Stimulation of saliva affects the release of aroma in wine; a study of microbiota, biochemistry and participant origin

Xinwei Ruan[1], Yipeng Chen[1], Aafreen Chauhan[1] and Kate Howell[1*]

[1]School of Agriculture, Food and Ecosystem Sciences, Faculty of Science, University of Melbourne, Parkville 3010, Australia

Xinwei Ruan: ORCID: 0000-0002-1963-3807

Yipeng Chen: ORCID: 0009-0002-7511-374X

Aafreen Chauhan: aschauhan@student.unimelb.edu.au

*** Corresponding author. khowell@unimelb.edu.au; ORCID: 0000-0001-6498-0472**

Highlights:

- **Saliva samples collected before, during and after stimulation are different in biochemical measures.**
- **Differences between three types of saliva were observed on the *ex-vivo* release of small aroma compounds from wine.**
- **Salivary microbiomes of Chinese and Australian participants are different.**
- **Specific correlations between salivary microbiomes and volatiles release from wine were found.**

Abstract

Saliva influences the release of aroma in the oral cavity. The composition of human saliva varies depending on stimulation; however, the compositional differences of saliva caused by stimulation and their influences on aroma release have not been fully evaluated. Humans display strong sensory preference for different aromas, with preference linked to country of origin, culture and personal history, yet how salivary composition is implicated in aroma release is not understood. In this study, we recruited healthy adults, of which 15 were Australian and 15 Chinese. Three types of saliva were collected from each participant: before, during, and after salivary stimulation. Ninety collected salivary samples were characterised by flow rate, total protein concentration, esterase activity and microbiome composition by full-length 16S rRNA gene sequencing. The saliva samples were mixed with wine to investigate the differences in released volatiles by headspace solid-phase microextraction gas chromatography - mass spectrometry. Differences in salivary composition and specific wine volatiles were found between Australian and Chinese participants, and amongst the three stimulation stages. Differential species were identified and significant correlations between the relative abundance of 3 bacterial species and 10 wine volatiles were observed. Our results confirm the influence of participant geographic origin and stimulation on salivary composition. Understanding the interactions of salivary components, especially salivary bacteria, on the release of aroma from wine allows nuanced appreciation of the variability of flavour perception in wine consumer groups.

Keywords: Microbiota, Salivary microbiome, Geographic variation, Aroma perception, Preference

1 Introduction

Saliva plays an important role in aroma release from foods and beverages (Feron et al., 2014; Genovese, Piombino, Gambuti, & Moio, 2009; Ployon, Morzel, & Canon, 2017). When mixing with saliva in oral cavity, volatile compounds are released from food matrix and then travel to olfactory receptors via a retronasal route. Physiochemically, saliva has a dilution effect on aroma due to continuous salivation (Taylor & Roozen, 1996). Saliva contains salt, sugar and the proteinaceous fraction of mucin, which may influence how volatiles partition in a solution (Friel & Taylor, 2001) and may exert further biochemical effects by degrading odorants or releasing aroma from aroma precursors (Buettner, 2002a, 2002b; Muñoz-González, Cueva, Ángeles Pozo-Bayón, & Victoria Moreno-Arribas, 2015). Two critical components of saliva, proteins and microbiota, are implicated with release or formation of aroma compounds and thus affect aroma release from food.

Proteins are able to bind, trap and interact with volatile compounds, thereby influencing the aroma release (Guichard, 2006). Two abundant proteins in whole human saliva, mucin and α-amylase, modify release of aroma compounds through hydrophobic interactions (Pagès-Hélary, Andriot, Guichard, & Canon, 2014). Pérez-Jiménez, Rocha-Alcubilla, and Pozo-Bayón (2019) demonstrated the efficacy of esterase in human saliva to degrade esters by comparing whole saliva with enzymatically inhibited saliva.

In contract to salivary proteins, the role of oral bacteria in aroma release is not well characterised. Oral microbiota contribute to the ability of human saliva to hydrolyse odourless grape glycosides (Muñoz-González et al., 2015). Piombino et al. (2014) identified the differences in salivary microbial profile between obese and normal-weight individuals, while no association was established with the differences in aroma release of wine. In recent years, the advances in culture-independent methods for oral microbiome identification have deepened our understanding of microorganisms in the oral cavity (Pedersen & Belstrøm, 2019; Wade, 2013) and reveal the salivary microbial composition with a stable core bacterial community shared by geographically diverse populations (Ruan, Luo, Zhang, & Howell, 2022). Increased accuracy of taxonomic identification would facilitate to study the role of salivary bacteria in aroma release.

Salivary protein and microbiota are likely to be dynamic during oral processing. Saliva production switches between stimulated and unstimulated depending on the presence of stimuli. During stimulation evoked by taste or mastication, the saliva secretion volume increases (Emmelin, 1972). More than 90% of saliva comes from three pairs of major salivary glands, including the submandibular, sublingual, and parotid glands. The salivary composition also varies due to the contribution of different glands during the stimulation (Proctor & Carpenter, 2007). It has been documented that parotid glands have a higher secretory rate under stimulation than in unstimulated conditions. Meanwhile, the submandibular and sublingual glands produce more saliva under

unstimulated conditions (Shannon, Suddick, & Chauncey, 1969). Recently, the published literature has offered contradictory findings about the changes in salivary bacterial profiles because of stimulation (Belstrøm et al., 2016; Goodstein, Bohlscheid, Evans, & Ross, 2014; Jo et al., 2019). Various salivary biochemical parameters have been observed between saliva collected before and during stimulation, including protein concentration, antioxidant activity (Neyraud, Palicki, Schwartz, Nicklaus, & Feron, 2012), esterase activity (Pérez-Jiménez, Muñoz-González, & Pozo-Bayón, 2021). Meanwhile, the composition of saliva collected after stimulation by food or other stimuli have not been characterised.

The microbiota and type of protein in saliva varies among populations and is shaped by the host characteristics, such as age (Mosca et al., 2019), gender (Bhuptani, Kumar, Vats, & Sagav, 2018), body mass index (BMI) (Burcham et al., 2020) and other lifestyle factors (Morita et al., 2005; Renson et al., 2019). The correlation of a host's ethnicity on the bacterial salivary composition has received considerable critical attention. Mason, Nagaraja, Camerlengo, Joshi, and Kumar (2013) investigated the oral microbial profile of 192 participants belonging to Chinese, Latinos, non-Hispanic whites, and non-Hispanic blacks, finding the lower alpha diversity of non-Hispanic blacks than the other three groups. Yang et al. (2019) observed the differences in overall microbial composition between African Americans and European Americans. A recent study documented different microbial alpha and beta diversity of supragingival plaque microbiome among 96 children from four ethnic groups, including African American, Burmese, Caucasian and Hispanic (Premaraj et al., 2020). A study conducted by our group demonstrated the differences between Australian and Chinese wine experts' salivary protein profile and their sensory responses (Luo et al., 2023). The mode of variation between Australian and Chinese saliva has not been established, but our preliminary data suggest that dietary differences might be correlated with these differences (data not shown).

Food preference is strongly influenced by a consumer's cultural, geographic and personal background (Kim, Petard, & Hong, 2018; Logue & Smith, 1986; Williamson, Robichaud, & Francis, 2012). The preferences of Australian and Chinese consumers for 14 red wines have been compared by Williamson et al. (2012), finding variance in some attributes for liking, such as "alcohol", "sweetness" and "dark fruit". Aroma perception is a prominent factor that influences the acceptability and preference of customers for different wines (Sáenz-Navajas et al., 2016). However, much uncertainty exists about the relationships between host factors, salivary composition, and aroma. We ask if the functional properties of diverse salivary microbiome communities can explain differing sensory perception amongst wine consumers.

To enhance our understanding of saliva composition and aroma perception, we analysed samples from 30 participants from Australia and China and mixed the saliva samples with wine to observe the ability of human saliva to release aroma compounds from wine. Each participant provided three types

of saliva samples collected before, during and after mechanical stimulation to extend our knowledge of reactions that occur in the oral cavity during stages of appreciating wine. Enzymatic activity tests and full-length 16S rRNA sequencing highlighted the differences of saliva type and origin of participants on salivary composition. Combining these findings with the wine aroma profile detected by headspace solid-phase microextraction gas chromatography-mass spectrometry (HS-SPME-GC-MS), we demonstrate differences in salivary proteins and microbiome due to the mode of salivary stimulation and how this varies based on geographic origin of the participant.

2 Material and Methods

2.1 Materials and reagents

All reagents and chemicals used in this study were of analytical grade. Methanol, citric acid, 4-Nitrophenyl acetate, sodium chloride, sodium phosphate, sodium bicarbonate, potassium chloride, potassium phosphate dibasic trihydrate, calcium chloride dihydrate, mucin from porcine stomach, phosphate-buffered saline, Tris-buffered saline, 4-octanol, amylase activity assay kit, and standards for volatile compounds detected in GC-MS analysis was obtained from Sigma-Aldrich (Castle Hill NSW, Australia). Micro bicinchoninic acid (BCA) protein assay kit was acquired from Thermo Fisher Scientific (Waltham, MA). Pure water was obtained from a Milli-Q system (Millipore Australia, Bayswater, Victoria, Australia). A Shiraz wine (Ladies Who Shoot Their Lunch; vintage 2019; 14.9% v/v alcohol) produced in Victoria, Australia, was purchased from a local wine producer, and used in all GC-MS assays with wine (section 2.6). This wine was selected because different aroma perception were observed between Chinese and Australian panellists in a previous sensory evaluation (Luo et al., 2023).

2.2 Study cohort

We recruited 30 healthy adults to meet the following criteria: (i) self-reported good systemic health (i.e., no diagnosed diabetes, cardiovascular diseases, endocrine disorders, or hypertension); (ii) no clinically diagnosed oral diseases; (iii) not receiving antibiotic or steroid hormone use within the previous three months; (iv) no smoking habits (less than several times a month); (v) not a regular drinker (less than several times a week); (vi) have a BMI index no higher than 24 kg/m^2. An online questionnaire was provided to all volunteers to ensure they met the inclusion criteria. Demographics were also acquired using self-reported questionnaires before the collection. Fifteen Chinese and fifteen Australian participants (aged 18-28) joined the study. The gender and BMI index of participants were balanced in each group **(Table S1)**. This study was approved by the University of Melbourne Human Research Ethics Committees (Review Reference Number: 2022-24220-29889-3).

