Research Article

Changes in Bacterial Composition of Zucchini Flowers Exposed to Refrigeration Temperatures

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Microbial spoilage is one of the main factors affecting the quality of fresh fruits and vegetables, leading to off-flavor, fermented aroma, and tissue decay. The knowledge of microbial growth kinetics is essential for estimating a correct risk assessment associated with consuming raw vegetables and better managing the development of spoilage microorganisms. This study shows, for the first time, that only a part of total microbial community, originally present on fresh harvested female zucchini flowers, was able to adapt itself to refrigerated conditions. Through the study of microbial growth kinetics it was possible to isolate forty-four strains belonging to twenty-two species of the genera Acinetobacter, Arthrobacter, Bacillus, Enterobacter, Erwinia, Klebsiella, Pantoea, Pseudoclostridium, Pseudomonas, Serratia, Staphylococcus, and Weissella, suggesting Enterobacteriaceae as potentially responsible for pistil spoilage.

1. Introduction

The fresh-like and healthy traits of ready-to-use (RTU) products such as leafy vegetables (lettuce, rocket, spinach, etc.) and fruits (melon, pineapple, apricot, peach, apple, etc.), with a minimal time of preparation before consumption, are characteristics sought by consumers [1–3]. Microflora causing release of off-flavors and tissue decay is well known in both RTU vegetables and fruits [4–9]. The dominating bacterial population during low temperature storage mainly consists of species belonging to the Pseudomonadaceae and Enterobacteriaceae; besides some species belonging to the lactic acid bacteria and moulds may be present at relatively lower numbers [4, 10–13].

The consumption of zucchini (Cucurbita pepo L.) flowers with the immature fruit attached is spread in many countries, specially in the Mediterranean area, but, due to their high perishability, they are in general marketed only locally.

Different authors [14, 15] studied the effects of cold storage on zucchini flowers processed as a new RTU vegetable; these papers showed that the shelf life of flowers was affected either by low storage temperatures and by cultivars. However, no study is reported in literature on the evolution under refrigerated conditions of natural microbial population occurring on female zucchini flowers that, composed by petals and pistil, presents different tissues and nutrient availabilities.

Aim of this work was to gain a better knowledge about the survival of natural microorganisms occurring on female zucchini flowers, potentially involved in vegetable spoilage, in order to lay the basis for the development of a new RTU vegetable.

2. Materials and Methods

2.1. Microbiological Analyses of Zucchini Flowers. Microbial evaluation on growth kinetics in zucchini female flowers (cv. Aquilone) was carried out in two consecutive years (trial A and B). Clean flowers, with their immature fruit attached, were harvested and immediately subjected to microbiological analyses.

At the same time, zucchini flowers were stored, as harvested, in gas permeable polypropylene open bags at 4°C ± 1°C for 11 days. Three replicate bags of eight zucchini flowers were prepared for each sampling time (4, 8, and 11 days).

Microbial analysis was carried out on about 25 g of petals and pistils transferred aseptically and separately to
a stomacher bag, containing 9 parts (w/w) of sterile saline solution (0.9% NaCl) and homogenized for 1 min using a stomacher (BagMixer, Intersience, St Nom, France). Petal and pistil dilutions were plated in triplicate on different agar media. Total mesophilic bacteria (Plate Count Agar, Oxoid S.p.A. Garbagnate, Milan, Italy) and Enterobacteriaceae (Violet Red Bile Glucose Agar) were counted after 24 h of growth at 30°C, yeasts and moulds (Potato Dextrose Agar) at 25°C for four days, whereas presumptive lactic acid bacteria (Rogosa agar under anaerobiosis) were enumerated after 48 h of growth at 30°C. In order to prevent yeast growth, 150 mg L\(^{-1}\) of cycloheximide were added to Rogosa and PCA, while to prevent bacterial growth 150 mg L\(^{-1}\) of chloramphenicol were added to PDA. Microbial biotyping and species identification were carried out on isolates from the dominant bacterial population.

For each storage time (0, 4, 8, and 11 days), about 10–15 well-isolated colonies were picked up from PCA plates with the highest number of colonies, inoculated in PC broth (Tryptone 5.0 g L\(^{-1}\), Yeast extract 2.5, Glucose 1.0 g L\(^{-1}\), Oxoid S.p.A. Garbagnate, Milano, Italy), incubated at 30°C for 24 h and frozen at −80°C for further analyses.

