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Volatiles and primary metabolites profiling in two *Hibiscus sabdariffa* (roselle) *cultivars via* headspace SPME-GC-MS and chemometrics

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ABSTRACT

Hibiscus sabdariffa (roselle) is a plant of considerable commercial importance worldwide as functional food due to its organic acids, mucilage, anthocyanins, macro and micro-nutrients content. Although *Hibiscus* flowers are emerging as very competitive targets for phytochemical studies, very little is known about their volatile composition and or aroma, such knowledge can be suspected to be relevant for understanding its olfactory and taste properties. To provide insight into *Hibiscus* flower aroma composition and for its future use in food and or pharmaceutical industry, volatile constituents from 2 cultivars grown in Egypt, *viz.* Aswan and Sudan-1 were profiled using solid-phase microextraction (SPME) coupled to GCMS. A total of 104 volatiles were identified with sugar and fatty acid derived volatiles amounting for the major volatile classes. To reveal for cultivar effect on volatile composition in an untargeted manner, multivariate data analysis was applied. Orthogonal projection to latent structures-discriminant analysis (OPLS-DA) revealed for 1-octen-3-ol *versus* furfural/acetic acid enrichment in Aswan and Sudan-1 cvs., respectively. Primary metabolites contributing to roselle taste and nutritional value *viz.* sugars and organic acids were profiled using GC-MS after silylation. The impact of probiotic bacteria on roselle infusion aroma profile was further assessed and revealed for the increase in furfural production with *Lactobacillus plantarum* inoculation and without affecting its anthocyanin content. This study provides the most complete map for volatiles, sugars and organic acids distribution in *two Hibiscus* flower cultivars and its fermented product.
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1. Introduction

Hibiscus sabdariffa L. (roselle; Malvaceae) is ranked among the most highly traded commercial and medicinal plants worldwide. Whether native to Africa or Asia, roselle is widely consumed in tropical countries as processed food, flavoring agent, traditional hot or cold beverage as well as a reputed herbal remedy for hypertension and hyperlipidemia (Hopkins, Lamm, Funk, & Ritenbaugh, 2013). Medicinal value of roselle has been verified by several biological studies exploiting its hepatoprotective, antioxidant, anti-inflammatory, antibacterial and cytotoxic properties (Ali, Ashraf, Biswas, Karmakar, & S., 2011; Hirunpanich *et al.*, 2006; Liu *et al.*, 2006; Lo, Huang, Lin, Chien, & Wang, 2007). The brilliantly red calyces of *Hibiscus* are rich in minerals, vitamin C and polyphenols (Ali, Wabel, & Blunden, 2005), however, it is the anthocyanin pigments that have drawn more attention in roselle owing to its antioxidant properties and health attributes (Du & Francis, 1973; Tsai, McIntosh, Pearce, Camden, & Jordan, 2002). Few studies have investigated the volatiles in roselle beverages (Chen, Huang, Ho, & Tsai, 1998; Gonzalez-Palomares, Estarrón-Espinosa, Gómez-Leyva, & Andrade-González, 2009; Jirovetz *et al.*, 1992). Furfural and 5-methyl furfural were detected in beverages prepared from dried hibiscus, while those prepared using fresh hibiscus were rich in linalool and 2-ethyl-1-hexanol. The appreciably variable aroma of roselle hot and cold extracts, comprise a total of 22 volatiles has been described as a combination of earthy, floral and fruity relevant to the extraction process (Ramírez-Rodrigues, Balaban, Marshall, & Rouseff, 2011). Nevertheless, the true aroma of roselle flower itself has yet to be reported. The appealing color and acidic sweet taste of roselle extract, provides a plausible substitute for grapes deficiency affecting the wine industry in the tropics passively (Maldonado, Rolz, & de Cabrera, 1975).

According to Food and Agriculture Organization (FAO), the world's best roselle originates from Sudan (Plotto, 1999). Egypt is also considered an important supplier, but most of its harvest is consumed domestically. The current study aims to investigate the volatile constituent profiles of two *Hibiscus* flower cultivars grown in Egypt using solid-phase microextraction (SPME) coupled to GCMS. This is the first report on roselle volatile analysis using SPME, and further unveiling differences intelligible to cultivar type *e.g.*, Aswan and Sudan-1 using chemometrics. SPME accentuates one true green revolution in volatiles sample preparation techniques gaining a broad spectrum of applications particularly in aroma profiling in herbal drugs over the past two decades (de Koning, Janssen, & Brinkman, 2009). Typical headspace SPME coupled to GC procedure implicates volatiles extraction and

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concentration on the polymeric coating of a silica fiber and the direct desorption of the loaded fiber into the GC injection port (Vas & Vékey, 2004).

Previous studies have reported the influence of incorporating roselle extracts on the biochemical and organoleptic properties of wine and yogurt (Alobo & Offonry, 2009; Iwalokun & Shittu, 2007). As a strong competing candidate in the field of functional foods, the impact of probiotic bacteria, *Lactobacillus plantarum* inoculation on the volatile profile of roselle infusion was also evaluated in this study. This study provides not only the first large scale volatiles profiling in roselle flower from 2 major cultivars in Egypt, but rather extend to determine changes impact of fermentation on *Hibiscus* infusion aroma.

2. Materials and methods

2.1. Plant material, SPME and chemicals

H. sabdariffa flower cultivars Aswan and Sudan-1 were purchased from Haraz Herbal Company (Cairo, Egypt) as dried calyx and epicalyx of *Hibiscus* flowers and devoid of other leaf and or floral parts. SPME holder and fiber coated with 50 μm/30 μm DVB–CAR–PDMS was supplied by Supelco (Oakville, ON, Canada). All other chemicals, volatile standards were provided from Sigma Aldrich (St. Louis, Mo., U.S.A.).

2.2. SPME volatiles isolation

Headspace volatiles analysis using SPME was adopted from (Farag & Wessjohann, 2012) with few modifications. Briefly, flowers were ground, and 3 g was placed inside 20 mL clear glass vials. (*Z*)-3-Hexenyl acetate absent from flower sample VOCs was used as an internal standard (IS), dissolved in water and added to each vial at a concentration of 1 μg/vial. Vials were then immediately capped and placed on a temperature controlled tray for 30 min at 50 °C with the SPME fiber inserted into the headspace above the flower sample. Adsorption was timed for 30 min. A system blank containing no plant material was run as a control.