2.3 Sample collection

Before collection, sampling procedures were explained in detail to all participants. Participants were asked to refrain from eating and drinking any beverage except water for an hour prior to providing saliva samples. Three types of saliva were collected before (Phase A), during (Phase B), and after (Phase C) stimulation. The collection procedure is illustrated in **Fig. S1**. For Phase A, participants naturally expectorated the unstimulated saliva into a 50 mL sterile collection tube until the volume reached 5 mL. For Phase B, participants were instructed to chew a piece of paraffin wax and expectorated the stimulated saliva into another collection tube in 5 minutes. Finally, after removing paraffin wax from the oral cavity, the participant repeated the saliva collection procedure described in Phase A. The saliva samples collected after stimulation were named as Phase C. Time was recorded in Phases A and C to calculate the flow rate as volume collected per minute (mL/min). For the same participant, Phase A, B, and C collections must be completed consecutively on the same day. Immediately after the collection, saliva samples were separated in smaller aliquots and kept frozen at -80 ^{o}C until analysis.

2.4 Biochemical analyses

Salivary total protein content, and enzymatic activities of 90 samples were tested individually to find the differences among different types of saliva. All analyses were performed in triplicates. Protein concentration of whole saliva samples was tested by Micro BCA™ Protein Assay Kit (Thermo Scientific™) using bovine serum albumin as standard for calibration.

For the enzymatic activity measurements, saliva samples were centrifuged at 15,000 ×g for 15 min at 4 °C to remove insoluble matter. All enzymatic activity assays were performed in triplicate. Total salivary esterase activity was conducted on individual samples according to the method from Pérez-Jiménez et al. (2021). α-Amylase activity assay was performed following the user manual of an Amylase Activity Assay Kit (Sigma-Aldrich), we pooled the samples for this assay because of the limited material for some participants.

2.5 Full length 16S rRNA gene sequencing

Bacterial genomic DNA was extracted from 90 whole human saliva samples using the QIAGEN® DNeasy® PowerSoil® Kit. The concentration of genomic DNA was measured using a Qubit HS DNA kit (Thermo Scientific™) and normalised to 1 ng/µL prior to Polymerase Chain Reaction (PCR). Following PacBio's recommended PCR protocol, full-length 16S amplicons were generated using the Platinum^{TM} SuperFi II High-fidelity PCR enzyme (Thermo Scientific™), with 2ng of input DNA. The amplicons generated were visualised on a 2% agarose gel and pooled. SMRTbell^{TM} cleanup beads (PacBio) were used to purify the pooled amplicons according to the PacBio protocol. The amplicon library was contracted with the SMRTbell® prep kit 3.0 library prep kit (PacBio) following

the protocol. The library was then polymerase-bounded using the Revio™ Polymerase Kit (PacBio) and loaded on a single-molecule real-time (SMRT) Cell 25M (PacBio). 16S rRNA full-length sequencing was performed for 30 hours on the PacBio Revio instrument.

2.6 Gas chromatography-mass spectrometry (GC-MS) analysis for whole human saliva

Different types of saliva samples were pooled together for the headspace solid-phase microextraction GC-MS analyses (HS-SPME-GC-MS) to overcome the inter-individual variation in saliva composition and the availability of saliva from each participant. Saliva was pooled based on stimulation phase, by participant origin, resulting in 6 groups of saliva samples; Australia-Phase A, Australia-Phase B, Australia-Phase C, Chinese-Phase A, Chinese-Phase B and Chinese-Phase C. Each sample type was measured in triplicate.

Three types of controls were used: the mixture of wine and water, the mixture of wine and artificial saliva, and the mixture of six pooled saliva samples and model wine. The mixture of wine and water were used to control for the dilution effect of saliva in the GC analysis matrix (Benjamin, Silcock, Beauchamp, Buettner, & Everett, 2012; Taylor & Roozen, 1996). An artificial saliva solution was spiked with wine to control for the effect of mucin and salts in aroma release. It was prepared according to the method of Genovese et al. (2009). The artificial saliva samples were freshly prepared by dissolving mucin (2.16 g/L), $NaHCO_3$ (5.208g/L), $K_2HPO_4 \cdot 3H_2O$ (1.369 g/L), NaCl (0.877 g/L), KCl (0.477 g/L) and $CaCl_2 \cdot 2H_2O$ (0.441 g/L) in Milli-Q water. The mixture of the model wine and six types of saliva were used to control for the confounder effect of the volatiles that may present in saliva itself. The model wine used in this study was prepared by saturating 12% ethanol (v/v) with potassium hydrogen tartrate, adjusting to pH 3.0 by 40% (w/v) tartaric acid. Each mixture was measured in triplicate.

The static headspace volatile extraction method was conducted following the method of Muñoz-González et al. (2014) with some modifications. The 20 mL GC vial was incubated at 37°C for 20 min to stabilise the temperature of the reaction environment. Then 5 mL of wine and 1 mL of pooled saliva were added to the vial with 10 μL of internal standard (100 mg/L 4-octanol). The wine-saliva mixture was incubated at 37 °C for 12 min. The headspace volatile extraction from the GC vial was performed by a 65 μL polydimethylsiloxane/divinylbenzene (PDMS/DVB) SPME fibre with 1 cm fibre length (Supelco, Bellefonte, PA) at 37 °C for 5 min.

GC-MS analysis was carried out with an Agilent 7890A GC system (Agilent Technologies, Mulgrave, VIC, Australia) coupled with an Agilent 5975C MS (Agilent Technologies). The fibre was desorbed at 220 °C in splitless mode at 8.7 psi for 30 s. Chromatographic separation of volatile compounds were performed with a DB-Wax polar capillary column (Agilent Technologies; 30 m × 0.25 mm × 0.25 μm film thickness). Helium was used as the carrier gas at a flow rate of 0.7 mL/min. After

initially holding the oven temperature at 40°C for 4 mins, it then ramped at 3°C/min to 220 °C, and held at 220 °C for 10 min. For the MS system, electron ionisation mode at 70 eV was used to record the electron impact mass spectra. The scanning mass range was from m/z 35 to 350. The temperature of the MS interface, MS source, MS quadrupole was 250, 230 and 150 °C.

The volatile compounds identification was based on the retention indices (RIs) with the facility of NIST library (version 11.0). RIs were obtained by calculating the retention times of alkane standards (C_5–C_{30}) on the same column. Peaks were semi-quantified using the internal standard, 4-octanol. The concentrations of volatiles were expressed as μg/L 4-octanol equivalence. Among the 27 compounds identified, sixteen compounds have also been quantified using external standards, facilitated by the calibration curve of standard compounds as:

$$Concentration\ (g/mL) = \left(\frac{peak\ area\ of\ identified\ compound}{peak\ area\ of\ internal\ standard} \times slope\right) + intercept$$

2.7 Statistical analyses

The data analyses of salivary biochemical parameters and the volatile compounds generated from the wine-saliva mixture were conducted in R (version 4.1.0). Student t-tests were used to examinate the significant ($p < 0.05$) differences between samples from Chinese and Australian participants. Two-way analysis of variance (ANOVA) with Tukey's comparison were performed to test the significant ($p < 0.05$) differences among saliva samples collected before, during and after stimulation. The Spearman's correlations were calculated with *rcorr* function in the R package *Hmisc* (Harrell, 2024).

For bioinformatic analyses, the demultiplexed PacBio HiFi full-length 16S rRNA gene sequences are quality filtered using QIIME2 (Bolyen et al., 2019). DADA2 (Callahan et al., 2016) was used to denoise the sequences into amplicon single variants (ASVs) using the function "dada2 denoise-ccs". A phylogenetic tree was generated from the *mafft* alignment. The naïve Bayesian machine learning based classification was performed using the expanded Human Oral Microbiome Database (eHOMD) version 15.23 (Escapa et al., 2018). If a species-level match was not found, it was further classified based on three other databases that successively refer to the next one. The three databases are GTDB r207, Silva v138 (Quast et al., 2013) and the NCBI RefSeq 16S rRNA database supplemented by the Ribosomal Database Project (RDP). The resulting objects were imported to R (version 4.1.0) for further analyses. Alpha diversity was calculated with Simpson's diversity index. Wilcoxon rank-sum tests were used to determine the statistically significant ($p < 0.05$) differences. Beta diversity was assessed using Bray-Curtis dissimilarly. Permutational multivariate analysis of variance (PERMANOVA) with 999 permutations was conducted to examine the statistically significant difference in beta-diversity using the *adonis2* function of *vegan* package (Oksanen et al., 2020). The differential taxa were defined from phylum level to species level by *metacoder* package (Foster, Sharpton, & Grünwald, 2017) with false discovery rate-adjusted Wilcoxon rank-sum test. ANCOM-

BC2 (Lin & Peddada, 2024) was performed to identify the differential species with the default parameters.

3 Results

3.1 Simulated saliva (Phase B) has a distinguishable protein and biochemical composition

3.1.1 Differences in saliva composition amongst the three phases

To determine whether human saliva produced before, during and after stimulation is different, the biochemical properties and microbial composition of human saliva samples were tested. The salivary flow rates were calculated as volume collected per minute, reflecting the speed of participants to produce saliva. *Ex vivo* measurements of α-amylase and esterase were conducted to assess enzymatic activity of the centrifuged saliva. The flow rate observed in Phase B and total salivary esterase activity are significantly higher than the other two phases, while Phase A and C saliva are not significantly different **(Fig. 1.A, E)**. For the total protein concentration of saliva samples, the value of Phase A is significantly higher than that of the other two phases **(Fig. 1.C)**. No significant differences were found among the three groups in the amylase activity **(Table S2)**.

Full-length 16S rRNA gene sequencing was used to detect the differences in the salivary microbiome. In contrast to the protein concentration and enzymatic activities, no significant differences were detected among Phases A, B and C regarding the alpha-diversity and beta-diversity of the microbial communities. Although several species have been identified as differential taxa **(Fig. S2)**. Most differential species have the lowest relative abundance in Phase B **(Fig. S2.A)**. The differential species identified in Phase A and C were clustered together in the heatmap **(Fig. S2.B)**, suggesting the microbial profile is distinct in Phase B at species level.

3.1.2 Effect of stimulation on the release of aroma from wine

To evaluate the ability of different saliva samples to release aroma compounds from wine, HS-SPME-GC-MS analyses were performed on the same wine sample spiked with saliva samples. Saliva samples were pooled into six groups: Australia-Phase A, Australia-Phase B, Australia-Phase C, Chinese-Phase A, Chinese-Phase B and Chinese-Phase C. After mixing the six pooled saliva samples with wine, twenty-seven volatile compounds were identified, consisting of 12 esters, 6 alcohols, 3 acids, 2 benzenoids and 4 terpenes **(Table 1)**. The wine sample was also mixed with water and artificial saliva as the control groups **(Fig. S3.A)**. For many compounds, especially esters, the addition of artificial saliva induced a significant decrease in their concentration compared to the wine-water mixture. Depending on the type of pooled saliva added, the decrease caused by artificial saliva can either be counteracted or further decreased **(Fig. S3.B)**. It suggests the whole human saliva induces other reactions with the wine matrix other than the effect of mucin.