2.2. Microbial Biotyping and Species Identification. DNA extraction was carried out using one mL of viable cell suspension by using Wizard Genomic DNA Purification kit (Promega Italia Srl, Milano, Italy) following manufacture’s instructions. DNA quantity and quality were determined by electrophoresis with known amounts of molecular weight marker (Marker IV, Roche Diagnostics, Milan, Italy) as a standard.

Isolates were submitted to the two-step RAPD analysis for biotyping [16] using the M13 primer (5’-GAGGGTGGC-GTTCT-3’) capable of characterizing bacterial isolates from apples [17]. After electrophoresis run (2% agarose in TBE buffer, QIAGEN, 70 V for 3–5 h), different isolates showing the same electrophoretic pattern were grouped. Isolates were clustered, sequenced, and identified as recently defined by Baruzzi et al. [18].

2.3. Evaluation of Spoilage Symptoms. Fruits and flowers were individually scored for spoilage symptoms (petal necrosis or water soaking, wilting, pistil acid rot, browning, and mycelia growth, etc.) by a group of six trained people using a 5 to 1 subjective scale, with 5 = excellent, no spoilage; 4 = very good, minor spoilage; 3 = fair, moderate spoilage; 2 = poor, major spoilage; 1 = inedible. A score of 3 was considered as the limit of marketability and a score of 2 as the limit of edibility [19].

3. Results and Discussion

3.1. Enumeration of Different Microbial Populations Occurring on Zucchini Flowers. With the aim to identify the microorganisms, which could influence the spoilage, different microbial populations occurring on pistils and petals were evaluated.

Separating microbial counts by pistils or petals, pistil microbial load was always higher than the corresponding petal microbial population by about 1 or 2 logarithmic unit. The greatest differences in microbial populations were found for total mesophilic aerobic bacteria; in particular, for each sampling time and for both experiments, total mesophilic bacteria on pistils were found to be from 10 to 1000 times more than on petals. The differences between pistils and petals for Enterobacteriaceae and presumptive lactobacilli were far less marked. The nutrient availability on petals can explain the higher microbial growth on pistils rather than on petals.

The growth kinetics of total aerobic bacteria, yeasts and moulds, presumptive lactobacilli, and Enterobacteriaceae on pistils during the storage at 4°C are shown in Figure 1. Table 1 shows the viable cell counts of total aerobic bacteria, yeasts and moulds, and Enterobacteriaceae showed a similar behavior for both trials; after an initial reduction, microbial counts remained stable for some days and then increased after eight days in storage, reaching the highest value recorded for flowers in refrigerated conditions at the end of the experiment when spoilage symptoms were detected. In particular, viable cell counts of total aerobic bacteria (Figure 1) decreased from 10^7 (trial A) to 100 (trial B) times reaching about 6 log cfu g\(^{-1}\) in both trials after eleven days of cold storage. The viable Enterobacteriaceae counts decreased from 10^7 × 10^5 to 2.4 × 10^4 cfu g\(^{-1}\) in trial A and from 1.6 × 10^6 to 8.2 × 10^3 cfu g\(^{-1}\) in trial B, increasing to about 5 log cfu g\(^{-1}\) at the end of the experiment for both trials. Total presumptive lactobacilli, usually known to be related to vegetable spoilage [10], showed counts (Figure 1) that remained relatively low, reaching 2.8 × 10^4 cfu g\(^{-1}\) only after eleven days of cold storage in trial B. Yeasts and moulds decreased 100 times in both trials with final values of 3.6 × 10^4 cfu g\(^{-1}\) and 5.6 × 10^2 cfu g\(^{-1}\) in trial A and B, respectively.

We can suppose that, after a selective effect of low temperatures on microflora, counts on fresh female zucchini flowers, surviving microorganisms fitted well with the new cold environment, starting to grow. Microbial growth kinetics resulted similarly in both experiments; in particular, total aerobic counts resulted in the dominant microflora, followed by Enterobacteriaceae, whereas yeasts and moulds and presumptive lactobacilli remained stable or increased slowly.