2.3. GC-MS volatile analysis

SPME fibers were desorbed at 210 °C for 1 min in the injection port of a Shimadzu Model GC-17A gas chromatograph interfaced with a Shimadzu model QP-5000 mass spectrometer (Japan). Volatiles were separated on a DB5-MS column (30 m length, 0.25 mm inner diameter, and 0.25 μm film (J&W Scientific, Santa Clara, CA, USA). Injections were made in the splitless mode for 30 s. The gas chromatograph was operated under the following conditions: injector 220 °C, column oven 38 °C for 3 min, then programmed at a rate of 12 °C/min to 180 °C, kept at 180 °C for 5 min, and finally ramped at a rate of 40 °C min^{-1} to 220 °C and kept for 2 min, He carrier gas at 1 mL min^{-1}. The transfer line and ion–source temperatures were adjusted at 230 and 180 °C, respectively. The HP quadrupole mass spectrometer was operated in the electron ionization mode at 70 eV. The scan range was set at *m/z* 40–500. Volatile components were identified using the procedure described in (Farag & Wessjohann, 2012) and peaks were first deconvoluted using AMDIS software (www.amdis.net) and identified by its retention indices (RI) relative to n-alkanes (C6-C20), mass spectrum matching to NIST, WILEY library database and with authentic standards when available.

2.4. GC-MS analysis of silylated primary metabolites

For analysis of primary metabolites (*viz*. amino acids, organic acids, and sugars), 100 μL of 70% aqueous extract (prepared by extracting 100 mg of dried flowers in 5 mL 50% MeOH with sonication for 30 min followed by centrifugation to remove plant debris) was evaporated under nitrogen till dryness. For derivatization, 150 μL of N-methyl- N-(trimethylsilyl)-trifluoroacetamide (MSTFA) was then added and incubated at 60 °C for 45 min. The samples were equilibrated at 28 °C and subsequently analyzed using GCMS. Silylated derivatives were separated on a Rtx-5MS (30 m length, 0.25 mm inner diameter, and 0.25 μm film) column. Injections were made in a (1:15) split mode and the GC was operated under the following conditions: injector 280 °C, column oven 80 °C for 2 min, then programmed at a rate of 5 °C/min to 315 °C, kept at 315 °C for 12 min. He carrier gas at 1 mL min^{-1}. The transfer line and ion–source temperatures were adjusted at 280 and 180 °C, respectively. The HP quadrupole mass spectrometer was operated in the electron ionization mode at 70 eV. The scan range was set at 50–650 *m/z*. Silylated compounds were identified as previously described under GC-MS volatile analysis, and their contents were determined based on peak areas relative to summed peak areas of identified metabolites.

2.5. Probiotic bacterial inoculation

A fresh plate was cultured from −70 °C freezer glycerol stock on MRS agar. A fresh *L. plantarum* colony was used to inoculate overnight in MRS broth until the OD600 was adjusted to give 10^9 CFU/mL. 2 mL of adjusted culture was centrifuged, washed twice, re suspended in PBS buffer pH 7.4 and used to inoculate roselle infusion (Infusion was prepared by adding 100 mL hot water containing 7% sucrose to 5 g of dried roselle powder and kept for 30 min at room temperature followed by filtration to remove plant debris). The initial pH for roselle infusion was adjusted to 4.8 ± 0.2 using $CaCO_3$ prior to sterilization. Sterilization was done in a 250 mL cotton plugged flask at 110 °C for 3 min, followed by rapid cooling in cold room. Samples were aliquoted in glass fitted tubes at 0, 24, 48 and 96 h for pH measurement, volatiles analysis and viable counts (CFU/mL) measurements. A 20 μL aliquot was taken at each time interval and serially diluted in 180 μL peptone saline diluents. 10 μL from each dilution was spotted on MRS agar plate and incubated for 48 h with bacterial count expressed as CFU/mL. Volatile analyses in fermented infusion was performed by placing 5 mL in 20 mL clear glass vials as described in (Sections 2.2 and 2.3) for volatile analysis from dried flowers. Volatiles were collected from two independent bacterial inoculated roselle juice cultures.

2.6. Multivariate data analyses

Principal component analysis (PCA) and hierarchical clustering analysis (HCA) were performed on the MS-data using custom script under the R 2.9.2 environment. Partial least squares-discriminant analysis (OPLS-DA) was performed with the program SIMCA-P Version 13.0 (Umetrics, Umeå, Sweden). Markers were subsequently identified by analyzing the S-plot, which was declared with covariance (p) and correlation (pcor). All variables were mean centered and scaled to Pareto variance. Distance to the model (DModX) test was used to verify the presence of outliers and to evaluate whether a submitted sample fell within the model applicability domain.

2.7. Anthocyanins quantification

For relative determination of anthocyanins in hibiscus fermented infusion at 0, 24, 48 and 96 h time points, 200 μL were aliquoted, and diluted with 200 μL of milliQ water. The mixture was vortexed vigorously for 1 min, centrifuged at 3000 g for 2 min and measured using UV spectrophotometer at 525 nm.

3. Results and discussion

3.1. Volatiles analysis

The goal of this study was to investigate the diversity of volatile profiles within 2 *Hibiscus* cultivars grown in Egypt, *viz*. Aswan and Sudan-1 using GCMS and chemometrics. To assess for biological variance, 3

biological replicates for each cultivar specimen were extracted and analyzed in parallel under identical conditions. GC-MS analysis of *Hibiscus* calyces samples from both cultivars resulted in the identification of 104 different volatile constituents, listed according to their origin (biosynthetic pathway) in Table 1. Aswan cultivar constituted 74 of the identified volatiles, compared to 71 in the Sudan-1 cultivar and with 41 constituent common in both cultivars. Identified volatiles accounted for 93% and 91% of the total volatile constituents in Aswan and Sudan-1, respectively, compared to 32 volatile components previously reported from roselle infusion (Ramírez-Rodrigues et al., 2011). Thoroughness and increase in volatiles identification was due to processing of MS files with AMDIS software that assists in adjacent peak de-convolution, background subtraction and increased detection limits (Halket et al., 1999). A representative gas chromatogram is shown in (Fig. 1**)** and indicates the differences in volatiles composition among the two cultivars. The qualitative composition of volatile profiles of Aswan and Sudan-1 *Hibiscus* was relatively similar, underlying mostly quantitative differences. Generally, *Hibiscus* volatile profile was dominated by fatty acid derived volatiles with regard to the number of identified constituents, but it was the sugar derived volatiles that comprised the dominant composition. Chemically, identified volatiles belonged to 6 major chemical classes *viz.* furans, terpenoids, aldehydes, acids, alcohols and esters, aside from 5 groups of minor abundance *viz.* aliphatic hydrocarbons, phenols/aromatics, ketones, naphthalenes, pyrans and pyrroles.

