IRIS - Archivio Istituzionale dell'Università degli Studi di Sassari

Isolation and characterization of microorganisms and volatiles associated with Moroccan saffron during different processing treatments

Questa è la versione Post print del seguente articolo:

*Original*
Isolation and characterization of microorganisms and volatiles associated with Moroccan saffron during different processing treatments / Fancello, Francesco; Petretto, Giacomo Luigi; Sanna, Maria Lina; Pintore, Giorgio Antonio Mario; Lage, Mounira; Zara, Severino. - In: INTERNATIONAL JOURNAL OF FOOD MICROBIOLOGY. - ISSN 0168-1605. - 273:(2018), pp. 43-49. [10.1016/j.ijfoodmicro.2018.03.014]

*Availability:*
This version is available at: 11388/205148 since: 2022-05-23T10:44:06Z

*Publisher:*

*Published*
DOI:10.1016/j.ijfoodmicro.2018.03.014

*Terms of use:*

Chiunque può accedere liberamente al full text dei lavori resi disponibili come “Open Access”.

*Publisher copyright*

note finali coverpage

(Article begins on next page)

25 May 2026

Elsevier Editorial System(tm) for
International Journal of Food Microbiology
Manuscript Draft

Manuscript Number:

Title: Isolation and characterization of microorganisms and volatiles associated with Moroccan saffron during different processing treatments

Article Type: Short Communication

Keywords: Saffron drying methods; Safranal; Microbial biodiversity; Bacillaceae

Corresponding Author: Dr. Severino Zara, Ph.D

Corresponding Author's Institution:

First Author: Francesco Fancello, Post Doc

Order of Authors: Francesco Fancello, Post Doc; Giacomo Petretto, Assistant professor; Maria Lina Sanna; Giorgio Pintore, Associate Professor; Mounira Lage; Severino Zara, Ph.D

Suggested Reviewers: Manuel Carmona
manuel.carmona@universidadeuropea.es
saffron expert

Hamid Reza Feili Reza Feili
hrfeili@gmail.com
saffron expert

Rafael Rotger Anglada
rrotger@farm.ucm.es

Vida Maghsoodi
maghsoodi@sharif.edu

**Università degli Studi di Sassari**
**DIPARTIMENTO DI AGRARIA**
Sezione Scienze e Tecnologie Ambientali e Alimentari

Viale Italia 39, 07100 Sassari
Tel. +39. 079 229386/17 Fax. +39. 079 229370
Partita IVA 00196350904

Sassari, 20 December 2017

Editorial office

*International Journal of Food Microbiology*

Dear Editor,

We would like to *re-submit the new version of the manuscript* "***Isolation and characterization of microorganisms and volatiles associated with Moroccan saffron spices during different processing treatments***" by Fancello et al., as a Short Communication to *International Journal of Food Microbiology.*

*The above manuscript has been rejected from your journal with the recommendation of the Editor Dr. Albert Mas to re-submit again the work after a serious revision that included some plagiarisms, the English language, and other major and minor criticisms.*

*The new version of the manuscript has been edited by an English language expert, the plagiarisms have been eliminated by adding the appropriate citations and the major and minor criticisms underlined by the Reviewers, solved.*

Saffron is the most expensive spice in the world and consists of the dried stigmas of *Crocus sativus L*. Saffron is exposed to a wide range of environmental microbial contamination during collection, processing, and in the retail markets by dust, wastewater, and animal excreta. Although saffron is added during cooking, so this risk is limited by the thermal processing of the food, some preparations involve cold infusion in water and oil extraction. For this reason, its addition to food may result in the proliferation of bacterial populations. This also in consideration that in many

producers countries, due to lack of techniques and hygiene during harvesting gathering, handling, draying, packaging and storage, causes contamination increasing and decreasing of the quality.
This preliminary study focused on the exploitation of natural microbiota present in saffron as well as to the targeted analysis of some carotenoid-derived aroma compounds. For this reason, three samples of saffron resulting from different dried system plus one sample of saffron from the market have been subjected to microbiological and chemical head-space analyses.

*Main findings of this study*

*Chemical composition:* The head-space chemical analysis show that the main components of saffron are carotenoid derived compounds, mainly represented by safranal. As previous documented in literature, the amount of volatiles of our samples depend, as well as the microbiota, on the drying method. Higher temperatures involve higher production of volatile organic compounds.

*Microbial biodiversity:* We found that the microbiota of saffron is dominated by bacteria belonging to *Bacillaceae* family and microflora is strongly influenced by the dried methods. Of particular interest *Dietzia maris* as possible potential pathogen and *Bacillus amyloliquefaciens* and *Bacillus aryabhattai* as biocontrol agents. Moreover, of particular interest could be *Carnobacterium maltaromaticum* a LAB also not previously isolated from saffron.

*Novelty:* To our knowledge this is the first report that investigated the microbiota of the edible part of Moroccan saffron.

*All the authors have approved the attached revised version of the manuscript.*

*We thank you in advance for your interest in our new submission of the revised version of the manuscript.*

Sincerely,
Dr. Severino Zara, PhD

**Responses to Technical Check Results**

Dear Reviewers,

Here below all the answers to the criticisms found in the manuscript. The English language has been edited by a specialized Company.

Sincerely,

Severino Zara

Ms. Ref. No.: FOOD-D-17-00981
Title: Isolation and characterization of microorganisms and volatiles associated with Moroccan saffron spices during different processing treatments
International Journal of Food Microbiology

Dear Dr. Severino Zara,

Unfortunately it has been concluded that your work is not acceptable for publication in the International Journal of Food Microbiology. However, you can resubmit your manuscript (as a new manuscript) if you rewrite seriously the whole manuscript. Besides the comments raised by the reviewers, especially reviewer 2, I have found additional shortcomings in your m manuscript. First of all, the extensive plagiarism that has been detected in the introduction and Material and Methods. For instance Lines 57--62 were identical to Cosano et, J. Food Protect, 2009
R. Modified and reference added as required (lines 50-56 new revised manuscript)
and Lines 67--72, identical to Del Campo et al et, J. Sci,. Food Agric., 2010.
R. Modified and reference added as required (lines 68-73 new revised manuscript)
Furthermore Lines 171-192, 267-269, 272-274 were identical to Mangia et al, J. Appl. Microbiol, 2016, article that was not mentioned and listed in references).
R. Modified and reference added as required (lines 174-202 new revised manuscript)
The highlights should be revised thoroughly (what are "saffron methods", maybe saffron drying methods?, among others.
R. Highlights have been revised as required
Check the symbol corresponding to "micro" (the text in this letter does not take the corresponding greek letter) throughout the manuscript.
R. Modified as required

For your guidance, the reviewers' comments are included below.

I hope that you understand the reasons for the decision made and I look forward to receiving other manuscripts from you in the future.

Yours sincerely,

Albert Mas, Ph.D.
Editor
International Journal of Food Microbiology

Reviewers' comments:

Reviewer #1: This work could be accepted with further review. The authors should review the following points:
Page 4 Line 66
Khazanei et al. 2006 in References figure 2011, the authors should clarify this.
R. It was 2011, we modified in the text as required.
Page 4 Line102
The authors must clarify that under the indicated conditions, the aromas are not generated.
Dear Reviewer, thank you for the suggestion, actually the experiments were carried out at 35°C following the procedure proposed by D'auria et al. 2004. Flav. Frag. J. 19, 17-23 with slight modification. The material and method section has been now corrected. Since the amount of detected volatiles is linked to drying temperature, according previous literature data, is possible suppose that the used temperature does not generate any aroma compounds.
Pages 7-8 Lines 133-156
Authors should separate paragraph 2.2 into two paragraphs where they clearly describe the two aspects. They must expand the description of the sampling, since it is an important fact for the objective pursued by the work; they should describe the drying conditions of each of the samples, especially they should indicate the drying temperature.
R. I think there is a misunderstood, in fact, the conditions cited here, in the paragraph 2.2, are related strictly only to saffron preparation for the isolation of microorganisms. As described, we diluted the saffron samples on Ringer solution in polypropylene tubes and we vortexed the samples in order to facilitate the microbial release. As written in the manuscript, we obtained the saffron directly from a co-author of the work, from local producers. In the new version of the manuscript we erased lines 134-136, in order to avoid confusion in the reader. For these reasons another paragraph was added at the beginning of the Material & Methods section whit the description of the sampling (lines 92-99 new version of the manuscript).
Page 9 Line 194
Authors should remove the underline in "Gordon et al. 1997"
R. Done as required
Page 11 Line 246

D'Auria et al. (2003) is not in References. According to Scopus, this author does not have publications on saffron in 2003. Authors should check this.
R. We modified in the text as required.
Page 12 Line 254
Change "D'auria" by "D'Auria".
R. We modified in the text as required.
Page 12 Line 264
Remove the point
R. We modified in the text as required.
Page 13 Line 286
Mia et al., 2017 appears in References in year 2016, the authors must correct this fact.
R. We modified in the text as required.
Page 14 Line 305
Change "table 2" by "Table 2".
R. We modified in the text as required.
Page 15 Lines 334-341
According to Guide for Authors, change "Figure legend" by "Figure captions".
R. We modified in the text as required.
Page 16 Line 349
According to Guide for Authors, change "Literature" by "References".
R. We modified in the text as required.
In the references section:
In Lines 365, 368, 383, 419 and 464. The authors must remove space between references.
R. We modified in the text as required.
Authors should clarify years of dating earlier said: D'Auria, M., et al 2003; Khazanei et al. 2006; Mia et al., 2017.
R. We modified in the text as required.
Authors should write well the header of Table 1.
R. We modified in the text as required.

