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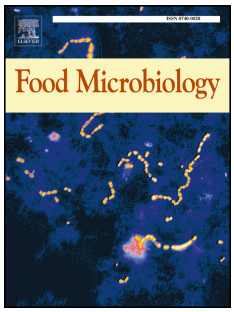

Efficacy of gaseous ozone to counteract postharvest table grape sour rot

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Efficacy of gaseous ozone to counteract postharvest table grape sour rot

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Abstract

This work aims at studying the efficacy of low doses of gaseous ozone in postharvest control of the **table grape sour rot**, a disease **generally attributed to** a consortium of non-*Saccharomyces* yeasts (NSY) and acetic acid bacteria (AAB). Sour rot incidence of wounded berries, inoculated with 8 NSYstrains, or 7 AAB, or 56 yeast-bacterium associations, was monitored at 25 °C up to six days. Sour rot incidence in wounded berries inoculated with yeast-bacterium associations resulted higher than in berries inoculated with one single NSY or AAB strain. Among all NSY-AAB associations, the yeast-bacterium association **composed of** *Candida zemplinina* CBS 9494 (Cz) and *Acetobacter syzygii* LMG 21419 (As) showed the highest prevalence of sour rot; thus, after preliminary *in vitro* assays, **this** simplified As-Cz microbial consortium was inoculated in wounded berries that were stored at 4 °C for ten days under ozone (2.14 mg m^{-3}) or in air. At the end of cold storage, no berries showed sour-rot symptoms although ozonation mainly affected As viable cell count. After additional 12 days at 25 °C, the sour rot index of inoculated As-Cz berries previously cold-stored under ozone or in air accounted for 22.6 ± 3.7 % and 66.7 ± 4.5 %, respectively. Molecular analyses of dominant AAB and NSY **populations** of both sound and rotten berries **during post-refrigeration period revealed** the appearance of new strains mainly belonging to *Gluconobacter albidus* and *Hanseniaspora uvarum* species, respectively. Cold ozonation resulted **an effective** approach to extend the shelf-life of table grapes also after **cold** storage.

Keywords: grape decay, microbial control, shelf-life, ethanol, acetic acid

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1.Introduction

Italy is one of the leading grape (*Vitis vinifera* L.) producers and consumers, and is also among the most important trade markets for this fruit in the world (USDA-FAS, 2014; 2015). The marketability of this product is strongly affected by postharvest microbial tissue decay resulting in undesirable physiological and chemico-physical changes **and shelf-life reduction**.

Sour rot is a grape disease (Guerzoni and Marchetti, 1987; Nigro et al., 2006) caused by opportunistic non-*Saccharomyces* yeasts (NSY) and acetic acid bacteria (AAB) (Barata et al., 2008; Barata et al., 2012b, c; Loureiro et al., 2012) mainly affecting late ripening cultivars under postharvest conditions (Hashim-Buckey et al., 2008; Puelles Tamsec and Sepulveda Ramirez, 2012). Rotten bunches **show** a strong and pungent odour of vinegar as the result of the production of microbial metabolites such as acetic acid, glycerol, ethyl acetate, ethanol, galacturonic acid, acetaldehyde and gluconic acid (Marchetti et al., 1984; Zoecklein et al., 2001). **In particular,** acetic and gluconic acid are usually considered chemical markers of sour rot development (Barata et al., 2012c).

The NSY-AAB consortium can be composed **of** different microbial species such as *Acetobacter malorum*, *A. cibinongensis*, *Gluconobacter oxydans*, *Pichia terricola*, *Hanseniaspora uvarum*, *Candida zemplinina*, and *Zygoascus hellenicus* as recently reported by Barata et al. (2012c).

The severity of grape sour rot is strongly promoted by the action of *Drosophila* spp. flies, attracted by volatile organic compounds released from sour rotten berries, that contribute to inoculate and disperse sour rot related microorganisms (Barata et al., 2012c).

To date, the control of microbial spoilage of table grapes under postharvest conditions is almost exclusively performed **by** using sulphur dioxide fumigation or applying SO_2-releasing pads (Lichter et al., 2006). However, excess of sulphur dioxide induces fruit and stem bleaching (Snowdown, 1990; Crisosto and Mitchell, 2002) and **may result in sulphite accumulation on table grape; thus, the content of sulphur dioxide residuals is internationally regulated (EPA, 1989; EU directive 2006/52/CE).** Therefore, alternative tools for controlling postharvest decays of table

grapes such as natural antimicrobials, decontaminating agents and physical methods have been recently proposed (Romanazzi et al., 2012). Among alternative postharvest decay control means (Feliziani et al., 2016; Pinto et al., 2015; 2016), ozone is increasingly gaining success owing to its broad antimicrobial spectrum and the lack of harmful residues, so as to be a compound generally recognised as safe (GRAS; Graham et al. 1997; FDA, 2001). **High** ozone concentrations were effective **in controlling** the germination of *Botrytis cinerea* conidia **reducing** grey mould development during grape cold storage (Gabler et al., 2010; Ozkan et al., 2011). Low doses of gaseous ozone (0.21-0.31 mg m^{-3}) were also **successfully used** to limit the spread of grey mould on grape during postharvest refrigerated period **(Feliziani et al., 2014). The antimicrobial action of ozone is generally attributed to its oxidant activity, also sustained by the release of reactive oxygen species, causing microbial cell membrane disruption, enzyme inactivation and nucleic acids damages (Hinze et al., 1987; Khadre et al., 2001).**

Despite these results, to the best of our **knowledge,** there are no studies reporting antimicrobial efficacy of ozone treatments against the **yeast-bacterium consortium** responsible for grape sour rot. **Thus, in this work one single yeast-bacterium consortium responsible for grape sour rot was selected. Then, ozone treatments were carried out to reduce viable load of selected microbial consortium when inoculated healthy berries to counteract** sour rot development during cold storage and post-refrigeration period.

2. Materials and Methods

The flow chart, describing the experiments carried out in this work, is depicted in Fig. 1.

2.1 *Yeast and bacteria strains and culture conditions*

In this work, eight yeast strains (*Candida vanderwaltii* CBS 5524, *C. zemplinina* CBS 9494; *Hanseniaspora guilliermondii* DSM 3432, *H. meyerae* CBS 8734, *H. uvarum* CBS 2585,

Zygoascus hellenicus CBS 6736, *Z. meyerae* CBS 4099, *Z. meyerae* CBS 6173) and seven AAB (*Acetobacter aceti* LMG 1504, *A. malorum* LMG 1746, *A. orleanensis* LMG 1583, *A. syzygii* LMG 21419, *Gluconacetobacter intermedius* LMG 18909, *Gl. saccharivorans* LMG 1582 and *Gluconobacter oxydans* LMG 1408) were chosen as representative strains of species usually identified in rotten berries and putatively involved in sour rot (Barata et al., 2012b; Barata et al., 2012c).

They were purchased from BCCM/LMG Bacteria Collection and CBS-KNAW Fungal Biodiversity Centre and Leibniz-Institut DSMZ, German Collection of Microorganisms and Cell Cultures, respectively. Bacteria were grown in YPM broth (D-mannitol 2.5 %; yeast extract 0.5 %; peptone 0.3 %) for 72 h at 30 °C in mild stirring whilst yeasts were grown in YPD broth (glucose 2 %; yeast extract 0.5 %; peptone 1%) at 30 °C for 48 h. *Gl. intermedius* LMG 18909 was grown in 10 mL of AE medium (glucose 1 %; yeast extract 0.2 %; peptone 0.3 %; acetic acid (100%) 1% v/v; ethanol (96%) 2% v/v) for 5 days at 30 °C. All strains were stored in Nutrient Broth (NB; Becton Dickinson Difco) with 20% glycerol at -80 °C in the Agro-Food Microbial Culture Collection (ITEM) at the Institute of Sciences of Food Production of Bari, Italy (http://server.ispa.cnr.it/ITEM/Collection/).

2.2 *Sour rot development assay*

The ability of NSY and AAB strains to cause sour rot on berries was evaluated as shown in Fig. 1 panel A1. Healthy grape berries of table grape cv. Sublima, received from the packing house Giuliano Srl (Apulia, Turi, Italy), were detached from the bunches, removing the pedicel, surface-sterilized with 2 % sodium hypochlorite for 5 min, rinsed with sterile tap water, air-dried for 30 min under a laminar flow cabinet and then inoculated with **1 x10^5** cfu berry^{-1} of **single** NSY strains (8), **or** AAB strains (7) **or** their associations (56). Control samples were represented by berries without pedicel amended or not with sterile saline solution, and by intact berries inoculated or not with strains. The **preparation of fresh microbial broth cultures for berry inoculation is** explained in the supplementary material section (SM 1).

Rotten berries, characterized by skin and pulp browning, dehydration, loss of grape juice and vinegar odor, were enumerated after 3 and 6 days of incubation at 25 °C. The assay was performed in triplicate (fifteen berries for each technical replicate; N = 3), measuring pH, redox potential and colorimetric coordinates as detailed in the supplementary material section (SM 1). At the end of **experiments,** the **yeast-bacterium** association showing the highest sour rot incidence was defined as a simplified microbial consortium and **subsequently evaluated**.

2.3 *Antimicrobial **assays** of gaseous ozone*

Antimicrobial assays were performed, as depicted in Fig. 1 panel A2, in a controlled ozone chamber endowed **with** 4 compartments with an internal volume of 0.038 m^3 and set at 4 °C. Ozone was generated via cold plasma using an OGS347 apparatus (Pipo 1962, Santa Giustina in Colle, PD, Italy) with a maximum production rate of 2.14 mg m^{-3} (1 ppm); the ozone emission and its concentration (every 10 minutes) were both monitored by a semiconductor SnO_2 probe and a specific data logger (Oneset Hobo datalogger, CapeCod, MA, USA). Further details related to the ozone chamber (Fig. 1S) are described in SM 2. Temperature and relative humidity **for each compartment** were measured every 30 min and recorded on Button® temperature/humidity logger model DS1923 (Maxim Integrated, San Jose, CA, USA).