Fatty acid derived volatiles comprised the largest number of volatile constituents (39 constituent, of which 35 were identified in the Aswan cv. compared to 27 in Sudan-1 cv) and amounting for *ca.* 27% and 18% of the volatiles present in both investigated samples, respectively. Volatile low molecular weight acids comprised an appreciable amount in *Hibiscus* volatile profile, amounting for 13.5% and 11% of the total identified volatiles in Aswan and Sudan-1 cultivars, respectively. Four short chain alcohols constituents were notably predominant in Aswan (4.3%) versus only one minor constituent in Sudan-1 (0.07%), with 1-Octen-3-ol (Matsutake alcohol) detected exclusively in Aswan cv. (3.9%), being almost absent in Sudan-1 and suggesting that it can be used as marker to distinguish between both cultivars. 1-Octen-3-ol is one of the chief components of mushroom aroma, with a characteristic odor described as moldy or meaty, that attracts insects (Fujioka et al., 2013). Furthermore, Aswan cv. samples were more enriched in aldehydes (4.7%) *vs.* (2.2%) in Sudan-1 cv., with n-nonanal as the major identified volatile (3.8% *vs.* 2.1%, respectively). Volatile ester derivatives were also slightly more enriched in Aswan roselle samples over Sudan-1 samples (1.2% vs. 0.8%, respectively) with methyl palmitate as the major constituent.

Sugar derived volatiles constituted *ca.* half the volatile profile of the investigated roselle samples, being more abundant in Sudan-1 cv. (50.6%) *vs.* Aswan cv. (41.7%). Volatile furan constituents, furfural (31.6% and 38.6%) and its 5-methyl derivative (3.4% and 2.7%) were common major components in Aswan and Sudan-1, respectively. In contrast, 2-(hydroxyacetyl) furan, another major furan, was detected exclusively in Aswan samples (4.5%), whereas methyl-3-furan carboxylate was only found in Sudan-1 cv (5.1%). Methyl-2-furan carboxylate isomer has been described as a tobacco/fungal odorant and is also recognized to contribute in coffee flavor (Flament & Bessière-Thomas, 2001; Burdock, 2009). Solerone, [5-acetyldihydro-2(3 H)-furanone], a substituted γ-lactone derivative characteristic for wine aroma (Augustyn, Wyk, Muller, Kepner, & Webb, 1971) was detected in Aswan and Sudan-1 (0.3% vs. 0.2%, respectively). Although these volatiles are often recognized as Maillard degradation products resulting from reducing sugars-amino acids interaction on drying at high temperatures, they have indeed been previously detected in air-dried roselles (Chen et al., 1998). Such an enrichment of furfural derivatives in *Hibiscus* volatile profile is likely to mediate for the sweet and roasted aroma in its flowers (Rowe, 1998).

22 terpenoids were identified in Sudan-1 vs. 12 in Aswan and accounting for 5.9% of the total volatiles profile compared to 3.3%, respectively, and with α-terpinene and β-phellandrene as the major forms in Sudan-1 cv. (*Z*)-geranylacetone is a carotenoid degradation product that was detected in Aswan (0.6%) and Sudan-1 (0.5%) cultivars

Phenylpropanoids and aromatic derivatives accounted for 15 to 18% of the volatile profiles from Aswan and Sudan-1, with (*E*)-cinnamaldehyde comprising *ca.* 14% of these total values in both cultivars. With regards to other less abundant volatile components, *p*-creosol followed by eugenol, were distinct in Aswan cultivar only at 1.1% and 0.8%, respectively.

Four amino acids derived volatiles were identified exclusively in Aswan cv, whereas only 2-acetylpyrrole was common in both cultivars. Total amino acid derived volatile constituents accounted for 2.9% and 0.5% of the total Aswan and Sudan-1 volatile profiles composition, respectively.

3.2. OPLS multivariate data analysis of volatiles

To identify cultivar differences (*i.e.* Aswan vs. Sudan-1) in *Hibiscus* volatile profile, PCA was performed on the volatile data, albeit no clear separation could be observed between cultivars, appearing to cluster altogether (data not shown).Consequently, supervised orthogonal projection to latent structures-discriminant analysis (OPLS-DA) was used to build a classification model for distinguishing between both cultivars; OPLS-DA also has greater potential in the identification of markers by providing the most relevant variables for the differentiation between two sample groups. Flower samples from both cultivars were modeled against each other using OPLS-DA with the derived score plot showing a clear separation between both samples (Fig. 2A). The OPLS score plot explained 99% of the total variance ($R^2 = 0.99$) with the prediction goodness parameter $Q^2 = 0.71$. A particularly useful tool that compares the variable magnitude against its reliability is the S-plot obtained by the OPLS model and presented in (Fig. 2B), where axes plotted from the predictive component are the covariance p[1] against the correlation p(cor)[1]. For the indication of plots with retention time *m/z* values, a cut-off value of $P < 0.05$ was used. Compared with Sudan-1 cv., Aswan contains more short chain alcohol, that is, 1-octen-3-ol, but less acetic acid and furfural volatiles and in agreement with results shown in Table 1. Generally, furfural, aldehydes *i.e.* cinnamaldehyde, acids *i.e.* acetic acid and the short chain alcohol 1-octen-3-ol appeared as the key constituents accounting for the differences among the 2 cultivars volatile profile. *Hibiscus* flavor has been previously described as a combination of sweet and tart (Wong, Yusof, Ghazali, & Che Man, 2003) which is in line with the data presented herein. However, the enrichment of acetic acid and furfural in Sudan cv. underlies an inferiority of its *Hibiscus* resource compared to that of Aswan and in agreement with its recognition in the market as a second rate *Hibiscus* source compared to that of Aswan.

3.3. GC-MS analysis of primary metabolites

To provide an overview of *Hibiscus* primary metabolites that could account for its taste *viz.* sugar and organic acids, GC-MS was adopted for primary metabolite profiling in the 2 cultivars following silylation. The analysis resulted in the detection of 73 metabolites, listed in **Suppl.**Table 1 and with GC chromatograms displayed in Fig. 3. Primary metabolites included saccharides (mono- and di-) as well as fatty, amino- and organic-acids. Glucose (monosaccharide) represented the major component (*ca.* 5–6% of the total ion count in both cultivars). Myo-Inositol, a sugar present in fruits, beans, grains, and nuts (Clements & Darnell, 1980), was also identified, albeit at relatively much lower levels. Sucrose was the only disaccharide detected with more than two fold levels in Aswan cv. (7.1%) vs. in Sudan-1 (1.9%) and confirming its better palatability as recognized by consumers. Malic acid is commonly found in unripe fruit and contributes to its sour taste (Hulme & Wooltorton, 1958), whereas citric acid is an antioxidant with the ability to chelate metals that could account for *Hibiscus* antioxidant activity (Zaheer et al., 2015). Malic acid represents 1.8% &

Table 1
Relative percentage of volatile components in *Hibiscus sabdariffa* cultivars Aswan and Sudan-1 using SPME-GC-MS. Results are derived from 3 replicates and expressed as average % ± standard deviation.

