Hydrosols of orange blossom (Citrus aurantium), and rose flower (Rosa damascena and Rosa centifolia) support the growth of a heterogeneous spoilage microbiota

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Hydrosols of orange blossom (*Citrus aurantium*), and rose flower (*Rosa damascena* and *Rosa centifolia*) support the growth of a heterogeneous spoilage microbiota

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A B S T R A C T

Hydrosols are hydrodistillation products of aromatic plants. They contain less than 1 g/L of dispersed essential oils giving organoleptic properties. Hydrosols are subjected to microbial proliferation. Reasons for spoilage have to be found in the nature of substrates supporting growth and of microbiological contaminants. The composition in essential oils and the microbiota of 22 hydrosol samples of *Citrus aurantium* L. ssp. *amara* L. (orange blossom), *Rosa damascena* Miller (rose D.), and *Rosa centifolia* L. (rose C.) flowers were analyzed to determine the factors responsible for decay. The median concentrations in essential oils were 677 mg/L for orange blossom hydrosols, 205 mg/L for rose D. hydrosols, and 116 mg/L for rose C. hydrosols. The dry matter content of these hydrosols varied between 4.0 mg/L and 702 mg/L, and the carbohydrate content varied between 0.21 mg/L and 0.38 mg/L. These non-volatile compounds were likely carried over during distillation by a priming and foaming effect, and could be used as nutrients by microorganisms. A microbial proliferation at ambient temperature and also at 5 °C has been observed in all studied hydrosols when stored in a non-sterile container. In contaminated hydrosols, maximal counts were about 7 $\log_{10}$ CFU/mL, while the French pharmacopeia recommends a maximal total bacterial count of 2 $\log_{10}$ CFU/mL. Neither yeast nor mold was detected. The isolated microbial population was composed of environmental Gram-negative bacteria, arranged in four major genera: *Pseudomonas* sp., *Burkholderia cepacia* complex, and presumably two new genera belonging to *Acetobacteraceae* and *Rhodospirillaceae*. Among those bacteria, *Burkholderia vietnamiensis* and *Novosphingobium capsulatum* were able to metabolize volatile compounds, such as geraniol to produce 6-methyl-5-hepten-2-one or geranic acid, or phenylethyl acetate to produce 2-phenylethanol. EO concentrations in hydrosols or cold storage are not sufficient to insure microbiological stability. Additional hurdles such as chemical preservatives or aseptic packaging will be necessary to insure microbial stability.

Keywords:
Orange blossom water
Rose water
Essential oils
Carbohydrate
Spoilage
Distillation

1. Introduction

Hydrosols are co-products from the steam distillation or hydrodistillation of aromatic plants for the production of the much less abundant but highly valuable essential oils (EOs). These are mainly used as food flavoring substances in a wide range of pastries and beverages of the Mediterranean basin and the Middle East. The steam distillation or hydrodistillation process of aromatic plants produces two nonmiscible phases: the EO phase containing the major part of volatile compounds, and the hydrosol phase composed of condensed water and of a low amount of dissolved EO (usually less than 1 g/L) that confers the organoleptic properties. At higher concentrations, EOs are not miscible and separate naturally from hydrosol (Fernandez, André, & Casale, 2014).

EOs in orange blossom and rose hydrosols are mostly composed of terpenoids and alcohols, such as linalool and α-terpineol in orange blossom, and 2-phenylethanol, citronellol, and geraniol in rose sp. hydrosols (Jeannot, Chahboun, Russell, & Baret, 2005; Ulusoy, Bosgelmez-Tinaz, & Secilmis-Canbay, 2009). Distillation of orange blossom (*Citrus aurantium* L. ssp. *amara* L.) results in the production of neroli EO (in a yield of about 0.1% of the distillation products) and of its co-product, the orange blossom hydrosol (also known as orange blossom water) in a yield of 99.9%. The major producers are Morocco and

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Tunisia, with annual productions of about 800 t and 300 t, respectively (Albert Vieille SAS unpublished data). Rose C. flower hydrosol (*Rosa centifolia* L.) is usually obtained by steam distillation, with quite a low yield in EO (about 0.01%). Most of the production comes from France. Rose D. flower (*Rosa damascena* Miller) hydrosol (Damascus rose water) is usually obtained by hydrodistillation, co-produced with 0.025% of EO, and most of the 1000 t annual world production comes from Bulgaria and Turkey (Fernandez et al., 2014).

Hydrosols contain EOs known for their antimicrobial effects, in particular against foodborne pathogens (Ait-Ouazzou et al., 2011; Al-Turki, 2007; Ammar et al., 2012; Burt, 2004; Chorianopoulos, Giaouris, Skandamis, Haroutounian, & Nychas, 2008; Fisher & Phillips, 2008; Sagdic, Ozturk, & Tornuk, 2013; Tornuk et al., 2011; Voon, Bhat, & Rusul, 2012). Moreover the same hydrosols must comply with professional microbiological standards that recommend a total bacterial count lower than 200 CFU/mL, a total mold and yeast count lower than 20 CFU/mL, and absence in 1 mL of the human pathogens *Staphylococcus aureus* and *Pseudomonas aeruginosa* (ANSM, 2012; Council of Europe, 2014). However, there is some concern about their microbiological stability, and about proliferation of bacteria or molds that contributes to hydrosol decay (Fernandez et al., 2014; Watt, 2015). EO concentrations may therefore not be high enough for microbiological control. The aim of this work was to characterize the microbiota of rose and orange flower hydrosols of diverse origins, its behavior in relation to chemical composition and storage conditions, and its impact on the aromatic properties of the products.

2. Materials and methods

2.1. Origin and preparation of the analyzed hydrosols

Samples of commercial rose (*R. damascena* Miller and *R. centifolia* L.) and orange blossom (*C. aurantium* L. ssp. *amara* L.) hydrosols were collected by Albert Vieille SAS (Vallauris, France) from different manufacturers at different locations in Europe and around the Mediterranean basin. Selected and examined hydrosols were in conformity with market practices. These hydrosols were produced by steam distillation or hydrodistillation of fresh flowers (Fig. 1). A second distillation of hydrosols may occasionally be performed, in case of non-compliance to microbiological or aromatic standards. For all hydrosols analyzed in the present work, volumes of 5 L to 10 L were sampled at different times of processing and storage (Table 1). Then, 50 mL to 100 mL aliquots of each hydrosol sample were aseptically separated for chemical and microbiological analysis. Among these, four hydrosol samples have been followed during a three-month storage period. Two rose C. samples (#18 and #22), and two orange blossom samples (#7 and #8) were collected in the industrial storage and collection tanks (therefore not maintained in a sterile environment and/or packaging during a few days after distillation) and poured into 10 L sterile containers stored at 5 °C (samples #18.1, #22.1, #7.1, and #8.1) or at ambient temperature (samples #18.2, #22.2, #7.2, and #8.2). Changes in bacterial populations were followed over a three-month storage period by sampling 50 mL volumes at regular time intervals. Two hundred grams of fresh rose C. flowers were collected before distillation (#17) for determination of microbiological counts.