Serial decimal dilutions in sterile saline solution of NSY and AAB strains (described in SM 1) were spread **onto YPD or YPM agar in** ventilated Petri dishes (Nuova Aptaca Srl, Canelli, Italy; 85 mm of diameter)**; the initial** estimated surface cell density **ranged** from 1 to 10000 cfu $plate^{-1}$. The inoculated plates were incubated for 10 days at 4 °C in the ozone chamber flowing the gas at 0 or 1.07 mg m^{-3}. **Petri** dishes were removed after 3, 7 and 10 days of ozone exposure, and incubated at 30 °C for 72 h in order to **allow microbial colony development and enumeration.**.

The effect of cold ozonation on cell viability for all strains was evaluated calculating the surface cell density, as reported in SM 2.

At the end of the experiments described in section 2.2, and the related supplementary content (SM 2), a single yeast-bacterium association was selected among 56 microbial associations assayed. This microbial consortium was additionally incubated **at 4 °C under ozonation at 2.14 mg m^{-3} for ten days.** Each assay was performed in triplicate (N = 3).

2.4 *Effect of gaseous ozone on contaminated table grape berries*

The **scheme** followed for the evaluation of sour rot development in berries after cold ozone treatment and during **the post-refrigeration period** is shown in Fig. 1, panel B.

2.4.1 Sample preparation and incubation conditions

Grape bunches cv. Italia were received from Giuliano Srl immediately after harvesting**, stored at 4 °C and processed within 48 h. Berries were inoculated with the selected AAB, or NSY strain** or with their association resulting from **the** previous sour rot screening, at *ca.* 10^4-10^5 cfu $berry^{-1}$, as described in section SM 1. The assay was repeated three times (N = 3). After the inoculum, berries were stored in a climate controlled storage chamber at 25 °C, 95% RH and at ventilation rate of 0.1 L min^{-1} for 48 h (sample T48h) in order to allow microbial cells to adapt to the berry environment and to simulate pre-harvest berry contamination before cold storage. After the adaptation period, samples were splitted in two **groups. The first group** of berries (3 replicates composed of 15 berries for each sample and sampling time) was kept in the climate controlled storage chamber for additional 12 days (samples T7 and T12)**. Conversely, the second group** (3 replicates composed of 15 berries for each sample, sampling time and incubation in air or under ozone) **was cold-stored** (4 °C). Refrigerated berries were incubated **in air or under** gaseous ozone (2.14 mg m^{-3}) for ten days (samples T10). After cold storage, **the** inoculated and **non-inoculated** berries, exposed or not exposed to cold gaseous ozone, **were further stored** in the climate controlled storage chamber (25 °C, 95% RH) **until 12 days** in order to simulate sale conditions (samples T17 and T22).

2.4.2 Evaluation of sour rot development, microbiological and molecular analyses, HPLC analyses of berry samples

The percentage of rotten berries and the disease severity **were recorded** according to an empirical scale with five degrees (Fig. 2S) **at each sampling time**. The empirical scale allowed the calculation of the McKinney's index, expressed as the weighted average of the disease on the maximum possible level (McKinney, 1923). AAB and total yeast loads were enumerated **immediately after the** inoculum (T0), after the adaptation period at 25 °C (T48h), after additional seven days at 25 °C (T7), at the end of cold storage (T10) and, during the post-refrigeration period at day seven (T17), as reported in SM 3.1.

Isolates from AAB and yeast populations from berry samples were characterized employing molecular biotyping techniques in order to ascertain their belonging to AAB and NSY strains inoculated in healty berries at T0. The composition of dominant AAB and total yeast populations was defined as reported in SM 3.1.

The extraction of sugars (glucose and fructose), organic acids (tartaric acid, malic acid, gluconic acid, acetic acid), ethanol and glycerol from grape samples was adapted from Mikulic-Petkovsek et al., (2012), as detailed in the SM 3.1.

2.5 *Statistical analysis*

A square root arcsin transformation (Sokal and Rohlf, 1995) was applied to percentages of spoiled berries and to McKinney's index values before **carrying out** the analysis of variance. Homogeneity of variance was assessed by Levene's test. The univariate General Linear Model (GLM) procedure, applying one- or two-way ANOVA ($P \leq 0.05$) through the SPSS software (SPSS, Inc., Chicago, IL), was used to evaluate the effects of the storage period and ozone treatment on the severity disease index values of berries and microbial cell counts and concentrations of different sugars and organic acids. Multiple comparisons among individual means of the severity index for each sample were made by Fisher's least significant difference (LSD) multiple range test at the 95% confidence

interval. Differences among mean values of yeasts and bacteria loads on sound and rotten berries **during post-refrigeration (samples T17)** were evaluated applying Tukey test ($P < 0.05$).

3. Results

3.1 ***Sour*** *rot development assay*

The development of sour rot in wounded berries inoculated with 8 NSYstrains, or 7 AAB, or 56 yeast-bacteria associations was monitored at 25 °C up to six days (Fig. 1, panel A1).

A significant ($P < 0.05$) increase in sour rotten berries, regardless microbial species or their associations, was registered for inoculated berries incubated at 25 °C and 95% RH (Table 1S); all control berry samples did not develop any sour rot symptom. NSY-AAB associations usually determined **a percentage of sour rot significantly higher than that recorded for** berries inoculated only with the NSY strain or the AAB strain (Table 1S). **After three days of incubation, a percentage of sour rotten berries higher than 50% was observed only in berries inoculated with associations composed of *C. zemplinina* CBS 9494 and *A. malorum* LMG 1746, or *A. syzygii* LMG 21419 or *Gl. saccharivorans* LMG 1582.** With the extension of the incubation period, also the remaining yeast-bacterium associations were able to develop sour rot in different extent as reported in SR 1. However, among all microbial associations, only those including *C. zemplinina* CBS 9494 were able to produce high **sour rot** percentage **starting from** three days of incubation **(Table 1S)**. At day six, the highest sour rot severity (scored four) was achieved **by combining** CBS 9494 with LMG 1746 or with LMG 21419 AAB strains**. However,** the 100% of rotten berries (15 berries for three replicates) was determined only together with the LMG 21419 strain.

Therefore, based on these results, the association *A. syzygii* LMG 21419 - *C. zemplinina* CBS 9494 (As-Cz) was selected as a simplified yeast-bacterium consortium for further experiments.

214 *3.2 **Evaluation of antimicrobial** activity of gaseous ozone against yeasts and bacteria*

215 The antimicrobial effect of gaseous ozone against 8 NSY and 7 AAB strains was monitored during
216 10 days of incubation (Fig. 1, panel A2).

217 Cold incubation determined a reduction in the surface cell density independently from ozone
218 exposure, depending on the NSY or AAB strain assayed; in addition, the inhibitory effect on cell
219 viability of these strains increased by combining low temperature and ozone treatment (Fig. 4S).
220 The survival of AAB at 1.07 mg m^{-3} cold ozonation was strain-dependent (Fig. 4S). In fact, *A. aceti*
221 LMG 1504 cells were not detected already after the third day of incubation, whereas *A. malorum*
222 LMG 1746 was found to be resistant until the end of cold ozonation period.

223 Conversely, NSY strains were overall found to be more resistant than AAB. After ten days of cold
224 storage, ozone flowed at 1.07 mg m^{-3}, was more effective against *C. zemplinina* CBS 9494 (Cz)
225 than *A. syzygii* LMG 21419 (As). Therefore, in order to reduce viability of both microbial strains, a
226 higher ozone concentration (2.14 mg m^{-3}) was assayed.

227 **The** surface cell density of Cz at the begin of incubation (15.25 ± 9.34 cfu cm^{-2}) decreased **after 10**
228 **days** to 9.81 ± 4.07 cfu cm^{-2} and to 1.14 ± 0.38 cfu cm^{-2} **in** air or **under** ozone, respectively. In the
229 case of As, initial surface cell density at 127.12 ± 28.38 cfu cm^{-2} was reduced to 103.32 ± 23.80 cfu
230 cm^{-2} when Petri dishes were **cold incubated** without ozone. **On the contrary, plates exposed to**
231 **ozone previously seeded with the lower microbial decimal dilution did not show colonies.**
232 Based on these results, the exposure for 10 days at ozone concentration **of** 2.14 mg m^{-3} was **used in**
233 **the subsequent experiments for the** control of sour rot under postharvest

234

235 *3.3 Effect of cold ozone treatment on berries inoculated with simplified yeast-bacterium association*

236 In order to stimulate the onset of sour rot, grape **berries, inoculated with As, or Cz, or the As-Cz**
237 **consortium,** were incubated at room temperature for 48 h. **After** the adaptation period, a group of
238 berries **were stored** in the climate controlled storage chamber whereas another group of berries

were **cold-stored** (4 °C) with or without ozonation (Fig. 1, panel B); **both group of samples were additionally incubated for 12 days (samples T17 and T22)**

3.3.1 Assessment of microbial viability and table sour rot development

At the end of **the** adaptation period (T48h at 25 °C)**,** no rotten berries **were found** (**herein and subsequently in the paper, when berries were scored with a McKinney's index ≥ 15%**). Viable cell **counts** of AAB and total yeast populations are shown in Table 1.

At the end of **the** adaptation period (T48h at 25 °C, Table 1) AAB population reached ***ca.*** 6.5 log cfu g^{-1} in sound berries (disease severity = 0) inoculated with As or the As-Cz association whereas AAB were not detected in remaining samples. Natural yeast **population** showed a moderate increase throughout nine days of incubation at 25 °C (sample SS in Table 1). **On the** contrary, in berries inoculated with Cz **(approximately at** 5 log cfu g^{-1}) total yeast population **increased their cell density by *ca.* two magnitude orders** within 48h. In comparison with natural yeast population enumerated in SS samples, berries inoculated with As, but **non-amended** with Cz, displayed an increase in total yeast population **by *ca.* three magnitude orders** at T48h. After nine days of storage at 25 °C (corresponding to T7 samples, Table 1) microbial population of sound berries was characterized by a decrease in AAB population (As and As-Cz samples) and an increase in yeast in SS control berries; samples inoculated with As or Cz showed values of viable yeasts close to those found at T48h. T7 samples displayed sour rot symptoms that, expressed as McKinney's index, were significantly ($P \leq 0.05$; LSD ± 13.7 %.) higher in As (23.9 ± 2.6 %) and As-Cz (39.4 ± 13.9%) inoculated berries than in those with Cz (7.8 ± 2.1); As, Cz and As-Cz berries showed different disease **severities** that, according to the 1-4 empirical scale, fell in the 1-2 range for Cz berries, 2 for As berries and 3 for As-Cz berries. In SS samples no rotten berries were detected.