Reviewer #2: The manuscript by Zara et al entitled 'Isolation and characterization of microorganisms and volatiles associated with Moroccan saffron spices during different processing treatments' reports the effect of post-harvest drying methods on the volatile composition and microbiota. Although a number of experiments have been carried out but the manuscript has soem shortcomings which are mentioned below for consideration of the authors

Major issues

1. The manuscript is poorly written.

R. The manuscript has been carefully edited by a specialized company

2. The authors seem to be confused as to whether the number 82 refers to the 82 isolates or microorganisms. As at one instance they mentioned 82 microorganisms and at the other 82 isolates. Given, the fact that many isolates may represent same microorganisms, I urge the author to clarify it.

R. Sorry for the mistake. Among the different colonies present in the plates, we randomly picked up 82 colonies that were representative of the different colony morphologies, particularly, based on the shape, color and size. The colonies were re-streaked and checked for purity on agar plates, and 82 isolates subjected to further analyses.

3. How do the authors overrule the possibility of contamination? I mean the authors state that they are reporting natural microbial biodiversity of saffron and at the same time they report the human pathogen *Dietzia maris*. How are the authors sure that the other microorganisms that they report are not contaminations?

R. Our study is an exploratory work, analyzing for the first time in depth the effect of drying treatment on natural microbiota of saffron. At the same time the study allowed us evaluate the presence of "undesirables" microorganisms (such as pathogens or spoilage bacteria) on saffron. *Dietzia maris* is not a human pathogen *sensu stricto* but as many bacteria is an opportunistic human pathogen. As example, many strains of *Escherichia coli* are recognized as human pathogens, but other strains are probiotic strains (marketed as Symbioflor (Beimfohr C., 2016)). Furthermore, *Escherichia coli* is known as a normal component of gut microbiota. The concept of contamination *per se* is an anthropocentric concept view. The microbial "contamination" of food can be considered "natural" because the ubiquity of bacteria. We get used to consider contamination in a food process everything that is not an ingredient, for example in a conventional brewery any kind of microorganisms that is not the starter yeast added to ferment the wort is considered a contamination and dangerous, while in the of sour beer the natural microbiota is central to the fermentation process. Based on the obtained clusters, the majority of the microorganisms isolated belonged to the *Bacillaceae* family, and mostly are typical from soil environment. We did not find any typical species related to human contamination as *S. aureus* or *Salmonella* etc. We described *Dietzia maris* not as human pathogen because previously isolated from humans, but because, based on the recent studies, could be a potential emergent pathogen for humans. The pathogenicity of a particular bacterial strains is related to their pangenome and then at presence of virulence gene, pathogenicity islands etc, In the bacteria kingdoms the horizontal gene transfer it is the normality not the exception, therefore even if a bacterium is considered harmless could become a fearsome pathogen. In accord with the above,

different author recently (Bera et al., 2017; Gusain et al., 2017, Akbari et al., 2017,) have proved that *Dietzia maris* is a promising bacteria for several biotechnological application.

4. The authors report that the effect of drying methods on the volatile composition. This has also been reported previously. Please see,
-Raina BL, Agarwal SG, Bhatia AK, Gaur GS. Changes in Pigments and Volatiles of Saffron (Crocus sativusL) During Processing and Storage. Journal of the Science of Food and Agriculture. 1996 May 1;71(1):27-32.
-Gregory MJ, Menary RC, Davies NW. Effect of drying temperature and air flow on the production and retention of secondary metabolites in saffron. Journal of agricultural and food chemistry. 2005 Jul 27;53(15):5969-75.
-Maghsoodi V, Kazemi A, Akhondi E. Effect of different drying methods on saffron (Crocus sativus L) quality. Iranian Journal of Chemistry and Chemical Engineering (IJCCE). 2012 Jun 1;31(2):85-9.

Given these published articles, what is the novelty and advancements in the present work that should justify the publication of the present work.

R. This is one of the few manuscripts that describes saffron microbiota in depth. To date (16 of November 2017) only 17 works, using as keywords *saffron, bacteria, microorganisms and Crocus sativus,* appeared on Scopus, and mostly are related to underground parts of Crocus and only few on the saffron as food ingredients. To our knowledge no other studies to date have deepen the saffron edible microbiota before us and using our procedures. So, we think that there is novelty and advancements in our work that widely justify the publication of the work, obviously after the revision process.

5. How do the authors justify the use of market saffron sample for comparison, given the fact that there is a possibility that one of the other drying methods described in the study could have been used for the market sample as well.

R. The saffron market was given as as sample for sure obtained as a mix of saffron dried with different methods. On the contrary, the other saffron were produced by craftsmen with certain drying processes.

Minor comments

6. I found the highlights of the work embodied in the paper poorly stated and the language is unacceptable at certain places/

R. We improved English language in the entire body of the manuscript included the highlights.

7. An abstract should contain a brief background, the aim of the study, methods used and the major results followed by a brief conclusion. The abstract of the present manuscript is poorly structured. There are so many syntax errors and there is use of non-scientific terms. For instance
-'after harvest should be 'post-harvest'.
-'Expolitation' should be 'expoloration' .

R. Thank you for the comment. We agree about some syntax errors and we tried to correct them. We don't agree about the abstract structure, there is no rule for them, particularly about backgrounds. We followed the guides for authors that cite: "*A concise and factual abstract is required. The abstract should state briefly the purpose of the research, the principal results and major conclusions*". Purpose of the research, methods used and results obtained have been showed.
We changed after harvest and exploitation as required.

-'This work presents the results of a study on the cultivable bacteria of saffron dried with different methods, namely in the shade (natural), under the sun, and in an oven'... This sentence needs consideration. There are several other such instances. I suggest the manuscript should be revised by a native English speaker or sent to a professional language editing company

R. English language has been revised by native English speaker as required

8. Similar to the abstract the language of the introduction is poor and unacceptable

R. English language has been revised by native English speaker as required

9. GC/MS and GC/FID methods are not properly described. Did the authors develop new methods or used previously developed methods. If so the authors should cite the appropriate references.

R. Thank you for the comment. In literature is reported a great variability in the condition for SPME of volatiles from saffron:
Manzo et al. J. 2015. Mt. Sci. 12, 1542-1550 exposed the fiber for 3h at 25°C
D'auria et al. 2004. Flav. Frag. J. 19, 17-23, exposed the fiber for 20min at 36°C
Lage et al. 2015. Am. J. Ess. Oil Nat. Prod. 2, 1-7, exposed the fiber for 1min and the temperature was not reported
Karabagias et al. 2017. Eur. Food Res. Tec. 243, 1577-1591, exposed the fiber for 10 min at 25°C
Therefore we carried out some experiments in order to obtain the bigger TIC of chromatogram (previous optimization).

10. Authors should properly describe the procedure used for

microbiota or cite the appropriate references. What was the control in each experiment?

R. We cited the appropriate reference in the new version of the manuscript (lines 148-149). We used the saffron market as comparison.

11. 'The DNA extraction from 82 isolates, from randomly isolated colonies'. What do the authors mean by this. I mean the DNA extract should have been done after pure culturing of the isolated colonies.

R. We modified the sentence in order to clarify the concept.

12. The discussion is poor and some of the similar recent studies have been ignored. There is no mention of how this study could be useful to the readers. Secondly, the discussion starts with the aim of the study which should not be the case. Major portion of the discussion is the repetition of the results and therefore I suggest rewriting of the discussion.

R. Thank you for the comment. We tried to cite all the recent literature, over 47 works, also in consideration that this is a short communication. Moreover, the section is “Results and discussion” together, so seems to us obvious that results were included in the discussion. We tried to improve the section as required.

13. In conclusion 'Market was the saffron with the most complex biodiversity, possibly because it had been manipulated more and obtained from mixtures of different saffrons' this sentence is grammatically incorrect. There are several other instances.

R. We erased the incorrected sentence.

14. The conclusion is highly speculative

R. We agree with the reviewer and changed the conclusion

15. I wonder how the composition of volatile compounds is mentioned as µg/mg (Table 1). Authors should clarify it.

R. Thank you for the comment. The most used approaches in order to obtain a quantitative data on the volatiles of a food matrix are: (i) relative percentage abundance, (ii) internal standard normalized percentage abundance (*normalized % abundance*); (iii) true quantitation of one or

more components (*true quantitation*) (Bicchi et al Flavour Fragrance Journal 2008; 23: 382–391).
In the case of solvent free extraction methods (such as SPME) more variables should be considered and the representation of data as relative percentages result not suitable. In the present work we carried out a true quantification with a calibration curve (as reported in material and method section) of pure standard for the main compounds commonly detected in the saffron samples, isophorone and safranal. In this latter cases the amount of the compounds was expressed as □g of the target compound for each mg of saffron sample, as well as relative percentages obtained by FID chromatogram.

16. It would be better to provide the GC/MS chromatogram than to provide the structures of the compounds only

R. The chromatograms images have been added as figure S1 in the new version of the manuscript.

## Highlights

Saffron subjected to different drying methods showed different microbiota.

The saffron microbiota was dominated by bacteria belonging to the *Bacillaceae* family.

The isolation of potential pathogenic bacteria was marginal.

