of the CFWs, for example, are normally not the typical products of a SCOBY-based fermentation but are eventually substrates. Moreover, the fermentation of these compounds should have led to a more significant qualitative and quantitative difference in the

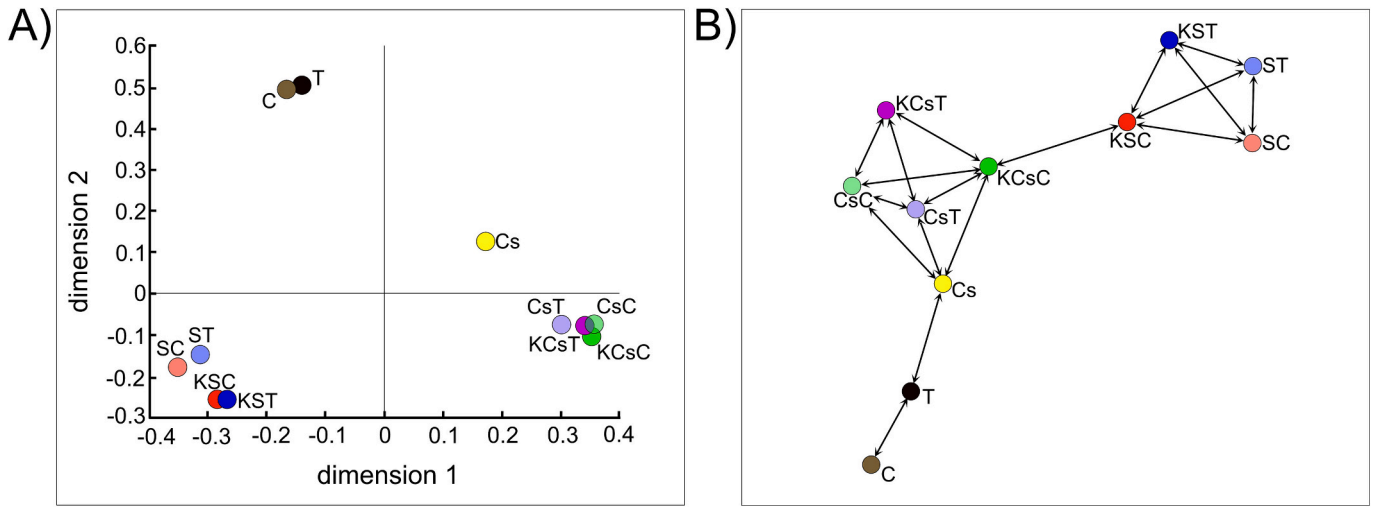

Fig. 7. Graphical visualization of the analyzed samples by **A)** MultiDimensional Scaling (MDS) and **B)** Maximal Regular Graph (MRG). The colored points represent the raw starting materials, the controls, and the kombucha samples, accordingly to the color code reported in Table 4.