As the volatiles may also have originated from the human oral cavity, another control was performed by mixing each pooled saliva sample with model wine. Among the detected volatiles, (–) – clovene was found in the mixture of saliva and wine and the mixture of saliva and model wine. Therefore, it was excluded from further analysis. For the 26 included volatiles, twenty-one volatiles were constantly detected across all six groups, while the other 5 volatiles were only occasionally found **(Table 1)**. Except for methyl hexanoate, the occasionally detected compounds are benzenoids and terpenes. These compounds are largely absent in unstimulated saliva in Phases A and C excepting limonene, which was not detected after mixing with Australian participants' Phase B saliva. The one-way ANOVA conducted with the concentration of 16 volatiles quantified using external standards is compatible with the results calculated using the internal standard equivalent concentration **(Table 1; Table S3)**.

The dataset was divided into two subgroups, Australian **(Fig. 2.A)** and Chinese **(Fig. 2.B)**, to show the changing trend for 21 constant-detected compounds among Phases A, B and C. The statistical analyses suggest that the Australian and Chinese groups individually have 9 and 8 compounds with significantly higher concentrations in Phase B than in the other two phases. Most of the distinguishing compounds are esters, while octanoic acid and 1,3-ditertiary butyl benzene show the same trend in either the Australian or both groups. In addition, several compounds demonstrated significant differences between Phase A and C, including diethyl succinate, 3-methylbutyl acetate, 3-ethyl-2-pentanol and acetic acid. 3-Ethyl-2-pentanol and acetic acid only showed a significant difference among phases in the Australian group, with significantly lower concentration in Phase A. Further, a significant difference was observed between the Australian and Chinese groups of these two compounds in Phase A **(Fig. 2.C)**.

To better understand the differential volatiles that contribute to the differences among the three phases, a principal component analysis (PCA) was performed on the 21 detected compounds shared amongst all samples **(Fig. 2.D)**. The internal standard equivalent concentration of the aroma compounds, (expressed as μg/L 4-octanol equivalents), distinguished the saliva samples from different phases. The first two principal components explained 68.57% of the variance. The first principal component (PC1) separated the samples of Phase B from those of Phase A and C. The samples of Phase B were highly associated with most esters, 1,3−ditertiarybutylbenzene and 3−ethyl−2−pentanol, positioned in the space defined by negative PC1. On the contrary, 1-butanol was positively related to PC1. The second principal component (PC2) was negatively associated with most alcohols, including 2-methyl-1-propanol, 3-methyl-1-butanol, 1-hexanol and 2,3-butanediol. Phase A and C samples were separated on PC2 with some overlap retained, suggesting the interaction between the two investigated covariates, phase, and subject origin **(Table S2)**. In addition, PCAs were conducted using a total 27 compounds and 16 compounds quantified using external standards, separately **(Fig. S4)**. These results are in agreement with the PCA constructed by the semi-quantified compounds.

3.2 The composition of saliva from Australian and Chinese participants is different

3.2.1 Differences in salivary proteins between two groups

The flow rate, total protein concentration, and activities of alpha-amylase and esterase of the saliva samples collected from Australian and Chinese participants were compared to investigate the influence of the host's cultural background on salivary composition. Although no differences were found between the flow rate of Australian and Chinese participants' saliva **(Fig. 1.B)**, the saliva of Australian participants demonstrated higher total protein concentration **(Fig. 1.D)**. For the enzymatic activities, the two groups demonstrated no statistical differences in either amylase or esterase activity **(Table S2; Fig. 1.F)**.

3.2.2 Differences in salivary microbiome profiles

For the comparison of the salivary microbial profile of participants from Australia and China, the full-length 16rRNA gene was sequenced. After denoising by DADA2, the samples were rarefied to 5,232, resulting in 11,052 amplicon sequence variants (ASVs). The Australian and Chinese groups differ in the alpha-diversity characterised by Simpson Index **(Fig. 4.A)**. Beta-diversity analysis conducted using Bray-Curtis dissimilarity also demonstrated the significant differences between these two groups (PERMANOVA, $p = 0.001$). The principal coordinate analysis (PCoA) result showed the separation between the microbial composition of samples from two groups **(Fig. 4.B)**.

When investigating the taxa that discriminate saliva samples from Australian and Chinese participants, we first compared the relative abundance of the two groups at phyla **(Fig. 3.A)** and genus levels **(Fig. 3.B)**. Firmicutes, the phylum with the highest relative abundance in both groups, showed higher relative abundance in the saliva samples from Australian participants. A higher relative abundance of *Veillonella* in Australian saliva was observed. We performed a Wilcoxon Rank Sum test with a false discovery rate correction to each taxon, spanning all taxonomic levels **(Fig. 3.C)**. Genus *Neisseria*, *Lautropia*, *Porphyromonas*, *Capnocytophaga*, *Cardiobacterium*, *Filifactor* and *Parvimonas* were higher in Chinese participants' saliva, with several species of these genre. All taxa belong to phyla Synergistetes and Spirochaetes, except the species were more prevalent in the Chinese group. In contrast, the saliva microbiota of Australian participants was distinguished mainly by taxa under the order *Veillonellales*, especially the genus *Veillonella* and *Megasphasera*. The genus with high abundances, such as *Streptococcus*, *Prevotella* and *Haemonphilus*, contain both species identified as biomarkers of Chinese and Australian participants.

We also used ANCOM-BC2 to identify the differential species between Australian and Chinese saliva to accomplish more precise results at the species level. We found 8 differential species for comparing two groups, mostly under the phylum *Bateroidetes* and *Firmicutes* **(Fig. 4.C)**. Among them, *Porphyromonas pasteri* and *Neisseria subflava* have higher relative abundance in the Chinese

group, which agrees with the results of the Wilcoxon Rank Sum tests **(Fig. 3.C)**. Similarly, the six differential species enriched in Australian samples identified by ANCOM-BC2 are also found in the Wilcoxon Rank Sum tests. In the Australian group, the differential species of Phase A and C were clustered together, suggesting they are closer to each other than Phase B. Meanwhile, the differential species in the Chinese Phase A and B samples were clustered.

3.2.3 Differences in saliva composition between groups are associated with aroma release

To investigate the aroma consequences of compositional differences in the saliva of saliva samples collected from Australian and Chinese participants, the volatile profile was collected by HS-SPME-GC-MS and compared. The result of two-way ANOVA showed that 11 compounds were released differently after mixing with saliva samples from two groups. Different concentrations due to the saliva samples collected at different phases were observed **(Fig. 4.E)**. All differential compounds showed higher concentration in the Chinese group, especially in unstimulated Phases A and C. Meanwhile, ethyl heptanoate released after mixing with Chinese saliva samples demonstrated higher concentration in Phases A and B.

We suspected that the salivary bacteria contributed to aroma release, and so we calculated the Spearman's rank correlation between the relative abundance of the species in pooled saliva samples and the aroma compounds released after mixing the wine samples with those saliva samples. We found three species significantly correlated to wine aroma profile, including *Streptococcus sp. HMT006*, *Prevotella shahii* and an unclassified species belongs to *Selenomonas* **(Fig. 4.D)**. The concentration of three differential esters, ethyl propanoate, 3-methylbutyl acetate and ethyl hexanoate, are positively associated with the relative abundance of *Streptococcus sp. HMT006*, while negatively associated with the relative abundance of the unclassified species of *Selenomonas* **(Fig. 4.F)**. The higher concentration of 3-ethyl-2-pentanol and several esters in the Chinese group has been positively correlated with the increased relative abundance of *Streptococcus sp. HMT006* in saliva samples from Chinese participants in Phase A. As the only benzenoid identified, the concentration of 1,3-ditertiarybutylbenzene showed a negative correlation with the unclassified species of *Selenomonas.* Although two-way ANOVA concluded that the concentration of 2,3-butanediol was not different between the Chinese and Australian groups, it was positively associated with the higher relative abundance of *Prevotella shahii* in the Chinese saliva.

4 Discussion

4.1 The composition of human saliva at different stimulation stages

Stimulated and unstimulated saliva were different in composition. We observed a higher total protein concentration in the saliva samples produced in Phase A. The lower protein concentration in Phase B

and C may be due to the dilution of the substantial water content secreted in Phase B (Lee & Linden, 1992). Interestingly, even though the flow rate dropped significantly after removing the stimulus from the oral cavity, the total protein concentration in saliva did not return to the level when the stimulus was not received. In contrast, a higher total salivary esterase activity was found in Phase B, while the esterase activity of Phases A and C had no significant difference. The esterase in saliva is an important enzyme for hydrolysing esters into carboxylic acids and alcohols (Buettner, 2002b). The higher esterase activity in stimulated saliva than in unstimulated saliva has also been observed by Pérez-Jiménez et al. (2021).

The association between oral microbiota and the type of saliva has been widely investigated but remains controversial. A previous study suggested a significant difference between unstimulated and stimulated saliva in both alpha and beta diversity (Gomar-Vercher, Simón-Soro, Montiel-Company, Almerich-Silla, & Mira, 2018). Meanwhile, Belstrøm et al. (2016) and Jo et al. (2019) reported no significant differences between the Shannon diversity index of bacteria in unstimulated and stimulated saliva samples. However, several species have been identified as differential among the mouth-rinse water, unstimulated saliva, and stimulated saliva by Jo et al. (2019), which agrees with our results. It is also possible that even if salivary microorganisms' composition differed during these three immediately adjacent phases, the differences could be too subtle to detect.

4.2 The influence of host origin on the composition of saliva

Emerging evidence has demonstrated the influence of the host's origin on salivary composition, especially the oral microbiome. To control the confounder effect of other host factors, we applied a series of selection criteria to ensure the two groups of participants have balanced gender and BMI. Although significant difference was identified in age of two groups, all participants in this study were aged between 18 to 28. The differences induced by host origin have been attributed to genetic or external factors, such as diet, altitude, climate, or other lifestyle factors (Hao et al., 2021; Liu et al., 2021; Luo et al., 2023; Ruan et al., 2022). Differences in cultural background and lifestyle habits between Asian countries and Western countries have led to interest in understanding the differences in salivary composition between different countries. Kim et al. (2009) compared the oral microbiome of 90 South Korean participants and 88 German participants, concluding that the microbial profiles of Caucasian and Asian ethnic-origin participants are significantly different. Our previous study collected salivary microbiome data from 47 publicly available datasets, reporting the differences in beta diversity of saliva samples from three geographic locations, including North America, Europe, and China (Ruan et al., 2022). Notably, the variance explained in the previous study could be confounded by external factors other than the differences among three populations, such as climate and collection methods. To better understand the differences between two participant groups, the current study effectively eliminated the variance caused by geographic location by collecting all the

saliva samples from the same location within a short period. *Veillonella* spp. was identified as the differential taxa that was higher in Western samples, which was consistent with the result of our current study. Among species in this genus, *Veillonella atypica* was identified as a biomarker to differentiate people with obesity from normal-weight individuals (López-Dávalos, Requena, Pozo-Bayón, & Muñoz-González, 2023) , underlying a potential association among diet, body weight and ethnicity.