Bacterial biodiversity indexes were inversely related to saffron drying methods.

*Manuscript with Line Numbers
Click here to view linked References

# Isolation and characterization of microorganisms and volatiles associated with Moroccan saffron during different processing treatments

Francesco Fancello[1§], Giacomo Petretto[2§], Maria Lina Sanna[1], Giorgio Pintore[2], Mounira Lage[3], and Severino Zara[1*]

[1]Dipartimento di Agraria, University of Sassari, Viale Italia 39 - 07100 Sassari. Italy

[2]Department of Chemistry and Pharmacy, University of Sassari - 07100 Sassari. Italy

[3]National Institute of Agronomic Research, Regional Center of Rabat, RU of Medicinal & Aromatic Plants and Origin-Linked Crops, Rabat-Institutes, Rabat Morocco

**Keywords**

Saffron drying methods; Safranal; Microbial biodiversity; *Bacillaceae*

**Highlights**

Saffron subjected to different drying methods showed different microbiota.

The saffron microbiota was dominated by bacteria belonging to the *Bacillaceae* family.

The isolation of potential pathogenic bacteria was marginal.

Bacterial biodiversity indexes were inversely related to saffron drying methods.

*Corresponding author

§ These authors contributed equally to this work

25

**ABSTRACT**

Saffron plants may be spoiled by a variety of microorganisms during cultivation, harvesting, and post harvesting. As saffron can be dried and stored in different ways, this preliminary study explored the natural microbiota present in Moroccan saffron when subjected to different drying techniques. An analysis of the carotenoid-derived compounds present in the saffron was also carried out. The culturable microbiota of the saffron samples dried using different methods, namely in the shade (also called natural), in the sun, or in the oven, were studied using classical and molecular approaches. The effect of the drying methods on head-space chemical volatiles was also determined. Eighty-two isolates grown in the different culture media were chosen from the colonies, and genotype analysis grouped the microorganisms into 58 clusters, revealing a wide diversity. Out of the 82 isolates, 75 belonged to the *Bacillaceae* family. The other isolates were distributed within the *Dietziaceae*, *Paenibacillaceae* and *Carnobacteriaceae* families. The dominant species was *Bacillus simplex*, which was detected in all samples, regardless of the drying method used. *Lysinibacillus macroides* was dominant in the sun-dried saffron. No pathogens were isolated, but an isolate belonging to *Dietzia maris*, a potential human pathogenic species, was detected. The biodiversity indexes were linked to the drying method and generally decreased as the intensity of the treatment increased. The results of this preliminary work show that the different drying methods strongly influenced the microbiota and affect the saffron volatile profile. Further analysis will be needed to determine possible effects of selected microbiota on saffron volatiles.

## 1. INTRODUCTION

Spices have been used for millennia to enhance the flavor of, and hide spoilage in, foods (Gohari et al., 2013). Saffron is the most expensive spice in the world and consists of the dried stigmata of *Crocus sativus L*. The plant is cultivated in many countries including Iran, Greece, Morocco, India, Spain and Italy, which are the most important producers of this spice. The properties which give saffron its strong aroma, its coloring and flavor, and make it extremely valuable are related to the presence of picrocrocin, safranal, and crocins (Carmona et al., 2006a; Carmona et al., 2007; Cosano et al., 2009).

Saffron production consists of distinct phases: harvesting, gathering, handling, drying, packaging, and storage (Cosano et al., 2009). As in the case of many other agricultural products, saffron, when collected, processed, and sold in retail markets, is susceptible to contamination by a wide range of environmental microbes due to its exposure to dust, wastewater, and animal excreta (McKee, 1995). Contaminated spices may cause microbiological problems, depending on their use. This risk is usually limited by the thermal processing of food as saffron is often added during cooking. However, some preparations of this spice involve cold infusion in water and oil extraction. In these cases, saffron addition to food may result in the proliferation of bacterial pathogens (Sagoo et al., 2009; Vij et al., 2006). Another relevant problem is the low hygiene standards of many producing countries in the processing techniques that may cause contamination and affect the quality of the product (Khazaei et al., 2011).

Depending on the country of origin, saffron is dehydrated in various ways, which differ according to the temperature used. In India, Iran, and Morocco it is usually sun-dried or dried at room temperature in air ventilated conditions; in Greece and Italy the process is conducted at a milder temperature, whereas in Spain the drying takes place at high temperature. In Iran and Morocco, the stigmata are spread on a cloth and dried in the sun for 2–6 h or in the shade

74 for 7–10 days (Del Campo et al., 2010). In India, the stigmata are dried for 3–5 days in the sun
75 until a reduction of moisture content below 8–10% is obtained (Del campo et al., 2010). An
76 alternative drying process involves using hot air or another heat source to obtain a
77 temperature of 45 to 50°C for 50–60 minutes (Carmona et al., 2005).
78 The components that give the aromatic properties of saffron are norisoprenoid compounds,
79 produced from the degradation of carotenoids. However, the main component of saffron's
80 characteristic aroma is produced from the presence of monoterpene aldehyde safranal, which
81 derives from the hydrolysis of picrocrocin. The degradation of carotenoids proceeds along
82 two main pathways: enzymatic and non-enzymatic cleavage. With respect to the non-
83 enzymatic cleavage, thermal degradation processes play a key role in the formation of the
84 aroma (Kanasawud and Crouzet, 1990).
85 The aim of this preliminary work was to study the natural microbiota present in saffron and
86 to carry out targeted analysis of some carotenoid-derived aromatic compounds. To this end,
87 we analyzed three samples of saffron obtained from different drying systems; a fourth sample
88 of saffron obtained from the farmer market and with unknown drying treatment was used for
89 comparison. The samples were subjected to microbiological and chemical head-space
90 analyses in order to isolate and identify their different microbial species and volatile organic
91 compounds.
92

93 **2. MATERIALS AND METHODS**

94 **2.1 Sample collections**

95 A total of 4 dried saffron samples were collected from local producers. They were placed in
96 sterile plastic bags and stored at room temperature in the dark until the analyses were
97 performed. Saffron samples were dried (i) in the sun for 4 hours (in this case the harvest took
98 place in late October, early November), (ii) in the shade for 8 days; or (iii) in the oven at 50°C

for 60 min. A sample of commercial saffron (a mixture of different saffron samples dried with the three different methods mentioned above) was purchased from a farmer market and used for comparison.

## 2.2 Chemical composition of saffron

### *2.2.1 Headspace Solid-Phase Microextraction (HS-SPME)*

A 100 µm PDMS/DVB/CAR (Polydimethylsiloxane/Divinylbenzene/Carboxen)-coated fiber 50/30 Stableflex (Supelco, Sigma Aldrich, St. Louis, Mo., USA) was preconditioned prior to use at 270°C for 1 h in a Gerstel MultiPurpose Sampler (MPS) bake-out station, according to the manufacturer's instructions. Two ml of saturated aqueous NaCl solution was added to 100 mg of the powdered saffron sample and placed in a SPME vial (20 ml, 75.5 × 22.5 mm), which was tightly closed with a septum and allowed to equilibrate for 15 min at 36°C (D'Auria et al., 2004).

The preconditioned fiber was then exposed to the headspace. The extraction time was fixed to 30 min, based on a previous optimization. All experiments were carried out under constant agitation. After the extraction, the fiber was desorbed for 5 min into the injector operating at 250°C in a splitless injection mode.

### *2.2.2 Qualitative analysis (Gas Chromatography-Mass Spectrometry, GC-MS)*

The qualitative analysis was performed using an Agilent 7890 GC equipped with a Gerstel MPS autosampler, coupled with an Agilent 7000C MS detector. Chromatographic separation was performed on a HP-5MS capillary column (30 m × 0.25 mm, film thickness 0.17 mm) and the following temperature program was used: 60°C held for 3 min, then increased to 210°C at a rate of 4°C/min, then held at 210°C for 15 minutes, then increased to 300°C at a rate of 10°C/min, and finally held at 300°C for 15 minutes. Helium was used as the carrier gas at a

constant flow of 1 ml/min. Data were analyzed using a MassHunter Workstation B.06.00 SP1. Identification of the individual components was made by comparison with co-injected pure compounds (isophorone) and matching the MS fragmentation patterns and retention indexes with the built-in libraries, literature data, or commercial mass spectral libraries (NIST/EPA/NIH 2008; HP1607 purchased from Agilent Technologies).

*2.2.3 Quantitative analysis (Gas Chromatography with a Flame Ionization Detector, GC-FID)*

The quantitative targeted analysis of the headspace compounds was carried out using an Agilent 4890N instrument equipped with an FID and a HP-5 capillary column (30 m × 0.25 mm, film thickness 0.17 mm). The column temperature was held at 60°C for 3 min, then increased to 210°C at a rate of 4°C/min and held at 210°C for 15 min, then increased to 300°C at a rate of 10°C/min, and finally held at 300°C for 15 min. Both injector and detector were held at a temperature of 250°C. Helium was used as carrier gas at a flow rate of 1 ml/min. The compound quantification in the headspace (HS) was carried out using the internal standard (n-decane) method.

A five-point calibration curve was constructed with isophorone and safranal; all the norisoprenoid compounds were also expressed as relative percentages obtained by internal normalization of the chromatogram.