Peak #	RT (min)	KI	Volatile compound	Egypt	Sudan
Fatty acid derived volatiles					
1	2.21	311	Acetic acid[a]	5.7 ± 1.2	6.97 ± 1.93
2	5.26	588	Hexanal[a]	0.22 ± 0.11	–
3	5.96	652	Vinyl acetate	0.11 ± 0.06	–
4	6.6	710	α-Angelica lactone	0.2 ± 0.14	–
5	7.02	749	Valeric acid	0.18 ± 0.04	0.15 ± 0.03
6	7.95	834	β-Angelica lactone	0.12 ± 0.03	0.1 ± 0.01
7	8.3	865	(*E*)-2-Heptenal	0.16 ± 0.09	0.05 ± 0.03
8	8.67	899	1-Octen-3-ol (Matsutake alcohol)	3.97 ± 2.5	–
9	8.75	908	Sulcatone	0.2 ± 0.07	0.13 ± 0.06
10	8.81	912	Methyl levulinate	–	0.13 ± 0.01
11	8.87	916	Caproic acid	3.47 ± 1.6	2.2 ± 0.88
12	9.09	936	(*Z*)-3-Hexen-1-yl acetate[a]	0.84 ± 0.02	0.59 ± 0.01
13	9.66	989	3-Octen-2-one	0.12 ± 0.04	–
14	9.71	994	Pantolactone	1.2 ± 0.53	0.88 ± 0.38
15	9.93	1014	Levulinic acid	0.19 ± 0.06	0.14 ± 0.03
16	9.97	1017	(*E*)-2-Octenal[a]	0.22 ± 0.08	–
17	10.1	1030	(*Z*)-2-Octen-1-ol	0.11 ± 0.05	–
18	10.24	1042	Heptanoic acid	0.52 ± 0.08	0.33 ± 0.05
19	10.64	1079	n-Nonanal[a]	3.81 ± 1.13	2.11 ± 1.06
20	10.82	1095	2-Ethylcaproic acid	0.17 ± 0.04	0.11 ± 0.03
21	11.48	1155	2-Nonenal	0.15 ± 0.04	–
22	11.49	1156	α-Undecene	–	0.11 ± 0.01
23	11.63	1169	Caprylic acid	1.64 ± 0.58	0.85 ± 0.15
24	11.98	1201	n-Undecane	–	0.24 ± 0.01
25	12.09	1209	Capric aldehyde	0.14 ± 0.03	0.11 ± 0.02
26	12.88	1270	n-Nonanoic acid	1.7 ± 0.9	0.93 ± 0.04
27	13.05	1282	(*E*)-2-Undecen-1-ol	0.12 ± 0.05	–
28	13.29	1301	n-Tridecane[a]	0.13 ± 0.05	0.1 ± 0.04
29	14.28	1866	Butanoic acid, butyl ester	0.08 ± 0.01	–
30	14.42	1386	(*Z*)-3-Tetradecene	0.05 ± 0.02	0.05 ± 0.0
31	14.52	1394	n-Tetradecane	0.36 ± 0.02	0.33 ± 0.04
32	14.66	1404	(*Z*)-2-Dodecenol	0.07 ± 0.01	0.07 ± 0.01
33	15.24	1448	2,6-Dimethylheptadecane	0.44 ± 0.11	0.39 ± 0.04
34	15.74	1487	n-Pentadecane[a]	0.25 ± 0.02	0.27 ± 0.03
35	17.09	1588	(*Z*)-1,4-Dimethylcyclooctane	0.13 ± 0.02	–
36	17.12	1590	1-Tridecene	–	0.13 ± 0.01
37	17.21	1599	n-Hexadecane[a]	0.27 ± 0.02	0.3 ± 0.05
38	22.37	1989	Methyl tridecanoate	0.05 ± 0.01	–
39	22.38	1990	Methyl palmitate[a]	0.1 ± 0.01	0.13 ± 0.03
Total fatty acid derived volatiles				(27.19)	(17.9)
Monoterpenes					
40	7.73	814	α-Thujene	–	0.19 ± 0.26
41	8.58	891	β-Thujene	–	0.43 ± 0.64
42	9.14	942	α-Phellandrene[a]	–	0.06 ± 0.04
43	9.32	959	α-Terpinene	–	0.54 ± 0.78
44	9.43	969	1,3,8-*p*-Menthatriene	0.42 ± 0.09	–
45	9.52	976	D-Limonene[a]	0.12 ± 0.05	–
46	9.56	981	β-Phellandrene[a]	–	0.63 ± 0.8
47	9.99	1019	γ-Terpinene[a]	–	0.42 ± 0.62
48	10.42	1059	Terpinolene	–	0.17 ± 0.22
49	11.05	1116	β-Pinene[a]	–	0.09 ± 0.12
50	12.74	1259	Crithmene	–	0.06 ± 0.01
Total monoterpenes				(0.54)	(2.59)
Sesquiterpenes					
51	14.83	1417	δ-Selinene	0.03 ± 0.02	–
52	14.84	1418	α-Gurjunene	–	0.02 ± 0.01
53	15.02	1431	β-Caryophyllene[a]	–	0.19 ± 0.12
54	15.19	1444	(*E*)-β-Famesene[a]	–	0.2 ± 0.05
55	15.97	1501	α-Muurolene	0.4 ± 0.13	0.3 ± 0.05
56	16.21	1522	δ-Cadinene	0.14 ± 0.02	–
57	16.3	1527	L-Calamenene	0.24 ± 0.06	0.23 ± 0.07
58	18.96	1730	Cadalene	0.08 ± 0.02	–
Total sesquiterpenes				(0.89)	(0.94)
Oxygenated monoterpenes					
59	10.2	1040	(Z)-Furan linalool oxide	0.24 ± 0.06	0.23 ± 0.11
60	10.44	1061	(*Z*)-Linaloloxide	0.11 ± 0.03	0.1 ± 0.04
61	10.71	1085	(*Z*)-Sabinene hydroxide	–	0.11 ± 0.16
62	10.86	1099	4-Terpineol[a]	–	0.47 ± 0.64
63	11.81	1190	α-Phellandrene-8-ol	0.44 ± 0.10	–
64	12.04	1205	α-Terpineol[a]	0.42 ± 0.07	0.52 ± 0.42
65	12.66	1252	Linalyl acetate[a]	–	0.17 ± 0.19

Table 1 (*continued*)