2.2. Chemical analysis

2.2.1. Extraction, identification and quantification of volatile compounds

Volatile compounds were extracted from 4 mL volumes of hydrosol by three successive liquid–liquid extractions with 1.5 mL of n-hexane (VWR International, Fontenay-sous-Bois, France). Water traces remaining in the organic phase were absorbed on Na_2SO_4 (VWR). Volatile compounds were analyzed and quantified with a gas chromatograph mass spectrometer (GCMS-QP2010; Shimadzu, Kyoto, Japan). Samples were

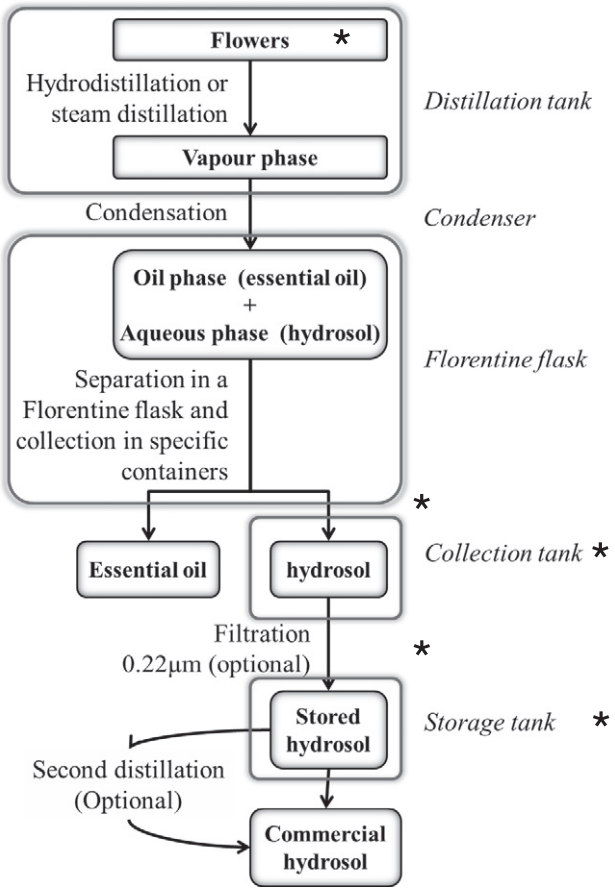

Fig. 1. Diagram of hydrosol production showing the product at different stages of processing, and physical and chemical processes applied to the product. Containers and process equipment are indicated in italics. Products and fractions are in bold characters. * indicates sampling locations.

injected with an auto-sampler (AOC-5000; Shimadzu) in splitless mode at 250 °C (purge opened after 0.5 min), then separated with a UBWAX column (30 m × 0.25 mm, 0.5 µm) (Interchim, Montluçon, France). The carrier gas was helium at a constant velocity of 35.5 cm/s. The oven program temperature was as follows: 50 °C, 4 °C/min to 230 °C and 5 min hold time. The mass spectrometer was operated in the electron impact mode at 70 eV in the *m/z* range 29–450 at a speed of 1.7 scans/s. The temperatures of the ion source and of the transfer line were respectively 200 °C and 250 °C. Mass spectral matches were performed by comparison of experimental mass spectra with the ones of the Wiley Mass Spectral library (8th edition) and NIST/EPA/NIH Mass Spectral Library (NIST 08). Experimental retention indices (RI) were determined for 31 constituents by injecting a series of n-alkanes (C7–C30) (Supelco, Bellefonte, USA) and were then compared to the values given in the literature (www.pherobase.com and www.flavornet.org) to confirm identification. Quantifications was done by area comparisons, using n-hexadecane (Sigma-Aldrich, Saint-Quentin-Fallavier, France) as internal standard, and measurement of the response factors of pure linalool, α-terpineol, citronellol, nerol, geraniol (all Sigma-Aldrich), and 2-phenylethanol (Merck, Darmstadt, Germany), representing respectively 85% and 75% of volatile compounds of *Rosa* sp. and orange blossom hydrosols. Volatile compound concentrations expressed in mg/L or % surface area were the mean of two replicate extractions. The reproducibility of extractions and analyses was estimated by % mean deviations. These were lower than 5.3% for EO concentrations and lower than 3.4% for peak areas of volatile compounds.

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Table 1
Description of orange blossom (*Citrus aurantium*), rose D. (*Rosa damascena*), and rose C. (*Rosa centifolia*) hydrosol samples.

Hydrosol	Sample #	Manufacturer; year of production; production country	Simple or double distilled	Filtration	Sampling location	Storage temperature (°C); storage duration before analysis	Storage conditions
Citrus aurantium	1	A; 2013; TN	Simple	NK	Storage tank	NK; 3 months	Not sterile
	2	B; 2013; FR	Simple	NK	Storage tank	NK; 20 days	Not sterile
	3	C; 2013; TN	Simple	NK	Storage tank	NK; 3 months	Not sterile
	4	D; 2013; TN	Simple	NK	Storage tank	Ambient; 3 months	Not sterile
	5	A; 2013; TN	Simple	NK	Storage tank	NK; 9 months	Not sterile
	6.1[a]	B; 2014; FR	Double	No	Collection tank	5 °C; 10 days	Not sterile
	6.2[a]	B; 2014; FR	Double	No	Collection tank	20 °C; 10 days	Not sterile
	6.3[a]	B; 2014; FR	Double	Yes	Storage tank	5 °C; 10 days	Not sterile
	7.1[a]	D; 2014; TN	Simple	No	Collection tank	5 °C; 3 days	Not sterile
	7.2[a]	D; 2014; TN	Simple	No	Collection tank	20 °C; 3 days	Not sterile
	8.1[a]	E; 2014; TN	Simple	No	Storage tank	5 °C; 7 days	Not sterile
	8.2[a]	E; 2014; TN	Simple	No	Storage tank	20 °C; 7 days	Not sterile
	9[a,b]	F; 2014; FR	Simple	No	Florentine flask	5 °C; 5 h	Sterile
	10	D; 2014; TN	Simple	NK	Storage tank	ambient; 2 months	Not sterile
Rosa damascena	11	G; 2013; MA	Simple	NK	Storage tank	NK; 4–6 months	Not sterile
	12	B; 2013; FR	Simple	NK	Storage tank	5 °C; 4 days	Not sterile
	13	H; 2013; BG	Simple	NK	Storage tank	NK; 4–6 months	Not sterile
	14	I; 2013; MA	Simple	NK	Storage tank	NK; 4–6 months	Not sterile
	15	J; 2013; TR	Simple	NK	Storage tank	NK; 4–6 months	Not sterile
	16	K; 2013; BG	Simple	NK	Storage tank	NK; 4–6 months	Not sterile
Rosa centifolia	17[a]	B; 2013; FR	Simple	No	Florentine flask	20 °C; 7 h	Sterile
	18.1[a]	B; 2013; FR	Simple	Yes	End of filtration	5 °C; 1 month	Not sterile
	18.2[a]	B; 2013; FR	Simple	Yes	End of filtration	20 °C; 1 month	Not sterile
	18.3[a]	B; 2013; FR	Simple	Yes	Storage tank	5 °C; 1 month	Not sterile
	19	B; 2012; FR	Simple	Yes	Storage tank	NK; 13 months	Not sterile
	20	B; 2012; FR	Simple	Yes	Storage tank	NK; 13 months	Not sterile
	21.1[a]	B; 2014; FR	Simple	No	Florentine flask	5 °C; 6 h	Sterile
	21.2[a]	B; 2014; FR	Simple	No	Florentine flask	20 °C; 6 h	Sterile
	22.1[a]	B; 2014; FR	Simple	Yes	Storage tank	5 °C; 1 month	Not sterile
	22.2[a]	B; 2014; FR	Simple	Yes	Storage tank	20 °C; 1 month	Not sterile

NK: not known. BG: Bulgaria; FR: France; MA: Morocco; TN: Tunisia; TR: Turkey.
[a] Samples analyzed at several time intervals during a 3-month storage.
[b] Laboratory (Albert Vieille SAS) distillation.

2.2.2. Determination of pH, carbohydrate and amino acid concentrations

The hydrosol pHs were measured at ambient temperature with a pH electrode (Hanna, Tanneries, France) and a pH meter (Mettler Toledo, Schwerzenbach, Switzerland). The pH meter was calibrated with freshly prepared buffers (pH 4.00 and pH 7.01) (Merck).

Carbohydrate concentrations have been determined by the phenol sulfuric-acid method (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956) on the residues of three replicate freeze-dried samples of 50 mL hydrosol volumes each solubilized again in 0.5 mL of Milli-Q Plus™ water (Merck Millipore, Billerica, USA), 0.5 mL of a 50 g/L phenol solution (Fisher Scientific, Illkirch, France) and 2.5 mL of pure sulfuric acid (VWR). After 30 min of reaction, the A_{485} was determined by spectrophotometric measurement (Xenius, Safas, Monaco). A calibration curve was set from 0.01 g/L to 0.08 g/L of a sucrose water solution (Sigma-Aldrich) ($R^2 = 0.99$).