**2.3 Saffron sample preparations and microbiological analyses**

Saffron samples were diluted in Ringer solution (Oxoid, England) and then subjected to microbial cell release as follows: 300 mg aliquots of saffron were diluted in 2700 µl of Ringer solution in 50-ml falcon tubes and vortexed for 30 s to obtain the total dissolution of saffron. The resulting solution was then allowed to settle for 60 min at room temperature in order to facilitate the release of the microorganisms from the stigmata. Once the saffron was dissolved,

149 serial dilutions were done adding 100 µL of the solution to 2-ml microfuges tubes (Eppendorf,
150 Germany) containing 900 µL of Ringer solution.
151 Six different culture media were used to study the microbial communities (Rantsiou et al.,
152 2005) of the different saffron samples. Plate Count Agar (PCA, Merck, Italy) kept at 30°C for
153 48 h was used as the general medium for the viable mesophilic bacteria population, PCA kept
154 at 7°C for 7 days was used for psychrophilic bacteria, and PCA kept at 80°C for 15 min was
155 used for spore forming bacteria. MRS (De Man Rogosa Sharpe, Merck, Italy) agar was used to
156 grow lactic acid bacteria at 30°C both in aerobiosis and anaerobiosis conditions (Thermo
157 Scientific™ Oxoid AnaeroGen, Basingstoke, UK). Mannitol Salt Agar (MSA, Oxoid, Milan, Italy)
158 at 37°C was used for gram-positive cocci both in aerobiosis and anaerobiosis conditions.
159 Violet Red Bile Glucose Agar (VRBG, Oxoid, Milan, Italy) was used at 37° C for 24 or 48 h for
160 total enterobacteria. Slanetz and Bartley Agar (Oxoid, Milan, Italy) was used for total
161 enterococci at 37°C for 48 h. Finally, YEPD was used at 30°C for 48 h for filamentous fungi and
162 yeasts. Analyses were performed in triplicate. Randomly selected colonies, representative of
163 the different colony morphologies (shape, color, dimension, etc.), were picked up, re-streaked
164 on PCA, stored at -80°C, and used for the experiments described in the following section.
165
166
167 **2.4 Molecular Analyses**
168 *2.4.1 DNA isolation*
169 The DNA of each single bacterial isolate (n=82) was extracted automatically using the
170 KingFisher Duo Prime Purification System (KingFisher, Westminster, United Kingdom) and
171 the MagJET Genomic DNA Kit (Thermo Scientific, Waltham, Massachusetts, USA) and
172 following the manufacturer's instructions.

The concentration and purity of the DNA samples were spectrophotometrically evaluated at 260 nm and 280 nm, respectively, by the LvisPLATE SpectroSTAR Nano (BMG Labtech, Ortenberg, Germany). DNA integrity was evaluated by electrophoresis in 1% agarose gel.

*2.4.2 Randomly amplified polymorphic DNA-polymerase chain reaction fingerprint analysis*

The randomly amplified polymorphic DNA-polymerase chain reaction (RAPD-PCR) was performed using the M13 sequence as primer (5′GAGGGTGGCGGTTCT-3′) (Huey and Hall, 1989). All reactions were performed in a 50 $\mu$L final volume according to Rossetti and Giraffa (2005) and Mangia et al., (2016) with a minor variation. Briefly, the reaction mixture contained: 1× PCR buffer (Life Technology, Carlsbad, CA), 5 mmoll$^{-1}$ $MgCl_2$, 200, $\mu$moll$^{-1}$ dNTPs, 2 $\mu$mol primer, 1.25 U TAQ polymerase® (Life Technology), and 50 ng bacterial DNA. PCR amplification was performed with a thermal cycler (MyCicler, Bio-Rad Laboratories, Hercules, CA) under the following conditions: initial denaturation at 94°C for 2 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 45°C for 40 s, elongation at 72°C for 2 min and final extension at 72°C for 10 min. PCR products were separated by electrophoresis for 3 h on 1.5% (w/v) agarose gel containing 0.1 $\mu$L SYBR safe (Life Technology) in 1× TAE (40 mmoll$^{-1}$ Tris-acetate, 1 mmoll$^{-1}$ EDTA, pH 8). A 100–10,000 DNA molecular mass marker (Euroclone, Milano, Italy) was used as a standard. Band patterns were captured using Chemidoc XRS (Bio-Rad Laboratories) for subsequent cluster analyses. Fingerprint reproducibility patterns of RAPD and rep-PCR methods were assessed using DNAs isolated from three randomly selected microbial colonies according to the method of Solieri et al. (2012).

*2.4.3 Sequencing of 16S rDNA of saffron isolates*

197 Isolated strains, representative of each cluster, were subjected to 16S rDNA sequencing. The
198 16S ribosomal DNA fragment (1,500 bp) was amplified using the universal primers W001 and
199 W002 as previously described by Godon et al., (1997). Subsequently, amplicons were
200 sequenced following purification using the QIAquick PCR Purification Kit (Qiagen GmbH,
201 Hilden, Germany) according to the manufacturer's instructions. Sequencing, using primers
202 W001 and W002, was performed at Macrogen (Hong Kong, China). Forward and reverse
203 sequences were aligned and managed using BIOEDIT (Hall,1999). An average of 800 bp per
204 sequence were obtained. They were compared with those present in the GenBank database
205 using the blast program (http://www.ncbi.nih.gov/BLAST/), and with those in the Ribosomal
206 Database project (https://rdp.cme.msu.edu/classifier/classifier.jsp ).
207
208 **2.5 Statistical analyses**
209 The gel images were acquired using the transilluminator ChemiDoc XRS system (Bio-Rad
210 Laboratories) and saved in *.tiff* format. Images were then analyzed using InfoQuest FP
211 software v.5.1 (Bio-Rad Laboratories). A similarity matrix of electrophoretic profiles was
212 calculated using Pearson's product-moment correlation coefficient. Cluster analyses of
213 similarity matrices were performed using the unweighted pair group method with arithmetic
214 averages (UPGMA). Degree of biodiversity was assessed using Past3 (Paleontological
215 Statistics, Version 3.16).
216
217 **3 RESULTS AND DISCUSSION**
218
219 **3.1 Head space analysis**
220 After a full scan GC-MS analysis of the head space of the samples, a total of 10 norisoprenoid
221 compounds were selected for the quali-quantitative analyses (Figures 1 and S1). Compounds

1-10 made up between 84% (SHADE sample) and 96% (OVEN sample) of the whole composition, based on the internal normalization of FID chromatograms. Safranal and isophorone were found to be the main compounds in all the samples, as already reported for saffron collected in Italy (Manzo et al., 2015). The safranal amount increased according to the temperature of the drying method (Table 1): OVEN sample (2.21 μg/mg) > SUN sample (1.05 μg/mg) > SHADE sample (0.91 μg/mg). Compound 10, tentatively identified as 4-hydroxy-2,6,6-trimethylcyclohex-1-enecarbaldehyde (also known as HTCC), is a precursor of safranal. When saffron is heated up, HTCC loses a molecule of water yielding safranal. Alonso et al., (2001) have linked the differences in safranal content to the different processing methods. Isophorone, the second most predominant compound after safranal, was found in low concentrations in the SHADE sample (0.19 μg/g) and in similar concentrations in the SUN and OVEN samples (0.32 and 0.27 μg/g respectively).

Considering that freshly harvested saffron is almost odorless and that the formation of the main compounds responsible for its aroma occurs during the drying process, our data confirm (Raina et al., 1996) that the thermal degradation of carotenoids is crucial for the generation of saffron's characteristic aroma. In line with this reasoning, it can be hypothesized that the drying temperature to which the MARKET samples were subjected was similar or slightly lower than that of OVEN samples. In fact, the amounts of safranal and isophorone found in the MARKET samples (1.98 μg/g and 0.43 μg/g, respectively) were similar to the concentrations of safranal and isophorone detected in OVEN samples (2.21 μg/g and 0.27 μg/mg, respectively).

In a study looking at several saffron samples from different countries, Carmona et al., (2006b) underlined that Moroccan and Iranian saffron are characterized by a high amount of acetic acid. Our results apparently contradict this finding as, in the HS of our samples, only traces of acetic acid were found. The fingerprint of our Moroccan samples looks similar to those of

247 other saffron samples from different countries, such as those studied by D'Auria et al., (2004)
248 and by Zarghami & Heinz (1971). Karabagias et al., (2017), in a recent study on saffron
249 samples from Greece, Spain, Iran, and Morocco, indicated several markers of Moroccan
250 samples. These included a compound named 5,5-dimethyl-2-methylenecyclohex-3-
251 enecarbaldehyde, which was also detected in our samples. Although quite unusual in saffron
252 samples (Manzo et al., 2015; Kanakis et al., 2004), compound 6, tentatively identified as 2,4-
253 cycloheptadien-1-one-2,6,6-trimethyl, also called eucarvone, was detected in all our Moroccan
254 samples, confirming the results reported in Karabagias et al., (2017). The presence of
255 eucarvone in Iranian saffron has been previously reported (Jalali-Heravi et al., 2009) and a
256 few eucarvone isomers were found by D'Auria et al., (2004, 2006) and by Tarantilis and
257 Polissiou (1997).
258
259 **3.2 Microbiological analyses**
260 Although the effect of drying methods on the chemical composition of saffron has long been
261 established (Nejad, 2004; Gregory et al., 2005; Carmona et al., 2005; Feili et al., 2012 Lage et
262 al., 2015), no information is available concerning the effect of the drying method on the
263 composition of its microbial community. As shown in Table 2, a total of 82 bacterial isolates
264 were obtained from our saffron samples using different culture media and conditions. The
265 microorganism total counts on the PCA medium showed the following concentrations: about
266 $9x10^2$/g in the sample dried in the sun, $4.4x10^3$/g in the sample dried in the shade, $6x10^2$/g in
267 the sample dried in the oven, and $6x10^2$/g in the market sample used for comparison.
268 Therefore, the drying treatment influenced the number of microorganisms found per gram of
269 saffron. In order to compare the genotypic redundancy among the strains, the RAPD-PCR
270 technique was used. This methodology showed a good reproducibility with a very similar
271 banding pattern for three independent DNA preparations obtained from three biological