Rotten berries showed AAB population in As inoculated samples close to 6 log cfu g^{-1}**,** whereas the same population increased to 6.97 ± 0.29 log cfu g^{-1} in As-Cz berries; these values were found significantly different, by Tukey test for $P \leq 0.05$, with those found in sound As and As-Cz

inoculated berries. At the same time, total yeast population**, occurring in these fruits,** were higher than 7 log cfu g^{-1}; in this case**,** significant differences were found only in the As inoculated samples. After 14 days of **storage at** room temperature (corresponding to T12 samples, see Fig. 1), disease severity for rotten berries showed values of 4 with McKinney's index of 37.8 ± 3.9, 22.2 ± 10.2, and 55.6 ± 13.9 (LSD = 13.7 %) for As, Cz and As-Cz inoculated berries, respectively. SS amended berries were still completely sound.

At the end of the adaptation period (T48h) **a group of berries was** incubated at 4 °C **in air or under** gaseous ozone (2.14 mg m^{-3}; Fig. 1, panel B), for ten days (samples T10). Changes in AAB and total yeast populations related to cold storage and gaseous ozone treatment of berries are shown in Table 2. AAB were not found in berries not inoculated with As, as already observed during the adaptation period (**48 h at 25 °C**). As concerns samples inoculated with As, AAB population of ozonated berries resulted significantly lower in comparison with the samples incubated **in** air after ten days of cold storage (Table 2, T10). In the case of yeasts, natural population in **control (SS)** and As samples resulted well controlled by ozone treatment. **On the contrary**, the same population enumerated in Cz inoculated berries incubated **in air and treated with ozone was not** significantly different.

After the end of cold storage**,** the additional storage at 25 °C resulted in sound and rotten berries (Table 2, T17). In particular, no sound **berries were** detected in samples, previously **cold-stored in air** (Table 2, T17: un = sound berries unavailable). AAB population enumerated in sound berries were found at values still close to 6 log cfu g^{-1} only in samples previously exposed to ozone. **Sound berries from both air and ozone cold-stored samples, did not show any significant difference in the density of viable yeast cells .**

The McKinney's index values redorded for T10, T17 and T22 berry samples are shown in Table 3. At the end of cold storage period (T10), no berry with sour rot symptoms was found independently by samples (SS, As, Cz and As-Cz) or storage conditions (Air vs Ozone). In addition, the visual appearance of berries did not show tissue damages potentially related to cold or cold/ozone

291 exposition. McKinney's indexes of SS berries increased significantly only at the end of post-
292 refrigration period (T22) without differences between air or ozone cold-stored berries. McKinney's
293 index of **the** remaining samples (inoculated berries at T17 and T22) was significantly lower in cold
294 ozonated berries than that recorded for fruits **cold-stored in** air.
295 Disease severity score of 3-4 was recorded for As and As-Cz inoculated rotten berries after seven
296 days in air at 25 °C, regardless the cold storage conditions previously applied.
297 On the contrary, disease severity of 1-2 or 2-3 distinguished Cz samples in relation to their previous
298 cold storage under ozone or in air, respectively. These results were similar to those previously
299 found at T7 and T12 in **non-refrigerated** berries stored at 25 °C under air when As inoculated
300 fruits always showed McKinney's indexes higher than Cz inoculated berries.
301 In rotten berries AAB population at T17 ranged from 6.40 ± 0.20 log cfu g^{-1} to 6.90 ± 0.31 log cfu
302 g^{-1} for As-Cz (cold-stored **in** air) and As (cold-stored under ozone), respectively; total yeast
303 population of rotten berries showed average values of 7.37 ± 0.15 log cfu g^{-1} .Tukey test revaled
304 that both microbial loads were significantly different in comparison to those found in sound berries
305 previously reported in Table 2 and subjected to the same treatments.
306
307 *3.3.2 Biotyping and taxonomic identification of microbial colonies isolated from sound and rotten*
308 *berries*
309 A total of 146 bacterial colonies and 175 yeast isolates were clustered by RAPD-PCR fingerprint
310 following the flow chart showed in Fig 1. The results of identification of colonies isolated from
311 sound berries are reported in Table 4. AAB isolates, collected from berries of samples T0, T48, T7,
312 T10 under air and T10 under ozone, generated only the banding pattern of *A. syzygii* LMG 21419
313 strain. Two new RAPD-PCR profiles from representative isolates of AAB population from T17
314 sound berries belonged to *Gluconobacter albidus* species. As reported in Tables 3 and 4, no sound
315 berries were found at T17 in samples previously cold-stored in air; for this reason it was possible to
316 characterize AAB dominant populations only in rotten berries. The analysis of isolates from rotten

berries of T17 samples showed, in addition to both *G. albidus* RAPD-PCR profiles, four new patterns belonging to *A. syzygii*, *A. pasteurianus/cerevisiae*, *G. oxydans*, and *G. frateurii* strains. AAB dominant populations of T17 As and As-Cz rotten berries were represented by new strains accounting**,** for the 73.2 ± 2.5 % in air samples and for 86.4 ± 6.4 % **on average,** in ozonated samples. The RAPD-PCR pattern of the LMG 21419 strain was identified only in T17 rotten As and As-Cz berries **in the range of 9-28% (regardless of previous refrigeration in air or under ozone).**

Yeast population, naturally occurring in sound berries, was mostly composed **of** *Hanseniaspora uvarum* and *Metschnikowia pulcherrima* strains**; these species were found in all control samples and in As inoculated berries**. *H. uvarum* strains dominated many of these samples, whereas the isolation of *Aureobasidium pullulans* strains was sporadic. **Sound berries inoculated with As showed** a new *C. zemplinina* strain **at T17** (indicated as Cz* in Table 4). Cz inoculated sound berries (Cz and As-Cz samples, Table 4) always showed dominant population largely represented by CBS 9494, although some samples showed few **isolates** belonging to *H. uvarum*.

Yeast populations of As and As-Cz rotten berries after 7 days at 25 °C (T7) were dominated **by** *C. zemplinina* strains.

In absence of sound inoculated As and As-Cz berries (Tables 3 and 4), As rotten berries showed only yeasts belonging **to** *H. uvarum**;*** the dominant yeast population of As-Cz rotten berries **was instead composed of** both *H. uvarum* (60%) and *C. zemplinina* species (40%). **On the** contrary, the dominant population of ozonated rotten berries, for both As and As-Cz samples, was composed only **of** *C. zemplinina* strains.

3.3.3 Identification and quantification of carbohydrates, alcohols and organic acids

Berry samples were analysed for reducing sugars, organic acids and alcohol **concentrations** (**expressed as** g per Kg of fresh weight) following the sampling **scheme** showed in Fig 1. **In particular, their content did not show significant (P< 0.05) differences beetween sound berries**

sampled at the end of the adaptation period (T48h) and after the additional (T7) incubation at 25 °C (Table 2S). However, acetic acid and glycerol were detected in As rotten berries (McKinney's index of 23.9 ± 2.6 %) at 3.84 ± 0.02 and 2.13 ± 0.37 g Kg^{-1}, **on average,** respectively; unexpectedly, these compounds were not detected in the As-Cz inoculated rotten berries that recorded a higher McKinney's index (39.4 ± 13.9%). No samples, including rotten berries, showed detectable traces of ethanol.

The severity of sour rots reached the highest values after 12 days at room temperature (samples T12, see Fig. 1); at the same time, glucose and fructose concentration halved (from 44.15 ± 0.73 and 116.65 ± 1.83 g Kg^{-1} to 27.47 ± 2.24 and 52.93 ± 5.33 g Kg^{-1}, for control and inoculated rotten samples, respectively) showing the appearance of ethanol, acetic acid and glycerol in As, Cz and As-Cz samples.

As and As-Cz rotten berries showed average values of 14.11 and 7.05 g Kg^{-1} of acid acetic and ethanol (rough ratio 2:1), respectively. **On the contrary, the average concentration of these metabolites showed a rough ratio 1:2 (acid acetic 5.04 g Kg^{-1}; ethanol 11.5 g Kg^{-1}) in rotten berries inoculated only with *C. zemplinina* CBS 9494.**

Malic acid concentration was found **to be** stable in sound berries **of control samples** at values close to 5 g Kg^{-1}. **In T12 rotten samples, the estimated gluconic acid concentration, a metabolite we can suppose produced only by AAB population, was significantly higher in As and As-Cz than in Cz samples ranged from 2.5 to 8.8 g Kg^{-1}.** Glycerol found in the range of 1.7 - 2.3 g Kg^{-1} did not seem related **to** As or Cz inoculum. Carbohydrate and organic acid content in sound berries after cold storage (T10 and T17, Table 3S) showed values similar to **the** sound berry samples previously analysed (Table 2S).

Different concentrations in acetic acid, ethanol and glycerol were found only in rotten berries after post-refrigeration period (T17, Table 5).

Rotten berries from As and As-Cz samples after 12 days at room temperature**,** as well as those from As and As-Cz at T17 refrigerated **in** air (Table 5)**,** showed higher concentration of acetic acid than

ethanol, so their ratio was always higher than 1**. In particular**, ratios higher than 2 were found in air samples inoculated with *A. syzygii* LMG 21419 at T17 (Table 5).

On the other hand, rotten berries from Cz samples after 12 days and ozonated rotten berries of T17 As samples showed higher **amounts** of ethanol compared to acetic acid, so the ratio of their concentration was less than 1.

In these latter samples, gluconic and malic acid concentrations were close to that of malic acid measured in sound samples; therefore the estimation of gluconic acid produced by AAB was considered not feasible.