Amino-acid concentration was determined using a method adapted from previously published ones (Frutos, Torrado, Perez-Lorenzo, & Frutos, 2000; Sun, Lin, Weng, & Chen, 2006). The calibration curve and the samples were prepared at the same time. For external calibration, final concentration ranged from 0.04 g/L to 0.07 g/L of glycin (Sigma-Aldrich) in Milli-Q Plus™ water ($R^2 = 0,99$). Each residue of three replicate 50 mL samples of freeze-dried hydrosols was dissolved in 1 mL of Milli-Q Plus™ water. A 400 μL aliquot of the dissolved residue was transferred into a screw-capped microtube. Then, 200 μL of 2% (wt/vol) Ninhydrine reagent (Sigma-Aldrich) in Milli-Q Plus™ water was added in all microtubes, heated in a dry bath (Lab-line, Maharashtra, India) at 95 °C for 15 min, and then cooled to room temperature in an ice bath. After 10 s vortexing, 300 μL of each sample was transferred into a 96-well microplate (Greiner Bio-One, Courtaboeuf, France). A_{570} was determined by spectrophotometric measurements (Xenius, Safas) within 20 min following heating, i.e., before initiation of a pronounced decrease in coloration.

2.2.3. Laboratory distillation of solutions of organic compounds

Solutions at 20 g/L of autolytic yeast extract (Biokar Diagnostic, Beauvais, France), glycin (Sigma-Aldrich) and sucrose (Sigma-Aldrich) were hydrodistilled in the laboratory in respectively 50 mL, 50 mL, and 100 mL of Milli-Q Plus™ water, using a 500 mL distillation flask heated in a heating mantle equipped with a magnetic stirrer, a still head equipped with a thermometer, and a condenser. Distillate products were collected in a graduated cylinder. The dry weight content in the distillation product (condensate) of the yeast extract solution was determined after three days drying in an oven at 73 °C. The amino-acid content in the glycin solution condensate and the carbohydrate concentration in the sucrose solution were determined using the previously described methods (Section 2.2.2).

2.3. Microbiological counts and changes with storage time

Microbial population was first determined in a sample of freshly picked rose flowers. Three replicates of 15 g fresh rose C. flowers were blended in 100 mL sterile demineralized water using a Stomacher® 400 blender (Seward, Worthing, UK) (2 × 60 s), and 100 μL of the appropriate decimal dilutions were spread onto Plate Count Agar (PCA) (Biokar Diagnostics) and Yeast Glucose Chloramphenicol agar (YGC) (Biokar Diagnostics), to enumerate, respectively, aerobic mesophilic bacteria and yeasts and molds. Counts of colony-forming units (CFU) of aerobic mesophilic bacteria in hydrosols were performed by spreading 100 μL of the appropriate decimal dilutions of hydrosols in sterile demineralized water onto 10 fold-diluted PCA, complemented with agar (Biokar Diagnostics) to get a final 12 g/L agar concentration (DPCA), unless otherwise specified. Previous tests in our laboratory consistently showed a better recovery on DPCA than on PCA (data not shown). All Petri dishes were incubated at 30 °C for two days before colony counts were recorded. Counts of microbial cells in hydrosols were

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performed using a Malassez counting chamber and an Olympus BX50 phase-contrast microscope at ×400 magnification.

2.4. Isolation and identification of bacteria

Approximately three to four colonies per hydrosol sample, from the ones grown on DPCA plates used for microbiological counts, were selected for their morphological differences, and observed for size and shape by phase-contrast microscopy at ×1000 magnification. All colonies observed on DPCA were composed of bacteria. They were then subcultured three times on DPCA for purity, and then picked and stored at −80 °C in a 400 mL/L glycerol solution (Sigma Aldrich).

In total 71 bacteria were isolated from hydrosols and were first analyzed by 16S ribosomal RNA (*rrs*) gene sequencing. Then, to determine clonal isolates, strains with similar *rrs* gene partial sequence isolated from the same hydrosol sample were typed by a molecular typing (M13-PCR). One representative strain of each clone was selected. To differentiate species of a same genus with non-discriminative *rrs* gene partial sequence, a phenotypic characterization was done. Detailed procedure is as follows.

DNA was extracted from purified colonies, suspended in 400 μL of ultrapure sterile water and grinded with 0.1 μm silica beads (Bio Spec, Bartlesville, USA) in screw-capped microtubes, and then mixed to 25 μL sodium dodecyl sulfate (200 g/L), 100 μL $NaClO_4$ (5 mol/L), and 600 μL of pure chloroform (all Sigma-Aldrich). Samples were kept on ice all along the extraction. After centrifugation (17,000 ×*g*), 1 mL of freezing absolute ethanol (Sigma-Aldrich) was added to the aqueous phase into a clean microtube. The DNA precipitate was retrieved and washed with 70% ethanol (Sigma-Aldrich), dried, and suspended in 100 μL ultrapure sterile water. RNAse (Roche Diagnostic, Mannheim, Deutschland) (0.5 mg/mL) was added and the sample was heated at 65 °C for 15 min, and then stored at −20 °C until further analysis.

The *rrs* gene corresponding to positions 9 to 1548 on *Bacillus subtilis rrs* gene was amplified using two modified primers (FD1 MOD: AGAGTT-TGA-TC(A,C)-TGG-CTC-AG) and (RD1 MOD: GG(A,C)-TAC-CTTGTT-ACG-A(T,C)T-TC) as previously described (Guinebretiere et al., 2001) with minor differences in the number of cycles (n = 35) and the use of a PCR 9700 thermocycler (Life Technologies, Carlsbad, USA). A positive amplification was checked by revelation of the expected size band in a 5 μL volume of each amplicon deposited onto a 100 mL electrophoresis gel of Tris–acetate–EDTA (TAE) buffer (0.5×) (Serva, Heidelberg, Germany) mixed with 10 g/L agarose (LonzaSeakem® LE, Basel, Switzerland). Gels were stained with 20 μL of ethidium bromide (MP Biomedicals Europe, Illkirch, France), and results were read after 30 min migration at 100 V in TAE (0.5×) electrophoresis buffer. PCR products were purified and sequenced by GATC Biotech® (Constance, Germany) in a Sanger ABI 3730xl, using the primer S6 (5′-GTATTACC GCGGCTGCTG-3′, position 518–534 of *Escherichia coli rrs* gene). Identifications of hydrosol bacteria were then performed by comparison of the resulting partial *rrs* sequences to the *rrs* gene sequences of type strains on the NCBI nucleotide BLAST library.

Bacterial clones among isolates were detected by M-13 PCR (Guinebretiere & Nguyen-The, 2003). Fragments were revealed on an electrophoresis gel made of 100 mL TAE buffer mixed with 15 g/L agarose and stained with 20 μL ethidium bromide, using the molecular weight marker 'Smart Ladder' (Eurogentec, Seraing, Belgium). A phenotypic characterization was performed to differentiate 15 strains of *Burkholderia* sp. having the same *rrs* gene partial sequence (*Burkholderia lata*, *Burkholderia cepacia*, and *Burkholderia vietnamiensis*; *Bu. lata* and *Burkholderia ambifaria*; *Bu. lata*, *Bu. cepacia* and *Burkholderia multivorans*) and 18 strains of *Pseudomonas* sp. (*Pseudomonas migulae* and *P. panacis*; *P. veronii* and *Pseudomonas extremaustralis*), using pure colonies subcultured on DPCA. *Burkholderia* species were identified by their differences in 42 °C growth capacity, hemolysis and gelatinase activity, NO_2 reduction and β-galactosidase activity (Vanlaere et al., 2009). *Pseudomonas* species were differentiated by gelatinase and hemolysis activity (Ivanova et al., 2002; Park et al., 2005). NO_2 reduction, β-galactosidase activity, and glucose metabolism were tested on API® 20 E (Biomerieux, Marcy l'Etoile, France) using NaCl (VWR) 8.5 g/L as cell medium. Gelatinase tests were performed on Petri dishes as previously described by Smith and Goodner (1958), using gelatin at 40.0 g/L (Biorad, Marnes-la-Coquette, France), and increased concentrations of autolytic yeast extract (3.0 g/L), and peptone (5.0 g/L) (Sigma-Aldrich). Hemolysis tests were performed on horse blood agar plates (BD Columbia, Le Pont de Claix, France) incubated at 30 °C, using *Bacillus cereus* ATCC14579 as positive control. Growth capacity at 42 °C was tested by formation of visible colonies on DPCA plates incubated for seven days.