The association between ethnicity and salivary protein composition is far from conclusive. Mosca et al. (2019) compared protein concentration, flow rate, amylolytic activity, and lipolytic activity of unstimulated saliva samples from 15 Chinese and 15 Caucasian participants living in the Netherlands. Like the current study, they focused on the difference in saliva samples between Chinese and residents of a country defined as "Western countries". However, they observed higher total salivary protein concentrations in Chinese than in Caucasian participants. This discrepancy between two studies could be attributed to the differences between the Western countries selected. Australian and Dutch residents could have specific differences in diets and lifestyles, leading to the different biochemical parameters of saliva. Meanwhile, no significant differences in flow rate and amylolytic activity was detected by Mosca et al. (2019), which is consistent with our findings. A recent study in our group demonstrated a significant difference in the concentration of proline-rich proteins and lipocalin-1 in saliva samples from Chinese and Australian participants (Luo et al., 2023). Further proteomic analyses on samples collected in the current study would facilitate the observation of the differences on specific protein concentrations; we suspect that differences in published studies might also be due to the collection of stimulated or unstimulated saliva (or merging of these two classes).

4.3 The contribution of saliva on the release of diverse aromas

This study demonstrates the association between host factors, salivary composition, and aroma release during wine consumption. For the two parameters controlled in this study, the phase of saliva production and the host's ethnic background, we simultaneously observed the differences in salivary composition and the aroma profiles of wine after mixing with saliva samples. The salivary stimulation stages and subject origin both influence aroma release ability as the composition of saliva is different.

The relationship between the stimulation stages and aroma release ability was found to be focused on the esters in wine and the esterase activity in human saliva. Esters are an essential group of aromatic compounds to contribute fruitiness to wine (Ferreira, López, Escudero, & Cacho, 1998). Most esters detected in this study have the odour activity values greater than 1 **(Table S3)**, meaning that these compounds are likely to have a sensory impact. Saliva samples produced in Phase B demonstrated the highest esterase activity and induced the highest concentration of esters released from wine samples. However, the presence of esterase was previously associated with the hydrolysis of esters (Ployon et al., 2017) and the generation of carboxylic acids (Pérez-Jiménez et al., 2019). Esters are relatively less

persistent in the oral cavity after swallowing, probably due to the modification by oral microbiota directly or with enzymatic action (Goodstein et al., 2014; Muñoz-González, Pérez-Jiménez, & Pozo-Bayón, 2020). This rather contradictory result may be explained by the presence of reactions other than enzymatic hydrolysis of the esters. In addition, the total salivary esterase activity could be attributed to different types of enzymes, such as carboxylesterases, carbonic anhydrases, trypsin, and cholesterol esterases (Pérez-Jiménez et al., 2021). The catalytic effect of specific enzymes could be accompanied by concurrent synthesis of esters. Interestingly, the same phenomenon was reported in our recent study by Luo et al. (2023), which strengthens our assumption that perhaps more than one type of reaction involving esterases occurs when human saliva mixes with esters in wine.

During retronasal perception of wine, two distinct types of aroma release are perceived by consumers: the immediate impression produced when swallowing and the prolonged perception after swallowing (Buettner, 2004). The first type of perception has been widely investigated by various studies, especially by *ex vivo* simulating the mixing procedure of human saliva and wine (Genovese et al., 2009; Mayr et al., 2014). The prolonged retronasal aroma perception, also called 'after-odour' or 'aroma persistence', has received increased attention. Several real-time *in vivo* monitoring methods, such as intra-oral SPME sampling (Esteban-Fernández, Rocha-Alcubilla, Muñoz-González, Moreno-Arribas, & Pozo-Bayón, 2016), have been introduced to measure the aroma release in this phase. Our study sheds light on the role of human saliva in immediate and prolonged retronasal perception of aroma and has introduced unstimulated saliva as a control in *ex vivo* experiments.

By comparing the saliva samples of Phase B and Phase C with the control, our study demonstrates that it is important to specify the type of saliva collected and used in *ex vivo* experiments. Stimulated saliva needs to be specified for the research targeting the reactions with stimulus. Meanwhile, although we demonstrated the different aroma release capacities of saliva samples collected during and after the mechanical stimulation, more complex mechanisms are introduced in aroma perception occurs during the wine tasting experience of human beings. Mixing the saliva samples collected after the mechanical stimulation with wine could not completely reflect the complexity and dynamics of "after-odour" occurring in human oral cavity. We set the stopping point for the collection of Phase C as the time that volume reaches 5 mL to allow enough time for saliva to change from "stimulated" to "unstimulated". However, after swallowing the wine, various amounts of odorants will be captured by oral mucosa and keep working as "stimulus" in mouth (Esteban-Fernández et al., 2016). Therefore, the changes of saliva from "stimulated" to "unstimulated" could not be as abruptly as what we mimicked using paraffin chewing. The proportion of wine and saliva used in our static headspace SPME method does not reflect all the consequences of oral processing to explain aroma production in the 'after-taste' period. Further analytic approaches to simulate the relationships between post-stimulated saliva and wine, include modelling the oral mucosa (Ployon, Brulé, Andriot, Morzel, & Canon, 2020), and should be used to further understand the role of saliva in the after-taste phase.

Our *ex vivo* results show that it is likely that Australian and Chinese have different aroma compounds released in the oral cavity. Our previous study reported that people from Australia and China have different sensory responses to the same wine (Luo et al., 2023) and reported increased levels of esters in the mixture of Chinese saliva and wine. According to the results of current study, the prevalence of *Streptococcus* sp. HMT 066 has been associated with the concentration of esters released. It has been reported that *Streptococcus* spp. have the ability to release aroma from odourless precursors (Muñoz-González et al., 2015). 2,3-butanediol is the second most abundant by-product generated during the alcoholic fermentation of wine, contributing to its bitterness (Romano, 1997). The higher 2,3-butanediol concentration found in our study using wine and saliva from the Chinese group positively correlates with the relative abundance of *Prevotella shahii* **(Fig. 4.F)**. *Prevotella* spp. has been described as a potential contributor to aroma perception by enzymatic activity (Parker et al., 2020). Nevertheless, within genera like *Streptococcus* and *Prevotella*, the function of different species varies. Functional annotation and taxonomic profiling with higher resolution at special or even stain level could be done to differentiate the bacteria belongs to these two genera. Further experiments, such as strain isolation, are required to verify the function of specific species. *Veillonella* spp. have been found different in relative abundance between hosts from China and Western countries in both a previous study (Ruan et al., 2022) and the current study. Although no correlation was found between *Veillonella* spp. and any aroma compounds in this study, more research on *Veillonella* could help understand the differences in aroma perception between Chinese and Western people.

5 Conclusion

The two critical components of human saliva, salivary proteins and microbiota, contribute to the aroma perception of wine. In this study, we demonstrated that saliva secreted before, during and after stimulation differs in composition and the ability of the three types of saliva to release aroma compounds from wine varies. Although Phase A and C have more in common than Phase B, there are still some differences in their composition and the role in aroma release. The enzymatic activity of esterase may explain the variation in the ability of human saliva to release aroma compounds attributed to stimulation. Another important finding of this study was the difference in salivary composition, especially salivary microbiota, between Australian and Chinese participants. Several differential species were identified by full-length 16S rRNA sequencing. We took samples from 30 people in this study, which is a relatively small number, yet our findings mirror data collected in other studies. For example, our findings of a differential relative abundance of *Veillonella* spp. contributes additional evidence to support this genus as a biomarker to differentiate people originating in different geographic areas. The volatiles released from the wine-saliva mixture of these two groups highlight the associations between salivary microbiota and aroma release. Our findings serve as a basis for future studies targeting the relationship between salivary composition, aroma perception, and wine preference. A better understanding of the variations in wine preference gives insights into designing

products for specific consumer groups and should be complemented in the future by sensory tests to verify the findings in the human oral cavity.

Ethics approval and consent to participate

The study is under the approval of the University of Melbourne Human Research Ethics Committees (Review Reference Number: 2022-24220-29889-3). All participants provided written informed consent.

Data availability

The sequencing data generated and analysed in the present study are available in NCBI BioProject database, PRJNA1077481(https://www.ncbi.nlm.nih.gov/bioproject/1077481).

CRediT authorship contribution statement

Xinwei Ruan: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Visualization, Writing – original draft, Writing – review & editing. **Yipeng Chen:** Data curation, Formal analysis, Investigation, Methodology, Writing – review & editing. **Aafreen Chauhan:** Data curation, Formal analysis, Investigation, Writing – review & editing. **Kate Howell:** Conceptualization, Methodology, Project administration, Supervision, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no competing financial interest.

Acknowledgements

This study was funded by the Faculty of Science at the University of Melbourne. XR and YC gratefully acknowledge a Melbourne Research Scholarship administered by the University of Melbourne. The authors would like to acknowledge all participants. The authors would also like to acknowledge the assistance of Zijian Liang to the sample collection process. The work of Dr Pangzhen Zhang to set up methodologies and verify volatile analysis is gratefully acknowledged.

Figure Legends

Fig. 1. The differences of salivary composition amongst three phases of collection and two participant groups. Boxplots showing the flow rate of (**A**) Phase A, B, C and (**B**) Australian and Chinese participants; the total protein concentration of (**C**) Phase A, B, C and (**D**) Australian and Chinese participants; the total salivary esterase activity of (**E**) Phase A, B, C and (**F**) Australian and Chinese participants. Significant differences ($p < 0.05$, Student's t-test; $p < 0.05$, one-way ANOVA) were tested and are marked with "*"; with NS denoting no significant differences observed.

Fig. 2. The aroma released from wine with saliva samples from three collection phases are different. Point plots with lines showing the change of each volatile released from (**A**) wine-Australia saliva mixture and (**B**) wine-Chinese saliva mixture. The green lines represent the compounds showing highest concentration in Phase B and have no significant differences between Phase A and C (one-way ANOVA, $p < 0.05$). Among them, light green lines represent compounds only significantly higher than Phase A, while dark green lines represent compounds significantly higher than both Phase A and C. The pink lines represent the compounds that also demonstrate significant differences between Phase A and C. The dark pink lines showing the compounds have significant differences among all three comparisons. The light pink lines showing the compounds have differential concentration between Phase A and B, and Phase A and C. The volatiles with significantly changes are indicate by letters: a) Ethyl acetate; b) Diethyl succinate; c) Ethyl hexanoate; d) 3-Methylbutyl acetate; e) Ethyl butanoate; f) Ethyl 3-methylbutanoate; g) Ethyl propanoate; h) Ethyl 2-methylbutanoate; i) Hexyl acetate; j) 3-Ethyl-2-pentanol; k) Acetic acid; l) 1,3-Ditertiarybutylbenzene; m) Octanoic acid. (**C**) Bar plots showing the concentration of 3-Ethyl-2-pentanol and Acetic acid in six subgroups. Significant differences ($p < 0.05$, Student's t-test) between Australian and Chinese group in Phase A are marked with "*". (**D**) Principal component analysis (PCA) plot demonstrating the wine aroma release after spiking with samples from three groups.