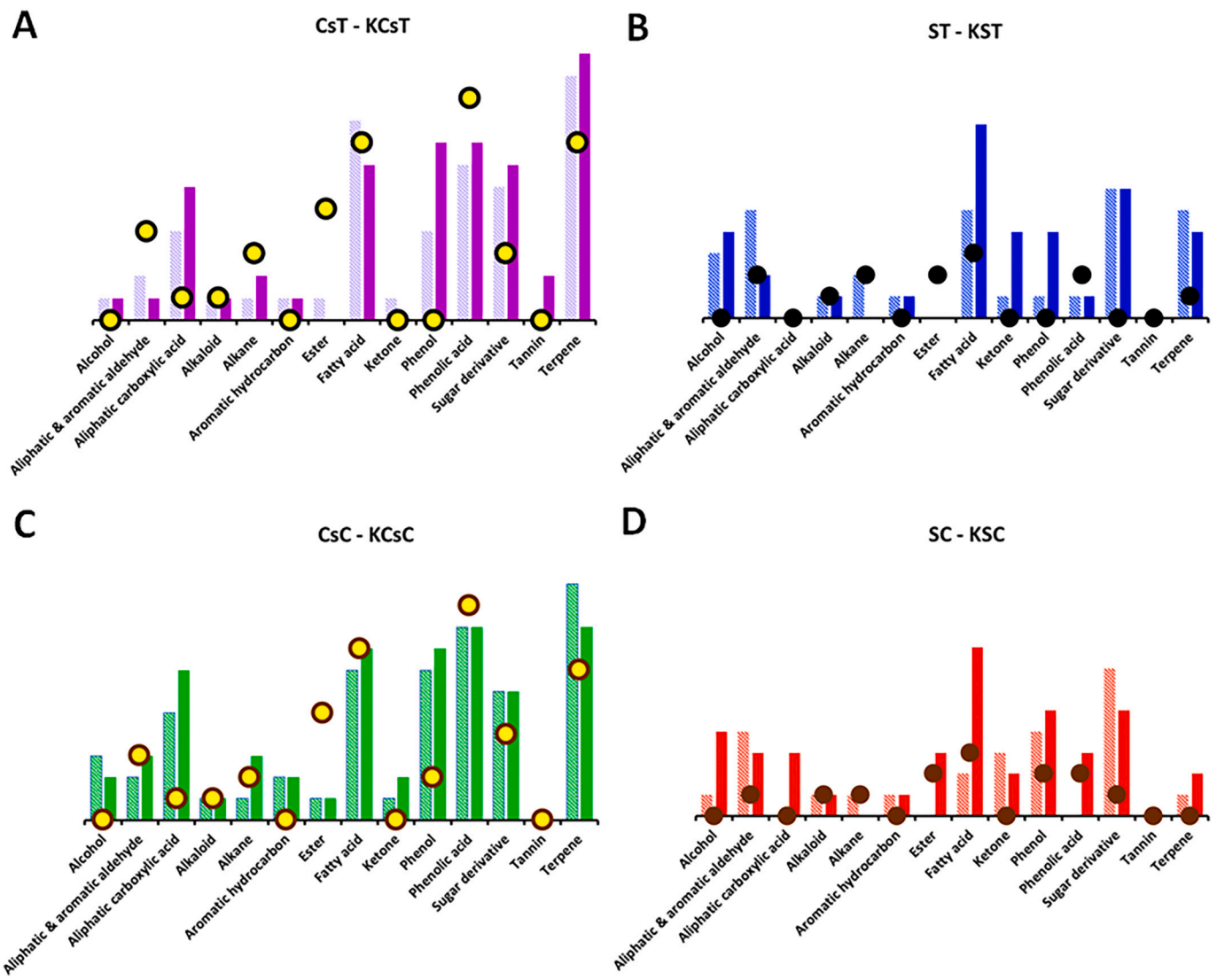

Fig. 8. Comparison of the composition in terms of compound class (x-assis) numerousness (y-assis) of the various kombucha preparations. The following couple of samples are compared: **A) CsT** – **KCsT**; **B) ST** – **KST**; **C) CsC** – **KCsC**; **D) SC** – **KSC**. Mixed colored dots represent the sum of individual compounds identified in respective starting ingredients, singly colored dots represent the numerousness of coffee and tea, respectively. Dotted lines are used for representing control samples.

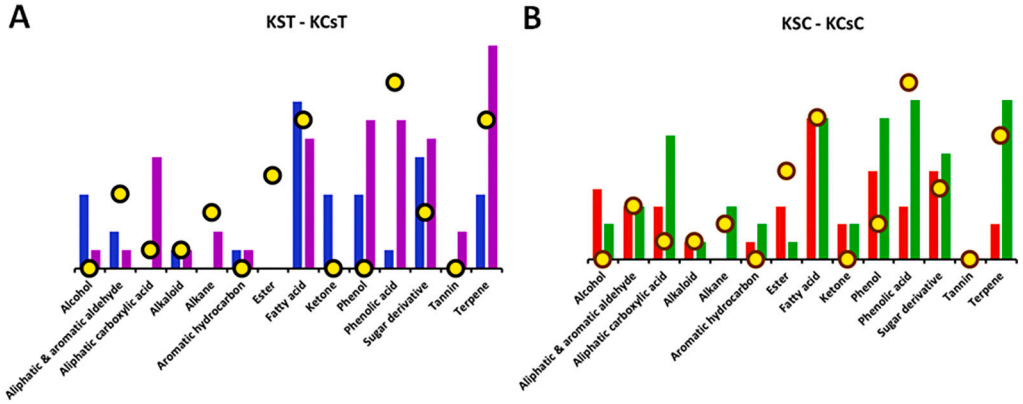

Fig. 9. Comparison of the composition in terms of compound class (x-assis) numerousness (y-assis) of the various kombucha preparations. The following couple of samples are compared: **A) KST** – **KCsT**; **B) KSC** – **KCsC**. Colored dots in A) and B) represent the sum of the individual compounds identified in the starting ingredients **Cs** and **T**, and **Cs** and **C**, respectively.