2.5. Changes in profiles of volatile compound in hydrosols inoculated with bacteria

Purified colonies of *Novosphingobium capsulatum* (strain IA_FO_33) and *Bu. vietnamiensis* (strain IA_FO_28) isolated from one hydrosol (sample #8.2 stored at ambient temperature) were subcultured overnight at 30 °C under shaking at 200 rpm into three replicate 5 mL volumes of a growth medium composed of 50 mg/L tryptone (Biokar Diagnostic), 25 mg/L autolytic yeast extract (Biokar Diagnostic) and 10 mg/L D-(+)-glucose (Sigma Aldrich). Then, each replicate of the previous pure culture was inoculated at a final concentration of approximately 10^3 CFU/mL into 5 mL volumes of filter-sterilized (0.22 μm) orange blossom or rose C. hydrosols. Orange blossom hydrosol was supplemented with 20 μL of a heat-treated (20 min at 121 °C) bacterial pellet obtained from 100 mL of the heavily contaminated hydrosol #8.2, washed and suspended in demineralized water to allow *N. capsulatum* growth. Concentrations in volatile compounds and microbial counts were determined at inoculation time (t0), and after 10-day incubation at 30 °C under shaking at 200 rpm as described in Sections 2.2.1 and 2.3.

2.6. Statistical analysis

The significance of the differences in the statistical distributions of two samples was evaluated with the Mann–Whitney test and using the XLstat software (Addinsoft, Paris, France).

3. Results and discussion

3.1. Hydrosol composition

3.1.1. Volatile compounds

EO concentrations and percentage area of 31 volatile compounds of nine orange blossom, six rose D., and eight rose C. hydrosols produced at different locations in Europe and around the Mediterranean basin are presented in Table 2. The EO concentration of orange blossom hydrosols analyzed in this work (median [minimal value; maximal value]) was 677 mg/L [276; 866]. The major compounds were linalool and α-terpineol (median surface areas at 44.1% and 23.7%, respectively), and to a lesser extent methyl anthranilate, phenylacetonitrile, nerol and geraniol (median surface areas at 4.2%, 2.9%2.5% and 1.6%, respectively). The EO concentrations of the rose hydrosols (median [minimal value; maximal value]) were 205 mg/L [132; 597] for rose D. hydrosols, and 116 mg/L [100; 482] for rose C. hydrosols. The major compounds were 2-phenylethanol (median surface areas at 25.0% and 45.6% for rose D. and rose C., respectively), citronellol (median surface areas at 20.9% and 24.6%, respectively), and geraniol (median surface areas at 21.2% and 11.3% respectively). Concentrations of a few compounds greatly varied among samples. For example, surface areas of 6-methyl-5-hepten-2-one varied between 0.01% and 22.0% in orange blossom hydrosols, surface areas of geraniol and 2-phenylethanol varied respectively between 0.6% and 30.7% and between 10.7% and 80.3% in rose D. hydrosols, and amounts of citronellol varied between 11.2% and 58.4% in rose C. hydrosols. Volatile profiles correspond to the ones already observed in previous studies and industrial samples of rose and

Table 2
Essential oil concentrations and area percentages of 31 volatile compounds of orange blossom (*Citrus aurantium*), rose D. (*Rosa damascena*), and rose C. (*Rosa centifolia*) hydrosols, and comparison with literature and industrial data.

			Hydrosols											
			Citrus aurantium				*Rosa damascena*				*Rosa centifolia*			
			This work		Literature and industrial data		This work		Literature and industrial data		This work		Literature and industrial data	
			n = 9		n = 43[a]		n = 6		n = 24[b]		n = 8		n = 15[c]	
			Median	[min; max]	Median	[min; max]	Median	[min; max]	Median	[min; max]	Median	[min; max]	Median	[min; max]
Essential oil concentration (mg/L)			677	[276; 866]	477	[210; 1075]	205	[132; 597]	240	[41; 830]	116	[100; 482]	165	[50; 519]
	RI[d]													
RI[d] and area % of aromatic compounds	1348	6-Methyl-5-hepten-2-one	0.2	[0.01; 22.0]	1.8	[0.03; 10.5]	0.2	[<0.01; 5.4] *	<0.01	[<0.01; 0.04]	0.01	[<0.01; 1.4]	0.2	[0.1; 1.5]
	1360	Rose oxide	<0.01	[<0.01; <0.01]	<0.01	[<0.01; <0.01]	1.2	[<0.01; 1.8]	0.3	[<0.01; 1.8]	0.7	[<0.01; 2.0] *	0.2	[<0.01; 0.4]
	1398	*cis*-3-Hexenol	0.02	[<0.01; 0.1]	<0.01	[<0.01; <0.01]	<0.01	[<0.01; <0.01]	<0.01	[<0.01; <0.01]	<0.01	[<0.01; <0.01]	<0.01	[<0.01; <0.01]
	1450	*cis*-Linalool oxide	5.7	[1.4; 9.6]	4	[1.0; 8.8]	<0.01	[<0.01; <0.01]	<0.01	[<0.01; 0.7]	<0.01	[<0.01; <0.01]	<0.01	[<0.01; 0.4]
	1473	6-Methyl-5-hepten-2-ol	0.4	[0.02; 2.2] *[e]	<0.01	[<0.01; 10.0]	0.04	[<0.01; 10.0]	<0.01	[<0.01; <0.01]	0.04	[<0.01; 0.6]	<0.01	[<0.01; <0.01]
	1483	*trans*-Linalool oxide	3.4	[0.7; 5.7]	2.4	[0.5; 5.7]	<0.01	[<0.01; <0.01]	<0.01	[<0.01; 0.5]	<0.01	[<0.01; <0.01]	<0.01	[<0.01; 0.2]
	1543	Benzaldehyde	<0.01	[<0.01; 0.1] *	0.4	[<0.01; 7.3]	<0.01	[<0.01; <0.01]	<0.01	[<0.01; 0.01]	<0.01	[<0.01; <0.01]	<0.01	[<0.01; <0.01]
	1563	Linalool	44.1	[24.9; 64.9]	45.4	[23.1; 67.3]	1.9	[0.6; 7.1]	3.1	[1.0; 8.0]	2.0	[0.4; 4.6]	1.0	[0.03; 5.0]
	1613	4-Terpineol	1.7	[0.4; 2.6]	1.5	[0.1; 3.5]	1.1	[<0.01; 1.9] *	0.4	[<0.01; 0.9]	0.1	[<0.01; 0.4]	0.2	[<0.01; 1.0]
	1670	2-Phenyl acetaldehyde	0.03	[<0.01; 2.6]	<0.01	[<0.01; <0.01]	0.04	[<0.01; 0.1]	<0.01	[<0.01; <0.01]	0.01	[<0.01; 1.2]	<0.01	[<0.01; <0.01]
	1690	Neral	0.03	[<0.01; 0.1]	<0.01	[<0.01; <0.01]	0.7	[<0.01; 1.8]	0.1	[<0.01; 1.0]	0.1	[<0.01; 1.0]	0.1	[<0.01; 0.5]
	1710	Alpha terpineol	23.7	[10.7; 38.0]	22	[11.3; 36.3]	0.9	[<0.01; 4.9]	1.3	[<0.01; 4.7]	0.6	[0.1; 11.4]	0.4	[<0.01; 1.7]
	1745	Geranial	0.1	[<0.01; 0.2]	<0.01	[<0.01; <0.01]	<0.01	[<0.01; <0.01] *	0.3	[<0.01; 1.3]	<0.01	[<0.01; <0.01] *	0.2	[<0.01; 3.5]
	1765	Neryl acetate	0.1	[<0.01; 0.8]	0.1	[<0.01; 0.4]	<0.01	[<0.01; <0.01]	<0.01	[<0.01; <0.01]	<0.01	[<0.01; <0.01]	<0.01	[<0.01; <0.01]
	1768	Geranyl acetate	0.1	[<0.01; 1.4]	0.1	[<0.01; 1.8]	<0.01	[<0.01; <0.01]	0.02	[<0.01; 0.3]	<0.01	[<0.01; <0.01]	0.1	[0.04; 1.9]
	1775	Citronellol	0.1	[<0.01; 0.7]	0.01	[0.01; 0.01]	20.9	[11.7; 27.3]	17.1	[2.3; 38.7]	24.6	[11.2; 58.4] *	16.5	[3.6; 22.8]
	1809	Nerol	2.5	[0.1; 2.8]	2.3	[0.1; 3.5]	10.8	[1.3; 15.1]	9.3	[<0.01; 16.5]	3.8	[2.0; 5.1]	4.0	[0.6; 5.7]
	1824	2-Phenetyl acetate	0.01	[<0.01; 0.1]	<0.01	[<0.01; 0.2]	<0.01	[<0.01; <0.01]	<0.01	[<0.01; 0.8]	<0.01	[<0.01; <0.01] *	0.2	[<0.01; 0.5]
	1857	Geraniol	1.6	[0.1; 8.0]	3.6	[0.1; 7.8]	21.2	[0.6; 30.7]	18.7	[<0.01; 31.3]	11.3	[6.4; 13.5]	7.4	[2.7; 15.8]
	1886	Benzylic alcohol	<0.01	[<0.01; 0.02]	<0.01	[<0.01; <0.01]	0.3	[0.1; 1.7]	0.1	[<0.01; 1.1]	0.4	[0.1; 0.7] *	<0.01	[<0.01; <0.01]
	1925	2-Phenyl ethanol	1.5	[0.2; 5.2]	2	[0.1; 7.9]	25.0	[10.7; 80.3]	32.8	[13.3; 85.4]	45.6	[24.2; 54.2] *	54.2	[40.0; 79.0]
	1940	Phenylacetonitrile	2.9	[0.6; 4.5] *	0.1	[<0.01; 5.0]	<0.01	[<0.01; <0.01]	<0.01	[<0.01; <0.01]	<0.01	[<0.01; <0.01]	<0.01	[<0.01; 0.3]
	1953	Jasmone	0.1	[<0.01; 0.1]	<0.01	[<0.01; <0.01]	<0.01	[<0.01; <0.01]	<0.01	[<0.01; <0.01]	<0.01	[<0.01; <0.01] *	0.6	[<0.01; 1.0]
	2024	Methyl eugenol	0.04	[<0.01; 1.3]	<0.01	[<0.01; <0.01]	2.7	[0.7; 5.6]	1.9	[<0.01; 4.3]	2.4	[2.0; 3.8] *	1.3	[0.1; 2.3]
	2053	Nerolidol	0.03	[<0.01; 1.6]	0.2	[<0.01; 1.4]	<0.01	[<0.01; <0.01]	<0.01	[<0.01; <0.01]	<0.01	[<0.01; <0.01]	<0.01	[<0.01; <0.01]
	2177	Eugenol	<0.01	[<0.01; 0.01]	<0.01	[<0.01; <0.01]	2.1	[0.1; 10.9]	3.2	[<0.01; 8.6]	3.1	[1.4; 3.5]	1.8	[0.4; 4.0]
	2211	4-Vinylguaiacol	0.1	[<0.01; 1.6]	<0.01	[<0.01; 1.4]	<0.01	[<0.01; <0.01]	<0.01	[<0.01; <0.01]	<0.01	[<0.01; <0.01]	<0.01	[<0.01; <0.01]
	2250	Methyl anthranylate	4.2	[2.8; 5.3]	3.4	[1.0; 8.6]	<0.01	[<0.01; <0.01]	<0.01	[<0.01; <0.01]	<0.01	[<0.01; <0.01]	<0.01	[<0.01; <0.01]
	2299	Geranic acid	0.1	[<0.01; 1.1]	<0.01	[<0.01; 2.0]	0.02	[<0.01; 0.1]	<0.01	[<0.01; 2.1]	<0.01	[<0.01; 0.2]	<0.01	[<0.01; 3.9]
	2429	Farnesol	0.1	[<0.01; 1.1]	0.2	[<0.01; 2.7]	<0.01	[<0.01; 0.2]	<0.01	[<0.01; 0.5]	<0.01	[<0.01; <0.01] *	0.2	[<0.02; 0.4]
	2455	Indole	0.8	[<0.01; 1.3]	0.8	[<0.01; 3.2]	<0.01	[<0.01; <0.01]	<0.01	[<0.01; <0.01]	<0.01	[<0.01; <0.01]	<0.01	[<0.01; <0.01]

