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Ecological linkages between biotechnologically relevant autochthonous microorganisms and phenolic compounds in sugar apple fruit (Annona squamosa L.)

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Corresponding Author:	PASQUALE FILANNINO, Ph.D. University of Bari Aldo Moro Bari, ITALY
First Author:	Ali Zein Alabiden Tlais
Order of Authors:	Ali Zein Alabiden Tlais
	Kalliopi Rantsiou
	PASQUALE FILANNINO, Ph.D.
	Luca Simone Cocolin
	Ivana Cavoski
	Marco Gobbetti
	Raffaella Di Cagno
Abstract:	Our study investigated the potential of Annona squamosa (L.) fruit as a reservoir of yeasts and lactic acid bacteria having biotechnological implications, and phenolics capable of modifying the ecology of microbial consortia. Only a single species of lactic acid bacteria (Enterococcus faecalis) was identified, while Annona fruit seemed to be a preferred niche for yeasts (Saccharomyces cerevisiae,Hanseniaspora uvarum), which were differentially distributed in the fruit. In order to identify ecological implications for inherent phenolics, the antimicrobial potential of water- and methanol/water-soluble extracts from peel and pulp was studied. Pulp extracts did not show any antimicrobial activity against the microbial indicators, while some Gram-positive bacteria (St. aureus, St. saprophyticus, L. monocytogenes, B. megaterium) were susceptible to peel extracts. Among lactic acid bacteria used as indicators, only Lactococcus lactis and Weissella cibaria were inhibited. The chemical profiling of methanol/water-soluble phenolics from Annona peel reported a full panel of 41 phenolics, mainly procyanidin and catechin derivatives. The antimicrobial activity was associated to specific compounds (procyanidin dimer type B [isomer 1], rutin [isomer 2], catechin diglucopyranoside), in addition to unidentified catechin derivatives. E. faecalis, which was detected in the epiphytic microbiota, was well adapted to the phenolics from the peel. Peel phenolics had a growth-promoting effect toward the autochthonous yeasts S. cerevisiae and H. uvarum.
Suggested Reviewers:	Emanuele Zannini University College Cork School of Food and Nutritional Sciences e.zannini@ucc.ie Expertise in food microbiology
	LUCA SETTANNI University of Palermo luca.settanni@unipa.it Expertise in Food Microbiology
	JOSÉ CURIEL GÁMIZ University of La Rioja jose-antonio.curiel@unirioja.es Expertise in Food Microbiology
	Luca Roscini University of Perugia luca.roscini@unipg.it

	Expertise in Food Microbiology

Bari, 18th November 2022

Dear Editor,

I would like to thank you and the referee for giving us the opportunity to improve the manuscript. Please, note that all the recommendations, none excluded, have been considered in the revised version. An itemized list of the revisions according to the referee's recommendations has been provided.

Kind regards,
Pasquale Filannino

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Point by point response to reviewers:

Reviewer #1

The manuscript entitled 'Ecological linkages between biotechnologically relevant autochthonous microorganisms and phenolic compounds in sugar apple fruit (Annona squamosa L.)' by XX et al. presents a nice overview of the microbial composition and bioactive compounds of A. squamosa.

Comments of the manuscript:

Section 3.1.2 - There is no data provided for this section, I suggest adding the PCR/RFLP profiles and the sequencing results to the supplementary data. How many representative isolates from each group were identified by sequencing? **Ok**, with regard to bacterial isolates, representative profiles of the 16S rRNA PCR products have been provided as supplementary material (please, see the new Supplementary Tables S1). With regard to yeasts, representative profiles of the ITS1-5.8S-ITS2 PCR products as well as RFLP profiles have been also provided as supplementary material (please, see the new Supplementary Table S3, and Supplementary Figures S2 and S3). In total 8 isolates were further subjected to D1/D2 loop sequencing and the sequencing results are shown in a supplementary table. One of the groups assembled consisted of 67 isolates out of which 3 were sequenced and resulted to be *Saccharomyces cerevisiae*. The second group consisted of 66 isolates out of which 4 were sequenced and resulted to be *Hanseniaspora uvarum*. The PCR-RFLP identification and the sequencing identification were concordant.

Section 3.2.2 - Can the criteria for the selection of bacterial and yeast strains in Table 1 be clarified, besides the common human pathogens? **Ok**, an explanation has been added within the manuscript (P15 L321-326). The antimicrobial activity of the extracts was assayed against a heterogeneous pool of indicator microorganism, both bacteria and yeasts isolated from different ecological niches (plants, insects, and humans), that included microorganisms of biotechnological interest and/or involved in complex relationships with the host organism (as pathogens, opportunists, or growth-

promoters). Such an assorted group was aimed to maximize the relevance of the connections covered by this study between microorganisms and phytochemicals.

Line 212 Add number of section (2.3.3?). **Ok**, the number of section has been added. I'm sorry for the omission (P10 L215).

Sentence starting in line 224 and ending on line 226 can be removes or included in results. **Ok**, in accordance with your suggestion, the sentence has been moved to the Results section (P16 L343-345).

Line 284: Do you mean 65 LAB isolates were identified by sequencing? **Yes**, all 65 presumptive epiphytic LAB were identified by partial sequencing of the 16S rRNA gene. Differentiation between *E. faecalis* and *E. faecium* species was carried out through ddl-specific primer pairs (Dutka-Malen, Evers, & Courvalin, 1995). The sentence has been revised to avoid misunderstanding (P13 L285-287).

Line 288: Remove 'of' between 'PCR' and 'product'. **Ok**, the sentence has been revised (P13 L291).

Section 3.2.1 - Is the data presented here not shown anywhere else? I think it would be good to have this summarized in a table (could be in supplementary data). **Ok**, a new table has been added to resume the results of the paragraph (please, see the new Table 3).

Line 318 - I recommend mentioning the number of strains that showed growth inhibition (and the total number of strains tested). **Ok**, the number of strains was included in the sentence (P15 L326-327). The extracts hindered the growth of only 6 strains out of a total of 26 indicator microorganisms tested.

Line 271 to line 380 - I think it would be easier for the reader if the authors presented first the identification of the compounds in the different fractions and then the inhibitory effect of such compounds. **Ok**, the paragraph has been rearranged according to the suggestion (P17 L382-388; P18 L389-391).

Check italics on species names. **Ok**, the species names have been verified throughout the manuscript.

Line 322 - The authors mention that the antimicrobial effect depends on the extraction solvent, could you elaborate on this in the discussion? Why is it? Due to different levels of extraction, or the possible impact of the solvent itself? **Ok**, the role of the solvent has been addressed within the manuscript (P20 L457; P21 L461). The varied chemical structure of phytochemicals determines their solubility. Thus, the type of solvent plays a major factor during the extraction of phytochemicals, both in terms of quantity and quality of solubilized compounds, with consequences on the bioactivities of the extract. The extraction yield depends greatly on polarity of solvent, and it generally increases in the order of methanol > ethanol > water. Concerning the potential interference of the solvent during the antimicrobial assays, the interference was evaluated and neutralized by appropriate technical expedients (e.g., evaporation of solvent, use of negative controls), thus the observed antimicrobial activity is not attributable to the solvent interference itself, but to the various solubilized phytochemicals.

The content of the discussion is appropriate, though I suggest tidying up a bit to facilitate the understanding. **Ok**, the discussion has been further rearranged as suggested for ease of reading and understanding.

Reviewer #2

This study provides information about the microbial and polyphenolic composition of Annona squamosa (sugar apple fruit). The authors concluded that this tropical fruit represents a very selective environment because only one species of lactic acid bacteria and two species of yeasts (S. cerevisiae and H. uvarum were isolated). The authors explained this limited microbial diversity to the antimicrobial activity of peel polyphenols. They evidenced the microbial activity of some of these polyphenols, previously separated of the total fraction. Beyond the fact that the microbiota and chemical composition of this tropical fruit has been scarcely studied, neither the microbiological nor the chemical study provide much novel information in terms of new microbial species or new phenolic compounds. A metagenomics study of the microbiota composition could have provided more novel information and the detection of new microbial groups. We appreciate the reviewer's critical analysis, and we are aware that culture-dependent methodologies have limitations and can provide a plausibly filtered view. However, such methodologies should not be underestimated in light of their valuable contribution in the study of microbial physiology and evaluation of specific patterns.

We have to point out that the aim of the work was not to identify new compounds or to give a comprehensive view about the Annona microbiota, **but rather to highlight links between Annona's phytochemicals and certain autochthons microorganisms of potential biotechnological interest. To this aim, it was essential to perform growth trials that cannot be performed without autochthonous pure strains that can only be obtained through culture-dependent approaches**.

For this reason, the applied culture-dependent analyses targeted only specific microbial groups (lactic acid bacteria and yeasts), and not all microbial groups potentially present in the fruit. An exclusively ecological study was not in ours mind and probably would have gone outside the scope of this Journal.

The novel findings that our study produced are clearly stated in the highlights; these findings are solidly supported by the implemented experimental design and, have not been described in previous studies (or in any case poorly treated, as also attested to by the teviewer):

(i) *A. squamosa* fruits had a specific spatial distribution of microbes and phenolics (with regard to the microbial groups under study: lactic acid bacteria and yeasts);
(ii) Peel phenolics had antimicrobial activity against several Gram-positive bacteria;
(iii) Antimicrobial activity of peel phenolics was associated to specific compounds;
(iv) *E. faecalis* was well adapted to the phenolics from the peel;
(v) Peel phenolics had a growth-promoting effect toward autochthonous yeasts.

Therefore, we believe that the applied approach is consistent with the purpose of the work, and a culture-independent approach (which also has limitations) can at best complement but not replace the approach used.

Some minor comments are:

Page 5, line 101. How long was the refrigeration period? A long period can exert a selective pressure in favor of some yeast and bacteria species (the less cryotolerant). **Ok**, a clarification has been added in the manuscript (P5 L100-102). Refrigeration conditions were strictly limited to the shipment (72 h). Fruits were subjected to microbiological analysis as soon as they were delivered to the laboratory. We would like to emphasize that the samples were not placed under freezing conditions

(before undergoing microbiological analysis) that could generate freezing damage or thermal shock to microbial cells.

Page 18, line 410. How is the mechanism or the via for S. cerevisiae to arrive to inner parts of the pulp? It is not to be expected a sterile environment in the center of a fruit? **Ok**, a clarification has been added in the manuscript (P19 L421-424). Detection of endophytic microorganisms (as S. ce*revisiae*) in fruits is not uncommon (Krishnan et al., 2012; Ren et al., 2019; Madbouly et al. 2020; Di Cagno et al., 2010). The modes of penetration may be various (Kumar et al., 2021), but the most likely is through the floral apparatus.