Fig. 3. The microbial profiles of Australian and Chinese participants' saliva samples are different. Comparison of the salivary microbial composition between two groups in stacked bar plots at (**A**) phylum level and (**B**) genus level. (**C**) Taxonomic hierarchies showing the enrichment of taxa from two groups from phylum to species level. The taxa with significant differences (false discovery rate-adjusted Wilcoxon rank-sum $q < 0.05$) were coloured with green (higher in Australian group) and pink (higher in Chinese group).

Fig. 4. The differences in salivary microbial composition between Australian and Chinese participants associate with the differences in aroma released after mixing with wine. (**A**) Comparison of the alpha-diversity between two groups, representing by Simpson's Diversity Index (Wilcoxon rank-sum test; $p = 0.029$). (**B**) Principal coordinate analysis (PCoA) with Bray-Curtis dissimilarity showing the difference between two groups (PERMANOVA; $p = 0.001$). (**C**) and (**D**) Heatmap represents the mean relative abundance of (**C**) eight differential species identified by ANCOM-BC2 and (**D**) three species that significantly correlated ($p < 0.01$, FDR-adjusted Spearman's rank correlation) with aroma compounds in six pooled saliva groups. (**E**) Bar plots showing the log-transformed concentration (μg/L 4-octanol equivalence) of aroma compounds detected in two groups. The coloured squares on the bottom indicate the result of two-way ANOVA. (**F**) Spearman's correlation heatmap between aroma compounds and relative abundance of species. Significant correlated pairs ($p < 0.01$, FDR-adjusted Spearman's rank correlation) are labelled with "*".

References

Belstrøm, D., Holmstrup, P., Bardow, A., Kokaras, A., Fiehn, N. E., & Paster, B. J. (2016). Comparative analysis of bacterial profiles in unstimulated and stimulated saliva samples. *J Oral Microbiol, 8*, 30112.

Benjamin, O., Silcock, P., Beauchamp, J., Buettner, A., & Everett, D. W. (2012). Tongue Pressure and Oral Conditions Affect Volatile Release from Liquid Systems in a Model Mouth. *Journal of Agricultural and Food Chemistry, 60*(39), 9918-9927.

Bhuptani, D., Kumar, S., Vats, M., & Sagav, R. (2018). Age and gender related changes of salivary total protein levels for forensic application. *J Forensic Odontostomatol, 36*(1), 26-33.

Bolyen, E., Rideout, J. R., Dillon, M. R., Bokulich, N. A., Abnet, C. C., Al-Ghalith, G. A., . . . Caporaso, J. G. (2019). Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. *Nature Biotechnology, 37*(8), 852-857.

Buettner, A. (2002a). Influence of Human Saliva on Odorant Concentrations. 2. Aldehydes, Alcohols, 3-Alkyl-2-methoxypyrazines, Methoxyphenols, and 3-Hydroxy-4,5-dimethyl-2(5H)-furanone. *Journal of Agricultural and Food Chemistry, 50*(24), 7105-7110.

Buettner, A. (2002b). Influence of Human Salivary Enzymes on Odorant Concentration Changes Occurring in Vivo. 1. Esters and Thiols. *Journal of Agricultural and Food Chemistry, 50*(11), 3283-3289.

Buettner, A. (2004). Investigation of Potent Odorants and Afterodor Development in Two Chardonnay Wines Using the Buccal Odor Screening System (BOSS). *Journal of Agricultural and Food Chemistry, 52*(8), 2339-2346.

Burcham, Z. M., Garneau, N. L., Comstock, S. S., Tucker, R. M., Knight, R., Metcalf, J. L., . . . Genetics of Taste Lab Citizen, S. (2020). Patterns of Oral Microbiota Diversity in Adults and Children: A Crowdsourced Population Study. *Scientific Reports, 10*(1), 2133.

Callahan, B. J., McMurdie, P. J., Rosen, M. J., Han, A. W., Johnson, A. J. A., & Holmes, S. P. (2016). DADA2: High-resolution sample inference from Illumina amplicon data. *Nature Methods, 13*(7), 581-583.

Emmelin, N. (1972). CONTROL OF SALIVARY GLANDS. In N. Emmelin & Y. Zotterman (Eds.), *Oral Physiology* (pp. 1-16): Pergamon.

Escapa, I. F., Chen, T., Huang, Y., Gajare, P., Dewhirst, F. E., & Lemon, K. P. (2018). New Insights into Human Nostril Microbiome from the Expanded Human Oral Microbiome Database (eHOMD): a Resource for the Microbiome of the Human Aerodigestive Tract. *mSystems, 3*(6), 10.1128/msystems.00187-00118.

Esteban-Fernández, A., Rocha-Alcubilla, N., Muñoz-González, C., Moreno-Arribas, M. V., & Pozo-Bayón, M. Á. (2016). Intra-oral adsorption and release of aroma compounds following in-mouth wine exposure. *Food Chemistry, 205*, 280-288.

Feron, G., Ayed, C., Qannari, E. M., Courcoux, P., Laboure, H., & Guichard, E. (2014). Understanding Aroma Release from Model Cheeses by a Statistical Multiblock Approach on Oral Processing. *PLOS ONE, 9*(4), e93113.

Ferreira, V., López, R., Escudero, A., & Cacho, J. F. (1998). The aroma of Grenache red wine: hierarchy and nature of its main odorants. *Journal of the Science of Food and Agriculture, 77*(2), 259-267.

Foster, Z. S. L., Sharpton, T. J., & Grünwald, N. J. (2017). Metacoder: An R package for visualization and manipulation of community taxonomic diversity data. *PLOS Computational Biology, 13*(2), e1005404.

Friel, E. N., & Taylor, A. J. (2001). Effect of Salivary Components on Volatile Partitioning from Solutions. *Journal of Agricultural and Food Chemistry, 49*(8), 3898-3905.

Genovese, A., Piombino, P., Gambuti, A., & Moio, L. (2009). Simulation of retronasal aroma of white and red wine in a model mouth system. Investigating the influence of saliva on volatile compound concentrations. *Food Chemistry, 114*(1), 100-107.

Gomar-Vercher, S., Simón-Soro, A., Montiel-Company, J. M., Almerich-Silla, J. M., & Mira, A. (2018). Stimulated and unstimulated saliva samples have significantly different bacterial profiles. *PLOS ONE, 13*(6), e0198021.

Goodstein, E. S., Bohlscheid, J. C., Evans, M., & Ross, C. F. (2014). Perception of flavor finish in model white wine: A time-intensity study. *Food Quality and Preference, 36*, 50-60.

Guichard, E. (2006). Flavour retention and release from protein solutions. *Biotechnology Advances, 24*(2), 226-229.

Hao, Y., Tang, C., Du, Q., Zhou, X., Peng, X., & Cheng, L. (2021). Comparative analysis of oral microbiome from Zang and Han populations living at different altitudes. *Archives of Oral Biology, 121*, 104986.

Harrell, F. E. J. (2024). Hmisc: Harrell Miscellaneous. Retrieved from https://CRAN.R-project.org/package=Hmisc

Jo, R., Nishimoto, Y., Umezawa, K., Yama, K., Aita, Y., Ichiba, Y., . . . Fukuda, S. (2019). Comparison of oral microbiome profiles in stimulated and unstimulated saliva, tongue, and mouth-rinsed water. *Sci Rep, 9*(1), 16124.

Kim, S., Petard, N., & Hong, J. (2018). What is lost in translation: A cross-cultural study to compare the concept of nuttiness and its perception in soymilk among Korean, Chinese, and Western groups. *Food Research International, 105*, 970-981.

Kim, T. S., Kang, N. W., Lee, S.-B., Eickholz, P., Pretzl, B., & Kim, C. (2009). Differences in subgingival microflora of Korean and German periodontal patients. *Archives of Oral Biology, 54*(3), 223-229.

Lee, V., & Linden, R. (1992). An olfactory-submandibular salivary reflex in humans. *Experimental Physiology, 77*(1), 221-224.

Lin, H., & Peddada, S. D. (2024). Multigroup analysis of compositions of microbiomes with covariate adjustments and repeated measures. *Nature Methods, 21*(1), 83-91.

Liu, K., Chen, S., Huang, J., Ren, F., Yang, T., Long, D., . . . Huang, X. (2021). Oral Microbiota of Children Is Conserved across Han, Tibetan and Hui Groups and Is Correlated with Diet and Gut Microbiota. *Microorganisms, 9*(5), 1030.

Logue, A. W., & Smith, M. E. (1986). Predictors of food preferences in adult humans. *Appetite, 7*(2), 109-125.

López-Dávalos, P. C., Requena, T., Pozo-Bayón, M. Á., & Muñoz-González, C. (2023). Decreased retronasal olfaction and taste perception in obesity are related to saliva biochemical and microbiota composition. *Food Research International, 167*, 112660.

Luo, J., Ruan, X., Ang, C.-S., Nolvachai, Y., Marriott, P. J., Zhang, P., & Howell, K. (2023). Variation of wine preference amongst consumers is influenced by the composition of salivary proteins. *npj Science of Food*, *7*(1), 51.

Mason, M. R., Nagaraja, H. N., Camerlengo, T., Joshi, V., & Kumar, P. S. (2013). Deep Sequencing Identifies Ethnicity-Specific Bacterial Signatures in the Oral Microbiome. *PLOS ONE*, *8*(10), e77287.

Mayr, C. M., Parker, M., Baldock, G. A., Black, C. A., Pardon, K. H., Williamson, P. O., . . . Francis, I. L. (2014). Determination of the Importance of In-Mouth Release of Volatile Phenol Glycoconjugates to the Flavor of Smoke-Tainted Wines. *Journal of Agricultural and Food Chemistry*, *62*(11), 2327-2336.

Morita, E., Narikiyo, M., Yokoyama, A., Yano, A., Kamoi, K., Yoshikawa, E., . . . Sasaki, H. (2005). Predominant presence of Streptococcus anginosus in the saliva of alcoholics. *Oral Microbiol Immunol*, *20*(6), 362-365.

Mosca, A. C., Stieger, M., Neyraud, E., Brignot, H., van de Wiel, A., & Chen, J. (2019). How are macronutrient intake, BMI, ethnicity, age, and gender related to the composition of unstimulated saliva? A case study. *J Texture Stud*, *50*(1), 53-61.

Muñoz-González, C., Cueva, C., Ángeles Pozo-Bayón, M., & Victoria Moreno-Arribas, M. (2015). Ability of human oral microbiota to produce wine odorant aglycones from odourless grape glycosidic aroma precursors. *Food Chemistry*, *187*, 112-119.