[a] Jeannot et al. (2005), and Albert Vieille SAS unpublished data.
[b] Ulusoy et al. (2009), Mahboubifar et al. (2014), and Albert Vieille SAS unpublished data.
[c] Aslam Khan & Shoaib Ur (2005) and Albert Vieille SAS unpublished data.
[d] Experimental retention index on polar column (UBWAX).
[e] For each hydrosol and each volatile compound, * indicates a significant difference between samples analyzed in the present work, and literature and industrial data at $P < 0.05$ (Mann–Whitney test).

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orange blossom hydrosols and differences in the concentrations were significant ($P < 0.05$) only for a few compounds. The major compounds in the samples analyzed in this work, i.e., linalool and α-terpineol in orange blossom hydrosols, 2-phenylethanol, citronellol and geraniol in both rose sp. hydrosols, were also in high amounts in hydrosols analyzed in previous studies, and variations in 6-methyl-5-hepten-2-one, linalool, α-terpineol, citronellol, geraniol, and 2-phenyl ethanol amounts among samples for example were also previously reported (Agarwal et al., 2005; Aslam Khan & Shoaib Ur, 2005; Jeannot et al., 2005; Mahboubifar, Shahabipour, & Javidnia, 2014; Ulusoy et al., 2009; Albert Vieille SAS unpublished data) (Table 2).

The major compounds contained in the EO phase of rose and orange blossom hydrosols are known for their antimicrobial properties. For example, linalool exhibits an inhibitory activity against *E. coli* and *Listeria monocytogenes* with minimal inhibitory concentrations (MICs) of 200 mg/L (Ait-Ouazzou et al., 2011), of 900 mg/L against some strains of *E. coli*, *S. aureus*, and *Ba. cereus*, and no inhibitory activity (MIC > 900 mg/L) against other strains of *E. coli*, *S. aureus*, *Ba. cereus*, and *L. monocytogenes*, *Salmonella* Typhimurium, *P. aeruginosa*, *Candida albicans*, and *Saccharomyces cerevisiae* (Cosentino et al., 1999); the MIC of 2-phenylethanol against *E. coli* and *Ralstonia solanacearum* is 200 mg/L (Zhu et al., 2011); the MICs of α-terpineol against *E. coli*, *Salmonella* Typhimurium, *S. aureus*, and *Ba. cereus* are between 225 mg/L and 900 mg/L (Cosentino et al., 1999); geraniol's MIC against *E. coli* and *Salmonella* Typhimurium is 500 mg/L, and 1000 mg/L against *L. monocytogenes* (Burt, 2004). EOs show better antibacterial activity against Gram-positive bacteria than Gram-negative bacteria (Burt, 2004). Linalool concentration observed in orange blossom hydrosol was close to the MIC against *E. coli* and *L. monocytogenes* (Ait-Ouazzou et al., 2011). However the concentrations of the other compounds in orange blossom, rose C. and rose D. flower hydrosols were 1.4 to >1000-fold lower than the reported MICs of pure compound solutions. These concentrations are likely not sufficient to allow microbiological stability, as already shown in rose flower hydrosol (Ulusoy et al., 2009).

3.1.2. Nonvolatile compounds and pH

Hydrosol pHs were comprised between pH 4 and pH 7 and were unrelated to area and year of production, and to type of distilled flower (Table 3). The dry matter contents were comprised between 95 and 702 mg/L, except in a laboratory distillate of orange blossom (*C. aurantium* # 9, Table 3) where it was 4.0 mg/L. Carbohydrates were detected at concentrations of (mean ± standard deviation, sd) 0.21 ± 0.01 mg/L and 0.25 ± 0.02 mg/L in two freshly distilled rose C. hydrosols, and of 0.38 ± 0.03 mg/L in a freshly distilled orange blossom hydrosol. In these hydrosols, organic and amino acid concentrations were lower than the threshold of detection, respectively 0.7 mg/L and 2 mg/L. The presence of low amounts of non-volatile compounds in freshly distilled hydrosols suggests their entrainment by steam. Laboratory distillations of solutions of pure non-volatile compounds of yeast extract, glycin, and sucrose, each at a concentration of 20 g/L, confirmed this hypothesis. Respectively 0.74 ± 0.4% (n = 3), 3.5 ± 0.4% (n = 3), and 0.31% (n = 1) of these compounds were found in the distillation products. The entrainment of water droplets during evaporation has been previously described (Cosandey & von Rohr, 2001; Nakoryakov, Misyura, & Elistratov, 2012; Prabhudharwadkar, More, & Iyer, 2010). Consequently the distillation process may enrich the hydrosols in some compounds other than EOs, which could be used as nutrients by microorganisms.