References:

Di Cagno et al. (2010). Taxonomic structure of the yeasts and lactic acid bacteria microbiota of pineapple (*Ananas comosus* L. Merr.) and use of autochthonous starters for minimally processing. Food Microbiology, 27(3), 381-389.

Krishnan et al. (2012). Isolation and functional characterization of bacterial endophytes from *Carica papaya* fruits. Journal of applied microbiology, 113(2), 308-317.

Ren et al. (2019). Endophytic bacterial communities of Jingbai Pear trees in north China analyzed with Illumina sequencing of 16S rDNA. Archives of microbiology, 201(2), 199-208.

Madbouly et al. (2020). Biocontrol of Monilinia fructigena, causal agent of brown rot of apple fruit, by using endophytic yeasts. Biological control, 144, 104239.

Kumar, A., Zhimo, Y., Biasi, A., Salim, S., Feygenberg, O., Wisniewski, M., & Droby, S. (2021). Endophytic microbiome in the carposphere and its importance in fruit physiology and pathology. In Postharvest pathology (pp. 73-88). Springer, Cham.

Do the authors have any hypothesis to explain yeast growth stimulation by some phenolic compounds? **Ok**, assumption have been included in the manuscript (P23 L510-521). Several mechanisms can be hypothesized. A protective effect against oxidative stress can be ascribed to the radicals-scavenging activity of phenolics (Belinha et al., 2007). Beyond the well-defined antioxidant potential, a role as signaling molecules was previously proposed for phenolic acids and flavonoids (Chabot et al., 1992). Absorption of phenolics on cell surface layer contribute to stimulate cellular metabolic activity by the binding of microelements, precursors, growth factors, and modifies the overall cell reactivity towards oxygen (Rapeanu et al., 2014; Salmon, 2006). We cannot rule out that carbohydrates moiety resulting from the hydrolysis of phenolic glycosides may represent an additional carbon source available for yeast growth.

Highlights

- *A. squamosa* fruits had a specific spatial distribution of microbes and phenolics;
- Peel phenolics had antimicrobial activity against several Gram-positive bacteria;
- Antimicrobial activity of peel phenolics was associated to specific compounds;
- *E. faecalis* was well adapted to the phenolics from the peel;
- Peel phenolics had a growth-promoting effect toward autochthonous yeasts.

Ecological linkages between biotechnologically relevant autochthonous microorganisms and phenolic compounds in sugar apple fruit (*Annona squamosa* L.)

Ali Zein Alabiden Tlais[a], Kalliopi Rantsiou[b], Pasquale Filannino[c]*, Luca Simone Cocolin[b], Ivana Cavoski[d], Marco Gobbetti[a], Raffaella Di Cagno[a].

[a]Faculty of Sciences and Technology, Libera Università di Bolzano, 39100 Bolzano, Italy

[b]Dipartimento di Scienze Agrarie, Forestali e Alimentari, Università di Torino, Torino, Italy

[c]Department of Soil, Plant and Food Science, University of Bari Aldo Moro, 70126 Bari, Italy

[d]CIHEAM-MAIB, Mediterranean Agronomic Institute of Bari, 70010 Valenzano, Bari, Italy

*Corresponding author: Pasquale Filannino. E-mail: pasquale.filannino1@uniba.it

Abstract

Our study investigated the potential of *Annona squamosa* (L.) fruit as a reservoir of yeasts and lactic acid bacteria having biotechnological implications, and phenolics capable of modifying the ecology of microbial consortia. Only a single species of lactic acid bacteria (*Enterococcus faecalis*) was identified, while Annona fruit seemed to be a preferred niche for yeasts (*Saccharomyces cerevisiae, Hanseniaspora uvarum*), which were differentially distributed in the fruit. In order to identify ecological implications for inherent phenolics, the antimicrobial potential of water- and methanol/water-soluble extracts from peel and pulp was studied. Pulp extracts did not show any antimicrobial activity against the microbial indicators, while some Gram-positive bacteria (*St. aureus, St. saprophyticus, L. monocytogenes, B. megaterium*) were susceptible to peel extracts. Among lactic acid bacteria used as indicators, only *Lactococcus lactis* and *Weissella cibaria* were inhibited. The chemical profiling of methanol/water-soluble phenolics from Annona peel reported a full panel of 41 phenolics, mainly procyanidin and catechin derivatives. The antimicrobial activity was associated to specific compounds (procyanidin dimer type B [isomer 1], rutin [isomer 2], catechin diglucopyranoside), in addition to unidentified catechin derivatives. *E. faecalis*, which was detected in the epiphytic microbiota, was well adapted to the phenolics from the peel. Peel phenolics had a growth-promoting effect toward the autochthonous yeasts *S. cerevisiae* and *H. uvarum*.

Keywords: antimicrobial activity; endophytes; epiphytes; yeasts; lactic acid bacteria.

Abbreviations: LAB, lactic acid bacteria; WSE, water soluble extract; MWSE, methanol/water soluble extract.

1. Introduction

Plants have long been a refuge for a wide array of epiphytic and endophytic microbiota (Bulgarelli et al., 2013; Yadav, 2020). These microbial communities are organized such that they create complex interconnected microbial networks that are essential for enhancing host development and ecosystem functioning, as well as being active in biocontrol of phytopathogens and improving the defense mechanism against insects and pests. In addition, they are efficient cell factories for the biosynthesis of phytohormones and other bioactive metabolites of major biotechnological or pharmaceutical significance (Pontonio et al., 2018; Sharma et al., 2017; Sushanto et al., 2016). Plant microbiota structure is highly changeable and affected by a variety of biotic and abiotic determinants, including host species, host developmental stage, and environmental conditions (Dudeja and Giri, 2014; Fierer, 2017). Despite the substantial amount of researches focusing on the dynamics, structure, and functional roles of plant microbial communities, the mechanisms of interactions and processes underlying plant microbiome modulation continue to be a scientific challenge, making it hard the identification of the fundamental ecological determinants (Bennett et al., 2019; Derocles et al., 2018; Toju et al., 2016). Several hypotheses have been proposed, most of which focus on the production of defensive compounds by the plant host or microbes, in response to the microbes or other stress condition (Bacon and White, 2016). In this scenario, compounds produced by the plant host, such as phenolic compounds, essential oils, and other secondary compounds, have been hypothesized to be major determinants in quorum quenching or, in more general terms, in changing the behavior of microbiota, often reducing microbial growth rates (Bacon and White, 2016). Further studies are required to prove this hypothesis and unravel the link between bioactive phytochemicals and plant microbiome.

Annona quamosa L. (Annonaceae), commonly known as sugar apple, represents an excellent source of bioactive molecules, including antibiotic, antiviral, and antifungal molecules, which highly qualify them as medicinal plants (Castronovo et al., 2021). It is native to tropical Central and South America, as well as West India. It is one of the exotic fruits prized for its very pleasant, soft, and unique sweet aroma (Manochai et al., 2018). Sugar apple fruit was recently introduced as a promising super fruit of the 21st century. Overall, at the plant host level a spatial tissue distribution of different classes of compounds is encountered. For instance, leaves of Annona contain several phytochemicals belonging to different chemical classes such as alkaloids, coumarins, tannins, cardiac glycosides, flavonoids, phenols, and saponins, which exert an antibacterial activity, notably against the enteric pathogens (Nguyen et al., 2020). The same compounds were found in alcoholic and ethyl acetate extracts of the fruit peel (Huang et al., 2010), and caryophyllene, diterpenes, phytols (precursors of many forms of vitamin E) and sterols were the main compounds isolated in the seeds (Bhardwaj et al., 2014). Phenolic compounds as free, bound and esterified form characterized the fruit pulp extracts (Baskaran et al., 2016). Although the screening of phenolic compounds and antibacterial efficacy of Annona fruits was largely investigated (Babawale et al., 2019; Bhardwaj et al., 2014; Cagnini et al., 2021; Folorunso et al., 2019; García-Salas et al., 2015; Neethu Simon et al., 2016; Nguyen et al., 2020; Patil et al., 2019; Santos et al., 2016), the relationship between these intrinsic phytochemicals and the structure of Annona microbiota and how this may be driven represents an intriguing field of ecological research and biotechnological applications. First, this study aimed to provide a framework on microbial and phytochemical distribution in sugar apple fruits, revealing possible correlations between intrinsic phenolic compounds and endogenous and exogenous microbiota. In particular, we focused our attention on autochthonous yeasts and lactic acid bacteria (LAB), which represent

two microbial groups whose biotechnological potential, including for food purposes, is well known. The discovery of interactions between phenolics and microbes (pathogenic, spoilage or pro-technology) could open up new scenarios for defining new biotechnologies based on both plant and microbial components (Chan et al., 2018; Zhang et al., 2021).

2. Materials and Methods

2.1. Sampling, handling and storage

Sugar apple fruits (*Annona squamosa* L.) were provided by a certified organic farm located in Trye (Lebanon). Harvesting concerned ca. 14 kg of fruits at physiological maturity, which underwent random selection from the orchard. Fruits were placed into sterilized bags, labelled, packed in refrigerated box for the shipment (72 h), and subjected to microbiological analysis as soon as they were delivered to the laboratory.

2.2. Microbiological analysis

2.2.1. Isolation of endophytic and epiphytic microorganisms

Sugar apple fruits were washed with tap water, in order to isolate the endophytic bacteria. Then they were submerged in 70% ethanol for 3 min, subsequently in 15% sodium hypochlorite for 15 min, and then in 70% ethanol for 30 s, following three successive washes in distilled sterilized water. After fruits sterilization, each fruit was peeled and subsequently divided into three sections: (i) outer pulp; (ii) middle pulp; and (iii) inner pulp (Figure 1). Sterile knifes under sterile conditions were used to prepare samples from each section. Ten grams of each sample were blended in sterile 0.9% (w/v) sodium chloride solution for 2 min at room temperature (Classic Blender, PBI International Milan, Italy). From each cell suspension, serial dilutions were prepared. Serially diluted aliquots were plated on different agar media. Mesophilic LAB were counted on MRS agar

(Oxoid Ltd, Basingstoke, Hampshire, England) supplemented with 0.1% (w v^{-1}) of cycloheximide (Sigma Chemical Co.) and incubated for 48 h at 30 °C under anaerobic conditions. Yeasts were enumerated on Yeast extract Peptone Dextrose Agar (YPD, Oxoid) supplemented with 150 ppm chloramphenicol after 72 h of incubation at 25 °C. Total mesophilic bacteria on PCA agar (Oxoid) and incubated for 48 h at 30 °C.