4. Discussion

Sour rot is an emergent grape disease distributed in different geographical areas such as USA, Chile, China and Europe (Barata et al., 2012b; Hall et al., 2015; Puelles Tamsec and Sepulveda Ramirez, 2012; Wei et al., 2015). **The etiology** of this grape rot was attributed to *Candida* spp., *Hanseniaspora* spp., *Issatchenkia* spp. and *Saccharomycopsis* spp. yeast species (Blancard et al., 2000; Guerzoni and Marchetti, 1987) **or** to AAB of the genera *Acetobacter*, *Gluconobacter* and *Gluconacetobacter* (Barata et al., 2012c; Blancard et al., 2000; Oliva et al., 1999). **Our results sustain the association between AAB and NSY as** the causal agent of grape sour rot development, **even though only certain AAB-NSY associations lead to a** high percentage of rotten berries**. These results are** consistent with those of Huber (2016) **reporting a strain-dependent** *Candida zemplinina* pathogenicity.

Furthermore, sour rot development in vineyard **was widely reported** associated with *Drosophila* spp. (Gravot et al., 2001; Marchetti et al., 1984)**. In** particular, Barata et al. (2012c) demonstrated that *Drosophila* spp. flies are the origin and/or the vector of AAB**; likewise, Hall et al. (2015) showed that axenic *Drosophila* spp. flies were unable to develop sour rot onto wounded berries**.

394 Since the aim of this work was to counteract table grape sour rot during postharvest refrigerated
395 storage, we were forced to find a yeast-bacteria association able to spoil berries in absence of flies.
396 Nevertheless, sour rot can also develop during and/or after cold storage thanks to a pre-harvest
397 latent infection. Thus, to simulate a field contamination, an incubation of two days (T48h samples)
398 at 25 °C, was considered useful for microbial adaptation to berry environment before the cold
399 storage.
400 Sour rot development assays allowed to select the *A. syzygii* LMG 21419 and *C. zemplinina* CBS
401 9494 as a simplified microbial consortium **model useful for carrying out** trials on table grape
402 under postharvest conditions. Likewise, *in vitro* assays informed that **strains belonging to** this
403 AAB-NSY association **were** controlled after 10 days of cold incubation under **continuous** ozone
404 flow at 2.14 mg m^{-3}. **The effect of gaseous ozone treatment against natural yeast population** on
405 table grape berry was slightly higher than that recently reported by Cravero et al. (2016) on wine
406 **grape** berries. **Moreover**, natural yeast **population load in berries inoculated with *A. syzygii***
407 **LMG 21419** increased significantly in comparison with **that enumerated in** control **berries,**
408 suggesting a possible role of this **bacterial** strain in promoting the growth of wild NSY.
409 The use of **ozone** for controlling both AAB and NSY **has been** rarely reported**. T**he main effect
410 **against *A. syzygii* LMG 21419** was recorded under ozone at 2.14 mg m^{-3}; however, this strain was
411 more efficiently controlled when **it was seeded onto Petri dishes than when it was** inoculated in
412 healthy berries. The reduction of antibacterial efficacy of ozone treatment could be due **to** several
413 factors, as the reaction with organic matter (de Candia et al., 2015; Sarig et al., 1996; Segat et al.,
414 2014), the reduced penetration in wounds and the leakage of ozone-reactive substances reducing the
415 ozone content (Smilanick et al., 1999). In addition, a different exposure of colonies **grown** in
416 wound or leaves has been reported to improve microbial resistance to ozone treatments (Palou et
417 al., 2002; Wani et al., 2016).

As concerns *C. zemplinina* CBS 9494, the low efficacy of ozonation of inoculated berries is in **accordance with the** results of Guzzon et al. (2013) **who** demonstrated the complete inactivation of several NSY **strains after ozonated water treatment** only at ozone concentration of 2.5 mg L^{-1}. However, **the** exposure to ozone affected both **berry** microbial composition and sour rot development. **After the refrigeration period, cold-stored non-ozonated** berries developed sour rot with a higher incidence than that found among ozonated samples. The highest McKinney's index was found in **As inoculated berries previously cold-stored in air.** On the other hand, *A. syzygii* LMG 21419 was not detected in AAB dominant population of ozonated As inoculated sound berries.

Even though dominant AAB population showed new **non-inoculated** strains, potentially responsible for an additional tissue decay, *A. syzygii* LMG 21419 was still found in both As and As-Cz rotten berries. Our results partially reflect changes in microbiota of sour rotten berries evaluated **under** pre-harvest conditions (Barata et al., 2012b).

These results suggest that the survival of *A. syzygii* LMG 21419 over cold storage, **under the experimental conditions here applied, plays** a pivotal role for the development of sour rot during **the** post-refrigeration period.

The isolation of both *C. zemplinina* and *H. uvarum* strains **immediately** after cold storage under ozone is in **accordance** with the survival of these species **naturally** occurring onto wine grape surface after ozonation **in gaseous and water form** (Cravero et al., 2016). **Thus, the growth of these new strains could be** co-responsible for sour rot development together with CBS 9494. The new *C. zemplinina* strain was isolated only from As rotten berries in which the strain *C. zemplinina* CBS 9494 was not inoculated. However, slight differences (>95% of similarity) were found between the molecular fingerprints of *C. zemplinina* isolates and that of the strain CBS 9494, confirming the low level of diversity found among *C. zemplinina* (synonym of *Starmerella bacillaris*, Duarte et al., 2012) strains also when different molecular-based methods were applied (Englezos et al., 2015; Masneuf-Pomarede et al., 2015).

Our assays showed that ozonation, thanks to the ozone antimicrobial activity, significantly reduced the extent of sour rot during the post-refrigeration period. However, other authors reported that ozone treatments induced a tissue response responsible for delaying or reducing the vegetable decay (Sarig et al., 1996; Rodoni et al., 2009). For these reasons, ozonation could control the table grape sour rot also thanks to additional non-antimicrobial effects.

In addition to **the** microbial composition, changes in main sugars, organic acids and alchohols during sour rot development were **monitored**. Reduction in sugar concentration cannot be an affordable marker for the evaluation of the early phases of sour rot, as significant changes were evident only in heavily rotten berries.

As concerns acetic acid and ethanol found in rotten berries, their concentration changed depending on the cold storage conditions applied. Conversely, the acetic acid concentration was lower in ozonated than in air **cold-stored** rotten berries of As and As-Cz samples; this result suggests that the release of acetic acid by microbial populations responsible for sour rot in table grape berries could be affected by cold ozonation.

In addition, in ozonated sound berries, as well as in Cz samples, ethanol was more concentrated than acetic acid, suggesting that it was not further oxidized to acetic acid and/or water and carbon dioxide (Guillamón and Mas, 2011). Conversely, samples cold-stored in air and displaying a high McKinney's index (>30%) were always characterized by an acetic acid to ethanol ratio higher than 1. Acetic acid, ethanol and glycerol found in berries inoculated only with *C. zemplinina* CBS 9494, suggested the ability of this yeast to produce these metabolites. However, **we could not ascertain if the low amount of acetic acid found in Cz rotten berries was released by *C. zemplinina* CBS 9494, as already reported for this species by Englezos et al. (2015), or, indeed, by the naturally occurring AAB**, mainly composed **of** *Gluconobacter* spp.. This latter bacterial genus was previously reported to be involved in grape sour rot (Gravot, 2001; Huber, 2016) and able to convert ethanol and glycerol into acetic acid and dihydroxyacetone, respectively (Guillamón and Mas, 2011).

Gluconic acid, an AAB metabolite (Couto et al., 2003) and a well- known marker of **grape** sour rot (Zoecklein et al., 2001), **was detected only in rotten berries scored with high McKinney's index,** as previously reported **in wine grapes** (Barata et al., 2012c; Zoecklein et al., 2001). In rotten berries**,** the glycerol to gluconic acid ratio was found to be close to that considered typical of sour rot (Zoecklein et al., 2001) and far from values recorded for *B. cinerea* ''noble rot''**,** characterized by **a** high level of glycerol and **a** low concentration of gluconic acid (Hausinger et al., 2015). Since AAB can release acetic and gluconic acids metabolizing ethanol and sugars (Lu et al., 1999; Mamlouk and Gullo, 2013), our findings suggest that **the direct oxidation of glucose into gluconic acid, carried out by AAB,** is not detectable until distinct and clearly recognizable sour rot symptoms can be observed.

In conclusion, ozone treatment**,** applied under cold storage**,** controlled sour rot of heavily contaminated berries during **the** post-refrigeration period and counteracted spoilage activity caused by AAB. Rotten berries**,** characterized by **high viable loads of** AAB and NSY populations, usually showed increasing concentrations **of a series of microbial metabolites represented by ethanol, then by acetic acid and finally by gluconic acid. T**his pattern, that needs to be validated after specific trials, reflected the grape sour rot severity and was modified under cold ozonation. Although ozone application needs larger scale trials, **cold storage of** table grape under low doses of gaseous ozone is a promising method for preventing microbial postharvest decay and **for replacing sulphur dioxide**–releasing systems.