Since total mesophilic aerobic bacterial population occurring in pistils was numerically dominant, it was considered, according to Ragaert et al. [2], responsible for female zucchini flower spoilage and then deeper investigated.

3.2. Microbial Biotyping and Taxonomic Identification. Biodiversity of aerobic mesophilic microflora from eight different pistil samples was studied analyzing RAPD-PCR profiles of 118 colonies from PCA plates. In general, every sample showed many different electrophoretic fingerprints, helping to identify the different strains present: clustering 118 colonies gave 44 strains. 16S rDNA analysis showed that in some cases different electrophoretic patterns belonged to the same species, confirming the ability of two-step RAPD analysis to differentiate isolates belonging to the same...
Figure 1: Growth dynamics of microbial populations from zucchini female flower pistils during cold storage at 4°C in trial A and B. Mean value ± standard deviation.

Figure 2: Distribution of bacterial genera and relative total load from zucchini female flower pistils in trial A and trial B after 0 (black bars), 4 (white bars), 8 (diagonal stripes bars), and 11 (grey bars) days of cold storage at 4°C. Bacterial genera are defined as: 1, *Acinetobacter* spp.; 2, *Serratia* spp.; 3, *Klebsiella* spp.; 4, *Pantoea* spp.; 5, *Erwinia* spp.; 6, *Arthrobacter* spp.; 7, *Staphylococcus* spp.; 8, *Weissella* spp.; 9, *Pseudoclavibacter* spp.; 10, *Enterobacter* spp.; 11, *Pseudomonas* spp.; 12, *Bacillus* spp.

species. The list of all strains isolated from pistils is presented in Table 1, whereas the distribution and the viable cell load of bacterial genera from pistils during trials A and B are shown in Figure 2. The cluster analysis carried out after RAPD-PCR and 16SrDNA analysis showed wide differences between trial A and B microflora. The trial A showed eight different fingerprints in naturally occurring bacteria on flowers, whereas isolates of trial B were clustered in four groups. During cold storage microbial isolates from trial A were grouped in six, eight, and fourteen RAPD-PCR cluster after four, eight, and eleven days of storage, respectively. The trial B was characterized by a lower level of microbial biodiversity showing six groups of isolates at any sampling time. The unstored flowers microflora was dominated by *Acinetobacter* sp. (Figure 2) in both trials, but the relative microbial load was higher in trial B. Together with this genus, after four days of storage other strains belonging to *Weissella* spp., *Erwinia* spp. and *Arthrobacter* spp. for trial A and *Pseudomonas* spp. and *Klebsiella* spp. for trial B appeared. After eight days, when the early damage symptoms became evident in zucchini flowers in the two experiments, other microbial genera were identified: *Pantoea* spp. in both trials, *Erwinia* spp., *Pseudoclavibacter* spp. and *Weissella* spp. in trial A or *Pseudomonas* spp. and *Bacillus* spp. isolated from trial B.

At the end of zucchini flowers cold storage, the dominant microflora in trial B were mainly *Klebsiella* and *Serratia* genera. In trial A, in addition to these genera, *Staphylococcus* spp., *Acinetobacter* spp., *Arthrobacter* spp., and *Erwinia* spp. were also detected. The microflora of both trials showed isolates belonging to *Acinetobacter*, *Serratia*, *Klebsiella*, and *Pantoea* genera. Isolates belonging to *Erwinia* spp. were always found in trial A and never in trial B, whereas, differently from trial A, no *Staphylococcus* strain was isolated from trial B samples.

Differences in microflora composition between trials could be also due to different environmental conditions during the growth of the zucchini flowers.

The strains isolated from both trials belonged to the following species: *Acinetobacter calcoaceticus*, *Staphylococcus succinus* and *S. xylosus*, *Arthrobacter nicotianae*, *Serratia marcescens*, *Enterobacter aerogenes*, *Pantoea agglomerans*, and *Klebsiella oxytoca*. The most frequently isolated species was
Acinetobacter calcoaceticus, present at each storing day in trial A and initially and after four and eight days in trial B. 