Aiming to isolate the epiphytic microbiota (Figure 1), entire fruit was placed in a sterile beaker with 1800 ml isotonic solution containing peptone (0.1%, w/v) and tween 80 (0.01%, w/v), and subjected to orbital shaking at 150 rpm for 1 h. Then, the suspension was centrifuged for 10 min at 10,000 rpm to collect the pellets before being resuspended in isotonic solution. As previously described, epiphytic total mesophilic bacteria, mesophilic LAB, and yeasts were enumerated on PCA, MRS, and YPD agar, respectively.

Furthermore, 50 g of each fruit parts or 50 ml of epiphyte suspension were mixed with 50 ml of MRS broth or YPD broth (enrichment medium) and incubated for 72 h at 30 and 25 °C, respectively. At each incubation time, mesophilic LAB and yeasts were enumerated as described above.

Isolation of LAB and yeasts was performed as described by Lhomme et al. (2015). Colonies were isolated from MRS and YPD plates of the highest dilution. Morphologically separated LAB and yeasts colonies were selected and purified by re-streaking onto the agar medium.

2.2.2. Molecular identification of LAB isolates.

Presumptive lactic acid bacteria isolates were identified by partial sequencing of the 16S rRNA gene. In detail, genomic DNA was extracted from bacteria using DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA), according to the manufacturer's instructions. Two primer pairs, LacbF/LacbR and LpCoF/LpCoR (Sigma-Aldrich), were used to amplify 16S rRNA gene

fragment of LAB (De Angelis et al., 2006). Differentiation between *Enterococcus faecalis* and *Enterococcus faecium* species was carried out through ddl-specific primer pairs (Dutka-Malen et al., 1995). Eurofins Genomics (Germany) carried out the sequencing of PCR products. Identification was performed by comparing the sequences of each isolate with those reported in the Basic BLAST database (http://blast.ncbi.nlm.nih.gov/Blast.cgi) (Altschul et al., 1997). Strains showing homology of at least 97% were considered to belong to the same species (Goebel and Stackebrandt, 1994).

2.2.3. Molecular identification of yeasts

Isolates, after streak in Wallerstein Laboratory Nutrient Medium (Oxoid, Milan, Italy), were grown overnight in 1 ml YPD medium at 25 °C. The broth culture was centrifuged at 14,000*g* for 5 minutes and the pellet was subjected to DNA extraction according to Cocolin et al. (2000). The extracted DNA was quantified using a Nanodrop ND-1000 spectrophotometer (Celbio, Milan, Italy) and standardized at 100 ng/µl. The DNA of all isolates was subjected to restriction fragment length polymorphism (RFLP) of the region ITS1-5.8S rRNA-ITS2 (ITS) (Esteve-Zarzoso et al., 1999). The ITS region was amplified with primers ITS1 (5'- TCCGTAGGTGAACCTGCGG -3') and ITS4 (5'-TCCTCCGCTTATTGATATGC 3') (White et al. 1990). The reaction mix was of 50 µl and contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM of deoxynucleoside triphosphates (dNTPs), 1.25 U of *Taq* Polymerase (Applied Biosystems, Milan Italy), 0.2 µM of each primer and 100 ng of template DNA. Amplification was carried out using a PTC-200 DNA Engine MJ Research thermal cycler (Biorad, Milan, Italy), as described by Esteve-Zarzoso et al. (1999) and the PCR products were checked by electrophoresis on 1.5% (w/v) agarose gel. The PCR products were subsequently digested with endonucleases *HinfI*, *HaeIII*, *CfoI* (Promega, Milan, Italy), according to the supplier's instructions. The restriction fragments were

separated by electrophoresis in 3% agarose gel and stained with ethidium bromide. PCR and RFLP fragment lengths were used for identification of yeasts by the comparison of the restriction bands with those available in literature (Esteve-Zarzoso et al., 1999; Granchi et al., 1999; Guillamon et al., 1998; Sabate et al., 2002). Identification to the species level was confirmed by sequencing the D1-D2 loop of the 26S rRNA encoding gene, after amplification using primers NL1/NL4 (Kurtzman and Robnett 1997) to obtain a polymerase chain reaction (PCR) product, which was sequenced by a commercial facility (Eurofins, Germany).

2.2.4. Total DNA extraction and direct analysis by PCR-DGGE

Samples of 5 g of fruit were diluted in 20 mL of Ringer's solution (Oxoid) and homogenized for 2 minutes in a stomacher (Interscience, Paris, France). The homogenate was further diluted by mixing an aliquot of 1 ml with 9 mL of Ringer's solution. One mL of the diluted homogenate was centrifuged at 14,000*g* for 5 min and the pellet was subjected to DNA extraction, using the Master Pure Complete DNA and RNA purification kit (Epicentre, Milan, Italy). The DNA extracted was quantified and standardized as described above. It was then used in a PCR amplification with primers NL1 (5′-GCC ATA TCA ATA AGC GGA GGA AAA G-3′) and LS2 (5′-ATT CCC AAA CAA CTC GAC TC-3′) (Cocolin et al. 2000). A GC-clamp (5′-CGC CCG CCG CGC CCC GCG CCC GTC CCG CCG CCC CCG CCC G-3′) was attached to the forward NL1 primer when the PCR product was destined for DGGE analysis (Sheffield et al. 1989). PCR was performed in a final volume of 25 mL containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.5 mM $MgCl_2$, 0.2 mM deoxynucleoside triphosphates (dNTPs), 1.25 U of Taq Polymerase (Applied Biosystems, Milan, Italy) and 0.2 mM of each primer. Amplifications were carried out in a PTC-200 DNA Engine MJ Research thermal cycler (Biorad, Milan, Italy). The amplification cycle was denaturation at 95 °C for 1 min, annealing at 42 °C for 1 min and extension at 72 °C for 1 min,

and the cycle was repeated 35 times. The cycle was preceded by an initial denaturation at 95 °C for 5 min and followed by a final extension at 72 °C for 7 min. After agarose gel electrophoresis (2% in 1.25 X Tris-Acetate- EDTA), PCR products were analysed by DGGE, using the D-Code universal mutation detection system (Bio-Rad Laboratories), with a 0.8-mm thick polyacrylamide gel (8% (wt/vol) acrylamide-bisacrylamide (37.5:1)). A 30–50% denaturing gradient (100% corresponds to 7 mol urea and 40% (wt/vol) formamide), increasing in the direction of the electrophoretic run, was used. The run was undertaken at 60 °C using 130 V for 270 min. Gels were stained for 20 min in 1.25 X Tris-acetate- EDTA containing 1 X SYBR Green (Sigma, Milan, Italy). They were visualised under UV light, digitally captured and analysed with the UVIpro Platinum 1.1 Gel Software (Eppendorf, Milan, Italy) for the recognition of the bands present. Selected bands were carefully excised from the gel, re-amplified using the conditions described above but with an NL1 primer that did not contain the GC-clamp. The PCR product was sequenced and the sequence compared to the National Center for Biotechnology Information database for the identification of the bands.

2.3. Phenolic compounds screening and characterization

2.3.1. Extraction

Fruit materials recovered from peel, and outer, middle and inner pulp (Figure 1) were extracted sequentially using distilled water (WSE) and methanol/water (MWSE). For WSE preparation, hundred grams of sample was suspended in 100 mL of water and then chopped using a blender rotator. Extraction continued for 1 h under stirring conditions at room temperature. The supernatant was recovered by centrifugation (12,000 x *g*, 20 min at 4°C), filtered by using Whatman filter paper (0.22 µm) and stored at −20°C until further use. The same procedure was

followed for MWSE, but the samples were mixed with 100 mL of methanol/water solution (70/30, v/v).

2.3.2. Total phenolic compounds

Total phenolics were assayed according to Folin-Ciocalteu method (Singleton and Rossi, 1965). For each type of extracts, an aliquot of extracts of 20 µL was added to 1.58 mL distilled water in a spectrophotometer cuvette, afterward 100 µL of Folin-Ciocalteu reagent was added. The solution was mixed and allowed to equilibrate. After 8 min, 300 µL of sodium carbonate solution was added. The mixture was shaken at 40°C for 30 min. The absorbance was measured at 740 nm using UV-Visible spectrophotometer (Shimadzu PharmaSpec UV 1700 Bouble Beam UV-Vis Spectrophotometer, Japan). Data were expressed as mg gallic acid equivalent (GAE).

2.3.3. Agar well diffusion assay for antimicrobial susceptibility test

Bacterial and yeast strains used as indicators in this study and growth conditions are shown in Table 1. The antibacterial activity of peel and pulp extracts was assayed through the agar well diffusion assay (Schillinger and Lücke, 1989). Briefly, analyses were carried out on 15 mL of agar-H_2O (2%, w/v) overlaid with 5 mL of different soft agar media, which contained ca. 4.0 log CFU/mL of an overnight culture of the indicator microorganisms. Wells (5 mm in diameter) were cut into agar plates, and subsequently 50 µL of each extract was added. Plates were stored for 1 h at 4 °C to permit the radial diffusion of extracts, and then incubation was allowed at 30 or 37 °C for 24 - 48 h.

2.3.4. Minimum inhibitory concentration (MIC)

The antimicrobial activity of MWSE from the peel was further investigated through the broth micro-dilution assay (Rizzello et al., 2013) against the indicator strains that showed susceptibility during the agar well diffusion assay. The MWSE was concentrated through a

SpeedVac Concentrator (Thermo Scientific) and then diluted in broth media to a final concentration of 2000 mg/L gallic acid equivalent. Serial 2-fold dilutions were made with broth media in sterile tubes. Logarithmic-phase cells (ca. 8 Log CFU/mL) of each indicator microorganism were harvested by centrifugation (8,000 g for 10 min), washed twice with 10 mM phosphate buffer, pH 7.0, and adjusted to ca. 5 Log CFU/mL. The final concentration of phenolics in sterile tubes ranged from 1 mg/L to 2000 mg/L gallic acid equivalent. After incubation, bacterial growth was determined by plating each dilution on agar media. The MIC was defined as the lowest concentration of phenolics required to completely block the growth of the indicator microorganisms. Control tubes contained all the components except MWSE, which was substituted with distilled water (positive control) or chloramphenicol (100 μg/mL) as a negative control.