Muñoz-González, C., Feron, G., Guichard, E., Rodríguez-Bencomo, J. J., Martín-Álvarez, P. J., Moreno-Arribas, M. V., & Pozo-Bayón, M. Á. (2014). Understanding the Role of Saliva in Aroma Release from Wine by Using Static and Dynamic Headspace Conditions. *Journal of Agricultural and Food Chemistry*, *62*(33), 8274-8288.

Muñoz-González, C., Pérez-Jiménez, M., & Pozo-Bayón, M. Á. (2020). Oral persistence of esters is affected by wine matrix composition. *Food Research International*, *135*, 109286.

Neyraud, E., Palicki, O., Schwartz, C., Nicklaus, S., & Feron, G. (2012). Variability of human saliva composition: Possible relationships with fat perception and liking. *Archives of Oral Biology*, *57*(5), 556-566.

Oksanen, J., Blanchet, F. G., Friendly, M., Kindt, R., Legendre, P., McGlinn, D., . . . Wagner, H. (2020). vegan: Community Ecology Package. R package version 2.5-7. Retrieved from https://CRAN.R-project.org/package=vegan

Pagès-Hélary, S., Andriot, I., Guichard, E., & Canon, F. (2014). Retention effect of human saliva on aroma release and respective contribution of salivary mucin and α-amylase. *Food Research International, 64*, 424-431.

Parker, M., Onetto, C., Hixson, J., Bilogrevic, E., Schueth, L., Pisaniello, L., . . . Francis, L. (2020). Factors Contributing to Interindividual Variation in Retronasal Odor Perception from Aroma Glycosides: The Role of Odorant Sensory Detection Threshold, Oral Microbiota, and Hydrolysis in Saliva. *Journal of Agricultural and Food Chemistry, 68*(38), 10299-10309.

Pedersen, A. M. L., & Belstrøm, D. (2019). The role of natural salivary defences in maintaining a healthy oral microbiota. *Journal of Dentistry, 80*, S3-S12.

Pérez-Jiménez, M., Muñoz-González, C., & Pozo-Bayón, M. Á. (2021). Specificity of Saliva Esterases by Wine Carboxylic Esters and Inhibition by Wine Phenolic Compounds Under Simulated Oral Conditions. *Frontiers in Nutrition, 8*.

Pérez-Jiménez, M., Rocha-Alcubilla, N., & Pozo-Bayón, M. Á. (2019). Effect of saliva esterase activity on ester solutions and possible consequences for the in-mouth ester release during wine intake. *Journal of Texture Studies, 50*(1), 62-70.

Piombino, P., Genovese, A., Esposito, S., Moio, L., Cutolo, P. P., Chambery, A., . . . Ercolini, D. (2014). Saliva from Obese Individuals Suppresses the Release of Aroma Compounds from Wine. *PLOS ONE, 9*(1), e85611.

Ployon, S., Brulé, M., Andriot, I., Morzel, M., & Canon, F. (2020). Understanding retention and metabolization of aroma compounds using an in vitro model of oral mucosa. *Food Chemistry, 318*, 126468.

Ployon, S., Morzel, M., & Canon, F. (2017). The role of saliva in aroma release and perception. *Food Chemistry, 226*, 212-220.

Premaraj, T. S., Vella, R., Chung, J., Lin, Q., Hunter, P., Underwood, K., . . . Zhou, Y. (2020). Ethnic variation of oral microbiota in children. *Scientific Reports, 10*(1), 14788.

Proctor, G. B., & Carpenter, G. H. (2007). Regulation of salivary gland function by autonomic nerves. *Autonomic Neuroscience, 133*(1), 3-18.

Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., . . . Glöckner, F. O. (2013). The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic Acids Res, 41*(Database issue), D590-596.

Renson, A., Jones, H. E., Beghini, F., Segata, N., Zolnik, C. P., Usyk, M., . . . Dowd, J. B. (2019). Sociodemographic variation in the oral microbiome. *Annals of Epidemiology, 35*, 73-80.e72.

Romano, P. (1997). *Metabolic characteristics of wine strains during spontaneous and inoculated fermentation*. Paper presented at the Food Technology and Biotechnology.

Ruan, X., Luo, J., Zhang, P., & Howell, K. (2022). The salivary microbiome shows a high prevalence of core bacterial members yet variability across human populations. *npj Biofilms and Microbiomes, 8*(1), 85.

Sáenz-Navajas, M.-P., Avizcuri, J. M., Echávarri, J. F., Ferreira, V., Fernández-Zurbano, P., & Valentin, D. (2016). Understanding quality judgements of red wines by experts: Effect of evaluation condition. *Food Quality and Preference, 48*, 216-227.

Shannon, I. L., Suddick, R. P., & Chauncey, H. H. (1969). Effect of atropine-induced flow rate depression on the composition of unstimulated human parotid fluid. *Archives of Oral Biology, 14*(7), 761-770.

Taylor, A. J., & Roozen, J. P. (1996). Volatile flavor release from foods during eating. *Critical Reviews in Food Science and Nutrition, 36*(8), 765-784.

Wade, W. G. (2013). The oral microbiome in health and disease. *Pharmacological Research, 69*(1), 137-143.

Williamson, P. O., Robichaud, J., & Francis, I. L. (2012). Comparison of Chinese and Australian consumers' liking responses for red wines. *Australian Journal of Grape and Wine Research, 18*(3), 256-267.

Yang, Y., Zheng, W., Cai, Q., Shrubsole, M. J., Pei, Z., Brucker, R., . . . Blot, W. J. (2019). Racial differences in the oral microbiome: data from low-income populations of African ancestry and European ancestry. *mSystems, 4*(6), 10.1128/msystems. 00639-00619.

Table 1 Aroma compounds detected by headspace solid-phase microextraction gas chromatography mass spectrometry (HS-SPME-GC-MS) from the mixture of six types of pooled saliva samples and wine.

No.	Aroma Compound	RI-NIST *	RI-Actual	Internal standard (4-octanol) equivalence (μg/L)					
				Phase A		Phase B		Phase C	
				Australian	Chinese	Australian	Chinese	Australian	Chinese
	Esters								
1	Ethyl acetate	888	887	4646.99 ± 691.59^{c}	7449.38 ± 35.97^{a}	8822.61 ± 158.22^{a}	8606 ± 759.79^{a}	5935.32 ± 842.17^{bc}	7368.83 ± 103.25^{ab}
2	Ethyl propanoate	960	958	60.23 ± 4.97^{bc}	74.47 ± 2.46^{ab}	89.82 ± 2.16^{a}	87.58 ± 10.23^{a}	56.58 ± 7.30^{c}	71.78 ± 2.21^{bc}
3	Ethyl isobutyrate	962	959	153.31 ± 37.03^{a}	171.05 ± 54.71^{a}	120.65 ± 38.81^{a}	128.48 ± 38.89^{a}	123.29 ± 34.79^{a}	70.75 ± 61.53^{a}
4	Ethyl butanoate	1039	1035	49.91 ± 43.35^{d}	121.16 ± 3.14^{b}	151.59 ± 5.06^{a}	149.92 ± 17.20^{a}	93.75 ± 11.98^{cd}	120.11 ± 4.30^{bc}
5	Ethyl 2-methylbutanoate	1055	1050	41.49 ± 6.72^{c}	79.85 ± 2.41^{b}	102.68 ± 4.39^{a}	102.34 ± 11.04^{a}	56.79 ± 8.78^{c}	76.28 ± 2.18^{b}
6	Ethyl 3-methylbutanoate	1072	1067	48.24 ± 9.48^{d}	107.18 ± 5.42^{b}	137.85 ± 2.7^{a}	134.99 ± 11.07^{a}	72.17 ± 13.35^{c}	101.04 ± 4.98^{b}
7	3-Methylbutyl acetate	1126	1122	545.84 ± 89.42^{d}	1084.34 ± 9.03^{bc}	1302.7 ± 21.06^{ab}	1347.1 ± 143.51^{a}	765.84 ± 108.53^{d}	1066.07 ± 59.63^{c}
8	Methyl hexanoate	1190	1184	n.d.	2.88 ± 0.57^{bc}	3.58 ± 0.37^{ab}	4.06 ± 0.39^{a}	0.96 ± 0.32^{d}	2.36 ± 0.25^{c}
9	Ethyl hexanoate	1235	1233	851.85 ± 223.93^{d}	1977.03 ± 53.02^{bc}	2391.76 ± 66.67^{ab}	2478.48 ± 252.05^{a}	1259.94 ± 253.43^{d}	1830.77 ± 80.92^{c}
10	Hexyl acetate	1235	1232	7.02 ± 6.64^{c}	15.17 ± 3.00^{abc}	20.62 ± 0.86^{a}	21.65 ± 1.20^{a}	10.06 ± 2.16^{bc}	16.05 ± 1.80^{ab}
11	Ethyl heptanoate	1334	1332	0.09 ± 0.01^{b}	4.29 ± 3.68^{ab}	2.7 ± 4.18^{ab}	8.01 ± 2.27^{a}	0.2 ± 0.11^{b}	0.21 ± 0.16^{b}
12	Diethyl succinate	1681	1681	2752.88 ± 136.55^{a}	2256.51 ± 215.43^{b}	2471.91 ± 151.36^{ab}	2314.02 ± 68.01^{ab}	2557.77 ± 315.12^{ab}	2695.89 ± 66.46^{ab}
	Alcohols								
13	2-Methyl-1-propanol	1098	1096	493.94 ± 24.95^{a}	468.86 ± 20.24^{a}	521.16 ± 20.25^{a}	492.19 ± 50.54^{a}	491.19 ± 35.89^{a}	515.29 ± 17.78^{a}
14	1-Butanol	1144	1141	12.36 ± 0.42^{a}	7.74 ± 6.67^{a}	8.97 ± 7.73^{a}	12.93 ± 1.23^{a}	12.3 ± 0.37^{a}	12.59 ± 0.86^{a}