Acinetobacter and Staphylococcus presented five strains; the remaining species showed one or two strains in both trials. As concerns strain growth kinetics, some strains (A. calcoaceticus, E. persicina, S. marcescens, K. oxytoca, and P. agglomerans) were recovered in more than one sample. The species present at the highest concentrations were A. calcoaceticus and Staphylococcus succinus (trial A) and A. calcoaceticus, S. marcescens, and K. oxytoca (trial B) isolated from fresh flowers in which total aerobic load showed the highest values. The strains with the lowest microbial load were A. calcoaceticus and K. oxytoca found at the end of storage in trial A and Pseudomonas sp., Pantoea sp., Bacillus megaterium, and S. marcescens detected in trial B. Even when it was possible to identify the same strain in more than one sample, no single strain (or single species) was ever identified as being entirely responsible for the spoilage symptoms showed. The microbial isolates identified during the two trials were quite similar, having many common species. The identification of Pantoea agglomerans, Erwinia persicina, Serratia marcescens, and Enterobacter aerogenes in all samples confirms the importance of Enterobacteriaceae in microbial spoilage of cold stored vegetables, according to previous indications [1, 10, 20].

A. baumannii and A. calcoaceticus form a phylogenetically complex of which only A. calcoaceticus is usually considered a nonpathogen soil-borne species [21]. We retained reliable the identification of Acinetobacter strains as belonging to A. calcoaceticus species, when the max score of alignment, calculated against sequences present in the Reference Sequence (RefSeq) database of other Acinetobacter species, was no higher than the 95% of that calculated for the top sequence match.

The microbial composition of zucchini flowers here reported partially resembles data from Janisiewicz and Buyer [22] relative to microflora associated with nectarine brown rot, that, in addition with strains belonging to other bacterial

| Strain | Taxonomic identification | Isolated in sample | Strain | Taxonomic identification | Isolated in sample |
|--------|--------------------------|--------------------|--------|--------------------------|--------------------|
| A5     | Acinetobacter calcoaceticus | T0                 | C35    | Acinetobacter calcoaceticus | T4                 |
| A12    | Acinetobacter calcoaceticus | T0, T4             | C36    | Acinetobacter calcoaceticus | T4                 |
| A13    | Acinetobacter calcoaceticus | T0, T8, T11        | C37    | Acinetobacter calcoaceticus | T4                 |
| A83    | Acinetobacter sp.         | T11                | C5     | Acinetobacter calcoaceticus | T0, T4, T8         |
| A63    | Arthrobacter arilaiti     | T11                | C3     | Acinetobacter sp.          | T0                 |
| A16    | Arthrobacter nicotianae   | T0                  | C86    | Bacillus Megaterium        | T8                 |
| A35    | Arthrobacter sp.          | T4                  | C107   | Enterobacter aerogenes     | T0                 |
| A80    | Arthrobacter sp.          | T0, T11            | C20    | Enterobacter aerogenes     | T0                 |
| A81    | Arthrobacter sp.          | T11                | C47    | Enterobacter sp.           | T8                 |
| A9     | Erwinia persicina         | T0, T4             | C107   | Klebsiella oxytoca         | T11                |
| A46    | Erwinia persicina         | T8, T11            | C38    | Klebsiella oxytoca         | T4, T11            |
| A38    | Erwinia sp.               | T4                  | C71    | Pantoea agglomerans        | T8                 |
| A64    | Erwinia sp.               | T8, T11            | C37    | Pantoea agglomerans        | T8                 |
| A65    | Klebsiella oxytoca        | T8                  | C51    | Pseudomonas sp.            | T4, T8             |
| A72    | Klebsiella sp.            | T11                | C122   | Serratia marcescens        | T11                |
| A42    | Pantoea agglomerans       | T4                  | C19    | Serratia marcescens        | T0                 |
| A57    | Pantoea agglomerans       | T8                  |        |                           |                    |
| A41    | Pantoea sp.               | T8                  |        |                           |                    |
| A48    | Pseudolavibacter helvolus | T8                  |        |                           |                    |
| A77    | Serratia sp.              | T11                |        |                           |                    |
| A65    | Staphylococcus saprophyticus | T11      |        |                           |                    |
| A17    | Staphylococcus succinus   | T0, T11            |        |                           |                    |
| A20    | Staphylococcus succinus   | T0                  |        |                           |                    |
| A67    | Staphylococcus succinus   | T11                |        |                           |                    |
| A62    | Staphylococcus xylosus    | T11                |        |                           |                    |
| A26    | Weissella sp.             | T4                  |        |                           |                    |
| A33    | Weissella viridescens     | T8                  |        |                           |                    |
genus, reported the isolation of Enterobacter, Erwinia, Panto-
toca, Pseudomonas, Serratia, and Staphylococcus strains asso-
ciated over a period of 7 weeks of nectarine development.