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644 **Figure Captions**

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647 **Fig. 1 Flow chart of the experiments carried out in this work.**

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SUPPLEMENTARY CONTENT

Efficacy of gaseous ozone to counteract postharvest table grape sour rot

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665 **Supplementary Methods (SM)**

666

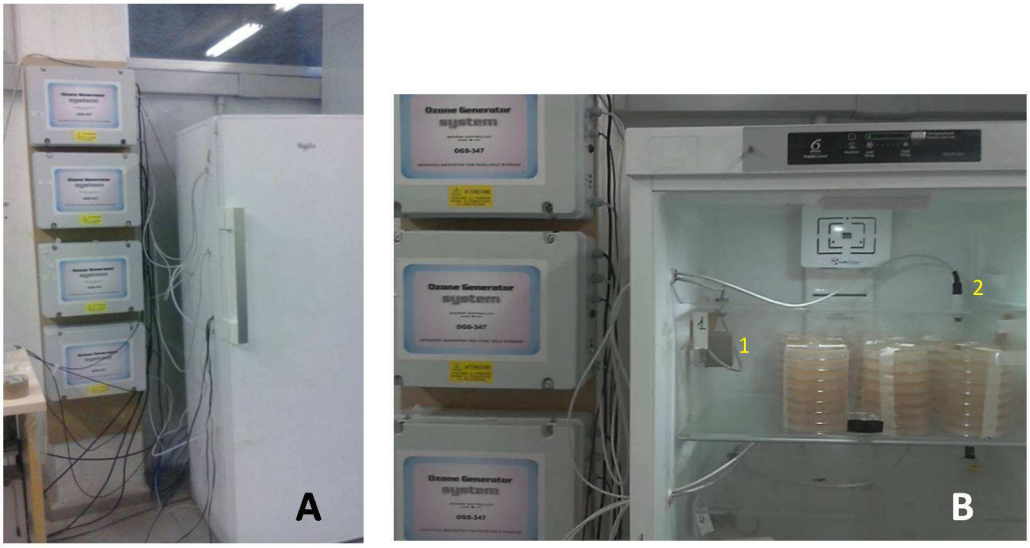
667 SM 1 - *Sour rot development assay*

668 Each **strain was cultivated inoculating a loopful of fresh yeast or bacterial cultures (24-48 h) in**
669 **10 mL of YPD or YPM, respectively. All cultures were incubated at 30 °C with mild shaking**
670 (120 rpm) for 48 h. **Then, microbial pellets** were harvested by centrifugation (13000 rpm for 5
671 min), **and** washed twice in sterile saline solution **(0.85% NaCl)**. The bacterial inoculum was
672 prepared from a cell suspension with an OD_{600} of 0.3 ± 0.05 **(corresponding to approximately 1**
673 **x10^8 cfu mL^{-1})** and diluted with sterile saline solution by reaching 10^7 cfu mL^{-1}; cellular density of
674 yeasts (10^7 cfu mL^{-1}) was assessed using a Thoma counting chamber (HGB Henneberg-Sander
675 GmbH, Lutzellinden, Germany). Cell suspension (10 µL) of each **strain** and their combinations
676 **were inoculated** in the pedicel cavity of 15 sterilized berries for each sampling time (final
677 concentration of **1 x**10^4-10^5 cfu berry^{-1}) in order to minimize the healing effect. Samples were
678 incubated in a climate controlled chamber for 6 days at 25 °C, 95% RH and at **a** ventilation rate of
679 0.1 L min^{-1} (Everlasting cabinet model 700 Glass, Everlasting S.r.l., Suzzara, Italy).
680 Color analysis of rotten berries was carried out determining colorimetric CIE (*Commission*
681 *Internationale de l'Eclairage*) coordinates L* (lightness), a* (redness) and b* (yellowness) on 3
682 random points of each sample. To this purpose**,** the ChromaMeter CR-400 (Konica Minolta, Osaka,
683 Japan) equipped with a D65 illuminant (6504 K), was used following the manufacturer's
684 instructions. The visible color differences (ΔE) **among** berries samples, recorded at the sixth day of
685 incubation, **were calculated** using the equation reported by Bai et al. (2013). The **measurements** of
686 pH and oxidation reduction potential in grape juice and grape pulp were also determined by a pH
687 meter (Model pH50 Lab pH Meter XS-Instrument, Concordia, Italy).
688 At the end of experiments, the **yeast-bacterium** association showing the highest sour rot incidence
689 was defined as a simplified microbial consortium and evaluated in **the subsequent experimental**
690 activities.

691

692 SM 2 - *Antimicrobial activity of gaseous ozone*

693 The ozone chamber was realised connecting a commercial refrigerator with four independent ozone
694 generators (Fig. 1S, panel A). The refrigerator included four independent airtight compartments,
695 with an approximate volume of 0.04 m^3; in each compartment, endowed with an independent probe
696 for monitoring ozone concentration, gas flowed from its own generator (Fig. 1S, panel B). Control
697 (unozonated) Petri dishes or berry samples were incubated in compartments whose generators were
698 switched off.



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700 Fig. 1S: The ozone chamber. Panel A, independent ozone generators connected with the
701 refrigerator. Panel B, a compartment in which the ozone inlet (1) and the probe for monitoring
702 ozone concentration (2) are present.
703

704 In order to evaluate the effect of cold ozonation, plates were inoculated with decimal dilutions of
705 broth cultures up to 10.000 times. After ozone exposure, plates seeded with different decimal
706 dilutions were incubated as described in Material and Methods section. Then, the number of cells,
707 occurring on the plate seeded with the broth culture (E), was estimated by enumerating colonies
708 developed. The E value was calculated as reported in the formula:

$$E = \frac{a10^{x1} + b10^{x2} + c10^{x3} + d10^{x4}}{n}$$

where, *a, b, c, d,* are the average number of colonies enumerated onto plates inoculated with different decimal dilutions, *x* is the exponent corresponding to the decimal dilution, and *n* is the number of dilutions showing countable colonies and used for the calculation of *E*.

Once the *E* value was calculated, the surface cell density was obtained dividing it by the plate surface (63.6 cm^2).

SM 3 - *Effect of gaseous ozone on contaminated table grape berries*

SM 3.1 Evaluation of sour rot development, microbiological and molecular analyses, HPLC analyses of berry samples

The empirical scale used for the calculation of incidence and severity, expressed as McKinney's index (McKinney 1923), is shown in Fig. 2S.

Fig. 2S Empirical scale with five degrees used for severity evaluation of sour rot: sound berries, 0; surface of berries spoiled from 1 to 10%, 1; surface of berries spoiled from 11 to 20%, 2; surface of berries spoiled from 21 to 50%, 3; more than 50% of spoiled surface, 4.

Microbiological analyses were carried out as follows: berries (15 g) were homogenized in 15 mL of sterile saline solution for 1 min at 11000 rpm using an **Ultra-Turrax T 25 digital (**IKA, Wilmington, NC); the slurries were decimal diluted in sterile saline solution and plated on GYC **(glucose 0.1%; yeast extract 0.1%; calcium carbonate 0.2%; agar 1.6%; pimaricin 100 mg L^{-1})** or YPDA medium **(YPD broth supplemented with agar 1.6% and amended with 100 mg L^{-1}** of cloramphenicol, Biolife Italiana Srl, Milan, Italy) for enumeration of AAB and total yeasts, respectively. Plates were incubated at 30 °C for 72 h for GYC plates and for 48 h for YPDA plates. The composition of dominant AAB and total yeast populations was defined by picking up a number of microbial colonies representing at least 10% of **the colonies enumerated on agar plates from**

735 **higher decimal dilutions**. Following this approach**,** 146 AAB isolates and 175 yeasts were
736 subjected to molecular typing. DNA from bacterial colonies was extracted according to Barata et
737 al. (2012b)**,** whereas DNA from yeasts was extracted using **the** Masterpure yeast DNA purification
738 kit (Epicentre Biotechnologies, WI, USA) according to manufacturer's instructions. Bacterial and
739 yeast isolates were typed applying different RAPD-PCR protocols (Baruzzi et al., 2015; Pfliegler et
740 al., 2014). Biotypes, representing different microbial clusters, were identified according to Mateo et
741 al. (2014) and Caputo et al. (2012) for bacteria and yeasts, respectively.
742 The extraction of sugars, organic acids and alcohols from grape samples was **carried out as**
743 **reported by Mikulic-Petkovsek et al. (2012) with minor changes**. Briefly, 15 grams of berries**,**
744 **were homogenized with 15 mL of bidistilled water for 1 min at 11000 rpm by using Ultra-**
745 **Turrax T-25 digital**. Then, the homogenate was centrifuged at 14000 rpm for 10 min at 4 °C.
746 Depending on the metabolites (organic acids and alcohols or sugars), supernatant from each sample
747 was appropriately diluted and filtered using 0.45 µm filters before the injection (20 µL) in the
748 HPLC system (Jasco Inc., Tokyo, Japan) equipped with a refractive index detector (RI-2031), an
749 UV detector (UV-2077) and an autosampler (AS-2057). JASCO ChromeNAVsoftware (Version
750 1.19.03 Build 6) was used for data acquisition, peak integration and standard calibration. Each
751 sample was analysed in triplicate and concentrations were expressed as g per Kg of fresh weight (g
752 Kg^{-1}).
753 Glucose and fructose were isocratically separated on a Rezex RCM monosaccharide Ca^{+2} column
754 (300 mm x 7.8 mm; 8µm; Phenomenex, Torrance, CA) preceded by a SecurityGuard Cartridge
755 (Carbo-H 4 x 3.0 mm; 8µm; Phenomenex). Elution was performed in MQ water at 0.6 mL min^{-1}
756 and 75°C. RI detector was set at 25 °C. A glucose and fructose calibration curve was built in the
757 range from 0.03 to 1%.
758 For the determination of organic acids and alcohols (tartaric acid, malic acid, gluconic acid, acetic
759 acid, ethanol and glycerol), the diluted berry supernatants were injected on a RezexROA H^+ 8%
760 column (300 mm × 7.8 mmid, 8µm; Phenomenex) preceded by a SecurityGuard Cartridge (Carbo-H

761 4 x 3.0 mm, 8 μm; Phenomenex). The elution of metabolites **was** performed isocratically with
762 sulphuric acid (0.005 N) at the flow rate **of** 0.6 mL min^{-1} and **at** 60 °C. RI detector was set at 40 °C.
763 Malic and gluconic acids (expressed as sum of malic and gluconic acid peak areas) and tartaric acid
764 were also UV detected at 210 nm after connecting UV and RI detectors in series. Calibration curves
765 (acetic acid, glycerol and ethanol in the range from 0.0625 to 1%; tartaric acid from 0.0078 to
766 0.125%; malic and gluconic acids from 0.0078 to 0.5%) were built to correlate the peak area with
767 each reference concentration. Due to gluconic acid and malic acid coelution in HPLC run, the
768 calculation of gluconic acid concentration of rotten berries was estimated by subtracting the malic
769 acid concentration measured in control (sound) berries.
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Supplementary Results (SR)

SR 1 - *Sour rot development assay*

Results of sour rot in berries inoculated with **single AAB (7) or NSY (8) strains** as well as their associations (56 combinations) are shown in Table 1S.