SCOBY-incubated samples also in terms of substances detectable in the gas-chromatographic analyses *versus* the untreated mixtures.

4. Conclusions and perspectives

The molecular composition of the kombucha-like beverage produced in this study using agricultural food wastes (AFRs) in form of CFWs is characterized by a rich composition in beneficial small organic molecules, as indicated by the list presented in Table 3. This richness goes beyond of what has been reported so far in the various works testing the effects of alternative starting ingredients as carbon and nitrogen sources (Table 1). The preparation of the kombucha from waste ingredients, omitting the addition of the traditional carbon source sucrose, yields for the first time a fermented drink that is rich in health-promoting small molecules using a single, yet more complex carbon source, *i.e.*, CFWs. In this respect, the high amounts of antioxidant species and natural flavors produced and/or liberated during the fermentation phase, represent an interesting distinguishing feature. In terms of potentially present inhibitors liberated upon fermentation as observed in some of the other works cited in Table 1, this study indicates that the fermentation process employing CFWs does actually not yield bacterial growth inhibitors. The present work hence demonstrates several key findings regarding the kombucha-like beverage produced using AFRs as starting point: i) untreated wastes enhance the chemical composition of kombucha-like beverages, adding flavors and health-promoting ingredients, aligning with growing interest in valorizing food waste as a sustainable resource for the production of functional beverages; ii) the ability of the kombucha microbial community to utilize alternative carbon and nitrogen sources from waste materials highlights its metabolic flexibility, with this capability ensuring not only sustained microbial growth but also the production/liberation of antioxidant bioactive molecules in the kombucha-like beverage, with some of them retained also in the BC matrix; iii) the SCOBY proved to be quite robust towards the antimicrobial compounds such as low molecular weight phenols released from agricultural and food residues, ensuring stable fermentation under challenging conditions. Future work will focus on optimizing the rather robust SCOBY-based fermentation for both the production of kombucha-like beverages and the formation of bacterial cellulose (BC) under different conditions using still alternative carbon and nitrogen sources as well as their combinations, preferentially from agricultural and food residues. Since it could be demonstrated that small molecules exhibiting antioxidant activity compounds remain entrapped within the BC, even after extensive washing, the selection of raw materials can tune the BC characteristics for specific applications, such as food packaging.

This research thus opens new avenues for the sustainable use of agro-industrial waste in producing bioactive-enriched kombucha and biopolymer materials.

CRediT authorship contribution statement

Francesca Sabatini: Writing – original draft, Methodology, Investigation, Conceptualization. **Emanuela Maresca:** Writing – original draft, Methodology, Investigation, Data curation. **Martina Aulitto:** Writing – review & editing, Methodology, Investigation, Conceptualization. **Veronica Termopoli:** Writing – review & editing, Methodology, Investigation, Conceptualization. **Arianna De Risi:** Methodology, Investigation, Conceptualization. **Monica Correggia:** Methodology, Data curation. **Gabriella Fiorentino:** Writing – review & editing, Investigation, Conceptualization. **Viviana Consonni:** Writing – review & editing, Investigation, Conceptualization. **Fabio Gosetti:** Writing – review & editing, Investigation, Conceptualization. **Marco Orlandi:** Writing – review & editing, Writing – original draft, Supervision, Investigation, Funding acquisition, Data curation, Conceptualization. **Heiko Lange:** Writing – review & editing, Methodology, Investigation, Conceptualization. **Patrizia Contursi:** Writing – review & editing, Writing – original draft, Supervision, Investigation, Funding acquisition, Data curation, Conceptualization.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this article.

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