Peak #	RT (min)	KI	Volatile compound	Egypt	Sudan
66	13.35	1305	4-Terpinenyl acetate	–	0.27 ± 0.34
67	15.13	1440	(*Z*)-Geranylacetone	0.62 ± 0.03	0.48 ± 0.10
Total oxygenated monoterpenes				(1.83)	(2.35)
Total terpenes				(3.26)	(5.88)
Phenylpropanoid derivatives/benzenoids					
68	8.4	874	Benzaldehyde[a]	0.19 ± 0.08	0.24 ± 0.07
69	9.44	970	*o*-Cymene	–	0.22 ± 0.11
70	9.76	998	α-Tolualdehyde	0.32 ± 0.12	–
71	10.85	1186	Phenylethyl alcohol[a]	0.03 ± 0.0	0.04 ± 0.01
72	11.56	1163	Benzoic acid	0.57 ± 0.07	–
73	11.71	1177	Acetic acid, phenyl-methyl ester	–	0.04 ± 0.01
74	11.92	1196	*p*-Creosol	1.12 ± 0.54	0.58 ± 0.37
75	13.11	1287	(*E*)-Cinnamaldehyde[a]	14.14 ± 2.86	13.87 ± 2.89
76	13.48	1314	α-Methylnaphthalene	–	0.08 ± 0.01
77	13.69	1330	β-Methylnaphthalene	–	0.17 ± 0.02
78	14.06	1358	Eugenol[a]	0.82 ± 0.11	–
79	14.63	1402	Orcinaldehyde	0.05 ± 0.0	–
80	15.91	1498	Unknown aromatic	0.14 ± 0.04	–
81	15.93	1500	Unknown aromatic	–	0.15 ± 0.02
82	16.34	1532	o-Methoxycinnamaldehyde	0.08 ± 0.01	–
83	20.77	1867	Benzyl Benzoate	0.12 ± 0.03	0.23 ± 0.06
Total phenylpropanoids/benzenoids				(17.58)	(15.62)
Sugar derived volatiles					
84	5.9	646	Furfural	31.66 ± 2.9	38.62 ± 2.6
85	7.45	789	2-Acetylfuran	–	0.96 ± 0.05
86	8.38	873	5-Methylfurfural	3.36 ± 0.34	2.67 ± 0.45
87	8.68	900	2,5-Dimethyl-2,4-dihydroxy-3(2 H)-furanone	–	1.59 ± 0.18
88	8.82	913	2-Pentylfuran	0.67 ± 0.26	–
89	10.36	1053	2-(Hydroxyacetyl) furan	4.5 ± 0.89	–
90	10.37	1054	Methyl-3-furancarboxylate	–	5.1 ± 0.72
91	10.91	1103	Maltol	0.18 ± 0.03	0.14 ± 0.01
92	10.99	1110	Solerone	0.3 ± 0.12	0.2 ± 0.09
93	11.3	1139	Pyranone	0.1 ± 0.02	0.05 ± 0.04
94	12.37	1231	5-Hydroxymethylfurfural	0.93 ± 0.27	1.24 ± 0.02
Total sugar derived volatiles				(41.7)	(50.57)
Amino acids derived volatiles					
95	6.92	740	Isopropyl alcohol[a]	1.24 ± 0.55	–
96	9.33	961	α-Pyrrolaldehyde	0.61 ± 0.08	–
97	10.12	1033	2-Acetylpyrrole	0.57 ± 0.18	0.48 ± 0.19
98	10.68	1083	6-Methyl-3,5-heptadiene-2-one	0.31 ± 0.06	–
99	11.97	1200	2,4-Dimethyl-3-hexanone	0.21 ± 0.01	–
Total amino acids derived volatiles				(2.94)	(0.48)
Unidentified volatiles					
100	10.56	1071	Unknown	–	0.7 ± 0.35
101	11.88	1192	Unknown	0.23 ± 0.05	–
102	12.31	1226	Unknown	–	0.15 ± 0.02
103	14.16	1366	Unknown	0.1 ± 0.01	–
104	14.33	1379	Unknown	0.1 ± 0.03	–
Total unidentified volatiles				(0.43)	(0.85)
Total volatile components (%)				93.1	91.3

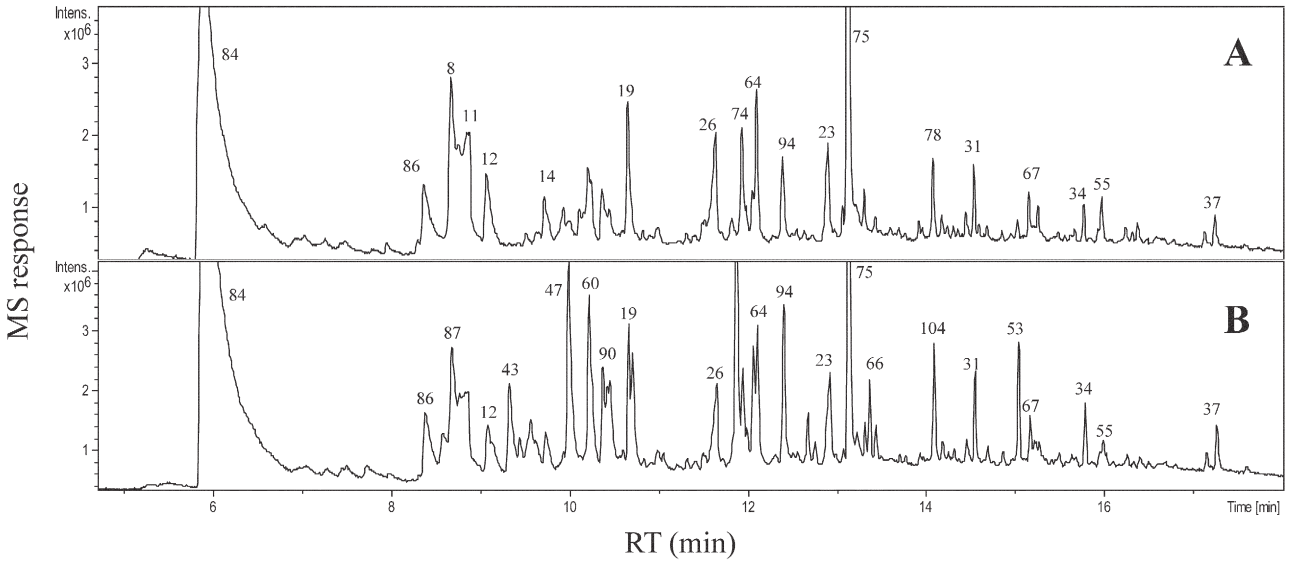
MS data for unknown volatiles: RI 1071, *m/z* (rel. int.): 165(5%), 85(22%), 43(76%), 57(100%); RI 1192: 156 (4%), 150 (2%), 141 (2%), 59(21%), 58(84%), 43(100%); RI 1226: 148(35%), 133(100%); RI 1366: 172 (13%), 157 (43%), 142 (24%), 128 (8%), 127(5%), 99 (9%), 85(100%),57(45%), 56(25%), 42(15%); RI 1379: 190 (26%), 175 (34%), 159 (15%), 157(7%), 147 (100%), 142 (12%), 105(97%). Compounds were identified by comparison of kovat index (KI) and mass spectral data with those of authentic compounds and by comparison of mass spectral data with those of NIST library. (−), not detected.