After three days of incubation**, only three out of the seven associations including** *C. zemplinina* CBS 9494 promoted a percentage of sour rotten berries higher than 50%. At day six, nine microbial associations were found to be responsible for a significant ($P < 0.05$) increase in the percentage of sour rotten berries in comparison with that recorded at day three. **In particular,** they were composed of *H. meyerae* CBS 8734 or *C. zemplinina* CBS 9494 associated with *Gl. saccharivorans* LMG 1582 or *Gl. intermedius* LMG 18909 or *A. malorum* LMG 1746, and *C. zemplinina* CBS 9494 associated with *A. syzygii* LMG 21419, and *H. guillermondii* DSM 3432 together with *A. malorum* LMG 1746 or *Gl. saccharivorans* LMG 1582.

At day three and six, berries inoculated with *G. oxydans* LMG 1408 as single culture showed **a high percentage of sour rot that was** not confirmed when it was associated with NSY strains. Rotten berries underwent grape pulp acidification and increase in the oxidation reduction potential value (data not shown), as well as they drastically turned **towards the red-brown with an** increase in a* opposed to the luminosity (L*) reduction (data not shown). **Examples** of sound and rotten berries are showed in Fig. 3S.

793 Table 1S Mean values (N = 3) of percentages of rotten berries after inoculum **with a single acetic acid bacteria (control A), or a**
794 **single yeast (control Y),** or their associations on grape cv Sublima seedless after 3 and 6 days at 25 °C

Day		Control A	Cv	Cz	Hg	Hm	Hu	Zh	Zm4	Zm6
3	Control Y		6.7 ± 11.5	6.7 ± 11.5	13.3 ± 11.5	6.7 ± 11.5	6.7 ± 11.5	13.3 ± 11.5	0.0 ± 0.0	0.0 ± 0.0
	Aa	20.0 ± 20.0	6.7 ± 11.5	6.7 ± 11.5	0.0 ± 0.0	0.0 ± 0.0	6.7 ± 11.5	0.0 ± 0.0	0.0 ± 0.0	6.7 ± 11.5
	Am	26.7 ± 11.5	6.7 ± 11.5	60.0 ± 20.0	20.0 ± 0.0	26.7 ± 11.5	26.7 ± 11.5	0.0 ± 0.0	13.3 ± 23.1	46.7 ± 11.5
	Ao	6.7 ± 11.5	6.7 ± 11.5	6.7 ± 11.5	6.7 ± 11.5	6.7 ± 11.5	13.3 ± 11.5	0.0 ± 0.0	6.7 ± 11.5	6.7 ± 11.5
	As	26.7 ± 11.5	13.3 ± 11.5	60.0 ± 0.0	20.0 ± 20.0	33.3 ± 23.1	6.7 ± 11.5	0.0 ± 0.0	0.0 ± 0.0	46.7 ± 11.5
	Gi	20.0 ± 20.0	6.7 ± 11.5	26.7 ± 11.5	26.7 ± 23.1	46.7 ± 23.1	6.7 ± 11.5	6.7 ± 11.5	0.0 ± 0.0	0.0 ± 0.0
	Go	53.3 ± 11.5	6.7 ± 11.5	26.7 ± 23.1	6.7 ± 11.5	13.3 ± 11.5	13.3 ± 11.5	0.0 ± 0.0	0.0 ± 0.0	6.7 ± 11.5
	Gs	20.0 ± 20.0	20.0 ± 20.0	66.7 ± 23.1	46.7 ± 11.5	40.0 ± 20.0	20.0 ± 20.0	0.0 ± 0.0	6.7 ± 11.5	33.3 ± 23.1

Day		Control A	Cv	Cz	Hg	Hm	Hu	Zh	Zm4	Zm6
6[1]	Control Y		11.1 ± 3.8	15.5 ± 3.9	17.8 ± 3.9	15.5 ± 3.9	17.8 ± 3.9	17.8 ± 3.9	0.0 ± 0.0	0.0 ± 0.0
	Aa	23.3 ± 15.3	6.7 ± 11.5	20.0 ± 20.0	46.7 ± 11.5	26.7 ± 23.1	20.0 ± 20.0	6.7 ± 11.5	0.0 ± 0.0	6.7 ± 11.5
	Am	26.7 ± 11.5	6.7 ± 11.5	80.0 ± 20.0	66.7 ± 11.5	86.7 ± 11.5	46.7 ± 11.5	0.0 ± 0.0	26.7 ± 23.1	46.7 ± 11.5
	Ao	23.3 ± 15.3	6.7 ± 11.5	6.7 ± 11.5	33.3 ± 11.5	6.7 ± 11.5	13.3 ± 11.5	0.0 ± 0.0	6.7 ± 11.5	6.7 ± 11.5
	As	26.7 ± 11.5	13.3 ± 11.5	100.0 ± 0.0	26.7 ± 11.5	40.0 ± 20.0	40.0 ± 20.0	0.0 ± 0.0	0.0 ± 0.0	53.3 ± 11.5
	Gi	26.7 ± 11.5	6.7 ± 11.5	80.0 ± 20.0	40.0 ± 20.0	86.7 ± 11.5	33.3 ± 11.5	13.3 ± 23.1	0.0 ± 0.0	0.0 ± 0.0
	Go	70.0 ± 10.0	6.7 ± 11.5	53.3 ± 11.5	13.3 ± 11.5	66.6 ± 11.5	26.6 ± 11.5	0.0 ± 0.0	6.7 ± 11.5	6.7 ± 11.5
	Gs	20.0 ± 11.5	20.0 ± 20.0	90.0 ± 10.0	66.7 ± 11.5	86.7 ± 11.5	33.3 ± 11.5	6.7 ± 11.5	6.7 ± 11.5	33.3 ± 23.1

AAB: Aa = *Acetobacter aceti* LMG 1504; Am = *A. malorum* LMG 1746; Ao = *A. orleanensis* LMG 1583; As = *A. syzygii* LMG 21419; Gi = *Gluconacetobacter intermedius* LMG 18909; Go = *Gluconobacter oxydans* LMG 1408; Gs = *G. saccharivorans* LMG 1582.

NSY: Cv = *Candida vanderwaltii* CBS 5524; Cz = *C. zemplinina* CBS 9494; Hg = *Hanseniaspora guillermondii* DSM 3432; Hm = *H. meyerae* CBS 8734; Hu = *Hanseniaspora uvarum* CBS 2585; Zh = *Zygoascus hellenicus* CBS 6736; Zm4 = *Z. meyerae* CBS 4099; Zm6 = Z. meyerae CBS 6173

1: The least significant difference comparison value (LSD, 95% confidence interval) expressed as % was calculated among the samples inoculated with yeasts, **or AAB,** or their associations: ± 46.22.

Control A: berries inoculated only with AAB strains; Control Y: berries inoculated only with NSY strains

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Fig. 3S Comparison among grape berries after 6 days of incubation at 25 °C. 1) wounded berries at the beginning of the assay; from 2 to 13 after 6 days at 25 °C; 2) **sound** berries; 3) wounded berries amended with sterile saline solution; 4) wounded berries **non-amended** with sterile saline solution; 5-9) berries inoculated with a single strain: 5) *C. zemplinina* CBS 9494; 6) *A. malorum* LMG 1746; 7) *A. syzygii* LMG 21419; 8) *Gl. saccharivorans* LMG 1582; 9) *Gl. intermedius* LMG 18909; 10-13) berries inoculated with *C. zemplinina* CBS 9494 associated with different AAB strains: 10) *A. malorum* LMG 1746; 11) *A. syzygii* LMG 21419; 12) *Gl. saccharivorans* LMG 1582; 13) *G. intermedius* LMG 18909.

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SR 2 - *Antimicrobial activity of gaseous ozone against yeasts and bacteria*

In the ozonation chambers temperature was stable at 4.61 ± 0.47 °C**,** and the average RH value was 65.24.± 2.15%, as recorded by different data loggers. The ozone concentration was affected by weak fluctuations in the order of 2 and 7% CV when ozone generator was set at 1.07 mg m^{-3} and 2.14 mg m^{-3}, respectively.

The results related to antimicrobial activity of 1.07 mg m^{-3} gaseous ozone against AAB and yeasts are shown in Fig. 4S. ***A. malorum* LMG 1746 resisted well to cold ozonation whereas *A. orleanensis* LMG 1583 was sensitive to ozone but resistant to cold storage conditions (Fig. 4S, panel A)**. Surface cell density of **the** remaining AAB strains was significantly ($P \leq 0.05$) affected by both (low temperature and ozone) storage conditions. With regards to yeasts (Fig. 3S, panel B), *H. meyerae* CBS 8734, *Z. meyerae* CBS 6173 and *C. vanderwaltii* CBS 5524 strains reduced significantly ($P \leq 0.05$) their surface cell density under ozone, whereas the viability of the remaining strains were mostly affected by the low temperature.

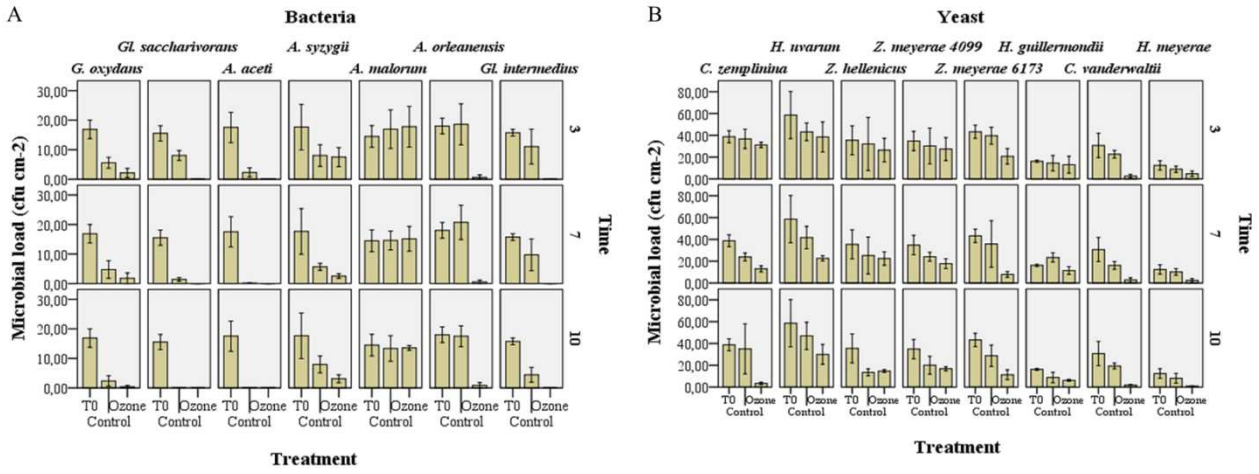

Fig. 4S. Mean values of surface cell density (expressed as cfu cm^{-2}) of AAB (A) and NSY (B) strains recorded after 3, 7 and 10 days of ozone exposure (1.07 mg m^{-3}) at 4°C (N = 3). LSD *G. oxydans* LMG 1408, 2.36; *Gl. saccharivorans* LMG 1582, 1.59; *A. aceti* LMG 1504, 2.98; *A. syzygii* LMG 21419, 4.84; *A. malorum* LMG 1746, 4.40; *A. orleanensis* LMG 1583, 3.59; *Gl. intermedius* LMG 18909, 2.84; *C. zemplinina* CBS 9494, 8.89; *H. uvarum* CBS 2585, 14.83; *Z. hellenicus* CBS 6736, 13.11; *Z. meyerae* CBS 4099, 8.90; *Z. meyerae* CBS 6173, 9.36; *H. guillermondii* DSM 3432, 4.29; *C. vanderwaltii* CBS 5524, 6.67; *H. meyerae* CBS 8734, 3.36.