Table 3
Composition, pH of, and bacterial counts in orange blossom (*Citrus aurantium*), rose D. (*Rosa Damascena*), and rose C. (*Rosa centifolia*) hydrosols from diverse origins and stored in different conditions.

Hydrosol	Sample[a] #	pH	Dry matter (mg/L)[b]	Carbohydrates (mg/L)	Microbiological counts	
					log_{10} CFU/mL	log_{10}cell/mL
Citrus aurantium	1	4.8	253 ± 12	NT	6.4	6.2
	2	4.2	319 ± 21	NT	4.9	4.6
	3	5.6	384 ± 6.7	NT	6	6
	4	4.7	95 ± 3.2	NT	4.1	4
	5	4.5	NT	NT	5.3	6.6
	6.1 [c]	6.8	164 ± 17	NT	4.5	4.7
	6.2 [c]	7	NT	NT	4.5	4.8
	6.3 [c]	6.5	NT	NT	4.2	ND
	7.1 [c]	4.1	NT	NT	6.1	6.1
	7.2 [c]	4.1	NT	NT	5.9	5.9
	8.1 [c]	4	NT	NT	4.5	6.1
	8.2 [c]	4.4	NT	NT	6.4	7.1
	9 [c,d]	4.7	4.0 ± 2.6	0.38 ± 0.03	<0.7	<4.00
	10	4	NT	NT	6.3	6.5
Rosa damascena	11	4.8	NT	NT	ND	5.1
	12	7	NT	NT	3.9	ND
	13	6.4	NT	NT	ND	6
	14	4	NT	NT	7	7
	15	4.7	NT	NT	5.5	5.5
	16	5.4	NT	NT	3.8	5.7
Rosa centifolia	17 [c]	6.9	348 ± 25	0.25 ± 0.02	<0.7	<4.00
	18.1 [c]	7.1	702 ± 88	NT	5.8	5.8
	18.2 [c]	7	641 ± 94	NT	5.8	5.8
	18.3 [c]	6.4	NT	NT	4.8	5.4
	19	4	NT	NT	5.5	6
	20	6.7	NT	NT	4.6	4.8
	21.1 [c]	4.8	281 ± 13	0.21 ± 0.01	<0.7	<4.00
	21.2 [c]	5.6	NT	NT	<0.7	<4.00
	22.1 [c]	5.9	NT	NT	7	6.6
	22.2 [c]	5.6	NT	NT	7.1	7.3

[a] See Table 1.
[b] Mean ± sd, n = 3 analytical replicates of each sample.
[c] Samples analyzed at several time intervals during a 3-month storage.
[d] Laboratory (Albert Vieille SAS) distillation; NT, not tested; ND, not determined.

3.2. Microbiological counts and changes with storage time

Microbiological counts in fresh rose C. flowers were 4.9 $\log_{10}$ CFU/g (±0.3) for bacteria and 3.9 $\log_{10}$ CFU/g (±0.5) for yeasts and molds. Approximately 300 kg of flowers and 750 L of water are mixed for distillation. At the end of the distillation, the microbial population in the hydrosol was lower than 0.7 $\log_{10}$ CFU/mL. The distillation process therefore achieved at least a 4 $\log_{10}$ CFU/mL (±0.3) reduction for bacteria and 3 $\log_{10}$ CFU/mL (±0.5) reduction for yeasts and molds. The hydrosols collected in a sterile container at the end of the distillation line (#9, #17, #21.1 and #21.2, Table 3) showed counts remaining low (<0.7 $\log_{10}$ CFU/mL) during a three-month storage period.

Two orange blossom hydrosol samples (#7 and #8) and two rose C. (#18 and #22) were stored at 5 °C (samples #7.1, #8.1, #18.1 and #22.1) or at ambient temperature (samples #7.2, #8.2, #18.2 and #22.2). Changes in bacterial populations were followed over a three-month storage period (Fig. 2). In orange blossom hydrosols, maximal bacterial populations were reached within 10 days at ambient temperature. The two samples of this hydrosol stored at 5 °C showed two different patterns: either bacterial counts that plateaued at 6 to 7 $\log_{10}$ CFU/mL over the examined storage period (Fig. 2, A), i.e., at the maximal counts observed in the other studied samples, or a pronounced decrease in the microbial counts, which were lower than the threshold of enumeration (i.e., <1 $\log_{10}$ CFU/mL) after 80 days (Fig. 2, B) (Table 3). In the two rose C. hydrosols, maximal bacterial populations (about 6 to 7 $\log_{10}$ CFU/mL) were reached within a few days when stored at ambient temperature, and in fifteen days to two months when stored at 5 °C (Fig. 2, C and D). For hydrosols analyzed at variable times during shelf-life, total bacterial counts were between 4 and 7 log10 CFU/mL (Table 3). High (or low) bacterial counts were not related with any manufacturer, year of production, pH, or EO concentration. Furthermore, a cold storage was not sufficient to insure stability. Most of the CFU counts were close to the cell counts. The slope of the linear regression curve between CFU counts and cell counts was 1.04, which was not significantly different from 1 ($P > 0.05$). In sample #8.1 stored at 5 °C, one of the few with cell counts markedly higher than CFU counts, a Live/Dead® BacLight viability kit assay (Molecular Probes, Eugene, USA) showed that most cells were not viable. As consequence the major part of viable micro-organisms present in hydrosols were likely in a culturable state.

3.3. Bacteria identification and phylogeny

Only bacteria were detected in the tested hydrosols, either spread onto DPCA or YGC agar. In total, 71 bacteria strains have been isolated from hydrosols. A molecular typing (M13-PCR) on 40 strains permitted eliminating 13 clones among isolates. In this way, we isolated 58 representative strains. All of them were Gram-negative species, known as environmental contaminants (Elomari et al., 1996; Lou, Zhang, Su, & Xie, 2007; Park et al., 2005), and belonged to a limited number of genera (Table 4). Phylogeny based on *rrs* genes showed an arrangement of the strains in four major branches: a *Pseudomonas* sp. branch, a *Bu. cepacia* complex branch, and two branches organized around undefined species belonging to *Rhodospirillaceae* and *Acetobacteraceae* (Supplementary data Fig. S1). The whole *rrs* genes of these two presumed new species are available under the accession numbers LN831188 and LN831189, respectively, in the European Nucleotide Archive (http://www.ebi.ac.uk/ena). Highly distant bacterial species were able to multiply in hydrosols. Many of them are described as acido-tolerant (Stopnisek et al., 2014) and are therefore rather adapted to survival and growth at the range of pHs of hydrosols (as low as 4.0 in some samples). They seem highly associated to hydrosols. For example *P. veronii* was isolated from hydrosols of different types and different areas and year of production (Table 4). *Pseudomonas* and *Burkholderia* species represent 39 out of the 58 hydrosol isolates. These species require low nutrients for growth, and are largely represented in water isolates (Elomari et al., 1996; Vanlaere et al., 2009). *N. capsulatum* isolated in a sample of orange blossom hydrosol has been first isolated from distilled water. Its colonies are yellow or whitish-brown (Leifson, 1962; Takeuchi, Hamana, & Hiraishi, 2001) and this could be in agreement with the yellow to brown sediment observed in contaminated orange blossom hydrosol containers (unpublished data). Neither yeast nor mold was detected in the studied hydrosols. Conditions of hydrosol production are very variable, but production in small units, filling of storage containers in the open air, and unhygienic practices are less