272 replicates of the same isolate. In fact, the reproducibility of the electrophoresis pattern was
273 91% (data not shown). The 82 isolates were grouped into 58 clusters at a cut off value of 85%.
274 Most of them were single cluster strains (Figure 2), highlighting the higher genotypic
275 variability of the isolates. No particular patterns were observed among the strains analyzed.
276 The partial sequence of the 16S rRNA genes from representative isolates of each cluster was
277 amplified by PCR and sequenced. As shown in Table 2, the culturable microbiota of saffron
278 was dominated by bacteria belonging to the *Bacillaceae* family and only one isolate belonged
279 to the *Actinobacteria* class.
280 In contrast to that found by Cosano et al., (2009), no pathogenic bacteria were isolated from
281 our saffron samples. Furthermore, of the species detected, none have been considered
282 medically relevant to date, although soil-related *Bacillus* and *Paenibacillus* species are being
283 increasingly implicated in various human diseases (Celandroni et al., 2016). The unique
284 isolate belonging to the Actinobacteria class was the *Dietzia maris* species. Some strains of
285 this species have been recognized as a potentially invasive human pathogen (Koerner et al.,
286 2009), although other reports suggest that the different species belonging to this genus may
287 have a wide range of medical, chemical, and food industry applications (Gharibzahedi et al.,
288 2014).
289 Many species of the *Bacillaceae* family have recently gained increased importance as
290 biological control agents, both for the management of plant pathogens and for plant growth
291 promotion (Mia et al., 2016). For example, *Bacillus amyloliquefaciens* and *Bacillus aryabhattai*
292 (Ambardar and Vakhlu, 2013), the species isolated in this work, are known for their potential
293 as biocontrol agents and as plant growth promoters. Furthermore, in light of recent findings
294 (Tanvir et al., 2017; Köberl et al., 2013), the interactive effects of these bacteria and saffron
295 on the generation of the aroma compounds and on the production of bioactive
296 phytochemicals should be more thoroughly investigated. Moreover, considering that Sharma

297 et al. (2015) recently showed *Bacillus* spp. to be the main genus of culturable endophytic
298 bacteria of saffron produced in the Kashmir valley, it is probable that the bacterial isolates in
299 this study are also endophytic bacteria.
300 Among the bacilli, we isolated 3 strains of *Carnobacterium maltaromaticum*, a lactic acid
301 bacteria (LAB), previously only isolated from food products and used as a ripening and
302 biopreservative flora in fermented food, but more recently also isolated from pollen and
303 wheat (Minervini et al., 2015; Obersteiner et al., 2016). Therefore, it is possible that
304 *Carnobacterium maltaromaticum* and other lactic acid bacteria species, occupy different
305 ecological niches (Duar et al., 2017); further studies would be necessary to elucidate the
306 biological role of *Carnobacterium maltaromaticum* strains in the saffron plant. Although a
307 recent work by Säde et al., (2016) showed that LAB are common contaminants in dried
308 vegetables and spices, our findings are consistent with earlier studies reporting that LAB are
309 rare contaminants in spices and dried herbs (Baxter and Holzapfel, 1982; Kneifel and Berger,
310 1994). However, the small number of LAB found in the present study probably reflects the
311 different drying methods used. In fact, as shown in Table 2, the oven drying method
312 drastically reduces the number of bacteria, with only two isolates being recovered from the
313 saffron surface. The drying methods also influence saffron biodiversity indexes: as shown in
314 Table 3, MARKET saffron showed the highest species richness followed by the samples dried
315 via the SHADE, SUN, and OVEN methods. Accordingly, the Shannon-Wiener diversity index
316 (H′) and Simpson's index (D) were higher in the MARKET and SHADE samples than in the SUN
317 and OVEN samples. In the oven-dried saffron, the biodiversity indexes were calculated using
318 only two strains. Presumably, the oven treatment eliminated the microbiota present almost
319 completely. The sun dried saffron was dominated by two species, *Lysinibacillus macroides* and
320 *Bacillus simplex*; this reflected the lower value of the evenness index compared with those for
321 the MARKET and SHADE methods. Conversely, the MARKET and SHADE saffron samples

showed a higher evenness, which reflects the higher number of species observed. The differences found in the saffron microbiota may be due to the differences in intensity of heat treatment, but also to differences occurring during the production processes.

## 4. Conclusions

This study of the microbiota of saffron dried with different methods allowed us to obtain at least three important results. The first, confirming previous findings in the literature, is that the presence of different concentrations of safranal and isophorone is strongly influenced by the drying method utilized. Indeed, the head-space chemical analysis showed that the main components of saffron are carotenoid derived compounds, mainly safranal, and that the amount of volatile substances depends on the drying method.

The second result, reported here for the first time, is that the microbiota of saffron is dominated by bacteria belonging to the *Bacillaceae* family and that the microflora is also influenced by the drying method utilized. In particular, the number of bacterial species isolated from the saffron samples is inversely related to the intensity of the heat treatment used, with a hotter treatment resulting in a lower number of bacterial species isolated.

The third result, concerning the presence of potential pathogenic bacteria, confirmed the works by Cosano et al. (2009) and Khazanei et al. (2011). These authors found that the presence of potential pathogenic bacteria was very low in the different saffron samples. Therefore, when considering the above mentioned results, it would be important to understand whether there are correlations between the microbiota and the type of volatiles present. It should also be pointed out, in fact, that many of the microorganisms found in this work are potential endophytes, i.e., microorganisms that somehow act in symbiosis with the host plant. In our opinion, having ascertained that safranal increases as the drying temperature increases, it remains to be determined whether, for the same heat treatment, the

variation of certain volatiles may also be attributed to the endophytic activity of certain bacteria or to the enzymes released by these bacteria after their death. To the best of our knowledge, the present study is the first in which a relationship between the saffron microbiota and the drying methods utilized for its preparation has been clearly identified.

## Figure Captions

**Figure 1**: Chemical structures of carotenoid derived compounds identified in the saffron samples

**Figure 2**. Genotyping by RAPD-PCR of isolates from saffron dried with different methods, in the shade, in the sun, and in the oven or purchased from the farmer market. The unweighted-pair-group method with arithmetic averages dendrogram is based on the Pearson correlation coefficient of the M13 profiles. The dashed line indicates the cluster cut-off at 85% similarity.

**Figure S1**: Raw chromatograms of the saffron samples. A, market; B, shade; C, sun; D, oven

## Acknowledgement

This study was conducted as part of the EMAP (Edible Medicinal and Aromatic Plants) project, under the Marie Curie action call (FP7-PEOPLE-2009-IRSES) N.247548. We also thank Stephanie Parsley for the English language revision.

372

373

374 **References**

375 Alonso, G.L., Salinas, M.R., Sanchez-Fernandez, M.A., Garijo, J. ,2001. Safranal content in
376 Spanish Saffron. Food Sci. Technol. Int. 7, 225-229.

377 Ambardar, S., Vakhlu, J., 2013. Plant growth promoting bacteria from Crocus sativus
378 rhizosphere. World J. Microb. Biot. 29, 2271-2279.

379 Baxter, R., Holzapfel, W.H., 1982. A Microbial Investigation of Selected Spices, Herbs, and
380 Additives in South Africa. J. Food Sci. 47, 570-574.

381 Carmona, M., Zalacain, A., Pardo, J.E., Alvarruiz, A. Alonso, G.L., 2005. Influence of different
382 drying and aging conditions on saffron constituents. J. Agric. Food Chem. 53, 3974–3979.

383 Carmona, M., Martinez, J., Zalacain,, A., Rodriguez-Mendez M.L., de Saja, J.A., Alonso, G.L.,
384 2006a. Analysis of Saffron volatile fraction by TD-GC-MS and e-noise. Eur. Food Res.
385 Technol. 223, 96-101.

386 Carmona, M., Zalacain, A., Sanchez, A. M., Novella, J. L., & Alonso, G. L., 2006b. Crocetin
387 esters picrocrocin and its related compounds present in *Crocus sativus* stigmas and
388 *Gardenia jasminoides* fruits. Tentative identification of seven new compounds by LC-ESI-
389 MS. J. Agr. Food Chem. 54, 973e979.

390 Carmona, M., Zalacain, A., Salinas, M. R., & Alonso, G. L., 2007. A new approach to saffron
391 aroma. Crit. Rev. Food Sci. 47, 145e159.

392 Celandroni, F., Salvetti, S., Gueye, S.A., Mazzantini, D., Lupetti, A., Senesi, S., Ghelardi, E.,
393 2016. Identification and pathogenic potential of clinical *Bacillus* and *Paenibacillus* isolates.
394 PLoS ONE 11, e0152831.

Cosano, I., Pintado, C., Acevedo, O., Novella, J.L., Alonso, G.L., Carmona, M., de la Rosa, C. Rotger, R., 2009. Microbiological quality of saffron from the main producer countries. J. Food Prot. 72, 2217–2220.