827 SR 3 - *Effect of cold ozone treatment on berries inoculated with **a** simplified yeast-bacterium*
828 *association*

829 SR 3.1 Biotyping and taxonomic identification of microbial colonies isolated from sound and
830 rotten berries

831 The seven AAB representative strains were typed, after the application of a rough DNA extraction
832 method from plate colonies (Barata et al., 2012b), by applying a RAPD-PCR protocol originally
833 defined for *Pseudomonas* spp. (Baruzzi et al., 2015). Electrophoretic patterns were well
834 distinguishable among strains and the molecular fingerprint of *A. syzygii* LMG 21419 was stable
835 using **amounts** of template within the range of 10-30 ng per reaction (data not shown). This method
836 was used to estimate the ratio of *A. syzygii* LMG 21419 strain on AAB population enumerated onto
837 GYC plates **after the adaptation period, during storage at 25 °C, after cold storage (in air or**
838 **ozone) and during the post refrigeration period.**
839 As regards molecular analysis of yeast isolates, 1283 and Rf2 primers were used for RAPD-PCR
840 protocols as described by Pfliegler et al., (2014). **However**, after the analysis of more than 50% of
841 isolates, Rf2 was preferred due to its **better** electrophoretic fingerprint.
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SR 3.2 Identification and quantification of carbohydrates, alcohols and organic acids

The concentrations of sugars, organic acids and alcohols registered in sound berries after the adaptation period (T48h) and after additional (T7) incubation at 25 °C are showed in Table 2S.

Table 2S Mean concentrations (g Kg^{-1}) of sugars and organic acids detected on berries amended with sterile saline solution (SS; control) or inoculated with *A. syzygii* LMG 21419 (As), *C. zemplinina* CBS 9494 (Cz), or both microorganisms (As-Cz) immediately after the inoculum (T0), after 48 h at 25 °C (adaptation period, T48h) and after seven days at 25 °C (T7).

	Sample	Glucose	Fructose	Tartaric acid	Gluconic/Malic acids
T0	SS	48.57 ± 4.41	117.75 ± 8.83	5.02 ± 0.33	4.79 ± 0.19
T48	SS	47.83 ± 1.47	117.75 ± 4.41	4.87 ± 0.36	3.87 ± 0.28
	As	49.31 ± 5.15	116.28 ± 12.51	4.94 ± 0.21	3.88 ± 0.20
	Cz	54.46 ± 5.15	124.37 ± 9.56	5.13 ± 0.44	4.47 ± 0.33
	As-Cz	48.94 ± 1.10	117.01 ± 2.20	6.41 ± 0.70	5.24 ± 0.45
T7	SS	66.60 ± 1.83	153.81 ± 5.88	7.61 ± 0.99	5.95 ± 0.33
	As	51.14 ± 7.72	115.54 ± 24.28	6.06 ± 0.54	5.34 ± 0.28
	Cz	60.34 ± 3.68	142.04 ± 11.77	6.29 ± 0.59	5.49 ± 0.25
	As-Cz	66.23 ± 4.41	148.29 ± 9.93	5.84 ± 0.23	5.41 ± 0.17

The least significant difference comparison values (LSD, 95% confidence interval , expressed as g Kg^{-1}) were calculated among the different samples at each sampling time and for each metabolite:
Glucose, T48h = 14.29; T7 = 18.45; Fructose, T48h = 31.07; T7 = 55.27;
Tartaric acid, T48h = 1.75; T7 = 2.44; Gluconic/Malic acids, T48h = 1.74; T7 = 0.99.

In Table 3S the concentrations of carbohydrates and organic acids in sound berries after the cold storage period (T10) and during the post-refrigeration (T17) are reported.

Tab. 3S Mean concentrations (g Kg^{-1} FW) of sugars, organic acids and alcohols detected on berries amended with sterile saline solution (SS; control) or inoculated with *A. syzygii* LMG 21419 (As), *C. zemplinina* CBS 9494 (Cz), or both microorganisms (As-Cz) at the end of cold storage period (T10) with or without gaseous ozonation (2.04 mg m^{-3}) and then incubated under air for seven days at 25 °C (T17).

Sample			Glucose	Fructose	Tartaric acid	Gluconic/Malic acids
T10	SS	Air	50.78 ± 2.94	118.85 ± 10.67	4.61 ± 0.29	4.57 ± 0.25
		Ozone	64.02 ± 7.36	152.71 ± 12.87	4.54 ± 0.33	4.10 ± 0.14
	As	Air	57.03 ± 1.83	135.41 ± 7.35	5.99 ± 0.56	5.33 ± 0.27
		Ozone	56.30 ± 6.99	121.80 ± 30.54	4.53 ± 0.25	4.17 ± 0.16
	Cz	Air	55.56 ± 5.52	122.17 ± 21.34	6.59 ± 0.70	5.75 ± 0.41
		Ozone	78.38± 6.25	180.31 ± 11.03	5.66 ± 0.60	5.85 ± 0.33
	As-Cz	Air	68.44 ± 1.47	150.50 ± 9.19	6.07 ± 0.74	4.88 ± 0.28
		Ozone	51.88 ± 3.31	129.53 ± 2.94	6.53 ± 1.01	5.87 ± 0.35
T17	SS	Air	44.15 ± 11.03	100.82 ± 25.02	4.88 ± 0.18	4.42 ± 0.19
		Ozone	67.70 ± 3.68	157.12 ± 8.46	6.99 ± 0.55	6.23 ± 0.34
	As	Ozone	68.07 ± 8.46	162.28 ± 19.50c	7.15 ± 0.80	4.61 ± 0.26
	Cz	Air	66.97 ± 3.68	121.43 ± 30.17	6.55 ± 0.57	5.25 ± 0.37
		Ozone	48.20 ± 4.04	112.97 ± 8.46	6.48 ± 0.14	5.90 ± 0.38
	As-Cz	Ozone	63.66 ± 7.72	116.65 ± 15.82	6.35 ± 0.28	4.53 ± 0.20

The least significant difference comparison values (LSD, 95% confidence interval, expressed as g Kg^{-1}) were calculated among the different samples at each sampling time and for each metabolite:
Glucose T10 = 12.17, T17 = 15.97; Fructose T10 =38.09; T17 = 44.76;
Tartaric acid T10 = 1.20; T17 = 1.47; Gluconic/Malic acids T10 = 0.69; T17 = 0.67.

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Tables

Table 1 Viable cell counts of acetic acid bacteria (AAB) and total yeasts (log cfu g^{-1} ± SD; N = 3) of sound grape berries amended with sterile saline solution (SS; control) or inoculated with *A. syzygii* LMG 21419 (As). or *C. zemplinina* CBS 9494 (Cz), or both microorganisms (As-Cz). immediately after the inoculum (T0), after 48 h a 25 °C (adaptation period, T48h) and after seven days at 25 °C (T7).

Sample	AAB			Total yeasts		
	T0	T48h	T7	T0	T48h	T7
SS	nd	nd	nd	3.15 ± 0.30	3.94 ± 0.08	5.01 ± 0.06
As	4.73 ± 0.31	6.75 ± 0.15	5.35 ± 0.24	3.07 ± 0.22	6.07 ± 0.21	5.96 ± 0.05
Cz	nd	nd	nd	5.24 ± 0.21	7.00 ± 0.15	7.30 ± 0.14
As-Cz	3.43 ± 0.06	6.45 ± 0.06	4.74 ± 0.06	5.20 ± 0.16	6.85 ± 0.18	6.88 ± 0.08

nd = not detected (detection limit 1.30 log cfu g^{-1}). The least significant difference comparison values (LSD, 95% confidence interval, expressed as log cfu g^{-1}) were calculated among the different samples for each sampling time: AAB: T0 = 0.74, T48h = 0.32. T7 = 0.32; Total yeasts: T0 = 0.91, T48h = 0.60. T7 = 1.12.

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Table 2 Viable cell counts of acetic acid bacteria (AAB) and total yeasts (log cfu g^{-1} ± SD; N = 3) on sound grape berries amended with sterile saline solution (SS; control) or inoculated with *A. syzygii* LMG 21419 (As). or *C. zemplinina* CBS 9494 (Cz), or both microorganisms (As-Cz) immediately at the end of cold storage period (T10) **in air or under ozone** (2.14 mg m^{-3}) **and subsequently incubated in air** for seven days at 25 °C (T17).

Sample	T10				T17			
	AAB		Total yeasts		AAB		Total yeasts	
	Air	Ozone	Air	Ozone	Air	Ozone	Air	Ozone
SS	nd	nd	4.67 ± 0.26	3.80 ± 0.21	nd	nd	5.33 ± 0.23	5.54 ± 0.12
As	6.96 ± 0.08	6.04 ± 0.06	6.24 ± 0.23	4.35 ± 0.08	un	6.06 ± 0.02	un	6.63 ± 0.24
Cz	nd	nd	6.89 ± 0.17	6.85 ± 0.12	nd	nd	6.49 ± 0.11	6.63 ± 0.25
As-Cz	6.02 ± 0.21	5.67 ± 0.04	6.81 ± 0.14	6.63 ± 0.24	un	5.91 ± 0.06	un	6.32 ± 0.16

nd = not detected (detection limit 1.30 log cfu g^{-1}); **un = unavailable sound berries**.