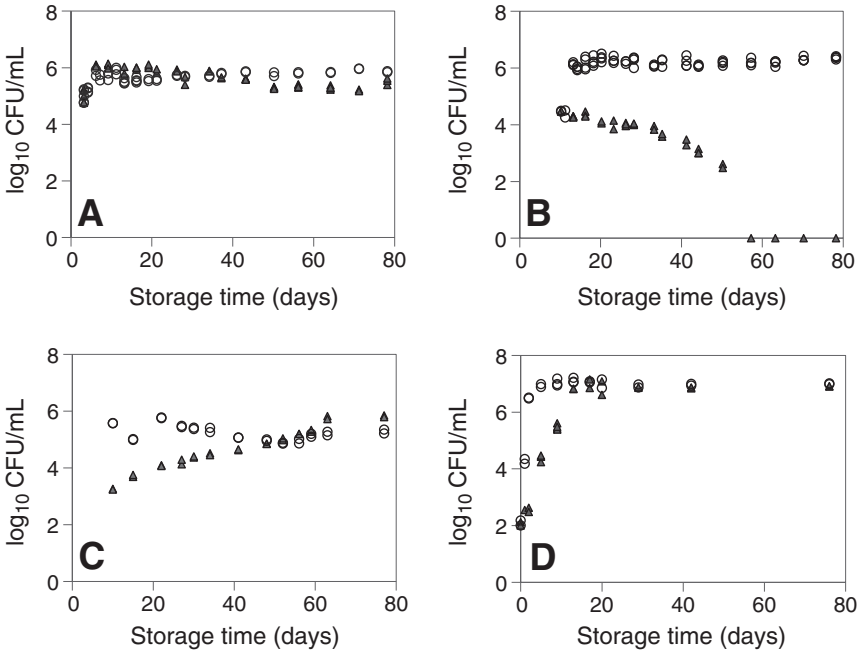

Fig. 2. Changes in bacterial counts at 5 °C (▲) and 20 °C (○) in orange blossom (*Citrus aurantium*) (A, B) and rose C. (*Rosa centifolia*) (C, D) in hydrosols from different origins and/or year of production. Three microbiological counts have been performed at each sampling date and each was represented by a dot. A) Hydrosols #7.1 and #7.2.; B) hydrosols #8.1 and #8.2; C) hydrosols #18.1 and #18.2; D) hydrosols #22.1 and #22.2 (Table 1).

Table 4
Identification of hydrosol bacteria.

Hydrosol	Identification	Identity (%) on NCBI [a]	Number of isolates [b]	Representative strain	Sample# [c]	Storage temperature (°C)	Manufacturer	Year	Production country
Citrus aurantium (orange blossom)	*Pseudomonas veronii*	99	1	IA_FO_02 [d]	2	ND	B	2013	France
	Pseudomonas veronii	99	1	IA_FO_03 [d]	2	ND	B	2013	France
	Pseudomonas veronii	99	1	IA_FO_04 [d]	2	ND	B	2013	France
	Pseudomonas panacis	99	1	IA_FO_19 [d]	6.1	5	B	2014	France
	Pseudomonas panacis	99	1	IA_FO_16 [d]	6.2	20	B	2014	France
	Pseudomonas panacis	99	1	IA_FO_20 [d]	6.1	5	B	2014	France
	Pseudomonas cedrina	99	1	IA_FO_01	2	ND	B	2013	France
	Burkholderia vietnamiensis	99	1	IA_FO_09 [d]	3	ND	C	2013	Tunisia
	Burkholderia vietnamiensis	99	3	IA_FO_21 [d]	7.1	5	D	2014	Tunisia
	Burkholderia vietnamiensis	99	3	IA_FO_28 [d]	8.2	20	E	2014	Tunisia
	Burkholderia vietnamiensis	99	1	IA_FO_29 [d]	8.1	5	E	2014	Tunisia
	Burkholderia vietnamiensis	99	1	IA_FO_31 [d]	8.1	5	E	2014	Tunisia
	Burkholderia lata	100	1	IA_FO_15 [d]	6.2	20	B	2014	France
	Burkholderia lata	99	1	IA_FO_23 [d]	7.1	5	D	2014	Tunisia
	Burkholderia lata	99	1	IA_FO_26 [d]	7.2	20	D	2014	Tunisia
	Burkholderia lata	99	1	IA_FO_34 [d]	10	Ambient	D	2014	Tunisia
	Burkholderia arboris	99	1	IA_FO_07	3	ND	C	2013	Tunisia
	Burkholderia ambifaria	99	1	IA_FO_08 [d]	3	ND	C	2013	Tunisia
	Azospirillum amazonense	95	1	IA_FO_05 [e]	0	ND	D	2012	Tunisia
	Azospirillum amazonense	95	1	IA_FO_12 [e]	1	ND	A	2013	Tunisia
	Azospirillum amazonense	96	1	IA_FO_10 [e]	4	Ambient	D	2013	Tunisia
	Azospirillum amazonense	95	1	IA_FO_18 [e]	5	ND	A	2013	Tunisia
	Azospirillum amazonense	95	1	IA_FO_22 [e]	7.1	5	D	2014	Tunisia
	Azospirillum amazonense	95	1	IA_FO_25 [e]	7.2	20	D	2014	Tunisia
	Acidomonas methanolica	97	1	IA_FO_13 [f]	1	ND	A	2013	Tunisia
	Acidomonas methanolica	96	1	IA_FO_11 [f]	4	Ambient	D	2013	Tunisia
	Acidomonas methanolica	95	1	IA_FO_17 [f]	5	ND	A	2013	Tunisia
	Novosphingobium capsulatum	99	1	IA_FO_33	8.2	20	E	2014	Tunisia
	Enterobacter cloacae	99	1	IA_FO_06	3	ND	C	2013	Tunisia
Rosa damascena (rose D.)	*Pseudomonas veronii*	99	2	IA_RD_06 [d]	15	ND	J	2013	Turkey
	Pseudomonas veronii	99	1	IA_RD_07 [d]	15	ND	J	2013	Turkey
	Pseudomonas veronii	99	1	IA_RD_01 [d]	16	ND	K	2013	Bulgaria
	Acidomonas methanolica	97	1	IA_RD_04 [f]	14	ND	I	2013	Morocco
	Achromobacter sp.	100	1	IA_RD_02	14	ND	I	2013	Morocco
	Gluconacetobacter sp.	100	1	IA_RD_03	14	ND	I	2013	Morocco
Rosa centifolia (rose C.)	*Pseudomonas protegens*	99	2	IA_RC_09	18.1	5	B	2013	France
	Pseudomonas protegens	100	1	IA_RC_10	18.2	20	B	2013	France
	Pseudomonas protegens	99	1	IA_RC_04	19	ND	B	2012	France
	Pseudomonas veronii	99	2	IA_RC_11 [d]	18.1	5	B	2013	France
	Pseudomonas veronii	99	2	IA_RC_01 [d]	19	ND	B	2012	France
	Pseudomonas veronii	99	1	IA_RC_05 [d]	19	ND	B	2012	France
	Pseudomonas veronii	99	3	IA_RC_22 [d]	22.1	5	B	2014	France
	Pseudomonas veronii	99	1	IA_RC_32 [d]	22.2	20	B	2014	France
	Pseudomonas veronii	99	1	IA_RC_40 [d]	22.2	20	B	2014	France
	Pseudomonas panacis	99	1	IA_RC_12 [d]	18.1	5	B	2013	France
	Pseudomonas panacis	99	2	IA_RC_13 [d]	18.1	5	B	2013	France
	Pseudomonas panacis	99	1	IA_RC_34 [d]	22.2	20	B	2014	France
	Burkholderia lata	99	1	IA_RC_17 [d]	18.2	20	B	2013	France
	Burkholderia lata	99	1	IA_RC_18 [d]	18.2	20	B	2013	France
	Burkholderia lata	99	1	IA_RC_24 [d]	22.1	5	B	2014	France
	Burkholderia lata	99	2	IA_RC_30 [d]	22.2	20	B	2014	France
	Burkholderia lata	99	1	IA_RC_39 [d]	22.2	20	B	2014	France
	Burkholderia diffusa	99	1	IA_RC_16	18.2	20	B	2013	France
	Azospirillum amazonense	96	1	IA_RC_03 [e]	19	ND	B	2012	France
	Acidovorax wautersii	99	1	IA_RC_06	20	ND	B	2012	France
	Sphingobium baderi	98	1	IA_RC_07	20	ND	B	2012	France
	Janthinobacterium lividum	100	2	IA_RC_14	18.1	5	B	2013	France
	Delftia acidovorans	99	1	IA_RC_08	20	ND	B	2012	France

[a] *rrs* gene sequence identity.
[b] Number of clonal isolates pointed out by molecular typing (M13-PCR).
[c] Same as Table 1.
[d] Species subjected to phenotypic tests for identification.
[e] Presumed new specie (accession number LN831188).
[f] Presumed new specie (accession number LN831189); ND: not determined.

than uncommon (Watt, 2015) and are the cause of contamination by the environmental bacteria identified in this work.