2.3.5. Identification and purification of phenolic compounds

Aiming at investigating the peel phenolic composition, the MWSE was analyzed through High Performance Liquid Chromatography (HPLC) using an Ultimate 3000 system equipped with a column Discovery C18 (250mm×4.6mm; 5μm). Solvent A (water/formic acid, 99.5/0.1, vol/vol) and B (methanol/water/formic acid, 99.5/0.1, vol/vol) were used for chromatographic separation. Samples were eluted with the following gradient: starting with A:B; 85:15 vol/vol, then linear gradient to 70% B in 25 min, then linear gradient till 95% B in 35 min maintained at 95% B for 5 min and equilibrate to initial mobile phase in 5 min. Twenty microliters of MWSE were injected, and elution was carried out at 35 °C with a flow rate of 1 mL/min. A scan mode ranging from 245 to 550 nm wavelength was used. Peaks were identified by comparison with relative retention times and UV spectra of pure standards and data reported on previous studies on sugar apples (Barreca et al., 2011; García-Salas et al., 2015; Huang et al., 2010; Santos et al., 2016). Calibration curves

were prepared using of pure standards. The calibration plots indicate good correlation between peak areas and analyte concentrations, and regression coefficients were higher than 0.99 in all cases. For those compounds where the standard was not available, concentrations were expressed as 4-hydroxybenzoic acid, tyrosol, catechin, quercetin, or rutin mg/L equivalents (García-Salas et al., 2015).

MESE obtained from peel was partially purified by reversed-phase high-performance liquid chromatography (RP-HPLC), using an Äkta purifier HPLC (GE Healthcare Bio-Sciences Corp., Piscataway, NJ) equipped with an Xterra MS C18 column (particle size, 5 µm; 4.6 by 250 mm; Waters, Brussels, Belgium) and a FRAC 920 automatic fraction collector (GE Healthcare). The gradient elution was at a flow rate of 0.8 mL/min, at room temperature. Eluent A consisted of 0.1% (vol/vol) formic acid in HPLC-grade water, and eluent B consisted of 0.1% (vol/vol) formic acid in acetonitrile (70%, vol/vol) and HPLC-grade water (29.9%, vol/vol). Extracts were eluted with the following gradient: 0% eluent B (10 min), 0 to 100% eluent B (120 min), 100% eluent B (8 min), 100 to 0% eluent B (5 min), and 0% eluent B (2 min). Sixty-eight fractions were recovered and subjected to antimicrobial activity using agar well diffusion assay. Fractions were further analyzed by HPLC-PDA for the identification and quantification of phenolics, as reported above.

2.4. Statistical analysis

All the microbiological and chemical analyses were carried out in triplicate for each batch of samples. Data were subjected to one-way ANOVA; pair-comparison of treatment means was achieved by Tukey's procedure at $p < 0.05$, using the statistical software Statistica for Windows (Statistica7.0 per Windows). Spearman's rank correlation matrix and p values were generated by cor.test and visualized by corrplot package (McKenna et al., 2016).

3. Results

3.1. Microbiological analysis

3.1.1. Enumeration of endophytic and epiphytic microorganisms

Epiphyte total aerobic bacteria and yeasts were detectable (1.9 ± 0.11 and 1.3 ± 0.12 Log CFU/mL, respectively) on peel of sugar apple fruit, whereas presumptive epiphyte lactic acid bacteria were not detectable in 10 g of sample (Table 2). When the peel was suspended in YPD and MRS and used as the enrichment media the number of epiphyte yeasts and presumptive LAB was ca. 7 and 8 Log CFU/mL, respectively. Cell densities of endophytes varied depending on fruit spatial distribution. Except for the outer of pulp, where no microbial group was detectable even after enrichment, both middle and inner fruit pulp harbored yeasts (Table 2).

3.1.2. Molecular identification of lactic acid bacteria and yeasts

All 65 presumptive epiphytic LAB were identified by partial sequencing of the 16S rRNA gene. Further differentiation between *E. faecalis* and *E. faecium* species was carried out through ddl-specific primer pairs (Dutka-Malen et al., 1995). *E. faecalis* was the only species found (Supplementary Tables S1). Whilst a total of 133 yeast isolates of which 66 as epiphytes were identified to the species level by PCR-RFLP of the ITS region and sequencing of the D1/D2 loop of the 26S rRNA encoding gene. By PCR amplification of the ITS region (comprising ITS1-5.8S rRNA-ITS2) all isolates gave a PCR product of either 850 or 750 bp length. The PCR products of 850 bp length, when subjected to digestion by restriction endonucleases *HinfI*, *HaeIII*, *CfoI* resulted in different profiles that allowed assemblage of the isolates in three groups. Representative isolates of each group were randomly chosen and subjected to sequencing. By sequence comparison 67 isolates from middle or inner pulp were identified as *Saccharomyces cerevisiae*. The PCR products of 750 bp in length from 66 epiphytic isolates, when subjected to digestion by

297 the same restriction endonucleases resulted in profiles that clustered the isolates in three groups.
298 Representative isolates of each group were randomly chosen and subjected to sequencing. By
299 sequence comparison the 66 isolates were identified as *Hanseniaspora uvarum*.

300 *3.1.3. PCR-DGGE analysis*

301 Due to a higher cell density and spatial distribution of yeasts compared to LAB, total DNA
302 extracted from different fruit parts was subjected to PCR-DGGE analysis to profile the yeast
303 populations harboring the fruit (Supplementary Figures S1-S3 and Supplementary Tables S2-S3).
304 Samples from inner pulp presented a band that by co-migration to a known reference was identified
305 as *S. cerevisiae*. *H. uvarum, Diaporthe maritime*, and *S. cerevisiae* were detected in samples from
306 outer and middle pulp. The bands of samples from peel were identified as *H. uvarum, D. maritime*
307 and *Penicillium* spp. Most samples presented two bands that corresponded to *Davidiella* spp. and
308 *Asimina triloba*.

309 **3.2. Phenolic compounds screening and characterization**

310 *3.2.1. Total phenolic compounds*

311 The analysis of total phenolic compounds was carried out on WSE and MWSE derived from pulp
312 and peel (Table 3). When water was used as extraction solvent, the total phenolic content in outer,
313 middle, and inner pulp extracts (370 ± 25 - 361 ± 42 mg/L gallic acid equivalent) was slightly, but
314 not significantly, higher ($P > 0.05$) than that in the peel (353 ± 70 mg/L gallic acid equivalent).
315 The use of methanol as solvent highly enhanced the extraction efficiency of the phenolic
316 compounds, notably in the peel extracts, which exhibited the highest amount (1043 ± 224 mg/L
317 gallic acid equivalent) ($P < 0.05$). The phenolic content in outer, middle, and inner pulp MWSE
318 (374 ± 53 - 363 ± 38 mg/L gallic acid equivalent) was almost similar to that of WSE ($P > 0.05$).

319 *3.2.2. Antimicrobial activity and minimum inhibitory concentration*

WSE and MWSE obtained from pulp or peel were also screened for their antimicrobial activity against a heterogeneous pool of indicator microorganism, both bacteria and yeasts isolated from different ecological niches (plants, insects, and humans), that included microorganisms of biotechnological interest and/or involved in complex relationships with the host organism (as pathogens, opportunists, or growth-promoters) (Table 1). Such an assorted group was aimed to maximize the relevance of the connections covered by this study between microorganisms and phytochemicals. The extracts hindered the growth of only 6 strains out of a total of 26 indicator microorganisms tested (Table 4). All extracts obtained from pulp did not show an inhibition zone when were incubated with all the indicators. A significant ($P < 0.05$) antibacterial activity of peel extracts was found towards *Staphylococcus aureus*, *Listeria monocytogenes*, *Bacillus megaterium* and *Lactococcus lactis* (Table 4). The antibacterial activity changed depending on the extraction solvent. Only MWSE extract, on the other hand, was able to inhibit *Weissella cibaria* and *Staphylococcus saprophyticus* (Table 4).

Based on the above findings, MWSE obtained from peel, which demonstrated the highest ($P < 0.05$) antimicrobial activity and total phenols content, was further investigated through the broth micro-dilution assay. The MWSE was concentrated until it reached a final concentration of 2000 mg/L gallic acid equivalent. The bacteriostatic effect of MWSE on pathogens, LAB and yeasts indicators was evaluated. After 48 h of cultivation on different agar media containing peel extract ranging from 1 to 2000 mg/L gallic acid equivalent, the MIC was determined. *Lc. lactis* was the most tolerant indicator to the peel extract (2000 mg/L gallic acid equivalent), followed by *St. aureus*, *L. monocytogenes*, *B. megaterium*, and *W. cibaria* which had the same MIC values of 1000 mg/L. The highest sensitivity to peel extract was found for *St. saprophyticus* (250 mg/L) (Table 4).

In order to highlight potential ecological implications for autochthonous yeasts, micro-dilution assay was also applied to 6 representative autochthonous yeast strains and 4 representative autochthonous lactic acid bacteria strains. Autochthonous endophytic yeast strains belonging to *S. cerevisiae* isolated from inner pulp (AN4Y30 and AN4Y7) and from middle pulp (AN3Y20 and AN3Y5) or epiphyte *H. uvarum* isolated from peel (AN1Y21 and AN1Y6), were also grown in YPD broth supplemented with concentrated MWSE from 1 to 2000 mg/L gallic acid equivalent. Compared to the control (only YPD broth), the low concentrations of MWSE did not significantly affect growth (data not shown), while the addition of MWSE at a concentration of 2000 mg/L gallic acid equivalent significantly ($P < 0.05$) increased the final cell density of *S. cerevisiae* AN4Y30, AN4Y7 and AN3Y5 by ca. two Log cycles, and *S. cerevisiae* AN3Y20, *H. uvarum* AN1Y21 and AN1Y6 by one Log cycle (Figure 2). No significant ($P > 0.05$) growth-promoting effect was detected against the 4 representative *E. faecalis* strains treated with MWSE (data not shown).