[a] Volatiles confirmed by co-analysis with standard.

0.7%, whereas citric acid stands for 3.1% & 0.3% of the total primary metabolites identified in Aswan and Sudan-1 cvs., respectively. Tartaric acid was also detected at higher levels (6–7%) in both cultivars. Tartaric acid accumulation during ripening occurs in a number of plant species, notably in the grapevine *Vitis vinifera*, where varying levels of tartaric acid account significantly for conferring grapes flavor (DeBolt, Cook, & Ford, 2006). Oxalic acid was also detected though at much lower levels, representing *ca.* 0.2% in both cultivars. Amino acids constituted 11–21% of cultivars primary metabolites composition detected using GCMS, with valine as a major amino acid (7–10%) in both cultivars. Saturated and unsaturated fatty acids constituted (13.3%) and (10.8%) of Aswan and Sudan-1, respectively, with linoleic acid as the major unsaturated fatty acid. Enrichment of roselle flowers with linoleic acid and other omega fatty acids accentuate its antioxidant properties (Fagali & Catalá, 2008) and might synergize polyphenols *i.e.* anthocyanins antioxidant effects. It is worth noting that the low pH of *Hibiscus* extracts, ranging from 2 to 2.5 implies for the abundance of acidic constituents, and being generally more enriched in Aswan compared to Sudan-1 cultivar.

3.4. Impact of probiotic bacterial inoculation on Hibiscus infusion

Increasing clinical evidence points to the myriad health benefits of fermented foods and beverages consumption in prevention of cancer, gastrointestinal disorders, hypertension and diabetes (Holzapfel, 2014; Tamang, 2015). As a worldwide highly consumed beverage, we



(8) 1-Octen-3-ol , **(11)** Caproic acid, **(12)** (*Z*)-3-Hexen-1-yl acetate, **(14)** Pantolactone, **(19)** n-Nonanal, **(23)** Caprylic acid, **(26)** n-Nonanoic acid, **(31)** n-Tetradecane, **(34)** n-Pentadecane, **(37)** n-Hexadecane, **(43)** α-Terpinene, **(47)** γ-Terpinene, **(53)** β-Caryophyllene, **(55)** α-Muurolene, **(60)** (Z)-Linalool oxide, **(64)** α-Terpineol, **(66)** 4-Terpinenyl acetate, **(67)** (Z)-Geranylacetone, **(74)** p-Creosol, **(75)** (E)-Cinnamaldehyde, **(78)** Eugenol, **(84)** furfural, **(86)** 5-Methylfurfural, **(87)** 2,5-Dimethyl-2,4-dihydroxy-3(2H)-furanone, **(90)** Methyl-3-furancarboxylate, **(94)** 5-Hydroxymethylfurfural, **(104)** Unknown.

Fig. 1. Representative SPME-GC-MS chromatogram of *Hibiscus sabdariffa* headspace volatiles derived from Aswan (A) and Sudan-1 (B) cultivars**.** The corresponding volatile names for each peak follow that listed in Table 1.

attempted to evaluate the impact of fermenting roselle infusion using *L. plantarum* on its volatile composition. Roselle infusion was inoculated with *L. plantarum* and examined for variation in pH and viable count over 0, 24, 48 and 96 h post inoculation, results are presented in *Suppl. Table 2*. It should be noted that pH of roselle infusion was initially raised to 4.8 using $CaCO_3$, prior to sterilization and bacterial inoculation as