The least significant difference comparison values (LSD, 95% confidence interval, expressed as log cfu g^{-1}) were calculated among samples at each sampling time. AAB: T10 = 0.14, T17 = 0.34.Total yeasts: T10 = 0.33, T17 = 0.34.

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Table 3 McKinney index (mean value ± SD; n = 3) recorded on grape berries amended with sterile saline solution (SS; control) or inoculated with *A. syzygii* LMG 21419 (As). or *C. zemplinina* CBS 9494 (Cz), or both microorganisms (As-Cz) immediately at the end of cold storage period (T10) **in air or under ozone** (2.14 mg m^{-3}) and then incubated **in air** for seven and 12 days at 25 °C (T17 and T22).

Sample	T10		T17		T22	
	Air	Ozone	Air	Ozone	Air	Ozone
SS	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	9.5 ± 4.1	11.9 ± 4.1
As	0.0 ± 0.0	0.0 ± 0.0	62.2 ± 7.5	28.9 ± 11.7	69.1 ± 4.1	29.2 ± 3.7
Cz	0.0 ± 0.0	0.0 ± 0.0	12.2 ± 7.5	5.6 ± 4.8	26.1 ± 12.5	20.2 ± 4.5
As-Cz	0.0 ± 0.0	0.0 ± 0.0	51.1 ± 3.5	18.3 ± 7.6	66.7 ± 4.5	22.6 ± 3.7

The least significant difference comparison values (LSD, 95% confidence interval) were calculated for each type of sample: SS = 3.4%; As = 2.9 %; Cz = 5.6 %; As-Cz = 1.7 %.

At T17 mean values (Air or Ozone) were additionally separated for the least significant difference value (LSD, 95% confidence interval): 7.69 %

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Tab. 4 Composition of dominant total non-*Saccharomyces* yeasts population (NSY) and acetic acid bacteria (AAB) isolated from sound grape berries amended with sterile saline solution (SS; control) or inoculated with *A. syzygii* LMG 21419 (As). or *C. zemplinina* CBS 9494 (Cz), or both microorganisms (As-Cz).

Sampling time: T0, after the inoculum; T48h, after 48 h a 25 °C (adaptation period); T7, after seven days at 25 °C; T10, at the end of cold storage period; T17, after seven days at 25 °C in air. Samples T10 and T17 were differentiated for cold storage **in air or under ozone** (2.14 mg m^{-3}).

Sample	Population	T0	T48h	T7	T10		T17	
					Air	Ozone	Air	Ozone
SS	NSY	Hu-Mp	Hu	Hu	Hu	Hu-Mp-Ap	Hu-Mp	Hu-Mp
	AAB	nd	nd	nd	nd	nd	nd	nd
As	NSY	Hu-Mp	Hu	Hu	Hu	Hu-Mp	un	Cz*
	AAB	As	As	As	As	As	un	Ga
Cz	NSY	Cz	Cz	Cz	Cz-Hu	Cz-Hu	Cz	Cz
	AAB	nd	nd	nd	nd	nd	nd	nd
As-Cz	NSY	Cz	Cz	Cz	Cz-Hu	Cz-Hu	un	Cz
	AAB	As	As	As	As	As	un	Ga

Legend of species:
Hu, *Hanseniaspora uvarum*; Mp, *Metschnikowia pulcherrima*; Ap, *Aureobasidium pullulans*; Cz, *Candida zemplinina* CBS 9494; Cz*, new *C. zemplinina* strain
As = *Acetobacter syzygii* LMG 21419; Ga = *Gluconobacter albidus*; un = sound berries unavailable;
nd = absence of presumptive AAB strains for undetectable level of AAB population
un = **unavailable sound berries**.

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Tab. 5 Mean concentrations (g Kg^{-1}) of acetic acid and alcohols detected in sound (s) and rotten (r) berries amended with sterile saline solution (SS; control) or inoculated with *A. syzygii* LMG 21419 (As), or *C. zemplinina* CBS 9494 (Cz), or both microorganisms (As-Cz) after seven days of post-refrigeration period (T17) **of samples previously cold-stored in air or under ozone (2.14 mg m^{-3})**.

Sample		Berries	Acetic acid	Ethanol	Glycerol
SS	Air	s	nd	nd	nd
	Ozone	s	nd	nd	nd
As	Air	r	3.63 ± 0.10	1.58 ± 0.18	1.07 ± 0.10
	Ozone	s	nd	nd	nd
		r	1.23 ± 0.31	3.64 ± 0.40	2.52 ± 1.46
Cz	Air	s	nd	nd	nd
	Ozone	s	nd	nd	nd
As-Cz	Air	r	2.53 ± 0.17	0.95 ± 0.22	nd
	Ozone	s	nd	nd	nd
		r	1.58 ± 0.10	nd	nd

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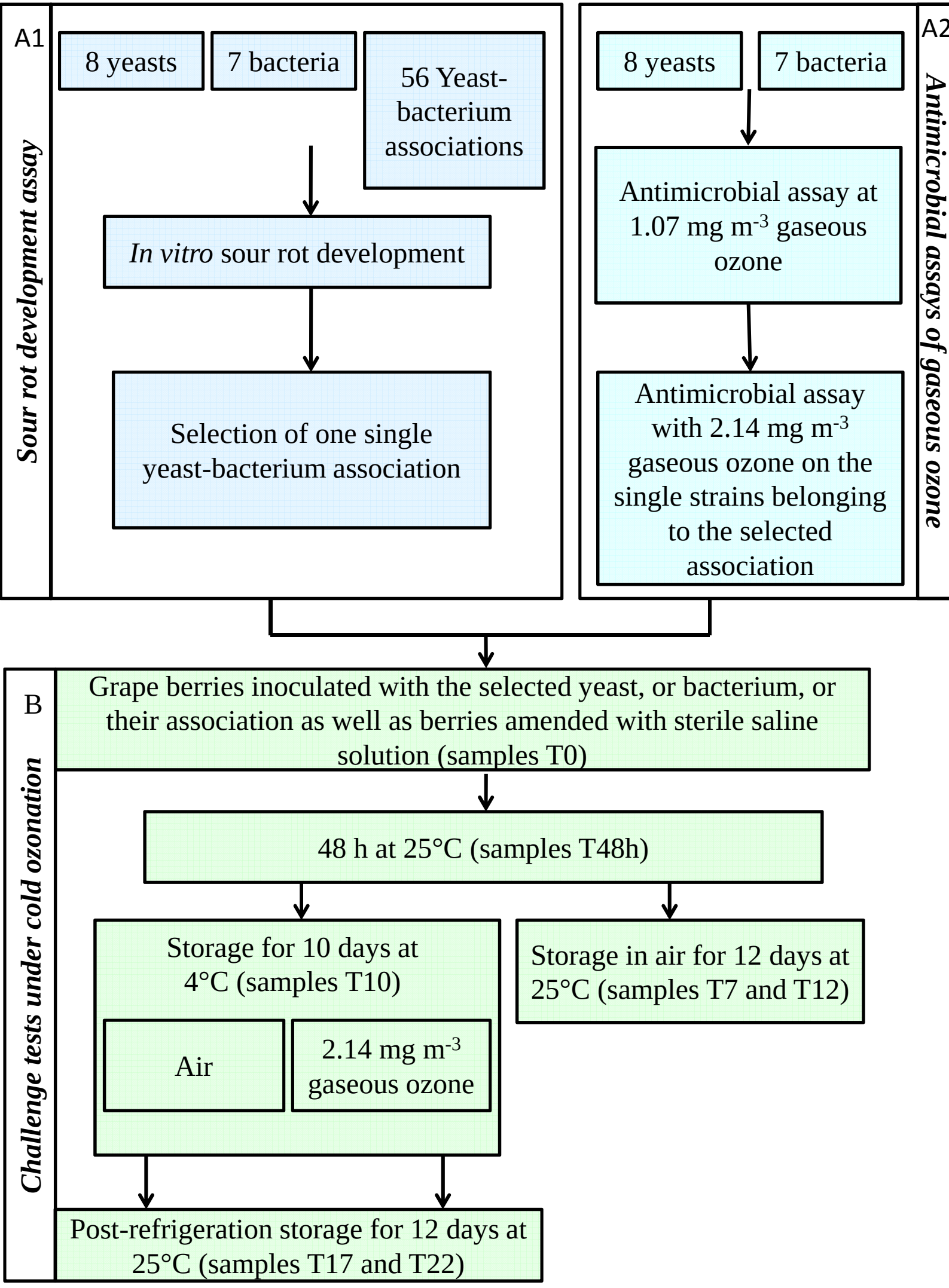
A1
Sour rot development assay
8 yeasts
7 bacteria
56 Yeast-bacterium associations
In vitro sour rot development
Selection of one single yeast-bacterium association
A2
Antimicrobial assays of gaseous ozone
8 yeasts
7 bacteria
Antimicrobial assay at 1.07 mg m-3 gaseous ozone
Antimicrobial assay with 2.14 mg m-3 gaseous ozone on the single strains belonging to the selected association
B
Challenge tests under cold ozonation
Grape berries inoculated with the selected yeast, or bacterium, or their association as well as berries amended with sterile saline solution (samples T0)
48 h at 25°C (samples T48h)
Storage for 10 days at 4°C (samples T10)
Air
2.14 mg m-3 gaseous ozone
Storage in air for 12 days at 25°C (samples T7 and T12)
Post-refrigeration storage for 12 days at 25°C (samples T17 and T22)

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Efficacy of gaseous ozone to counteract postharvest table grape sour rot

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Highlights

- Development of table grape sour rot is specific for different AAB-NSY associations
- *A. syzygii* LMG 21419 and *C. zemplinina* CBS 9494 constituted a simplified microbial consortium
- Cold ozonation at 2.14 mg m^{-3} reduced significantly the LMG 21419 viability
- Cold ozonation delayed sour rot development also in post-refrigeration period.

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