3.2.3. Identification and purification of phenolic compounds

Among low molecular weight polyphenols, 41 compounds were quantified in MWSE obtained from the peel (Table 5). Separation of phenolic compounds was carried out through HPLC-PAD. The highest peaks were identified based on their retention time, UV spectra, comparison with external standards and the related literature data (Barreca et al., 2011; García Salas et al., 2015; Huang et al., 2010; Santos et al., 2016). The most abundant family of phenolic compounds available in MWSE obtained from peel was flavan-3-ols. In particular, procyanidins, representing a group of condensed flavan-3-ols, were detected at high concentrations and with distinct types (A and B), forms (dimer, trimer and tetramer) and isomers. Fourteen procyanidins were quantified accounting for 399 ± 8.9 mg/L. Among procyanidins, tetramer type B (isomer 2) was the most

abundant (62.7 ± 1.1 mg/L), followed by dimer type B (isomer 1) (56.9 ± 1.2 mg/L) and trimer type B (isomer 3) (52.2 ± 0.9 mg/L). Other procyanidins were found at lower concentrations. Epicatechin (76.4 ± 1.4 mg/L) was the major flavan-3-ols derivative quantified in the MWSE from peel. Catechin (23.3 ± 0.9 mg/L) and catechin derivatives were also found at lower amounts (0.9 ± 0.0 to 53± 1.1 mg/L). Among flavonols detected in the MWSE from peel, the most abundant was rutin (isomer 2) (12.6 ± 0.8 mg/L), followed by calabricoside A (isomer 1) (7.14 ± 0.8). Other flavonols identified at low content were 3-betaglucopyranosyloxy-2-hydroxy-1-(4-hydroxy-3-methoxyphenyl) - propan-1-one rutin (isomers 1, 2 and 3) and rutin (isomer 1). Four compounds belonging to phenolic acids and their derivatives were identified, with lariciresinol-glucopyranoside (9.4 ± 0.9 mg/L) as the most abundant. Low amounts of sibiricose A, quinic acid,vanillic acid hexoside, and phenylethanoids were also identified (Table 5).

Aiming at identifying the phenolic compounds responsible for antimicrobial activity, MWSE obtained from the peel was subjected to RP-FPLC fractioning. In total, sixty-eight fractions were collected and evaluated by agar well diffusion assay for their efficacy toward *St. aureus*, which was the indicator that showed the greatest susceptibility to extracts (Table 4). Only nine fractions (4, 22, 23, 24, 25, 26, 28, 31 and 32) showed inhibition zones ranging from 0.33 ± 0.20 to 2.53 ± 0.26 mm (Figure 3A). Fractions were subjected to HPLC-PDA analysis. Fraction 31 contained catechin diglucopyranoside and rutin (isomer 2). Fraction 28 showed the presence of unidentified catechin derivatives (compounds 2 and 5), calabricoside A (isomer 2), and procyanidin dimer type B (isomer 1). Citric acid (isomer2), catechin, and 3-betaglucopyranosyloxy-2-hydroxy-1-(4-hydroxy-3-methoxyphenyl) - propan-1-one (isomers 2) were detected in fraction 4. Several catechin derivatives and the highest procyanidins content were attributed to fraction 26. Other fractions showed varying concentrations among phenolic acids, procyanidin derivatives and

389 catechin derivatives. The strongest antimicrobial activity was assigned to fraction 31, followed by
390 fractions 28 (1.84 ± 0.27 mm), 4 (1.67 ± 0.24 mm) and 26 (1.51 ± 0.28 mm), whereas the lowest
391 activity was found for fraction 24.

392 *3.2.4. Correlation between phenolic compounds and antimicrobial activity*

393 Aiming to determine the correlations between the identified phenolic compounds in the fractions
394 and the antimicrobial activity against *St. aureus*, a correlation matrix was established based on
395 spearman correlation coefficients (Figure 3B). Most procyanidins demonstrated a negative
396 correlation with the antimicrobial activity, particularly procyanidin trimer and tetramer type B
397 (isomer 2) whereas only procyanidin dimer type B (isomer 1) had a strong positive correlation.
398 Catechin derivatives, as predominant compounds among the fractions, showed different
399 correlations. Epicatechin was strongly and negatively correlated with the antimicrobial activity
400 whereas unidentified catechins derivatives (2 and 6) and catechin diglucopyranoside showed an
401 opposite correlation. Rutin (isomer 2) exhibited a significant ($P < 0.05$) positive correlation with
402 the antimicrobial activity. A positive but not significant ($P > 0.05$) correlation was found for citric
403 acid (isomer 2) whereas a negative correlation was observed for calabricoside A (isomer 1) and
404 lariciesinol-glucopyranoside (Figure 3B).

405

406 **4. Discussion**

407 Advances in plant-microbe interactions have shown that plants do not interact randomly with
408 microbes but choose their specific partners for interaction (Bulgarelli et al., 2012; Chan et al.,
409 2018; Pontonio et al., 2018; Tlais et al., 2022). Being a treasure house of bioactive phytochemicals,
410 sugar apple fruit deserves to be fully explored especially in view of the essential interaction
411 between microbiota and phytochemicals, contributing to plant health, management of postharvest

412 diseases and plant therapeutic properties, as well as for innovative food applications (Baskaran et
413 al., 2016; Leite et al., 2021; Manochai et al., 2018). To best of our knowledge, this is the first study
414 reporting a full panel of phenolics from *A. squamosa* as drivers that may affect the epiphyte and
415 endophyte fruit microbiota.
416 With respect to the large microbial biodiversity of tropical fruits (Ruiz Rodríguez et al., 2019;
417 Tenea et al., 2020), only a single species of epiphytic lactic acid bacteria, *Enterococcus faecalis,*
418 was identified from sugar apple fruit after enrichment. *E. faecalis* is usually found in the most
419 common tropical fruits (Di Cagno et al., 2013; Filannino et al., 2020; Thligene et al., 2015). On
420 the other side, sugar apple fruit seemed to be a preferred niche for yeasts. *S. cerevisiae* occupied
421 the middle and inner pulps of *A. squamosa.* Detection of endophytic microorganisms in fruits is
422 not uncommon (Di Cagno et al., 2010; Krishnan et al., 2012; Madbouly et al. 2020; Ren et al.,
423 2019;). The modes of penetration may be various (Kumar et al., 2021), but the most likely is
424 through the floral apparatus. *S. cerevisiae* has high capacity to cope with harsh environmental
425 circumstances due to the various survival strategies adopted by this species (Parapouli et al., 2020),
426 and it was previously reported as a vital yeast species due its prominent role as plant growth
427 promoting and biocontrol agent for sustainable agriculture in harsh conditions (Mukherjee et al.,
428 2020). *H. uvarum* was detected as epiphyte on the peel of *A. squamosa*. Among non-
429 *Saccharomyces*, *H. uvarum* is the frequent yeast epiphyte inhabitant in various plant trees and
430 fruits (e.g., plum, cherry, apple, apricot, peach, and grapes) (Jolly et al., 2014; López et al., 2015),
431 and its significant antagonistic traits against molds causing fruit deterioration are well known
432 (Albertin et al., 2016; Apaliya et al., 2017). When it comes to microbiota associated with Annona
433 plant, only studies regarding the characterization of endophyte fungi have been reported. Several
434 parts of the plant harbors different fungal species that have the potential to produce bioactive

435 metabolites with therapeutic features (Kouipou Toghueo and Boyom, 2019; Minarni et al., 2017;
436 Ola et al., 2014, 2020; Yunianto et al., 2012). Based on our direct analysis of samples by PCR-
437 DGGE, *Davidiella* spp., *Diaporthe maritime*, and *Penicillium* spp. were found in sugar apple fruit.
438 The diversity of endophytic fungi isolated from sugar apple is highly dependent on the plant part
439 and the growing season. For instance, *Diaporthe* spp. were identified mainly in leaves and twigs
440 of Annona (Lin et al., 2010), while *Penicillium* spp. were the dominant fungi in the root bark of
441 the plant (Kouipou Toghueo and Boyom, 2019). Most of these fungi are well known as fungal
442 pathogens of plants. The pathogenicity of endophytes may be related to various biotic interactions,
443 environmental factors, and, in particular, metabolites that would be evolved probably modifying
444 the behavior of harmful microbial symbionts to maintain them in a nonpathogenic state (Babalola
445 et al., 2020; Bacon and White, 2016). Chemical analysis on *Diaporthe* spp. isolated from Annona
446 squamosa revealed the detection of known flavomannin-6,6'-di-O-methyl ether, which strongly
447 inhibited the growth of *Staphylococcus pneumonia* and showed moderate activity against the
448 multidrug-resistant clinical isolate *St. aureus* (Ola et al., 2014). Whilst an extract of *Penicillium*
449 spp. isolated from Annona root bark showed potent anti-plasmodial potential against *Plasmodium*
450 *falciparum* strains, the most lethal *Plasmodium* species in humans (Kouipou Toghueo and Boyom,
451 2019; Wells, 2013).
452 Based on our findings, we hypothesized that the microbiota of *A. squamosa* results from the
453 interaction of several drivers, which include also phenolics (Filannino et al., 2019). The total
454 phenolics content in the fruit pulp and peel, as determined by Folin-Ciocalteu assay, revealed a
455 distinct content and distribution of phenolics. Although the water-soluble extract yielded almost
456 same values in the pulp and peel, the methanol/water soluble extract exhibited considerably higher
457 values at the peel level. The varied chemical structure of phytochemicals determines their

458 solubility. Thus, the type of solvent plays a major factor during the extraction of phytochemicals,
459 both in terms of quantity and quality of solubilized compounds, with consequences on the
460 bioactivities of the extract. The extraction yield depends greatly on polarity of solvent, and it
461 generally increases in the order of methanol > ethanol > water (Sridhar et al., 2021). It is worth
462 noting that the peel of edible and medicinal fruit plant species, as primary defense of fruit against
463 the outside environment, possessed higher levels of phenolics, carotenoids and vitamin C,
464 comparing to other fruit parts (De Pascual-Teresa et al., 2010). In accordance with our results, the
465 peel of different *Annona* spp. exhibited higher content of phytoconstituents compared to the pulp
466 (Akomolafe and Ajayi, 2015; García-Salas et al., 2015; Shehata et al., 2021).
467 Sugar apple pulp extracts did not show any inhibition against all indicators used in this study. Our
468 culture dependent approach revealed the absence of culturable LAB and a limited diversity of
469 yeasts in the pulp, conveying the hypothesis of pulp as a hard to access or hostile environment for
470 bacterial and yeast, although this is not attributable to phenolic compounds. In contrast to our
471 result, previous study assigned to the extracts from sugar apple pulp a moderate antimicrobial
472 activity against various pathogens, including *Escherichia coli* and *St. aureus* (Shehata et al., 2021).
473 On the other hand, the same study, credited a stronger antimicrobial activity to the peel extract
474 than pulp extract, which is highly consistent with our finding. Under the condition of our study,
475 some Gram-positive bacteria (*St. aureus*, *St. saprophyticus*, *L. monocytogenes*, *B. megaterium*)
476 were susceptible to peel extracts, although among LAB only *Lactococcus lactis* and *Weissella*
477 *cibaria* were inhibited.
478 Despite its exceptional antimicrobial features, information on phenolics pattern of sugar apple peel
479 is limited when compared to other parts of *A. squamosa* plant such as leaves, pulp and seeds or
480 other *Annona* spp. (Cagnini et al., 2021; Patil et al., 2019). At this regard, we investigated the

phenolics profile of sugar apple peel through HPLC-PAD analysis. Most of them were previously reported as constituents of *Annona cherimola* peel (Barreca et al., 2011). Huang et al. (2010) identified few isomers of procyanidins, catechins and epicatechins in the peel extract of sugar apple. Based on our finding, more procyanidin and catechin derivatives were detected at relatively high levels together with calabricoside A and some rutin derivatives. Procyanidins and catechins contain benzene rings and other functional groups which might explain their bactericidal effect (Gopal et al., 2016; Taylor et al., 2005). Their potential effect was confirmed by positive spearman correlation with the antimicrobial activity against *St. aureus*. The same features are attributed to epicatechin which was major unit components in the tannins inhibiting the growth of *St. aureus* (Jie Shi et al., 2020), although our spearman matrix showed a negative correlation. Besides, rutin and citric acid, as constituents of sugar apple peel phenolic profile, were highly reported in literature as effective inhibitor of *St. aureus* and *E. coli* (Al-Shabib et al., 2017; Bai et al., 2019; Blando et al., 2019).