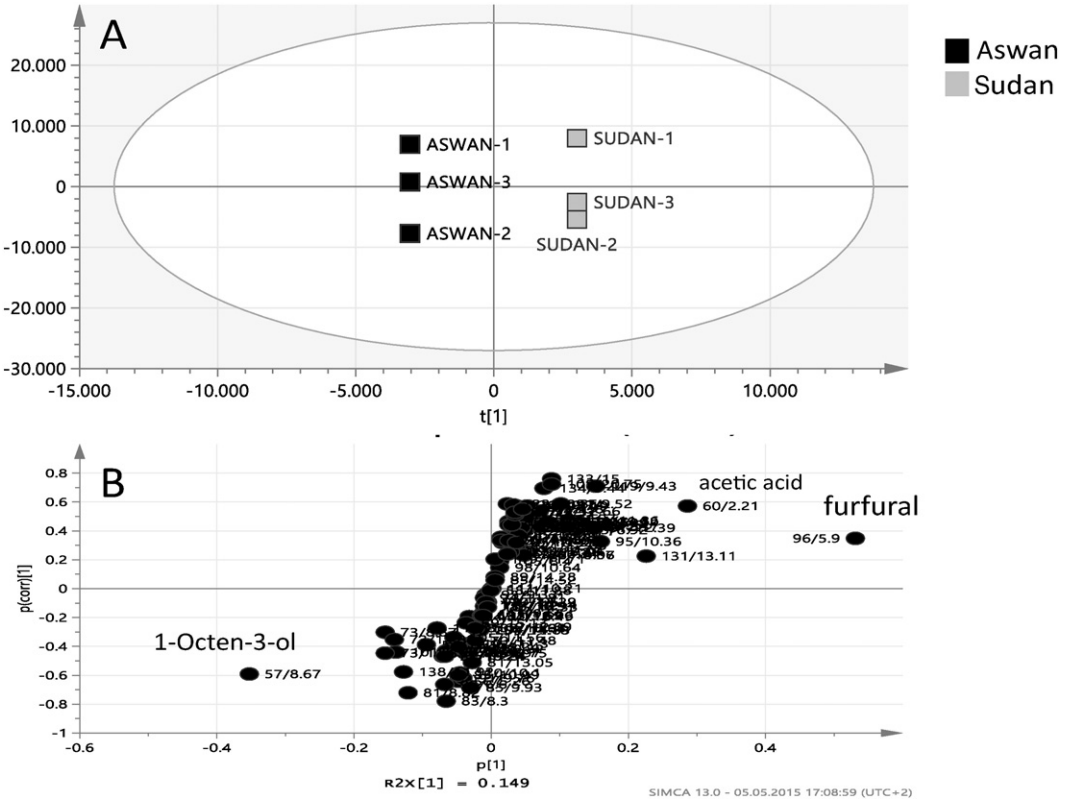
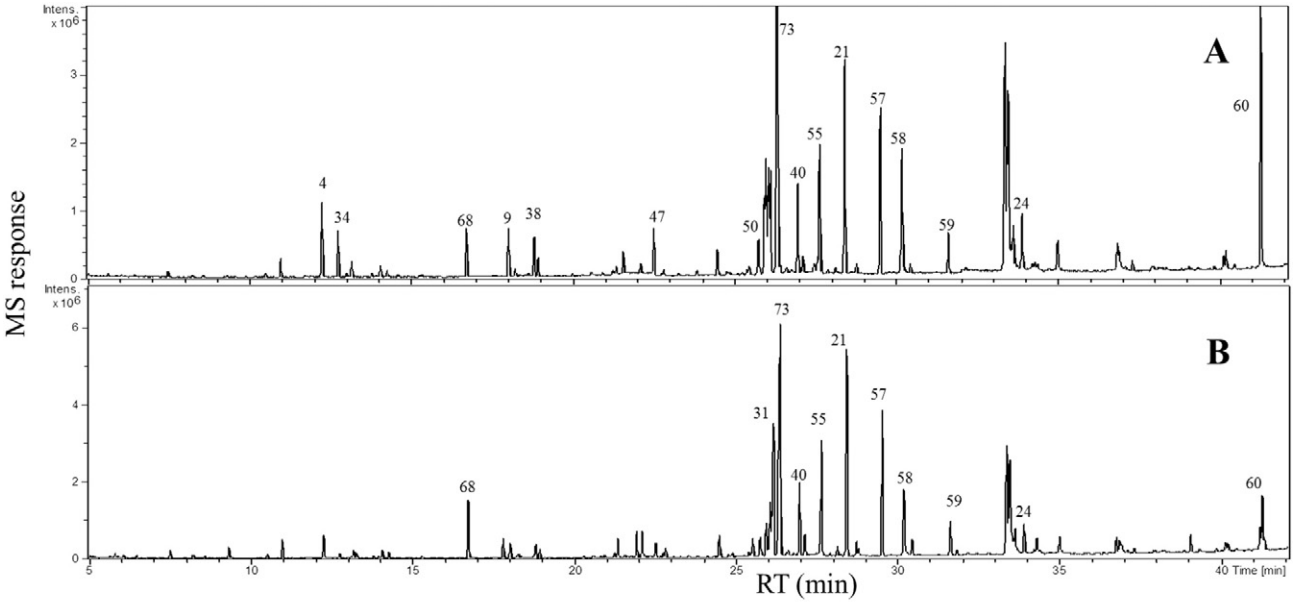


Fig. 2. (A) OPLS-DA score plot and (B) loading S-plots derived from modeling Aswan cv. (■) against Sudan-1 cv. (▭). The S-plot shows the covariance p[1] against the correlation p(cor)[1] of the variables of the discriminating component of the OPLS-DA model. Cut-off values of $P < 0.01$ were used; variables selected are highlighted in the S-plot with m/z retention time in seconds, KI values and identifications are discussed in text.



(4) Phosphoric acid,tris-(TMS), **(9)** Malic acid, O-bis(TMS)-ester, **(21)** Tartaric acid, bis(TM)-ester, **(24)** Linoleic acid TMS ester, **(34)** Proline, N-(TMS)-ester, **(38)** Aspartic acid, N-TMS-, bis(TMS)-ester, **(40)** Threonic acid,**(47)** Arabinofuranose, 1,2,3,5-tetrakis-O-(TMS), **(50)** Fructofuranose, pentakis(TMS)- ether, tris(TMS)-ester, **(55)** D-Glucose, 2,3,4,5,6-pentakis-O-(TMS)-ether, **(57)** β-D-Glucopyranose-(TMS), **(58)** Inositol, 1,2,3,4,5,6-hexakis-O-(TMS)- ether, **(59)** myo-Inositol -(6TMS), **(60)** Sucrose, octakis-O-(TMS), **(68)** Unknown, **(73)** Unknown.

Fig. 3. GC-MS chromatograms of silylated *H. sabdariffa* flower 50% methanol extract from Aswan-1cv. (A) and Sudan cv. (B). The corresponding metabolite names for peaks are shown in Table S1.

infusion original pH, being distinctly acidic (pH 2.4) was found discouraging for the bacterial viability (data not shown). Unexpectedly, no significant change was observed in the pH of roselle infusion (4.9, at 0 h) post inoculation for samples harvested at 24, 48 and 96 h with only slight increase at 24 h reaching a pH of 5.0. Bacterial viable count at 0 time was 2×10^7 (CFU/mL) in both samples and survival of the *Lactobacillus* bacteria increased throughout fermentation period, reaching its peak after 48 h (viable count $= 3 \times 10^8$ CFU/mL), a drop in the culture growth was then observed at 96 h (viable count $= 1 \times 10^8$ CFU/mL), *Suppl. Table 2*. It should be noted that roselle infusion maintained its characteristic red color throughout the fermentation period and by measuring anthocyanins absorption (*Suppl. Table 2*) in infusion at different time points post inoculation, suggesting no degradation of its anthocyanins content. Detailed secondary metabolites profiling of fermented roselle infusion could provide better evidence on fermentation impact on non-volatile metabolome of roselle extract.