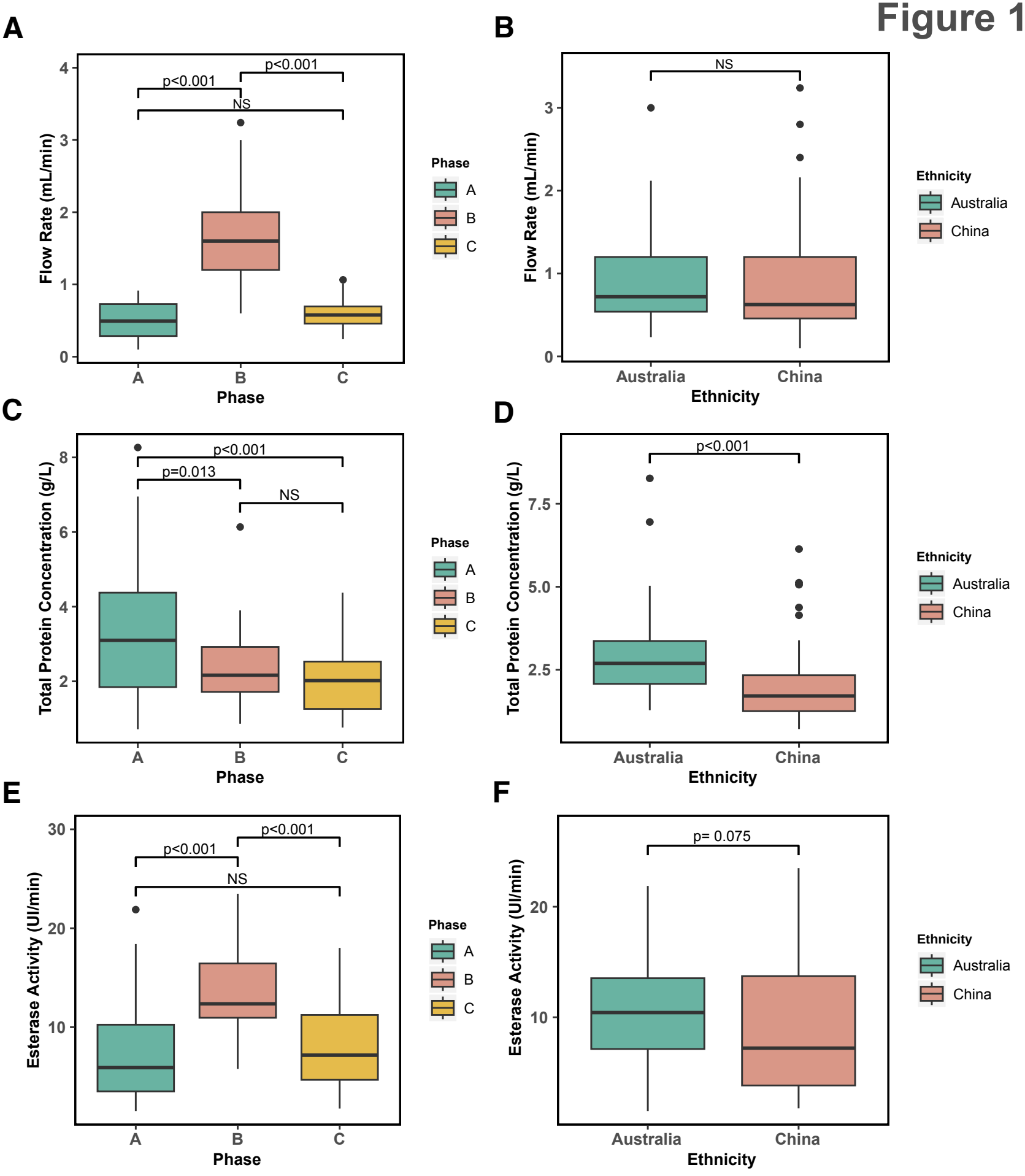
15	3-Methyl-1-butanol	1204	1205	10562.24 ± 491.79[a]	9444.18 ± 259.00[a]	10666.84 ± 311.22[a]	10051.84 ± 1014.17[a]	10159.14 ± 712.50[a]	10557.02 ± 315.42[a]
16	1-Hexanol	1348	1346	319.34 ± 15.53[a]	305.42 ± 12.75[a]	346.57 ± 13.21[a]	331.82 ± 31.04[a]	315.04 ± 19.65[a]	335.08 ± 13.11[a]
17	3-Ethyl-2-pentanol	N/A	1531	19.18 ± 1.58[b]	32.62 ± 2.77[a]	40.21 ± 6.14[a]	39.85 ± 5.86[a]	29.76 ± 4.30[ab]	36.01 ± 4.12[a]
18	2,3-Butanediol	1542	1540	43.59 ± 1.18[a]	43.61 ± 13.28[a]	37.71 ± 5.61[a]	47.51 ± 6.63[a]	41.67 ± 6.48[a]	51.4 ± 14.36[a]
	Acids								
19	Acetic acid	1461	1454	94.23 ± 21.35[c]	165.63 ± 34.94[b]	221.29 ± 12.79[ab]	205.82 ± 25.96[ab]	228.81 ± 13.09[ab]	230.26 ± 23.85[a]
20	Pentanoic acid	1744	1732	8.81 ± 0.10[a]	7.26 ± 0.85[a]	8.37 ± 0.78[a]	8.39 ± 0.49[a]	7.94 ± 0.63[a]	8.4 ± 1.55[a]
21	Octanoic acid	2087	2079	3.91 ± 0.82[a]	5.9 ± 2.16[a]	7.74 ± 0.66[a]	8.52 ± 2.42[a]	4.63 ± 1.53[a]	7.33 ± 3.15[a]
	Benzenoid								
22	Styrene	1256	1254	n.d.	2.56 ± 0.23[b]	4.47 ± 0.42[a]	4.79 ± 0.77[a]	n.d.	1.19 ± 1.03[b]
23	1,3-Ditertiarybutylbenzene	N/A	1424	17.57 ± 1.55[bc]	32.37 ± 3.73[b]	56.56 ± 4.08[a]	69.43 ± 4.61[a]	14.76 ± 9.41[c]	29.05 ± 7.84[bc]
	Terpenes								
24	D-Limonene	1199	1196	4.5 ± 3.55[a]	n.d.	n.d.	0.92 ± 0.70[a]	n.d.	n.d.
25	p-&m-Cymene	1269	1272	n.d.	n.d.	5.16 ± 0.65[a]	6.96 ± 1.21[a]	n.d.	n.d.
26	(-)-Clovene	N/A	1304	190.44 ± 38.62[a]	40.73 ± 9.73[b]	45.69 ± 19.52[b]	65.47 ± 12.48[b]	16.35 ± 0.60[b]	27.51 ± 3.92[b]
27	1,1,6-Trimethyl-1,2-dihydronaphthalene (TDN)	1712	1704	22.88 ± 5.95[a]	15.8 ± 1.76[ab]	15.54 ± 1.19[ab]	22.88 ± 5.95[a]	n.d.	9.23 ± 1.57[b]

Superscripts letters for the same compound indicate significant differences ($p < 0.05$; Tukey test) on the aroma release from wine after spiking with different saliva samples.

RI = Retention index.

*RI-NIST was obtained from NIST Webbook (2018).

Figure 1


A
Flow Rate (mL/min)
p<0.001
p<0.001
NS
Phase
A
B
C
B
Flow Rate (mL/min)
NS
Ethnicity
Australia
China
C
Total Protein Concentration (g/L)
p<0.001
p=0.013
NS
D
Total Protein Concentration (g/L)
p<0.001
E
Esterase Activity (UI/min)
p<0.001
p<0.001
NS
F
Esterase Activity (UI/min)
p= 0.075

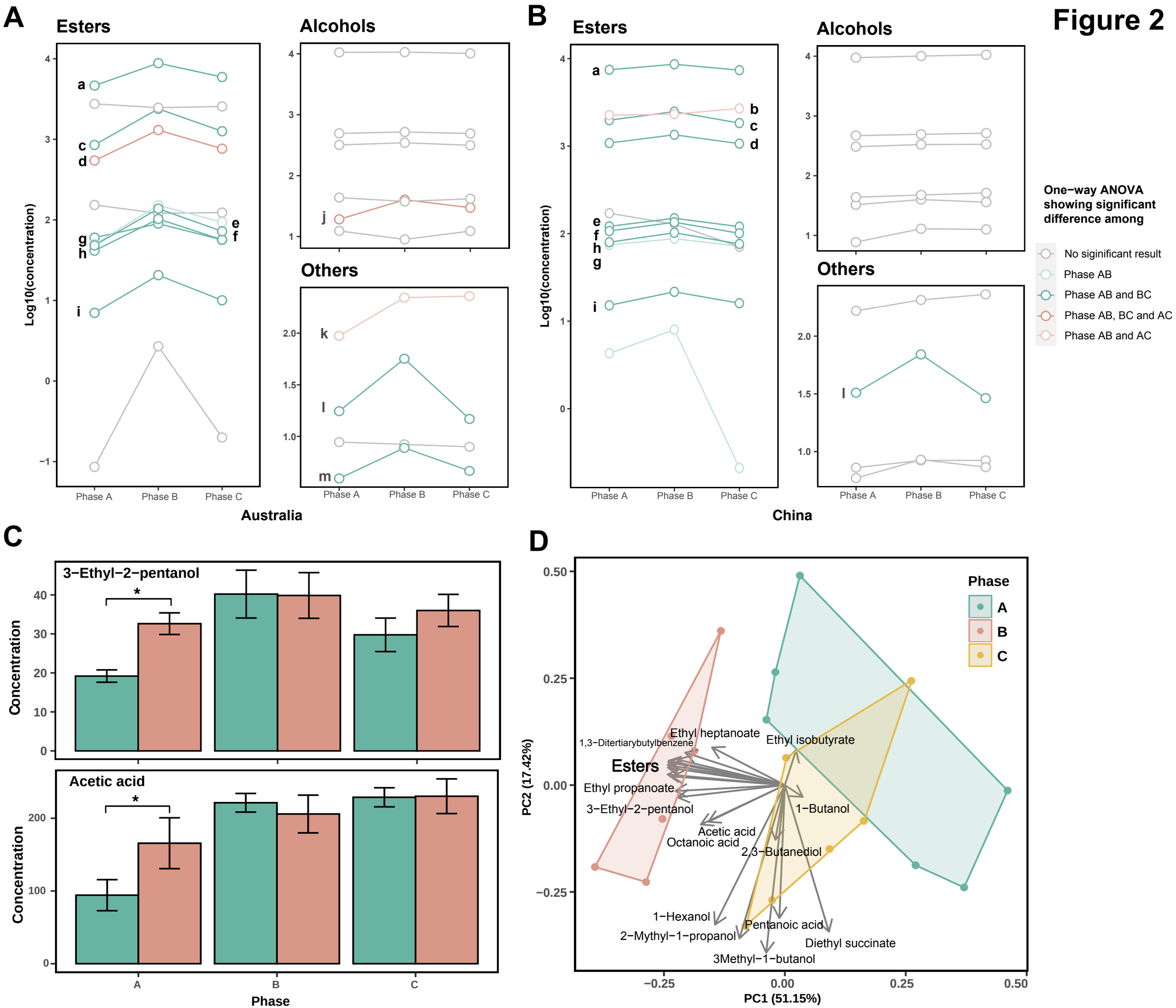


Figure 2
A
Esters
Alcohols
Others
Log10(concentration)
Phase A
Phase B
Phase C
Australia
B
China
One−way ANOVA showing significant difference among
No siginificant result
Phase AB
Phase AB and BC
Phase AB, BC and AC
Phase AB and AC
C
3−Ethyl−2−pentanol
Acetic acid
Concentration
Phase
D
Phase
A
B
C
Ethyl heptanoate
1,3−Ditertiarybutylbenzene
Ethyl isobutyrate
Esters
Ethyl propanoate
3−Ethyl−2−pentanol
1−Butanol
Acetic acid
Octanoic acid
2,3−Butanediol
1−Hexanol
2−Mythyl−1−propanol
Pentanoic acid
Diethyl succinate
3Methyl−1−butanol
PC1 (51.15%)
PC2 (17.42%)