E. faecalis, which was detected in the epiphytic microbiota of *A. squamosa* was well adapted to the phenolics from the peel. The antimicrobial potential of phenolics on LAB has been less thoroughly investigated than the effects on pathogenic bacteria and has been variously described by the available studies. Phenolic rich extracts of oregano, Japanese knotweed, and pomegranate peel did not show any inhibitory effect on LAB except for *Lactobacillus bulgaricus* (Chan et al., 2018). Ten wine phenolic compounds were claimed to have different inhibitory effect against *Lactiplantibacillus plantarum* strains (Landete et al., 2007). On the contrary, pomegranate juice rich in antioxidant phenolics, was reported to be suitable for LAB fermentation especially with *L. plantarum* strains (Filannino et al., 2013). In addition, the antibacterial effects of several pure phenolic compounds, mainly benzoic acid, catechin and epicatechin on LAB resulted as species-

and strain-dependent (Cueva et al, 2010). Similarly, the effect of phenolics on yeast survival is still controversial (Kimani et al., 2021; Tlais et al., 2021), suggesting that the adaptation of yeasts to fruit ecosystems is species and strains-specific, and markedly varies depending on the plant niche. Under the condition of our study, the phenolics of *A. squamosa* peel extract did not show any inhibitory response against different yeast species. On the other hand, concentrated peel extract stimulated the planktonic growth of autochthonous *S. cerevisiae* and *H. uvarum* yeasts during growth in YPD medium. By examining the conflicting information available in the literature (Anghel, 2019; Mekoue Nguela et al., 2019; Orozco et al., 2012; Rapeanu et al., 2014), we can assume that the bio-stimulant effect of phenolics on yeast is strongly dependent on the concentration and type of compounds. With regards to the mechanism underlying such effect, it remains unresolved, as several mechanisms can be hypothesized. A protective effect against oxidative stress can be ascribed to the radicals-scavenging activity of phenolics (Belinha et al., 2007). Beyond the well-defined antioxidant potential, a role as signaling molecules was previously proposed for phenolic acids and flavonoids (Chabot et al., 1992). Absorption of phenolics on cell surface layer contribute to stimulate cellular metabolic activity by the binding of microelements, precursors, growth factors, and modifies the overall cell reactivity towards oxygen (Rapeanu et al., 2014; Salmon, 2006). We cannot rule out that carbohydrates moiety resulting from the hydrolysis of phenolic glycosides may represent an additional carbon source available for yeast growth.

Under the Annona fruits ecosystems, phenolics likely play multiple roles: growth-promotion of yeasts, and inhibition of some Gram-positive bacteria, which can result in selective pressure in favor of phenolics-adapted lactic acid bacteria. Focusing on the consequences for the host plant, phenolics of sugar apple peel are hypothesized to favor growth-promoting microorganisms, as well to protect the plant from microbial pathogens colonization. Accordingly, these phenolics,

particularly procyanidins, catechin and epicatechin have recently been demonstrated as promising natural biocontrol agents.

5. Conclusion

Our study provided a comprehensive view about the microbial and chemical composition of sugar apple fruit, revealing it as a highly selective environment owing to a specific spatial distribution of microorganisms and phenolic compounds. While we confirmed the inability of sugar apple phenolics to inhibit the growth of many LAB and yeasts, we also demonstrated peel phenolics as an effective weapon in hindering several of several Gram-positive bacteria, some of which are pathogenic for humans, and promoting the growth of autochthonous yeasts on the other. We related the antimicrobial activity of methanol/water soluble phenolics from peel to specific compounds (procyanidin dimer type B [isomer 1], rutin [isomer 2], catechin diglucopyranoside), in addition to unidentified catechin derivatives.

Figure captions

Figure 1. Extraction of microbiota from sugar apple fruit (*Annona squamosa* L.). Endophytic microbiota were isolated from outer pulp (1), middle pulp (2) and inner pulp (3). Epiphytic microbiota were isolated from the surface of the peel (4).

Figure 2. Growth (Log CFU/mL) assay of autochthonous yeast strains isolated from sugar apple fruit (*Annona squamosa* L.) in Yeast extract Peptone Dextrose broth supplemented with peel methanol/water-soluble (MWSE) extract (2000 mg/L gallic acid equivalent). Data are the means (± SD) of three independent experiments analyzed in triplicate. Data were subjected to one-way ANOVA followed by Tukey's procedure at $P < 0.05$. Bars and line with different superscript letters differ significantly ($P < 0.05$).

Figure 3. Halo of inhibition diameter (mm) evaluated through the well diffusion assay of partially purified fractions (FR) obtained from the methanol-water soluble extract of peel from sugar apple fruit (*Annona squamosa* L.) (A). Data are the means (± SD) of three independent experiments analyzed in triplicate. Data were subjected to one-way ANOVA followed by Tukey's procedure at $P < 0.05$. Bars and line with different superscript letters differ significantly ($P < 0.05$). Spearman's rank correlation matrix between the compounds identified in the partially purified fractions obtained from the methanol-water soluble extract of peel from sugar apple fruit and the antimicrobial activity (B). Large and small circles indicate strong and weak correlations, respectively. Colors of the scale bar describe the type of correlation: 1 indicates a perfect positive correlation (dark blue) and -1 indicates a perfect negative correlation (dark red) between two microbial populations. The significance P values were not corrected by FDR and were represented by (*) < 0.05, (**) < 0.01, (***) < 0.001.

Supplementary material

Figure S1. DGGE gel of PCR products, obtained with primers NL1GC-LS2 from DNA extracted directly from the fruit samples. H: PCR product obtained from a pure culture of *Hanseniaspora uvarum*; S: PCR product obtained from a pure culture of *Saccharomyces cerevisiae,* used as reference. Numbers indicate bands that were excised from the gel and subjected to sequencing. Identification results are presented in Supplementary Table S2.

Figure S2. Representative profiles of PCR products obtained by amplification of the ITS1- 5.8S-ITS2 region. Lanes 1 and 13: 1 Kb molecular weight marker, Lanes 2, 4, 5, 12: PCR products with approximate size of 750 bp, Lanes 3 and 6-11: PCR products with approximate size of 850 bp. All isolates gave PCR products that were of either 750 bp or 850 bp size. All PCR products were

subsequently subjected to digestion by restriction enzymes as described in the materials and methods section.

Figure S3. Representative electrophoretic profiles obtained after digestion with three restriction endonucleases of the ITS1- 5.8S-ITS2 PCR product. Panel A: Restriction endonuclease *Hinf* I; two electrophoretic profiles were obtained among all isolates tested. Panel B: Restriction endonuclease *Hae* III; two electrophoretic profiles were obtained among all isolates tested. Panel C: Restriction endonuclease *Cfo* I; two electrophoretic profiles were obtained among all isolates. The molecular weight marker is 1Kb while the blue and yellow lines highlight the bands that allow grouping in different electrophoretic profiles for each restriction endonuclease used. Based on the results obtained from the digestion with the three restriction endonucleases, isolates were assembled in 3 groups.

Table S1. Results of partial sequencing of the 16S rRNA gene for representative lactic acid bacteria isolates.

Table S2. Identification results, based on sequence comparison to NCBI database, for bands excised from DGGE gel shown in Supplementary Figure S1.

Table S3. Results of sequencing of the D1/D2 loop of the 26S rRNA encoding gene for representative isolates from each of the two groups previously assembled by PCR-RFLP of the ITS1-5.8S-ITS2 region.

CRediT author statement

Ali Zein Alabiden Tlais: Investigation, Writing - Original Draft, Formal analysis. **Kalliopi Rantsiou:** Methodology, Investigation. **Pasquale Filannino**: Conceptualization, Methodology,

Project administration; Writing - Review & Editing. **Luca Simone Cocolin:** Methodology, Writing - Review & Editing. **Ivana Cavoski:** Conceptualization, Investigation, Formal analysis. **Marco Gobbetti:** Funding acquisition, Writing - Review & Editing. **Raffaella Di Cagno:** Conceptualization, Methodology, Supervision, Writing - Review & Editing.

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 paper.

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Table 1. Microorganisms and growth conditions used in this study as indicators to assay the antimicrobial activity of extracts obtained from pulp and peel of sugar apple fruit (*Annona squamosa* L.).