3.4. Impact of bacteria on volatile compounds

The analyses of EO composition of hydrosols during shelf life showed that the volatile profile may change with time. For example, rose C. hydrosols in which *Pseudomonas* sp., *Bu. lata*, *Burkholderia diffusa*, and *Janthinobacterium lividum* were the most common isolates (#18), or not contaminated (#17) (Table 3), stored at 5 °C as well as ambient temperature, kept the same volatile profile during at least six months of storage. In contrast pronounced changes were observed in the orange blossom hydrosol #8.2 stored at 20 °C dominantly contaminated by *Bu. vietnamiensis* and *N. capsulatum*, in which linalool and geraniol

concentrations markedly decreased (respectively from 343 mg/L to 146 mg/L and from 61 mg/L to 2.2 mg/L) and concomitantly 6-methyl-5-hepten-2-one concentration increased from 1.3 mg/L to 128 mg/L.

Analysis of the EO composition of rose C. and orange blossom hydrosols (Fig. 3), after inoculation with a strain of *Burkholderia vietnamiensis* (strain IA_FO_28), at final bacteria concentrations of respectively 8.0 (±0.1) CFU/mL and 7.0 (±0.2) CFU/mL, showed that this species was able (i) to metabolize geraniol and to synthesize citronellol and geranic acid in the rose C. hydrosol and (ii), to convert geraniol and phenylethyl acetate into respectively geranic acid and 2-phenylethanol in the orange blossom hydrosol (difference between sterile control and inoculated samples significant at $P < 0.01$). Similarly, *N. capsulatum* (strain IA_FO_33), at final bacterial concentrations of respectively 7.0 (±0.1) CFU/mL and 6.6 (±0.04) CFU/mL, was able (i) to convert geraniol into 6-methyl-5-hepten-2-one in both hydrosols, (ii) to convert citronellol into citronellic acid in the sterile rose C. hydrosol, and (iii) to metabolize α-terpineol, methyl anthranilate and phenylethyl acetate which was finally converted into 2-phenylethanol in the orange blossom hydrosol (difference between sterile control and inoculated samples significant at $P < 0.01$). Metabolization of terpenoids by micro-organisms follows different pathways (Demyttenaere, 2001; Marmulla & Harder, 2014). For example *Pseudomonas putida*, thanks to a plasmid, and *Penicillium digitatum* convert geraniol into 6-methyl-5-hepten-2-one (Vandenbergh, 1989; Wolken & van der Werf, 2001). Moreover *P. putida* can use geraniol (or citronellol) as the sole carbon source (Vandenbergh & Cole, 1986; Vandenbergh & Wright, 1983). Similarly the bacterium *Castellaniella defragrans* converts geraniol into geranic acid using a geraniol and geranial dehydrogenase, with a gain of two NADH molecules (Lüddeke et al., 2012). *P. aeruginosa* converts citronellol into citronellic acid by successive enzymatic reactions involving the geraniol dehydrogenase and the citronellal dehydrogenase (Förster-Fromme et al., 2006). Further research would be necessary to identify such enzymatic activities in those strains *N. capsulatum* and *Bu. vietnamiensis* representative of the hydrosol microbiota.

4. Conclusion

Hydrosols of orange blossom, rose C., and rose D harbors a heterogeneous microbiota able to proliferate to relatively high numbers (up to 10^6–10^7 CFU/mL) despite the presence of EOs. Some of the strains contaminating hydrosols during process operations have an impact on volatile profile. The production of 6-methyl-5-hepten-2-one in hydrosols was specific to the presence of *N. capsulatum* and could be used as a chemical marker for such contamination. All the identified bacterial contaminants could be eliminated by pasteurization (no spore-forming bacteria identified), but with a possible alteration of the aromatic profile (Baydar, Kuleasan, Kara, Secilmis-Canbay, & Kineci, 2013). In the absence of an aseptic packaging at the end of the distillation line and/or filtration, and because of the relative inefficiency of long term storage at low temperature, stability has to be obtained with chemical preservatives, which must comply with sanitary rules

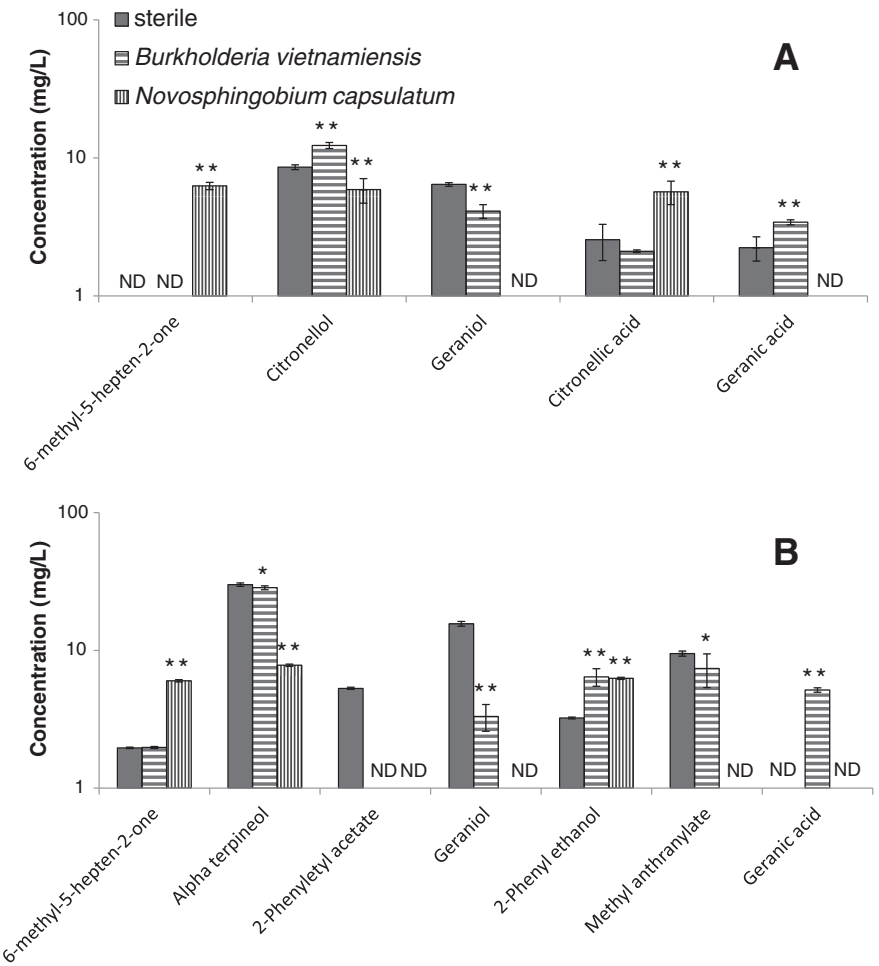

Fig. 3. Impact of *Bu. vietnamiensis* and *N. capsulatum* on aromatic compounds in *Citrus aurantium* (A) and *Rosa centifolia* (B) hydrosols after 10 days of incubation. Full gray: sterile hydrosol; horizontal stripes: hydrosol inoculated with *Burkholderia vietnamiensis* strain IA_FO_28; vertical stripes, hydrosol inoculated with *Novosphingobium capsulatum* strain IA_FO_33. ND: not detected, concentration lower than the detection threshold limit (about 1 mg/L). Errors bars surrounding mean values represent sd (n = 3). ** (*) indicates a significant difference between the sterile control and the hydrosol inoculated with each strain at $P < 0.01$ ($P < 0.05$) (Mann–Whitney test).

and also keep the physical, chemical and delicate sensory quality of the products.

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Contributors

CL carried out most of the experimental work, analyzed and interpreted the data and wrote the initial versions of the manuscript. CG advised on chemical analysis and interpretation of chemical data. MHG contributed to design bacterial identifications and phylogeny approaches. CR made a substantial contribution to the conception and design of the experimental work. CC coordinated the collection of the analyzed samples and advised about the hydrosol process and characterization. FC supervised the project and revised the initial versions of the paper. All authors commented on the manuscript.

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Appendix A. Supplementary data

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