3.5. Multivariate data analysis of infusion volatiles in response to probiotic bacteria

We assessed the effect of probiotic inoculation on *Hibiscus* infusion volatile profile. Inoculated *Hibiscus* infusion samples were aliquoted at 0, 24, 48 and 96 h post inoculation and further subjected to SPME volatile analysis (following the procedure described in Section 2.3). Of the 104 volatiles detected from the dried flower hibiscus sample, 22 were only detected in the infusion flower samples at 0 h and with furfural still amounting for the major volatile form (*Suppl.*Fig. 1). To determine the effect of *Lactobacillus* growth on *Hibiscus* infusion in an untargeted way, volatiles analyzed using SPME were subjected to principal component analysis (PCA) and hierarchical clustering analysis (HCA). Hierarchical cluster analysis (HCA) was performed to define both similarities and differences across samples in a fairly intuitive graphical way, showing two major clear clusters, (Fig. 4A) referred to as groups 1a and 1b. Volatiles collected from samples at 0 h were clustered separately in one group 1a and distinct from other time points (24, 48 & 96 h) being clustered altogether as evident from their more or less comparable volatiles profile, and differing from that of non-inoculated *Hibiscus* infusion. Principal component analysis (PCA) was further performed to explore the relative variability within *Hibiscus* infusion volatiles upon bacterial inoculation. The PC1/PC2 scores plot (Fig. 4B) shows that 2 major, distinct clusters were formed corresponding to the 4 different sampling time mostly along PC1 and PC2 overall explaining 96% of the variance. Except for volatiles collected at 0 time point, duplicate measurements from the same sample were found to be highly reproducible, as the scores of replicate measurements were more or less superimposed. On the right side of the plot, 24, 48 and 96 h samples are positioned (positive PC1 values), whereas on the far left side, un inoculated or samples harvested at 0 h are located (negative PC1 values). The metabolite loading plot (Fig. 4C), which exposes the most important components with respect to scattering behavior, reveals that δ-cadinene, isopropyl alcohol and hexadecane contributed the most, negatively along PC1. The second group had a positive effect on PC1, mostly from furfural enrichment in inoculated roselle infusion. Although furan derivatives, namely furfural and 5-methylfurfural, are known to inactivate fermentation processes of *Saccharomyces cerevisiae* and cell replication by inhibiting specific enzymes related to glycolysis (Palmqvist & Hahn-Hägerdal, 2000), furfural production was found to affect positively the growth of *Lactobacillus* in roselle infusions under investigation. This effect could be dependent on the type of probiotic used, previous work on *Lactobacillus reuteri* exploited its capacity to consume reactive aldehydes and ketones as electron acceptors for balancing their redox metabolism (van Niel, Larsson, Lohmeier-Vogel, & Rådström, 2012). Furfural enhanced the growth rate of *Lactobacillus reuteri* by about 25% and biomass yield by 15% (van Niel et al., 2012), which might account for its increase in *Lactobacillus* fermented roselle infusion, acting as a positive feedback growth regulator.

4. Conclusions

Volatiles were extracted from 2 *Hibiscus* flower cultivars using SPME and analyzed by GC/MS. A total of 104 compounds were detected of which only 32 were previously reported and with furans and aldehydes

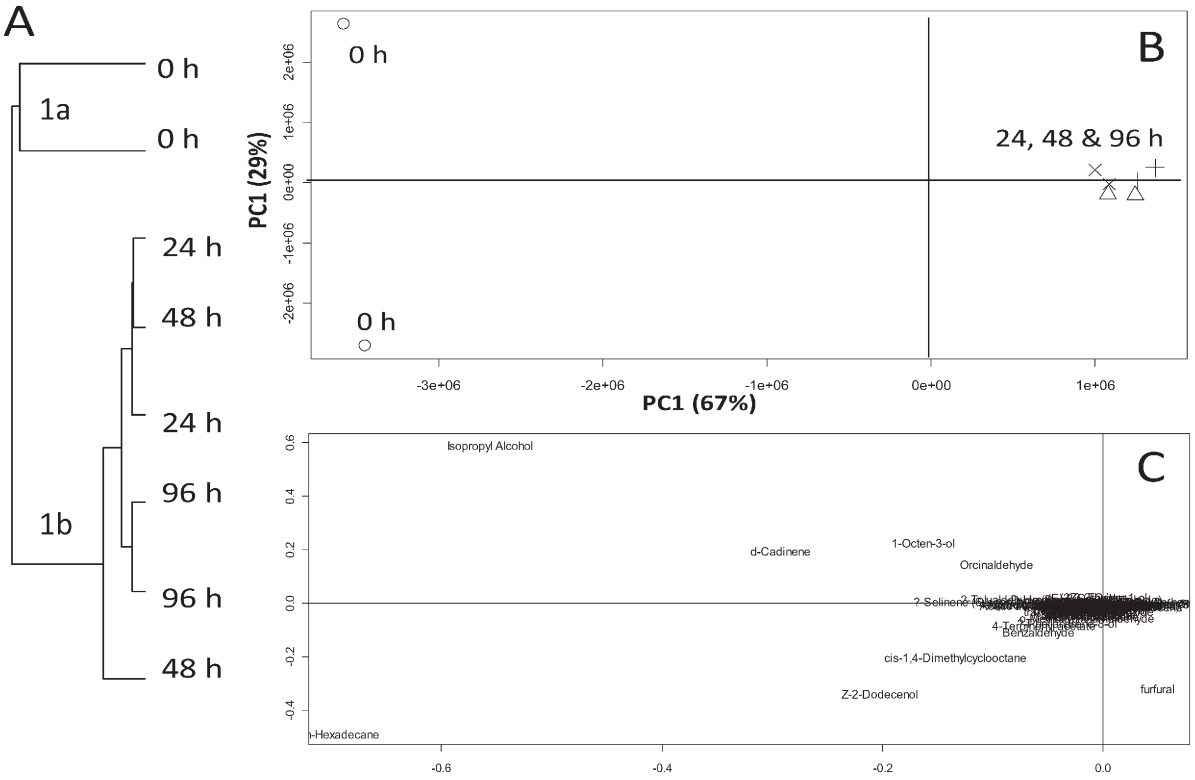


Fig. 4. Hierarchical clustering and principal component analysis of SPME extracted volatile from *Hibiscus* infusion inoculated with *Lactobacillus casei* and harvested at 0, 24, 48 and 96 h post inoculation (n = 2). (A) HCA plot (B) Score plot of PC1 *vs.* PC2 scores. (C) Loading plot for PC1 & PC2 contributing volatiles and their assignments. The metabolome clusters are located at the distinct positions in two-dimensional space described by two vectors of principal component 1 (PC1) = 67% and PC2 = 29%.

amounting for the major volatile classes in both cultivars.. The most notable difference in composition was the higher levels of acetic acid and furfural in Sudan cultivar, compared with high short chain alcohols abundance *i.e.* 1-octen-3-ol in Aswan cultivar. Analysis of roselle flower primary metabolites revealed the enrichment of both cultivars in organic acids *viz.* tartaric, malic and citric acids asides from monosaccharides as major sugar forms. Our multiplex approach targeting both volatile and primary metabolite analyses in parallel to multivariate data analyses provided the first comprehensive volatiles, sugars profile in *Hibiscus* flowers which can be further applied for investigating other factors on metabolites composition for example, seasonal variation, growth stage, and or storage conditions. Drying temperature of *Hibiscus* flowers could be considered crucial on aroma and flavor of the product, favoring the preservation of short chain alcohols and aldehydes or the accumulation of Maillard degradation products. Development of *Hibiscus* probiotic beverage, specifically using *L. plantarum* with its unique metabolic capacity for consumption of deleterious low molecular weight aldehydes, extends additional *in vivo* health value to roselle medicinal merit.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.foodres.2015.09.024.

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