Indicator microorganism	Source	Culture collection	Growth conditions
Staphylococcus aureus DSM 20231	Human pleural fluid	DSMZ[a]	Trypticase soy yeast extract medium at 37°C
Staphylococcus saprophyticus PP2	*Opuntia ficus indica* L.	DISSPA[b]	Trypticase soy yeast extract medium at 37°C
Listeria monocytogenes ATCC 19115	Human	ATCC[c]	Brain heart infusion medium at 37°C
Escherichia coli DSM 30083	Human urine	DSMZ	Luria-Bertani broth at 37°C
Bacillus megaterium F6	Fresh vegetables	DISSPA	Luria-Bertani broth at 30°C
Lactococcus lactis C3	Cheese	DISSPA	M17 medium at at 30°C
Weissella cibaria SD21	Sourdough	DISSPA	MRS broth at 30°C
Leuconostoc lactis DSM 20202	Milk	DSMZ	MRS broth at 30°
Lactiplantibacillus plantarum POM1	*Solanum lycopersicum* L.	DISSPA	MRS broth at 30°C
Levilactobacillus brevis POM4	*Solanum lycopersicum* L.	DISSPA	MRS broth at 30°C
Furfurilactobacillus rossiae 2MR8	*Ananas comosus* (L.) Merr.	DISSPA	MRS broth at 30°C
Leuconostoc mesenteroides KI6	*Actinidia chinensis* Planch.	DISSPA	MRS broth at 30°C
Enterococcus faecalis PP6	*Opuntia ficus indica* L.	DISSPA	MRS broth at 30°C
E. faecalis PP7	*Opuntia ficus indica* L.	DISSPA	MRS broth at 30°C
Enterococcus casseliflavus DR5	*Drosophila* spp. gut	DISSPA	MRS broth at 30°C
E. casseliflavus DR2	*Drosophila* spp. gut	DISSPA	MRS broth at 30°C
Pantoea agglomerans SD48	Sourdough	DISSPA	Nutrient broth at 30°C
Serratia marcescens DR8	*Drosophila* spp. gut	DISSPA	Nutrient broth at 30°C
S. marcescens DR10	*Drosophila* spp. gut	DISSPA	Nutrient broth at 30°C

Table 1. Continued

Indicator microorganism	Source	Culture collection	Growth conditions
Debariomyces hansenii SD24	Sourdough	DISSPA	Sabouraud dextrose broth at 25°C
Hanseniaspora uvarum SD36	Sourdough	DISSPA	Sabouraud dextrose broth at 30°C
Pichia kudriavzevii SD16	Sourdough	DISSPA	Sabouraud dextrose broth at 30°C
Kodamaea ohmeri SD31	Sourdough	DISSPA	Sabouraud dextrose broth at 30°C
Saccharomyces cerevisiae SD30	Sourdough	DISSPA	Sabouraud dextrose broth at 30°C
Wickerhamomyces anomalus SD53	Sourdough	DISSPA	Sabouraud dextrose broth at 30°C
Torulaspora delbrueckii SD12	Sourdough	DISSPA	Sabouraud dextrose broth at 30°C

[a]DSMZ, Leibniz Institute German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany).

[b]DISSPA, Department of Soil, Plant and Food Sciences (University of Bari Aldo Moro, Bari, Italy).

[c]American Type Culture Collection (Manassas, VA, USA)

Table 2. Cell densities of total mesophilic bacteria, presumptive lactic acid bacteria and yeasts before and after enrichment counted from different parts of sugar apple fruit (*Annona squamosa* L.). Data are the means (± SD) of three independent experiments analyzed in triplicate.

Parts	Microbiota	Total aerobic bacteria (Log CFU/g or Log CFU/mL)	Presumptive lactic acid bacteria* (Log CFU g^{-1})	Presumptive lactic acid bacteria** (Log CFU/mL)	Yeasts* (Log CFU/g or Log CFU/mL)	Yeasts** (Log CFU/mL)
Peel	Epyphitic	1.9 ± 0.11	n.d.	8.78 ± 0.57	1.3 ± 0.12	7.4 ± 0.40
Outer pulp	Endophytic	n.d.	n.d.	n.d.	n.d.	n.d.
Middle pulp	Endophytic	1.7 ± 0.88	n.d.	n.d.	1.6 ± 0.75	6.0 ± 0.49
Inner pulp	Endophytic	2.1 ± 0.59	n.d.	n.d.	1.7 ± 0.89	6.0 ± 0.41

*, before enrichment; **, after enrichment; n.d., not detected in 10 g

925 **Table 3.** Total phenolics content (mg/L gallic acid equivalent) of water- (WSE) and methanol/water-soluble (MWSE) extracts obtained
926 from different parts of sugar apple fruit (*Annona squamosa* L.). Data are the means (± SD) of three independent experiments analyzed
927 in triplicate.

Parts	Total phenolics content (mg/L gallic acid equivalent)	
	WSE	**MWSE**
Peel	353 ± 70[b]	1043 ± 224[a]
Outer pulp	370 ± 25[b]	374 ± 53[b]
Middle pulp	363 ± 33[b]	368 ± 41[b]
Inner pulp	361 ± 42[b]	363 ± 38[b]

928 [a-b]Means with different superscript letters differ significantly ($P < 0.05$).

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Table 4. Antimicrobial activity[a] of different extracts from sugar apple fruit (*Annona squamosa* L.) and the minimum inhibitor concentration (MIC)[b] as mg/L gallic acid equivalent of the water- (WSE) and methanol/water-soluble (MWSE) extracts obtained only from peel and pulp.

Indicator microorganism	WSE		MWSE		MIC mg/L peel MWSE
	Peel	Pulp	Peel	Pulp	
	Well diffusion assay				Microdilution assay
Staphylococcus aureus DSM 20231	+++	-	+++	-	1000
St. saprophyticus PP2	-	-	+	-	250
Listeria monocytogenes ATCC 19115	++	-	+++	-	1000
Bacillus megaterium F6	++	-	+++	-	1000
Lactococcus lactis C3	+	-	++	-	2000
Weissella cibaria SD21	-	-	++	-	1000

[a]Inhibitory activity was scored as follows: -, no inhibition; +, halo of inhibition diameter of < 0.5, ++ halo of inhibition diameter of 0.5 to 2 mm; +++, halo of inhibition diameter of 2 to 4 mm.

[b]The final concentration of the total phenolics from MWSE ranged from 1 to 2000 mg/L gallic acid equivalent. Further details are included in Material and Methods. The data are from three independent experiment.

Table 5. Quantification of phenolics and other polar compounds (mg/L) by HPLC-PAD in methanol/water-soluble extracts (MWSE) from peel of sugar apple fruit (*Annona squamosa* L.).

	Compounds	**(mg/L)**
Phenolic acids and derivatives	Quinic acid	3.27 ± 0.6
	Vanillic acid hexoside	0.48 ± 0.01
	Lariciresinol-glucopyranoside	9.43 ± 0.9
	Sibiricose A3	3.41 ± 0.5
	Total	16.59 ± 1.2
Phenylethanoids	Hydroxytyrosol hexoside	1.52 ± 0.1
	Tyrosol hexoside pentoside	0.46 ± 0.1
	Total	1.98 ± 0.1
Procyanidins	Procyanidin dimer type A (isomer 1)	18.55 ± 0.5
	Procyanidin dimer type B (isomer 1)	56.87 ± 1.2
	Procyanidin dimer type B (isomer 2)	48.66 ± 1.1
	Procyanidin dimer type B (isomer 3)	40.86 ± 1.1
	Procyanidin trimer type B (isomer 1)	18.12 ± 0.4
	Procyanidin dimer type B (isomer 4)	2.95 ± 0.2
	Procyanidin tetramer type B (isomer 1)	22.80 ± 0.8
	Procyanidin tetramer type B (isomer 2)	62.73 ± 1.1
	Procyanidin trimer type B (isomer 2)	5.32 ± 0.2
	Procyanidin tetramer type B (isomer 3)	5.85 ± 0.2
	Procyanidin trimer type B (isomer 3)	52.24 ± 0.9
	Proanthocyanidin dimer type B (isomer 4)	33.17 ± 0.8
	Proanthocyanidin tetramer type B (isomer 4)	29.46 ± 0.4
	Procyanidin dimer type B (isomer 5)	1.39 ± 0.1
	Total	398.97 ± 8.9

946 **Table 5.** Continued

	Compounds	**(mg/L)**
Catechins and epicatechins	Catechin	23.32 ± 0.9
	Catechins derivatives-unidentified (1)	1.89 ± 0.2
	Catechins derivatives-unidentified (2)	4.41 ± 0.2
	Epicatechin	76.42 ± 1.4
	Catechins derivatives-unidentified (3)	1.19 ± 0.0
	Catechins derivatives-unidentified (4)	0.89 ± 0.0
	Catechins derivatives-unidentified (5)	53.11 ± 1.1
	Catechins derivatives-unidentified (6)	13.75 ± 0.8
	Catechins derivatives-unidentified (7)	1.68 ± 0.1
	Catechins derivatives-unidentified (8)	3.30 ± 0.2
	Catechins derivatives-unidentified (9)	1.42 ± 0.1
	Catechin diglucopyranoside	11.97 ± 0.4
	Total	193.35 ± 5.3
Quercetin and derivatives	3-betaglucopyranosyloxy-2-hydroxy-1-(4-hydroxy-3-methoxyphenyl)-propan-1-one (isomer 1)	2.47 ± 0.2
	3-betaglucopyranosyloxy-2-hydroxy-1-(4-hydroxy-3-methoxyphenyl)-propan-1-one (isomer 2)	3.17 ± 0.6
	3-betaglucopyranosyloxy-2-hydroxy-1-(4-hydroxy-3-methoxyphenyl)-propan-1-one (isomer 3)	1.33 ± 0.1
	Rutin (isomer 1)	0.71 ± 0.1
	Rutin (isomer 2)	12.60 ± 0.8
	Calabricoside A (isomer 1)	7.14 ± 0.8
	Calabricoside A (isomer 2)	0.81 ± 0.0
	Total	28.23 ± 1.9
Other polar compounds	Citric acid (isomer 1)	9.27 ± 0.8
	Citric acid (isomer 2)	2.65 ± 0.5