The microbial data showed many species and sometimes
different strains of a single species (Table 1); since some
strains were found both initially and at the end of cold
storage, whereas other grew only during storage, it is possible
to assume that only a part of total microbial community,
originally present on flowers, was adaptable to refrigerated
conditions.

The strain kinetic during cold storage highlighted the
microbial biodiversity of flowers, underestimated in fresh
flowers that usually are not subjected to severe changes in
environmental conditions.

The Acinetobacter strains are often associated with food
spoilage as previously demonstrated for meat, milk, and
cheeses [18, 23, 24].

The finding of some potentially pathogenic bacteria—
such as Acinetobacter spp. [25], K. oxytoca, Enterobacter aer-
ogenes [26], and Serratia spp. [27, 28]—commonly found on
vegetables, does not represent a risk for consumer health,
since these vegetables are usually cooked before eating. Nota-
bly, in some cases (Erwinia persicina, Enterobacter aerogenes,
Klebsiella oxytoca, and Serratia marcescens) the counts for
single strains or species, from PCA plates, was significantly
higher (more than 10 times) than Enterobacteriaceae counted
on VRBGA plates. In addition, in both trials the shape of the
Enterobacteriaceae growth curve appears the same, but with
lower values, of total aerobic bacteria curve.

The results of this work seem to indicate that VRBGA
is excessively selective for Enterobacteriaceae present on the
flowers, leading us to underestimate this microbial load. The
presence of 1.5 g L⁻¹ of bile salts, needed to evaluate the col-
iforms present in milk and dairy products, could be selective
against Enterobacteriaceae naturally occurring on vegetables
where bile salts are absent. If similar results will be obtained
for other vegetables, then the use of VRBGA for evaluating
Enterobacteriaceae in these foods should be avoided.

3.3. Evaluation of Spoilage Symptoms. During both experi-
ments (trial A and trial B), petals and pistils showed different
types of spoilage. Generally, petals remained intact but their
orange-yellow color lost natural brightness. In addition,
petals turgidity reduction (score 3 or less) was observed after
eleven days of refrigerated storage. Pistil spoilage occurred
quickly just after eight days of cold storage, when about 80%
of pistils appeared brown with gelatinous materials on the
surface. Even though we did not perform assays direct to
differentiate physiological from microbial spoilage, symp-
toms on petals remain physiological and biochemical
tissue disorders caused by storage at low temperature for
long time [29, 30], whereas pistil spoilage resembled soft rot
caused by pectolytic and macerating enzymes of many plant
pathogenic bacteria on different organs and succulent leaves
of vegetables [31, 32]. The evaluation of visual symptoms
occurring on petals, pistils, and fruits of female zucchini
flowers leads us to identify the pistils as the main perishable
part limiting the marketability during the cold storage.

4. Conclusions

In this work we evaluated, for the first time, the microbial
kinetics of natural occurring microflora of zucchini flowers
developing under cold storage conditions; the most perish-
able part of female zucchini flower was represented by pistils,
well known to have high amount of free simple sugars, in
comparison with petals, with limited nutrient availability.
Molecular tools enabled us to isolate forty-four strains from
complex microflora developing on pistils suggesting Enter-
obacteriaceae as potentially responsible for pistil spoilage.
However, due to the high microbial biodiversity found in
all samples of both trials, the joint responsibility of other
bacterial genera cannot be excluded.

A parallel result coming from data comparison from
strain biotyping, taxonomic identification, and microbial
isolation of different media is that the VRBGA media is exces-
sively selective to evaluate Enterobacteriaceae from vegetables.

Microbial data from this work could be useful to set tools
and technologies able to preserve the most delicate part of
zucchini flower, the pistil, against spoiler microorganisms.

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