D'Auria, M., Mauriello, G., Rana, G.L., 2004. Volatile organic compounds from Saffron. Flavour Frag. J. 19, 17-23.

D'Auria, M., Mauriello G., Racioppi, R, Rana, G.L., 2006. Use of SPME–GC–MS in the study of time evolution of the constituents of saffron aroma: modifications of the composition during storage. J. Chromatogr. Sci, 44, 18-21.

Del Campo, C.P, Carmona, M., Maggi, L., Kanakis, C.D., Anastasaki, E.G., Tarantilis, P.A, Polissiou, M.G., Alonso, G.L., 2010. Effects of mild temperature conditions during dehydration procedures on saffron quality parameters. J. Sci. Food Agr. 90, 719-725.

Duar, R.M., Lin, X.B., Zheng, J., Martino, M.E., Grenier, T., Pérez-Muñoz, M.E., Leulier, F., Gänzle, M., Walter, J., 2017. Lifestyles in transition: evolution and natural history of the genus *Lactobacillus*. FEMS Microbiol. Rev. 41, S27-S48.

Feili, H.R., Aghaee, E.M., Taslimi, A., 2012. Study of chemical and microbiological properties of saffron dehydrated by using solar drying system. Int. J. Renew. Ener. Res. 2, 627-630.

Gharibzahedi, S.M.T., Razavi, S.H., Mousavi, S.M., 2014. Characterization of bacteria of the genus *Dietzia*: an updated review. Ann. Microbiol. 64, 1-11.

Godon, J. J., Zumstein, E., Dabert, P., Habouzit, F., Moletta, R., 1997. Molecular microbial diversity of an anaerobic digestor as determined by small-subunit rDNA sequence analysis. Applied and Environmental Microbiology, 63(7), 2802–2813.

Gohari, A. R., Saeidnia, S., & Mahmoodabadi, M. K. (2013). An overview on saffron, phytochemicals, and medicinal properties. *Pharmacognosy Reviews*, *7*(13), 61–66.

418 Gregory, M.J., Menary, R.C., Davies, N.W., 2005. Effect of drying temperature and air flow
419 on the production and retention of secondary metabolites in saffron. J. Agr. Food Chem. 53,
420 5969-5975.

421 Hall, T.A., 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis
422 program for Windows 95/98/NT. Nucl. Acids. Symp. Ser. 41. 95-98.

423 Huey, B., Hall, J., 1989. Hypervariable DNA Fingerprinting in *Escherichia coli* - Minisatellite
424 Probe from Bacteriophage-M13. J. Bacteriol. 171, 2528-2532.

425 Jalali-Heravi, M., Parastar, H., & Ebrahimi-Najafabadi, H., 2009. Characterization of volatile
426 components of Iranian saffron using factorial-based response surface modeling of
427 ultrasonic extraction combined with gas chromatography-mass spectrometry analysis. J.
428 Chromatog. A, 1216, 33.

429 Karabagias, I. K., Koutsoumpou, M., Liakou, V., Kontakos, S., & Kontominas, M. G., 2017.
430 Characterization and geographical discrimination of saffron from Greece, Spain, Iran, and
431 Morocco based on volatile and bioactivity markers, using chemometrics. Eur. Food Res.
432 Technol. 243, 9.

433 Kanakis, C.D., Daferera, D.J., Tarantilis, P. A., Polissou, M.G., 2004. Qualitative determination
434 of volatile compounds and quantitative evaluation of safranal and 4-Hydroxy-2,6,6-
435 trimethyl-1-cyclohexene-1-carboxaldehyde (HTCC) in Greek saffron. J. Agric. Food Chem.
436 52, 4515-4521.

437 Kanasawud, P., Crouzet, J. C., 1990. Mechanism of Formation of Volatile Compounds by
438 Thermal Degradation of Carotenoids in Aqueous Medium. 1. β-Carotene Degradation. J.
439 Agric. Food Chem. 38(1).

440 Khazaei, N., Jouki, M., Kalbasi, A., Tavakolipour ,H., Rajabifar, S., Motamedi Sedeh, F., Jouki,

441 A., 2011. Study of microbial critical points of saffron from farm to factory in Iran world.
442 World Acad. Sci. Eng. Technol. 5, 293-296.
443 Kneifel, W., Berger, E., 1994. Microbiological criteria of random samples of spices and
444 herbs retailed on the Austrian market. J. Food Protect. 57, 893-901.

445 Köberl, M., Schmidt, R., Ramadan, E., Bauer, R., Berg, G., 2013. The microbiome of medicinal
446 plants: diversity and importance for plant growth, quality and health. Front. Microbiol. 4.

447 Koerner, R.J., Goodfellow, M., Jones, A.L., 2009. The genus *Dietzia*: a new home for some
448 known and emerging opportunist pathogens. FEMS Immunol. Med. Mic. 55, 296-305.

449 Lage M., Melai B., Cioni P.L., Flamini G., Gaboun F., Bakhy K., , Zouahri A., Pistelli L., 2015.
450 Phytochemical composition of Moroccan saffron accessions by headspace solid-phase-
451 microextraction. Am. J. Essent. Oil. Nat. Prod 2, 1-7.

452 Manzo, A., Panseri, S., Bertoni, D., Giorgi, A., 2015. Economic and Qualitative Traits of
453 Italian Alps Saffron. J. Mount. Sci. 12, 1542-1550.

454 McKee, L. H., 1995. Microbial contamination of spices and herbs: a review. Lebensm.-Wiss.
455 Technol. 28, 1–11.
456 Mangia, N. P., Fancello F., and Deiana P.. 2016. Microbiological characterization using combined
457 culture dependent and independent approaches of Casizolu pasta filata cheese. J Appl Microbiol
458 120, 329-345.
459 Mia M.A.B., Naher U.A., Panhwar Q.A., Islam M.T. (2016) Growth Promotion of Nonlegumes
460 by the Inoculation of *Bacillus* Species. In: Islam M., Rahman M., Pandey P., Jha C., Aeron A.
461 (eds) Bacilli and Agrobiotechnology. Springer, Cham.

462 Minervini, F., Celano, G., Lattanzi, A., Tedone, L., De Mastro, G., Gobbetti, M., De Angelis, M.
463 2015. Lactic Acid Bacteria in Durum Wheat Flour Are Endophytic Components of the Plant
464 during Its Entire Life Cycle. Appl. Environ. Microbiol. 81, 6736–6748.

Nejad, M.H., 2004. Evaluation of quality characteristics and microbial contamination of saffron samples dried by microwave, Acta Hortic. 650, 345-353.

Obersteiner, A,. Gilles, S., Frank, U., Beck, I., Häring, F., Ernst, D. Rothballer, M., Hartmann, A., Traidl-Hoffmann, C., Schmid, M., 2016. Pollen- associated microbiome correlates with pollution parameters and the allergenicity of pollen. PLoS ONE 11, e0149545.

Raina, B.L., Agarwal, S.G., Bhatia, A.K., Govind, G.S., 1996. Changes in pigments and volatiles of Saffron (*Crocus sativus* L.) during processing and storage. J. Sci. Food Agric. 71, 27-32.

Rantsiou, K., Urso, R., Iacumin, L., Cantoni, C., Cattaneo, P., Comi, G., & Cocolin, L. (2005). Culture-Dependent and -Independent Methods To Investigate the Microbial Ecology of Italian Fermented Sausages. *Applied and Environmental Microbiology*, *71*(4), 1977–1986.

Rossetti, L., Giraffa, G., 2005. Rapid identification of dairy lactic acid bacteria by M13-generated, RAPD-PCR fingerprint databases. J. Microbiol. Meth. 63, 135-144.

Säde, E., Lassila, E., Björkroth, J., 2016. Lactic acid bacteria in dried vegetables and spices. Food Microbiol. 53, 110-114.

Sagoo, S. K., Little, C. L., Greenwood, M., Mithani, V., Grant, K. A., McLauchlin, J., de Pinna, E., Threlfall, E.J., 2009. Assessment of the microbiological safety of dried spices and herbs from production and retail premises in the United Kingdom. Food Microbiol. 26, 39-43.

Sharma, T., Kaul, S., Dhar, M.K., 2015. Diversity of culturable bacterial endophytes of saffron in Kashmir, India. Springerplus 4, 1-13.

Solieri, L., Bianchi, A., Giudici, P., 2012. Inventory of non-starter lactic acid bacteria from ripened Parmigiano Reggiano cheese as assessed by a culture dependent multiphasic approach. Syst. Appl. Microbiol. 35, 270-277.

Tanvir, R., Javeed, A., Bajwa, A.G., 2017. Endophyte bioprospecting in South Asian

488 medicinal plants: an attractive resource for biopharmaceuticals. Appl. Microbiol. Biot. 101,
489 1831-1844.

490 Tarantilis, P. A., Polissiou, M. G. ,1997. Isolation and Identification of the Aroma
491 Components from Saffron (*Crocus sativus*). J. Agric. Food Chem., 45, 459-462.

492 Vij, V., Ailes, E., Wolyniak, C., Angulo, F. J., & Klontz, K. C., 2006. Recalls of spices due to
493 bacterial contamination monitored by the U.S. Food and Drug Administration: the
494 predominance of *Salmonellae*. J. Food Protect., 69, 233-237.
495 Winterhalter, P., Rouseff, R., 2001. Carotenoid-derived aroma compounds. ACs symposium
496 series; American Chemical Society. Washington, DC.

497 Zarghami, N.S., Heinz, D.E., 1971. Monoterpene aldehydes and isophorone-related
498 compounds of saffron. Phytochemistry 10, 459-562.

499

**Table 1.** Main constituents of the head space fraction of saffron based on targeted gas-chromatography analysis

| Chemical compounds | | Sun | | Shade | | Oven | | Market | | RI |
|---|---|---|---|---|---|---|---|---|---|---|
| | | µg/mg | A% | µg/mg | A% | µg/mg | A% | µg/mg | A% | |
| 5,5-dimethyl-2-methylenecyclohex-3-enecarbaldehyde | **1** | nq | 2.42 | nq | 3.00 | nq | 1.15 | nq | 2.34 | 1111 |
| isophorone | **2** | 0.32 | 14.77 | 0.19 | 11.7 | 0.27 | 8.60 | 0.43 | 11.3 | 1125 |
| 4 ketoisophorone | **3** | nq | 7.45 | nq | 3.6 | nq | 2.75 | nq | 10.7 | 1149 |
| 1,4-cyclohexanedione, 2,6,6-trimethyl- | **4** | nq | 8.33 | nq | 4.5 | nq | 2.80 | nq | 6.3 | 1179 |
| safranal | **5** | 1.05 | 49.01 | 0.91 | 56.0 | 2.21 | 69.87 | 1.98 | 52.3 | 1210 |
| 2,4-Cycloheptadien-1-one, 2,6,6-trimethyl- | **6** | nq | 1.08 | nq | 0.7 | nq | 0.41 | nq | 0.8 | 1222 |
| 2-hydroxy-3,5,5-trimethylcyclohex-2-ene-1,4-dione | **7** | nq | 1.01 | nq | 0.8 | nq | 0.27 | nq | 0.9 | 1235 |
| 4-hydroxy-3,5,5-trimethylcyclohex-2-enone | **8** | nq | 0.76 | nq | 0.9 | nq | 0.23 | nq | 0.7 | 1312 |
| 4-hydroxy-2,6,6-trimethyl-3-oxocyclohexa-1,4-dienecarbaldehyde | **9** | nq | 2.58 | nq | 1.7 | nq | 4.73 | nq | 3.7 | 1393 |
| 4-hydroxy-2,6,6-trimethylcyclohex-1-enecarbaldehyde | **10** | nq | 0.79 | nq | 0.6 | nq | 5.19 | nq | 1.0 | 1425 |

Results are expressed as µg/mg of sample as well as relative proportions percentages of the norisoprenoid constituents obtained by FID peak area normalization (A%). RI= retention index, nq: not quantified

**Table 2.** Bacterial isolates from saffron treated with different drying methods

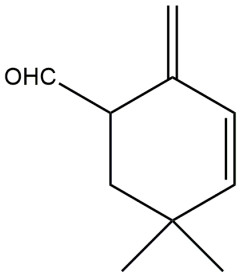
| **Species** | **Dry methods** | | | | |
|---|---|---|---|---|---|
| | **Sun** | **Shade** | **Oven** | **Market** | **Tot** |
| *Bacillus spp.** | 1 | 2 | 0 | 6 | 9 |
| *Bacillus simplex** | 7 | 4 | 1 | 2 | 14 |
| *Lysinibacillus macroides** | 12 | 1 | 0 | 1 | 14 |
| *Bacillus aryabhattai** | 1 | 1 | 0 | 5 | 7 |
| *Bacillus niacini** | 1 | 3 | 1 | 3 | 8 |
| *Gracilibacillus dipsosauri** | 0 | 3 | 0 | 0 | 3 |
| *Oceanobacillus picturae** | 0 | 3 | 0 | 0 | 3 |
| *Oceanobacillus spp.** | 0 | 3 | 0 | 0 | 3 |
| *Bacillus amyloliquefaciens** | 0 | 1 | 0 | 1 | 2 |
| *Bacillus endophyticus** | 0 | 0 | 0 | 2 | 2 |
| *Bacillus megaterium** | 0 | 1 | 0 | 1 | 2 |
| *Bacillus pumilus** | 0 | 1 | 0 | 1 | 2 |
| *Paenibacillus spp.*[§] | 0 | 0 | 0 | 2 | 2 |
| *Bacillus badius** | 0 | 0 | 0 | 1 | 1 |
| *Bacillus jeotgali** | 0 | 0 | 0 | 1 | 1 |
| *Bacillus licheniformis** | 1 | 0 | 0 | 0 | 1 |
| *Bacillus subtilis** | 0 | 0 | 0 | 1 | 1 |
| *Virgibacillus carmonensis** | 0 | 1 | 0 | 0 | 1 |
| *Lysinibacillus chungkukjangi** | 0 | 0 | 0 | 1 | 1 |
| *Solibacillus silvestris*** | 0 | 0 | 0 | 1 | 1 |
| *Carnobacterium maltaromaticum*[#] | 1 | 2 | 0 | 0 | 3 |
| *Dietzia maris*°* | 0 | 0 | 0 | 1 | 1 |
| | 24 | 26 | 2 | 30 | 82 |

**Families:** *Bacillaceae; **Unassigned (order Bacillales); [§] Paenibacillaceae; [#]Carnobacteriaceae; °Dietziaceae

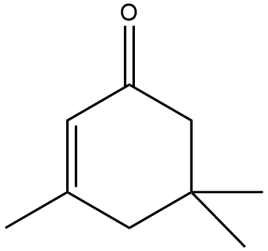
**Table 3**. Indexes of biodiversity calculated for single saffron treated with different drying methods.

| **Biodiversity indexes** | **Sun** | **Shade** | **Oven** | **Market** |
|---|---|---|---|---|
| Taxa_S | 7 | 13 | 2 | 16 |
| Isolates | 24 | 26 | 2 | 30 |
| Simpson's index_D | 0,344 | 0,104 | 0,500 | 0,111 |
| Shannon's index_H' | 1,368 | 2,398 | 0,693 | 2,494 |
| Evenness | 0,561 | 0,846 | 1,000 | 0,757 |

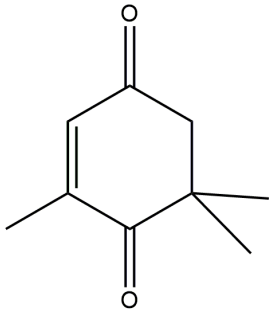
Figure 1



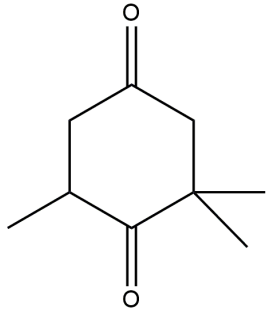

**1**
5,5-dimethyl-2-methylenecyclohex-3-enecarbaldehyde



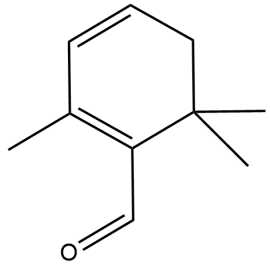

**2**
Isophorone



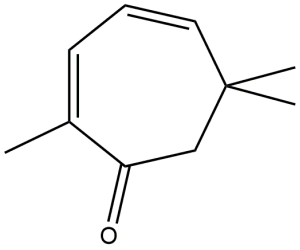

**3**
Isophorone 4 keto



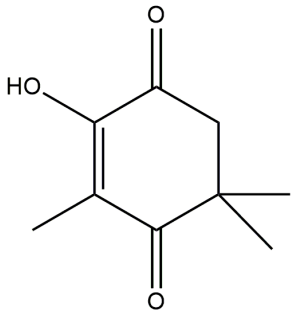

**4**
1,4-Cyclohexanedione, 2,6,6-trimethyl-



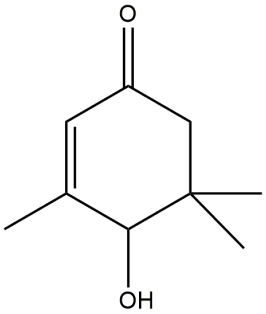

**5**
Safranal



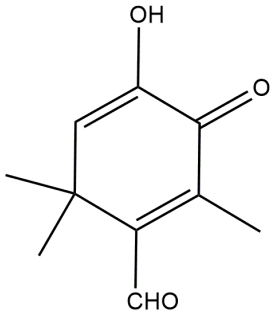

**6**
2,4-Cycloheptadien-1-one, 2,6,6-trimethyl-



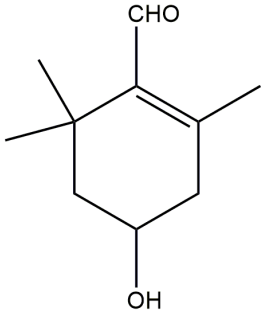

**7**
2-hydroxy-3,5,5-trimethylcyclohex-2-ene-1,4-dione




**8**
4-Hydroxy-3,5,5-trimethylcyclohex-2-enone




**9**
4-hydroxy-2,6,6-trimethyl-3-oxocyclohexa-1,4-dienecarbaldehyde

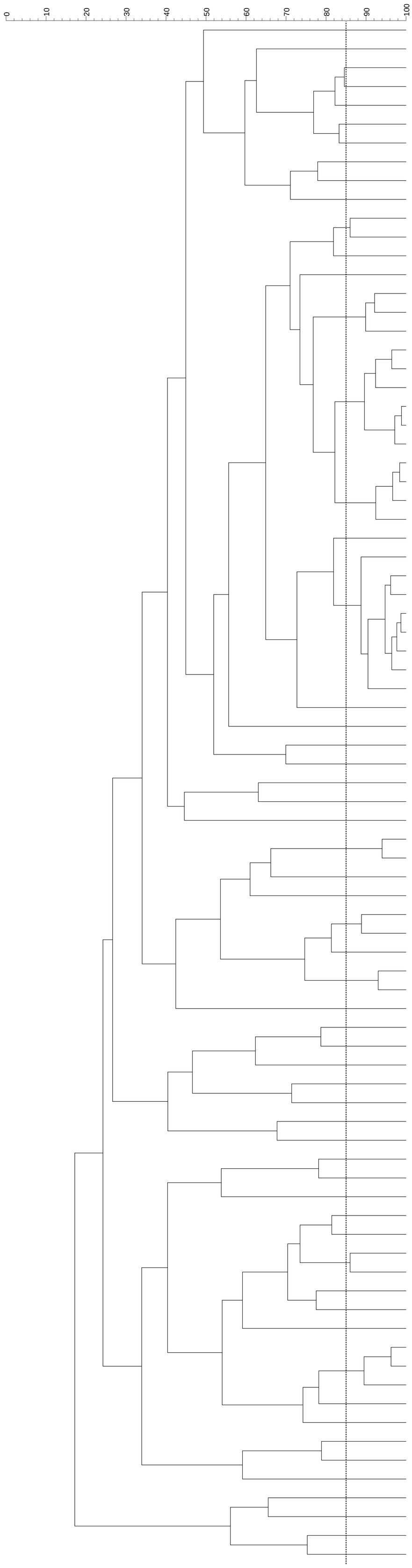



**10**
4-hydroxy-2,6,6-trimethylcyclohex-1-enecarbaldehyde

**Figure 2**

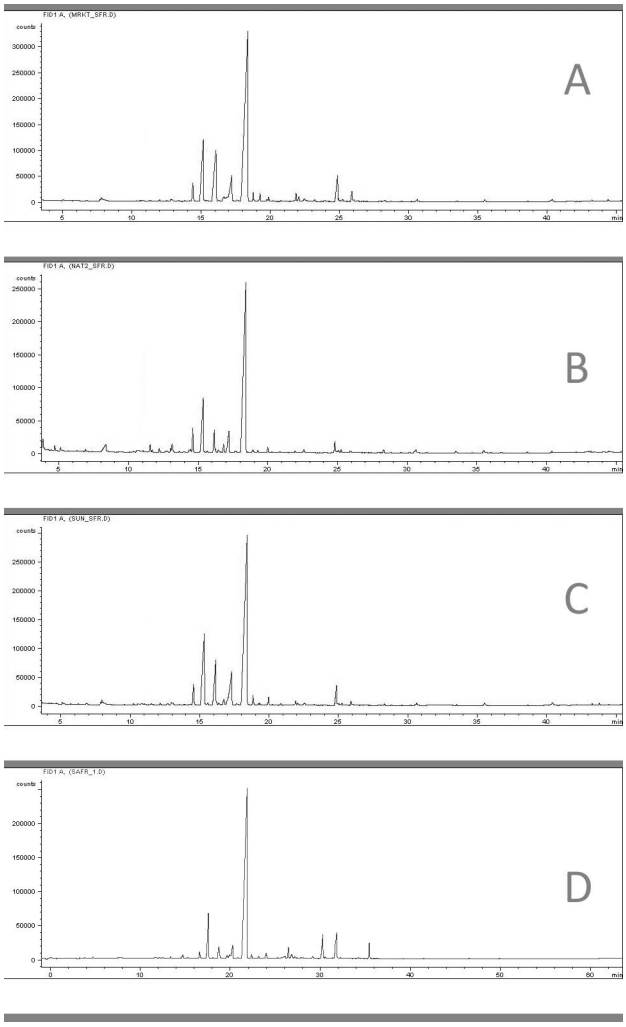
Pearson correlation (Opt:1.25%) [0.0%-100.0%]

**M13**




| DRYING METHOD | Genus | Species | CLUSTER |
|---|---|---|---|
| SHADE | Gracilibacillus | dipsosauri | 0001 |
| SHADE | Bacillus | aryabhattai | 0002 |
| MARKET | Bacillus | aryabhattai | 0003 |
| MARKET | Bacillus | aryabhattai | 0004 |
| MARKET | Bacillus | aryabhattai | 0005 |
| SUN | Bacillus | simplex | 0006 |
| MARKET | Bacillus | niacini | 0007 |
| MARKET | Bacillus | megaterium | 0008 |
| MARKET | Bacillus | jeotgali | 0009 |
| MARKET | Bacillus | spp. | 0010 |
| MARKET | Bacillus | spp. | 0011 |
| SHADE | Bacillus | spp. | 0011 |
| MARKET | Solibacillus | silvestris | 0012 |
| SHADE | Bacillus | spp. | 0013 |
| MARKET | Bacillus | simplex | 0014 |
| SHADE | Bacillus | simplex | 0014 |
| SUN | Bacillus | simplex | 0014 |
| SUN | Lysinibacillus | macroides | 0015 |
| SHADE | Lysinibacillus | macroides | 0015 |
| MARKET | Lysinibacillus | macroides | 0015 |
| SUN | Lysinibacillus | macroides | 0015 |
| SUN | Lysinibacillus | macroides | 0015 |
| SUN | Lysinibacillus | macroides | 0015 |
| SUN | Bacillus | simplex | 0016 |
| SUN | Bacillus | simplex | 0016 |
| SUN | Baciilus | simplex | 0016 |
| SUN | Bacillus | simplex | 0016 |
| SUN | Bacillus | spp. | 0017 |
| SUN | Lysinibacillus | macroides | 0018 |
| SUN | Lysinibacillus | macroides | 0018 |
| SUN | Lysinibacillus | macroides | 0018 |
| SUN | Lysinibacillus | macroides | 0018 |
| SUN | Lysinibacillus | macroides | 0018 |
| SUN | Lysinibacillus | macroides | 0018 |
| SUN | Lysinibacillus | macroides | 0018 |
| SUN | Lysinibacillus | macroides | 0018 |
| MARKET | Paenibacillus | spp. | 0019 |
| SUN | Bacillus | simplex | 0020 |
| SHADE | Bacillus | pumilus | 0021 |
| MARKET | Bacillus | badius | 0022 |
| SHADE | Oceanobacillus | picturae | 0023 |
| SHADE | Oceanobacillus | picturae | 0023 |
| SHADE | Gracilibacillus | dipsosauri | 0024 |
| SHADE | Gracilibacillus | dipsosauri | 0025 |
| SHADE | Oceanobacillus | spp. | 0026 |
| SHADE | Oceanobacillus | spp. | 0026 |
| SHADE | Oceanobacillus | spp. | 0027 |
| MARKET | Paenibacillus | spp. | 0028 |
| SHADE | Bacillus | simplex | 0029 |
| MARKET | Bacillus | simplex | 0029 |
| SHADE | Bacillus | simplex | 0030 |
| SHADE | Bacillus | simplex | 0031 |
| OVEN | Bacillus | simplex | 0031 |
| MARKET | Bacillus | endophyticus | 0032 |
| MARKET | Bacillus | spp. | 0033 |
| MARKET | Bacillus | pumilus | 0034 |
| MARKET | Lysinibacillus | chungkukjangi | 0035 |
| SHADE | Bacillus | niacini | 0036 |
| SUN | Bacillus | licheniformis | 0037 |
| SHADE | Oceanobacillus | picturae | 0038 |
| SHADE | Oceanobacillus | picturae | 0039 |
| SHADE | Carnobacterium | maltaromaticum | 0040 |
| SHADE | Carnobacterium | maltaromaticum | 0040 |
| SUN | Carnobacterium | maltaromaticum | 0041 |
| SHADE | Carnobacterium | maltaromaticum | 0042 |
| MARKET | Bacillus | subtilis | 0043 |
| MARKET | Bacillus | endophyticus | 0044 |
| MARKET | Bacillus | aryabhattai | 0045 |
| SUN | Bacillus | aryabhattai | 0045 |
| MARKET | Dietzia | maris | 0046 |
| MARKET | Bacillus | spp. | 0047 |
| MARKET | Bacillus | aryabhattai | 0048 |
| SHADE | Bacillus | niacini | 0049 |
| SHADE | Bacillus | niacini | 0049 |
| MARKET | Bacillus | niacini | 0049 |
| SUN | Bacillus | niacini | 0050 |
| SHADE | Virgibacillus | carmonensis | 0051 |
| SHADE | Bacillus | megaterium | 0052 |
| MARKET | Bacillus | niacini | 0053 |
| SHADE | Bacillus | spp. | 0054 |
| OVEN | Bacillus | niacini | 0055 |
| MARKET | Bacillus | spp. | 0056 |
| MARKET | Bacillus | amyloliquefaciens | 0057 |
| MARKET | Bacillus | spp. | 0058 |

**Figure S1**


FID1 A, (MRKT_SFR.D)
counts
A
FID1 A, (NAT2_SFR.D)
counts
B
FID1 A, (SUN_SFR.D)
counts
C
FID1 A, (SAFR_1.D)
counts
D