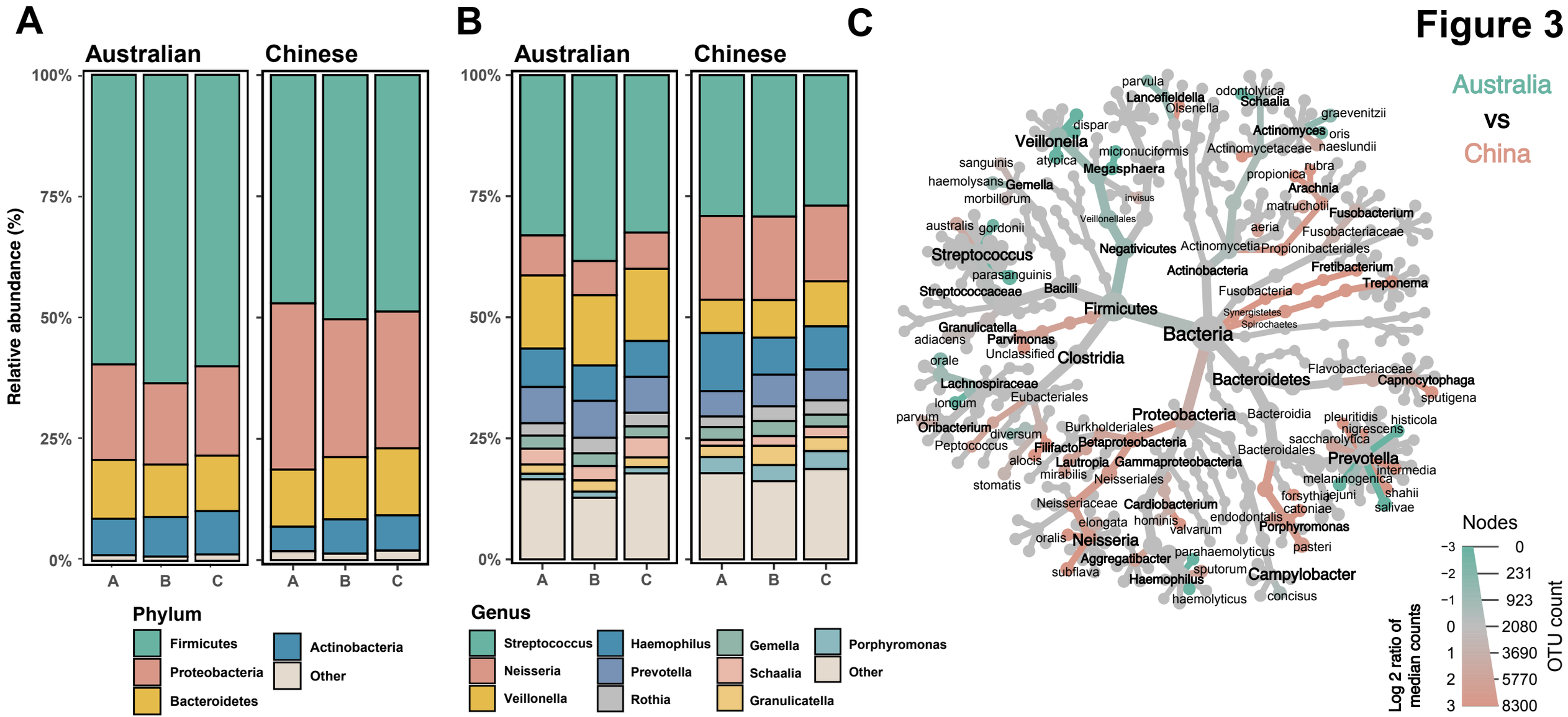
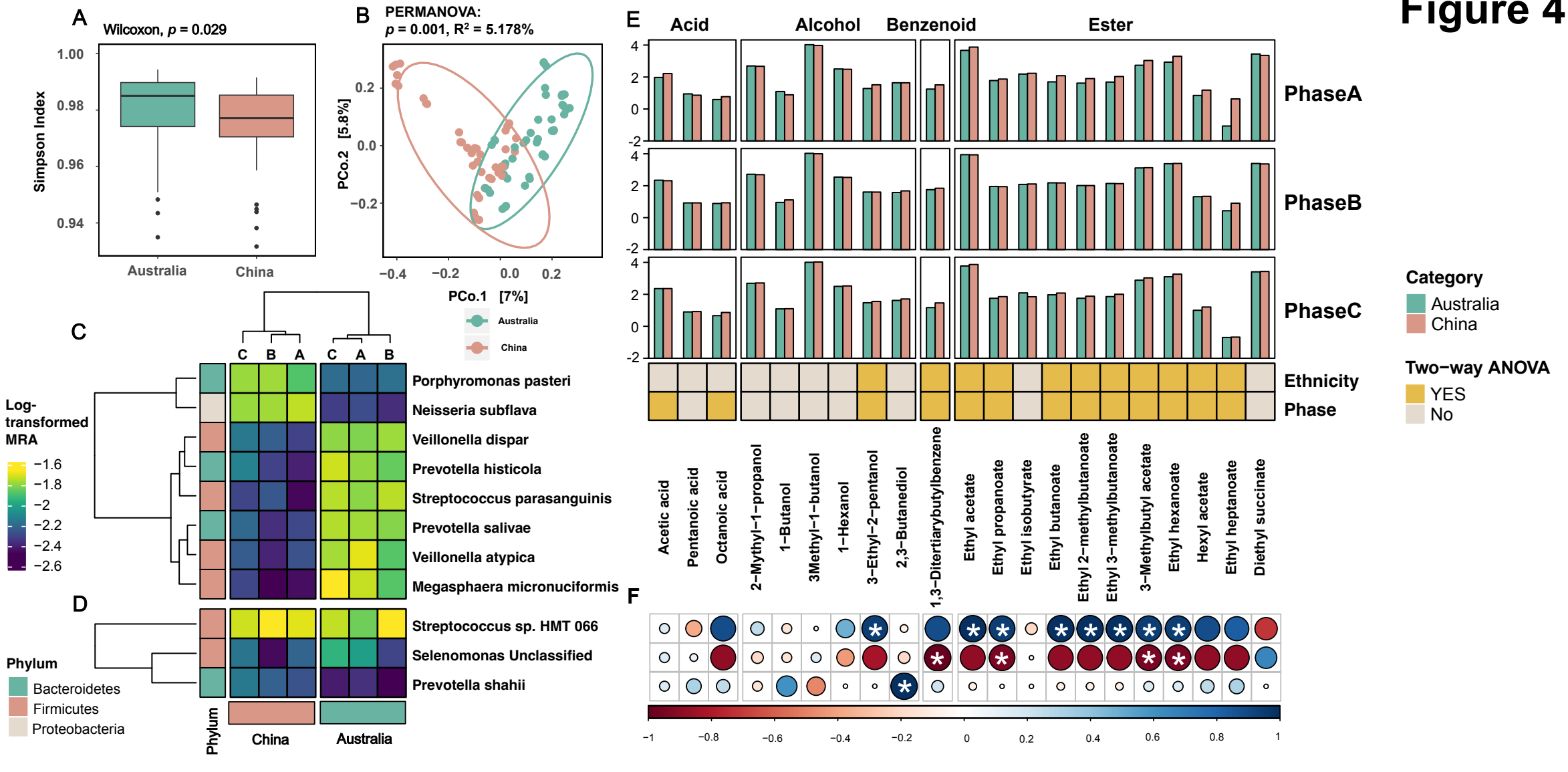


Figure 3
A
Australian
Chinese
Relative abundance (%)
100%
75%
50%
25%
0%
A
B
C
Phylum
Firmicutes
Proteobacteria
Bacteroidetes
Actinobacteria
Other
B
Genus
Streptococcus
Neisseria
Veillonella
Haemophilus
Prevotella
Rothia
Gemella
Schaalia
Granulicatella
Porphyromonas
Other
C
Australia
vs
China
Nodes
Log 2 ratio of median counts
OTU count
-3
-2
-1
0
1
2
3
0
231
923
2080
3690
5770
8300
Bacteria
Firmicutes
Bacteroidetes
Proteobacteria
Actinobacteria
Negativicutes
Clostridia
Bacilli
Veillonellales
Veillonella
Streptococcus
Streptococcaceae
Lachnospiraceae
Eubacteriales
Granulicatella
Parvimonas
Oribacterium
Peptococcus
Filifactor
Lautropia
Burkholderiales
Betaproteobacteria
Gammaproteobacteria
Neisseriales
Neisseriaceae
Neisseria
Aggregatibacter
Haemophilus
Cardiobacterium
Campylobacter
Porphyromonas
Prevotella
Capnocytophaga
Flavobacteriaceae
Bacteroidia
Bacteroidales
Fusobacteriaceae
Fusobacteria
Fusobacterium
Fretibacterium
Treponema
Propionibacteriales
Actinomycetia
Actinomyces
Actinomycetaceae
Arachnia
Schaalia
Lancefieldella
Megasphaera
Gemella
Synergistetes
Spirochaetes

Figure 4


A
Wilcoxon, p = 0.029
Simpson Index
1.00
0.98
0.96
0.94
Australia
China
B
PERMANOVA:
p = 0.001, R² = 5.178%
PCo.2 [5.8%]
PCo.1 [7%]
Australia
China
C
Log-transformed MRA
−1.6
−1.8
−2
−2.2
−2.4
−2.6
Porphyromonas pasteri
Neisseria subflava
Veillonella dispar
Prevotella histicola
Streptococcus parasanguinis
Prevotella salivae
Veillonella atypica
Megasphaera micronuciformis
D
Phylum
Bacteroidetes
Firmicutes
Proteobacteria
Streptococcus sp. HMT 066
Selenomonas Unclassified
Prevotella shahii
Phylum
China
Australia
E
Acid
Alcohol
Benzenoid
Ester
PhaseA
PhaseB
PhaseC
Ethnicity
Phase
Acetic acid
Pentanoic acid
Octanoic acid
2−Mythyl−1−propanol
1−Butanol
3Methyl−1−butanol
1−Hexanol
3−Ethyl−2−pentanol
2,3−Butanediol
1,3−Ditertiarybutylbenzene
Ethyl acetate
Ethyl propanoate
Ethyl isobutyrate
Ethyl butanoate
Ethyl 2−methylbutanoate
Ethyl 3−methylbutanoate
3−Methylbutyl acetate
Ethyl hexanoate
Hexyl acetate
Ethyl heptanoate
Diethyl succinate
F
Category
Australia
China
Two−way ANOVA
YES
No