947 n.d., not detected

Figure 1

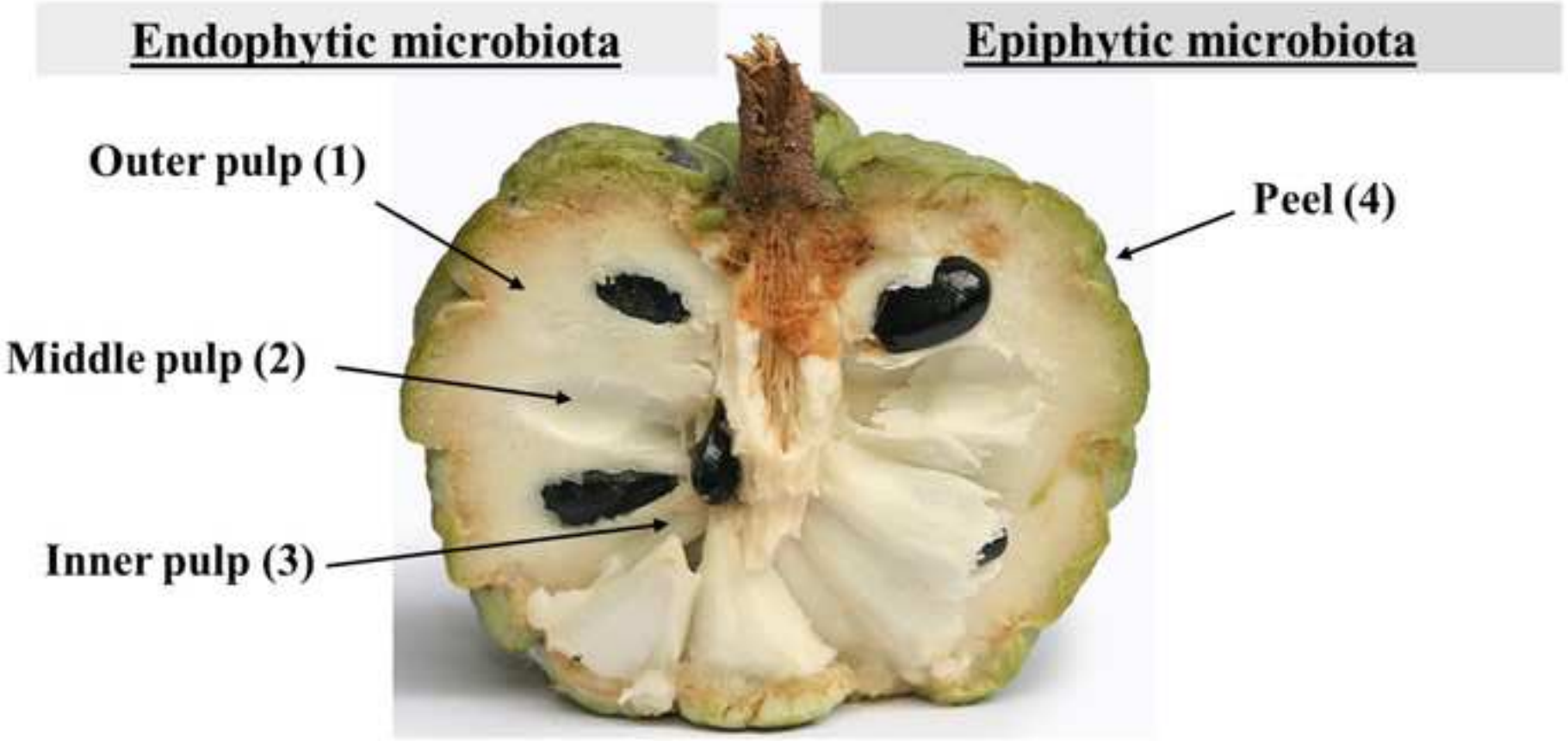


Figure 2

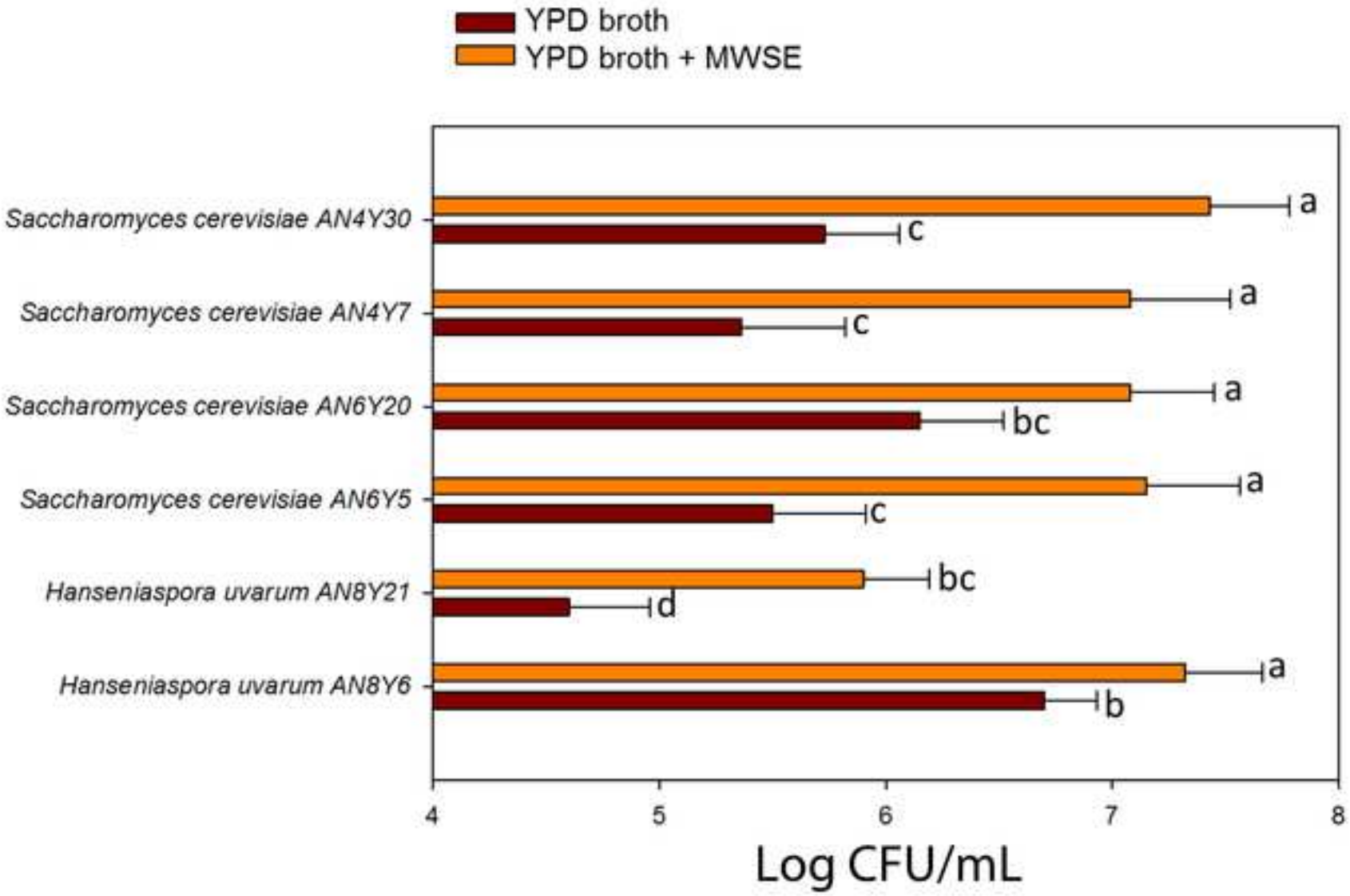
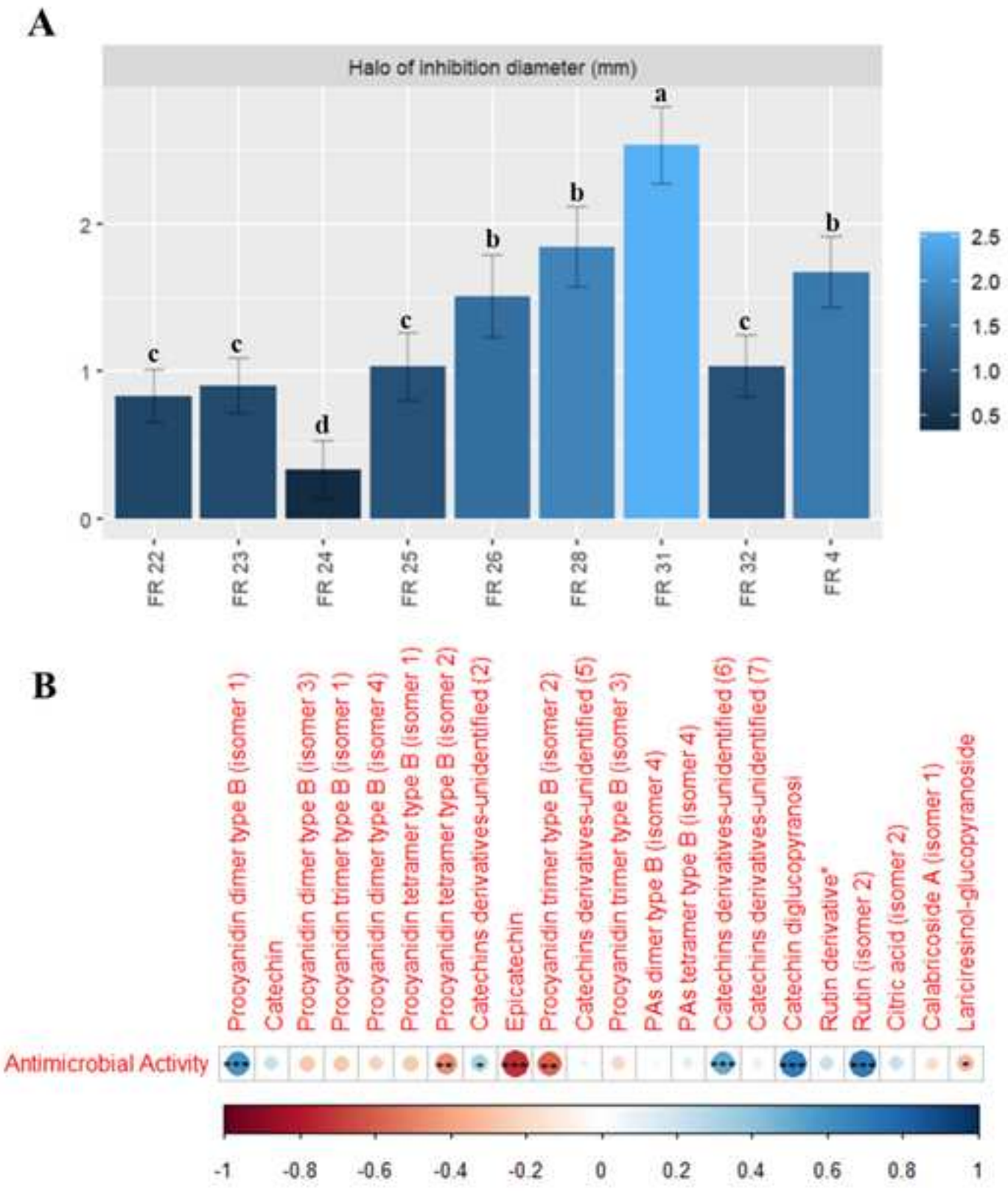


Figure 3



*, 3-betaglucopyranosyloxy-2-hydroxy-1-(4-hydroxy-3-methoxyphenyl) - propan-1-one (isomers 2)

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Supplementary Interactive Plot Data (CSV)

Supplementary Material.pdf

Declaration of interests

x 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 paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:
