International Journal of Food Microbiology 296 (2019) 65–74

Contents lists available at ScienceDirect

International Journal of Food Microbiology

journal homepage: www.elsevier.com/locate/ijfoodmicro

Growth and metabolite production of a grape sour rot yeast-bacterium consortium on different carbon sources

L. Pinto[a,*], M. Malfeito-Ferreira[b], L. Quintieri[a], A.C. Silva[b], F. Baruzzi[a]

[a] Institute of Sciences of Food Production, National Research Council of Italy, Via G. Amendola 122/O, 70126 Bari, Italy
[b] Linking Landscape, Environment, Agriculture and Food (LEAF) Research Center, Instituto Superior de Agronomia, University of Lisbon, Tapada da Ajuda, 1349-017 Lisbon, Portugal

ARTICLE INFO

Keywords:
Co-culture
Sour rot
Oxidative fermentation
Temporal succession
Gluconic acid
Acetic acid

ABSTRACT

The present study was designed to evaluate possible sugar-based trophic interactions between acetic acid bacteria (AAB) and non-*Saccharomyces* yeasts (NSY) involved in table grape sour rot, a disease in which berries spoilage is caused by the accumulation of several microbial metabolites. *Acetobacter syzygii* LMG 21419 (*As*) and *Candida zemplinina* CBS 9494 (*Cz*), a simplified AAB-NSY association responsible for table grape sour rot, grew differently in a minimal medium (YP) supplemented with glucose, ethanol, acetic and gluconic acid under monoculture conditions. In *As* -*Cz* co-culture media, after 24 h of incubation, *As* showed high relative abundance in YP-ethanol, whereas *Cz* was the dominant strain in YP-glucose medium. Co-culture in YP-glucose showed that glucose was converted into ethanol by *Cz* that, in turn, promoted the growth of *As* population. Gluconic acid was the main bacterial metabolite from glucose in monoculture, whereas acetic acid putatively derived from ethanol oxidation was found only in co-culture. However, gluconic acid showed inhibitory effect against *As* whereas acetic acid mainly inhibited *Cz*. Negative effects of both metabolites were mitigated in the glucose-supplemented medium.

The results suggest a possible metabolic- based temporal succession between AAB and NSY during grape sour rot development. At the begin of sour rot, low glucose concentration promotes NSY producing ethanol, then, the AAB could take advantage from the oxidation of ethanol into acetic acid, becoming the dominant microbial sour rot population during the late stages of the process.

1. Introduction

Acetic acid bacteria (AAB) and non-*Saccharomyces* yeast (NSY) species, alone or in association, are responsible for grape (Barata et al., 2012b; Pinto et al., 2017), soft drinks (Kregiel et al., 2018) and cider (Carr and Whiting, 1971) spoilage.

It is well known that the rate of acidification in stuck fermentations during wine production depends on species belonging to the yeast-bacterium association (Alexandre and Charpentier, 1998). Previously, the growth of AAB strains in grape juice was positively affected by co-culture with yeasts leading to an incomplete fermentation with high residual concentration of sugar, and the production of low amount of ethanol and high amounts of both acetic and gluconic acid (Drysdale and Fleet, 1989). Rotten fruits represent another example of food spoilage caused by yeast-bacterium association based on competition for simple sugars (González et al., 2018). However, cross-feeding interactions during food spoilage have been only scarcely investigated in comparison with those occurring in food fermentations (Moens et al., 2014; Nguyen et al., 2015). To this aim, sour rot, a disease of wine and table grapes, well explains this scenario. Microbial spoilage activity on grapes is attributed to a NSY-AAB consortium, composed by different microbial species, (Barata et al., 2012b; Pinto et al., 2017) converting sugars into alcohols, aldehydes, esters and organic acids (Marchetti et al., 1984; Zoecklein et al., 2001). Among these metabolites, acetic and gluconic acids are usually considered chemical markers of sour rot development (Barata et al., 2012b). In our previous work, we demonstrated that ethanol was the first detected metabolite, followed by acetic acid and finally by gluconic acid in the late stages of table grape sour rot (Pinto et al., 2017). These results could support the assumption of a temporal succession between NSY and AAB, reported during sour rot development in wine red grapes (Barata et al., 2012a, 2012b), but not sustained by microbial metabolites quantification.

Abbreviations: As, *Acetobacter syzygii* LMG 21419; *Cz*, *Candida zemplinina* CBS 9494

* Corresponding author.
E-mail address: loris.pinto@ispa.cnr.it (L. Pinto).

https://doi.org/10.1016/j.ijfoodmicro.2019.02.022
Received 4 October 2018; Received in revised form 23 January 2019; Accepted 28 February 2019
Available online 01 March 2019
0168-1605/

The distinctive metabolic profile of *Saccharomyces-Acetobacter* co-culture was able to drive olfactory and egg-laying behaviours of *Drosophila* flies (Fischer et al., 2017), in turn responsible for dissemination of sour rot related microorganisms among berries (Barata et al., 2012b).

Once berry wounds are colonized by microorganisms, microbial growth is sustained by nutrients occurring in grape berries (Shiraishi et al., 2010). By this way, the NSY-AAB consortium is the player of its own persistence and spreading in the vineyard showing the existence of a complex ecological metabolite-based interaction with wild insects.

To the best of our knowledge no study reported how the availability of specific carbon sources or the accumulation of certain microbial metabolites could delay, or eventually promote, the growth of some of the microorganisms belonging to the microbial consortium. Therefore, in this work, a simplified microbial consortium responsible for table grape sour rot was employed, under laboratory conditions, to draw microbial kinetics in minimal medium supplemented with different carbon sources; the release of microbial metabolites was also investigated to understand the trophic relationships between NSY and AAB populations involved in grape sour rot.

2. Materials and methods

The modular approach followed to define potential trophic relationships between strains belonging to the simplified AAB-NSY association is depicted in Fig. 1.

2.1. Yeasts and bacteria culture conditions

Acetobacter syzygii LMG 21419 (*As*) and *Candida zemplinina* CBS 9494 (syn. *Starmerella bacillaris*, *Cz*) were previously selected as the members of a simplified microbial model of table grape sour rot (Pinto et al., 2017). Both strains were stored in the ITEM (Agri-Food Toxigenic Fungi Culture Collection, Bari, Italy) microbial collection of the Institute of Sciences of Food Production (Bari, Italy; http://server.ispa.cnr.it/ITEM/Collection/) under the numbers 17,387 and 17,538, respectively.

Each strain was cultivated inoculating a loopful of fresh yeast or bacterial cultures (24–48 h) in 50 mL of YPD (yeast extract 5 g/L, peptone 5 g/L, glucose 20 g/L) or GY (glucose 50 g/L, yeast extract 10 g/L), respectively. All cultures were incubated at 25 °C with mild shaking (120 rpm) for 48 h.

Inocula for subsequent fermentation assays were prepared as follows: microbial pellets were harvested by centrifugation (13,000 rpm for 5 min), and washed twice in sterile saline solution (0.9% *w/v* NaCl). The bacterial inoculum was prepared from a cell suspension with an optical density at 600 nm (OD_{600}) of 0.3 ± 0.05 (corresponding to 7–8 log cfu/mL), whereas yeast cell density was set at about 1×10^7 cfu/mL by using a Thoma counting chamber (HGB Henneberg-Sander GmbH, Lutzellinden, Germany).

2.2. Mono- and co-culture growth kinetics on single carbon sources

Single carbon sources were added to a minimal medium composed of soluble extract of autolysed yeast cells and from enzymatic digestion of selected fresh beef meat (YP: Yeast extract and Peptone bacteriological, respectively, Biolife Italiana Srl, Milan, Italy). In order to define the optimal formulation of YP able to sustain the growth of both target strains, yeast extract and peptone were combined at different concentrations ranging from 0.125% *w/v* to 0.5% w/v and from 0.25% w/v to 1% w/v, respectively. Final concentrations of both ingredients were defined monitoring the growth of microbial strains spectrophotometrically at 600 nm.

Once YP formulation was optimized, single carbon sources were added. Among different carbon source involved in grape sour rot, acetic acid is most responsible in the decrease of pH values, and, for microbial growth inhibition. The acetic acid concentration lower than minimal inhibitory concentration (within the 0.1–1% *v*/v range), was set spectrophotometrically for both strains. Then, the concentration of each carbon source was defined according to the selected acetic acid concentration. Thus, YP was supplemented with glucose (YPG), ethanol (YPE), acetic acid (YPA) and gluconic acid (YPGA, supplemented as sodium gluconate) at equimolar concentrations, and employed for growth kinetics experiments. Except for YPA, showing a pH value of 4.9, remaining supplemented YPs registered pH values of 7.3, on average.

In the case of mono- and co-culture growth kinetics, a volume of 1.5 mL of each cell suspension, was transferred to 500 mL cotton-plugged Erlenmeyer flasks filled with 150 mL of YP, YPG, YPE, YPA or YPGA, to obtain an initial cell density of ca. 5 log cfu/mL (5.0 ± 0.3 log cfu/mL) for each strain.

All fermentations were carried at 25 °C for 144 h with mild shaking (120 rpm) under aerobic conditions.

Growth kinetic of co-culture was additionally monitored in YP supplemented with 110 mM of glucose up to 96 h at 25 °C under aerobic conditions. YP inoculated with microbial strains and non-supplemented with glucose was also used. Initial cell density for each strain was set up at ca. 4 log cfu/mL (3.9 ± 0.1 log cfu/mL).

Enumeration of viable and culturable cells of both microorganisms for these and for the following trials was carried out as follows: *A. syzygii* LMG 21419 was enumerated on glucose yeast extract calcium carbonate agar (GYC, glucose 5 g/L, yeast extract 1 g/L, calcium carbonate 3 g/L, agar 20 g/L, pH 7) after incubation at 30 °C for 5 days, whereas *C. zemplinina* CBS 9494 was counted after incubation at 30 °C for 48 h on yeast extract peptone dextrose agar (YPD, yeast extract 5 g/L, peptone 5 g/L, glucose 20 g/L, agar 20 g/L).

In order to count microorganisms during co-culture experiments, YPD medium was supplemented with chloramphenicol (100 mg/L; Biolife Italiana Srl, Milan, Italy), whereas GYC medium with Delvocid® (200 mg/L; DSM, Delft, Netherlands containing 100 mg/L natamycin). Microbial load was expressed as log cfu/mL ($N = 3$, three repetitions).

All chemicals were obtained from VWR International (Darmstadt, Germany).

2.3. Evaluation of antimicrobial effect of microbial metabolites on monoculture growth kinetics

On the basis of the results obtained during growth kinetics of monocultures, the antimicrobial effect of some single carbon sources against *As* and *Cz* was checked spectrophotometrically. Briefly, YPG (110 mM of glucose) supplemented with 0, 55, 110, 250, 350, 450 and 550 mM of sodium gluconate was inoculated with 4 log cfu/mL of *A. syzygii* LMG 21419 or *C. zemplinina* CBS 9494. Microbial growth was monitored by measuring OD_{600} every 10 min with the Varioskan Flash spectrofluorimeter (ThermoFischer Scientific, Waltham, MA, USA) at a wavelength of 600 nm up to 96 h at 30 °C. Positive control was represented by inoculated YPG not supplemented with sodium gluconate whereas negative control was non-inoculated YPG. Each assay was performed in triplicate.

The antimicrobial effect of acetic acid (36.6 and 55 mM) was also checked against target strains in YPG (110 mM of glucose). The antimicrobial activity was determined by calculating the Inhibition Index turbidity ratio (II_{TR}) and, at the end of incubation, the Inhibition Index (II) using plate counting as described by Baruzzi et al. (2015).

2.4. Mono and co-culture growth kinetics with multiple carbon sources

A. syzygii LMG 21419 and *C. zemplinina* CBS 9494 were mono- and co-cultured in YP medium supplemented with both glucose (YPG, 110 mM) and sodium gluconate (YPGA, 55 mM). Viable and culturable cells of both strains (see Section 2.2), as well as chemical quantification of released metabolites (see Section 2.5), were monitored during

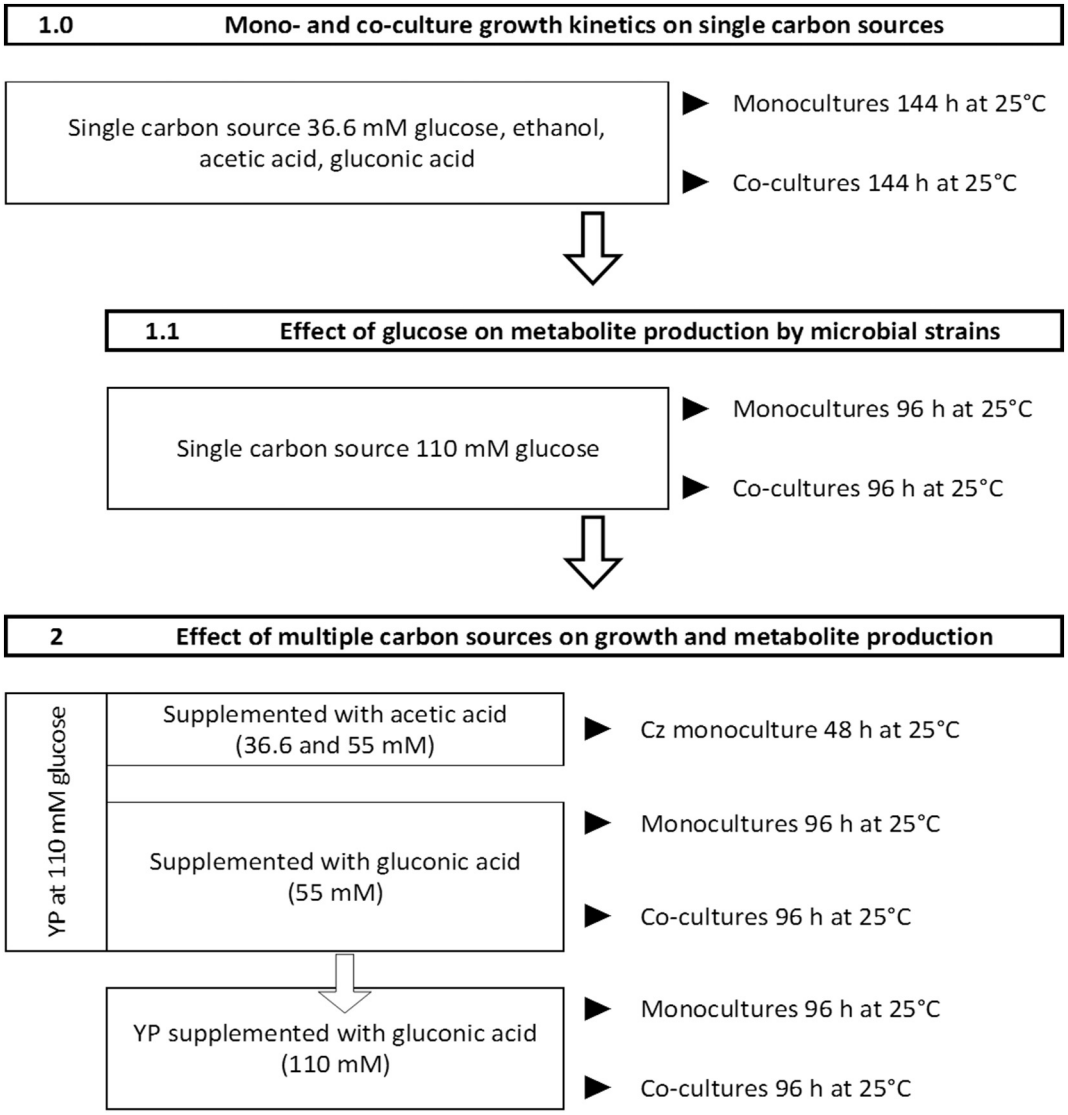

Fig. 1. Overview of the modular design of experiments carried out in this work.

incubation at 25 °C for 96 h under aerobic conditions. *As* and *Cz* were additionally co-cultured in YP supplemented with sodium gluconate (110 mM) as a single carbon source following the same incubation conditions described above. Initial cell density for each strain was set up at ca. 4 log cfu/mL (3.9 ± 0.1 log cfu/mL).

The measurement of pH and Oxidation Reduction Potential (ORP) values in different media was also determined by a pH/ORP meter (Model pH 50 Lab pH Meter XS-Instrument, Concordia, Italy).

2.5. HPLC determinations

The concentration of glucose, organic acids and alcohols in mono and co-culture condition was determined following the chromatographic method described by Pinto et al. (2017). Analytical columns used for the analyses were Rezex RCM monosaccharide Ca^{+2} for sugars and Rezex ROA H^+ 8% (Phenomenex, Torrance, CA) for organic acids and alcohols. Broth aliquots at each sampling time (at 0, 24, 36, 48, 72 and 96 h) were centrifuged (14,000 rpm, 10 min at 4 °C) and the supernatant was diluted in H_2SO_4 0.005 M or MQ water, centrifuged again and filtered through a 0.45 µm cellulose ester filter. Glucose, organic acids and alcohols were quantified using the external standard method. Standard curves were prepared in minimal medium and fell in the range of concentration 0.0625–1% *v*/v for acetic acid, ethanol and glycerol, and 0.0078–0.5% *w/v* for gluconic acid. Standard curve for glucose fell in the range of concentration 0.03125–1% w/v. Each sample was analysed in triplicate and the concentrations were expressed as mmol/L.

2.6. Statistical analysis

The univariate General Linear Model (GLM) procedure, applying one- or two-way ANOVA ($P \leq 0.05$), performed on SPSS software (SPSS, Inc., IBM Corp., Chicago, IL, USA), was used to examine the effect of the incubation period and type of carbon source on viable cell counts of *C. zemplinina* CBS 9494 and *A. syzygii* LMG 21419 during incubation and to estimate the effect of time and sample on sugar, alcohols and organic acids concentration. Multiple comparisons among individual means for each sample were made by Fisher's least significant difference (LSD) multiple range test at the 95% confidence interval.

3. Results and discussions

3.1. Viability of strains on single carbon sources

The rationale of trials reported in this section (Fig. 1, step 1) was to

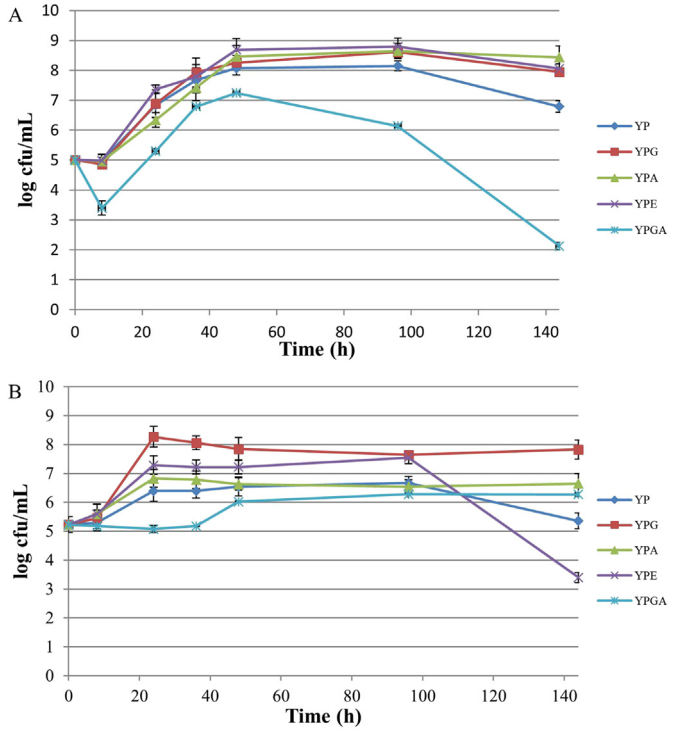

Fig. 2. Microbial growth kinetic of monocultures of *A. syzygii* LMG 21419 (panel A) and *C. zemplinina* CBS 9494 (panel B) during 144 h of aerobic incubation in YP medium supplemented with glucose (YPG), ethanol (YPE), acetic acid (YPA) or gluconic acid (YPGA) at equimolar concentrations (36.6 mM). Two way- ANOVA was applied to estimate the effect of time and type of carbon source on *As* and *Cz* viable cell counts; the least significant difference comparison values (LSD, 95% confidence interval, expressed as log cfu/mL) were calculated among samples for each strain. *As*: 0.58 (time), 0.46 (sample); *Cz*: 0.58 (time), 0.47 (sample). Mean values were separated using lowercase and capital letters as shown in Table 1S of Supplementary Materials.

understand if, under co-culture conditions, the occurrence of a single carbon source, supplemented at low but not limiting concentration, stimulated differently, or not, the growth of *As* and *Cz* during 144 h of incubation.

The minimal medium allowing the growth of both strains, defined after spectrophotometric readings (data not shown), was composed by 0.25% *w/v* yeast extract and 0.25% w/v peptone. The highest acetic acid concentration that did not inhibit the growth of *A. syzygii* LMG 21419 (*As*) and *C. zemplinina* CBS 9494 (*Cz*) strains was 0.2% *v*/v (36.6 mM). Therefore, glucose (0.66% *w/v*), ethanol (0.17% *v*/v) and gluconic acid (0,72% w/v, supplemented as sodium gluconate) concentrations in YP were defined at 36.6 mM.

Results of viable cell counts of *As* and *Cz* monocultures on single carbon source during 144 h at 25 °C are shown in Fig. 2. Two-way ANOVA analysis showed that the effect of incubation time, type of carbon source and their interaction affected significantly ($P \leq 0.05$) viable cell count of both microorganisms.

Acetobacter syzygii LMG 21419 is able to growth on glucose, ethanol and acetate as single carbon source producing gluconic acid, acetic acid and CO_2 and water, respectively (Lisdiyanti et al., 2001). As regards the use of gluconic acid as carbon source, *As* did not produce 2-keto-D-gluconate, 5-keto-D-gluconate and 2,5-diketo-D-gluconate (Lisdiyanti et al., 2001). It is possible to guess the use of gluconate for the production of ribulose-5-phosphate in the pentose phosphate pathway (Velasco-Bedrán and López-Isunza, 2007).

All carbon sources promoted the growth of *As*, showing values equal or significantly higher than YP medium, except for gluconic acid (YPGA) (Fig. 2A). The highest cell count was recorded after 96 h on YPE (8.8 log cfu/mL); this value differed significantly ($P \leq 0.05$) from cell loads detected in YP but not from that found on YPA and YPG. A growth delay was observed in YPGA in comparison to YP medium during the first 24 h of incubation (Fig. 2A). In addition, after 48 h, *As* viable cell counts showed a constant decrease by reaching the value of ca. 2 log cfu/mL at the end of the experiment (Fig. 2A). Growth kinetics of *As* monoculture showed that ethanol, followed by acetic acid and glucose significantly promoted the growth of this strain; on the contrary, gluconic acid always determined lower cell densities than those detected in minimal medium.

Growth and metabolic behaviour of *C. zemplinina* strains on selected carbon sources was well characterized. These strains produced ethanol, glycerol and low amounts of acetic acid and acetaldehyde from glucose as carbon source. Different strains were also able to growth on ethanol medium up to 14% *v*/v (Englezos et al., 2015). For these yeasts, information about the growth and metabolite production on acetic and gluconic acid are limited.

C. zemplinina CBS 9494 grew on all carbon sources (Fig. 2B); this strain reached the highest cell load (8.3 log cfu/mL) in YPG after 24 h of incubation. The growth on YPA did not differ significantly in comparison to YP, whereas a significant increase in *Cz* cell load was recorded on YPE during 96 h of incubation (Fig. 2B). As observed in the monoculture growth curves of *As* in YPGA, a growth delay was also observed for *Cz* in the same medium during the first 36 h of incubation. However, in this case, viable cell count values remained stable until the end of incubation without recording any further reduction. At the end of incubation the pH values of YPGA for both *As* and *Cz* was close to 7.30, suggesting that sodium gluconate, supplemented to YP was present under its dissociated gluconate form (pKa 3.86). At the end of incubation, *Cz* viability decreased significantly ($P \leq 0.05$) in YPE, reaching a final cell load of 3.4 log cfu/mL (Fig. 2B). Overall, data of *Cz* monocultures showed that the carbon sources that significantly promoted the growth of this strain were glucose followed by ethanol; acetic acid did not affect growth in comparison to minimal medium, whereas gluconic acid had an inhibitory effect during early stages of incubation.

Viable cell counts of both *As* and *Cz* during co-culture fermentation were significantly affected by the incubation time, the carbon sources as well as their interaction ($P \leq 0.05$). Viable loads of *As* and *Cz* co-cultivated in YP medium did not show significant differences with values found for monoculture conditions. The body of results suggested that the growth of both strains, and in particular of *As*, was largely sustained by yeast extract.

On the contrary, the addition of a carbon source to YP affected *As* and *Cz* growth curves as shown in Fig. 1S.

The addition of glucose during *Cz* co-culture, did not affect the growth of the yeast in comparison to viable loads enumerated for monoculture in YPG; in fact, *Cz* reached the highest cell yield (7.9 ± 0.5 log cfu/mL) after 36 h at 25 °C (Fig. 1S), a value close to that found after the same incubation period in monoculture. The *Cz* viable cell count remained stable up to the end of the incubation period at values above 7 log cfu/mL, whereas *As* viability was negatively affected by YPG co-culture conditions; in fact, it reached the stationary phase at 48 h of incubation (6.5 ± 0.1 log cfu/mL) and cell densities ranged from 6.5 to 6.7 log cfu/mL up to the end of incubation (Fig. 1S). Viable cell counts detected were quite lower than that found under monoculture conditions (Fig. 2A).

In YP supplemented with ethanol (YPE), *A. syzygii* LMG 21419 reached the highest cell yield (8.7 ± 0.4 log cfu/mL) after 48 h of incubation, showing a decrease in cell viability to 7.3 ± 0.1 log cfu/mL at 144 h. In YP supplemented with acetic acid (YPA) *As* reached the stationary phase at 48 h when a viable cell load of 8.5 ± 0.3 log cfu/mL was detected; then, the viable cell counts values did not differ significantly up to the end of incubation (Fig. 1S).

In these media *Cz* reached the stationary phase (mean values of viable cell counts of 7.0 log cfu/mL in YPE and 7.2 log cfu/mL in YPA) at 24 h; then, cell densities values on both media did not change

significantly up to 96 h. Afterwards, at 144 h of incubation in both media, *Cz* viable cell count dropped to on average value of 2.7 log cfu/mL (Fig. 1S).

It is possible to conclude that the co-culture growth kinetic of *As* and *Cz* in YP supplemented with ethanol or acetic acid did not differ significantly ($P \leq 0.05$) in comparison with those recorded under monoculture conditions.

Growth kinetic of *Cz* in YPGA co-culture media showed that the yeast did not increase initial cell load throughout the incubation, showing mean values of viable cell counts in the range 4.2–5.6 log cfu/mL. *As* population in the same media showed an initial viability decrease at 8 h of incubation (mean viable cell count of 3.8 ± 0.1 log cfu/mL); then, this strain grew reaching the highest cell count value at 48 h (7.2 ± 0.1 log cfu/mL). Afterwards, *As* viable cell count dropped to on average value of 2.2 ± 0.2 log cfu/mL at the end of incubation (Fig. 1S).

Gluconic acid showed the same negative effects for both strains already found under monoculture conditions.

Based on viable cell count values recorded, it was possible to found a different balance between *As* and *Cz* populations depending on the carbon source supplied (Fig. 3).

In YP supplemented with glucose, *Cz* was the dominant strain at each sampling time; indeed, after 36 h *Cz* reached the maximum viable concentration of 7.9 log cfu/mL (99.5% of the total microbial population of 8.6×10^7 cfu/mL). On the contrary, *As* reached 6.7 log cfu/mL (6.1% of total microbial population of 8.9×10^7 cfu/mL) only after 96 h of incubation.

This result supports the hypothesis that at the beginning of sour rot, simple sugars released by wounded berries are quickly metabolized by non-*Saccharomyces* yeasts rather than AAB.

On the contrary, in YP-ethanol *As* represented > 90% of the total microbial population from 24 to 144 h of incubation (mean value of the total microbial population of 2.8×10^8 cfu/mL) with an average concentration of 8.4 ± 0.6 log cfu/mL. In fact, it is well known that *Acetobacter* species prefer ethanol as carbon source in comparison to glucose (De Ley et al., 1984); this alcohol also stimulated dehydrogenase activity in cells under stationary phase of growth (Shafiei et al., 2017).

The occurrence of acetic acid delayed the growth of both strains in comparison with values recorded in YPG and YPE. However, *As* grew sharply from 24 h to the end of incubation by reaching the maximum value of 8.7 ± 0.1 log cfu/mL at 96 h (98% of the total microbial population of 4.6×10^8 cfu/mL), whereas *Cz* remained stable at ca.7 log cfu/mL from 24 to 96 h, and dropped down at 2.7 ± 0.3 log cfu/mL at 144 h. Relative abundance of *Cz* population in YPA decreased from 74.7% at 24 h (total population of 2.0×10^7 cfu/mL) to 2.4% at 96 h (total population of 4.6×10^8 cfu/mL) whereas *As* population increased from 25.3% at 24 h to 97.6% at 96 h (Fig. 3).

It can be argued that ethanol produced during early phases of sour rot (Pinto et al., 2017) could be produced by yeasts, and then it could be oxidized by AAB to acetic acid. In addition, the oxidation of ethanol to acetic acid, typical of *Acetobacter* species, could sustain *As* growth by the oxidation of acetic acid to CO_2 and water through TCA cycle (Mamlouk and Gullo, 2013).

In monoculture experiments, gluconic acid had an inhibitory effect on the growth of both strains; in fact, at each sampling time, viable cell densities of both strains were lower than those found in YP, by 1 to 2 log cfu/mL. During co-culture growth kinetic, *As* was able to grow slowly by reaching its maximum value of ca. 7 log cfu/mL at 48 h of incubation (97.9% of the total microbial population of 2×10^7 cfu/mL), whereas *Cz* did not grow throughout the incubation period remaining at the initial cell load. However, at 144 h, when microbial population was represented for > 99% (total microbial population of 3.6×10^5 cfu/mL) by *Cz* viable cells (Fig. 3), *As* decreased its viable cell count < 3 log cfu/mL.

3.2. Effect of glucose on metabolite production by microbial strains

At the end of incubation, the analysis of YPG for both monoculture and co-culture trials (Section 3.1), showed the absence of detectable glucose (data not shown). Thus, a new growth kinetic of *As*, *Cz* and their co-culture was drawn in YP increasing glucose concentration to 110 mM and reducing incubation period to 96 h, as outlined in the step 2 of Fig. 1. In order to avoid growth limiting conditions, glucose concentration was increased to 110 mM that is the concentration usually found in several microbiological media amended with glucose 20 g/L.

The ORP values registered during mono- and co-culture fermentations were found to be always higher than 200 mV, characteristic of aerobic conditions.

As concerns viable cell loads (Table 1), monoculture of *As* in YPG reached its highest concentration of 8.4 ± 0.3 log cfu/mL within 48 h, remaining in the stationary phase until the end of incubation. No significant differences were found in viable cell concentration of *As* when it was co-cultured together with *C. zemplinina* CBS 9494 in YPG.

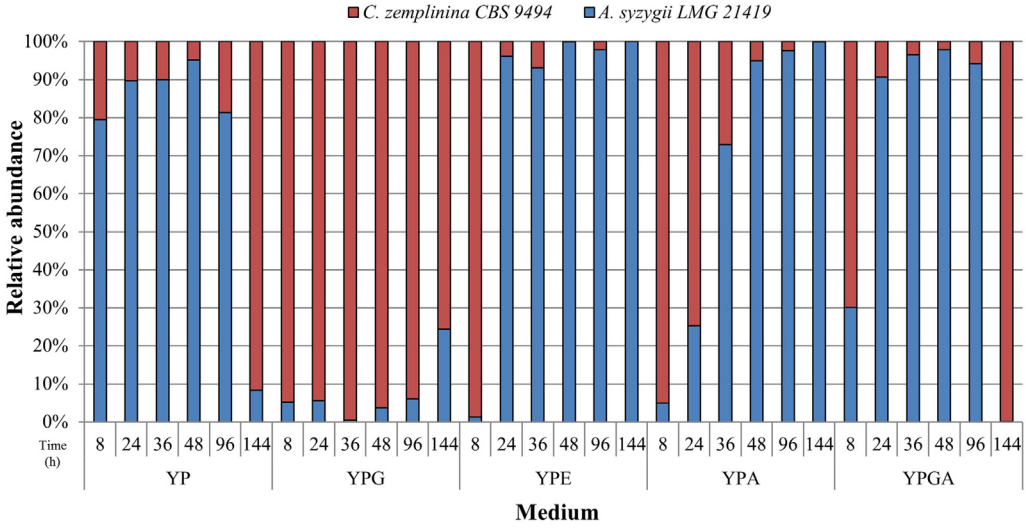

Fig. 3. Relative abundance (% of viable cell count calculated on the basis of cfu/mL values) of *A. syzygii* LMG 21419 (blue bars) and *C. zemplinina* CBS 9494 (red bars) co-culture during 144 h of aerobic incubation in YP medium supplemented with glucose (YPG), ethanol (YPE), acetic acid (YPA) or gluconic acid (YPGA) at equimolar concentrations (36.6 mM). Two way- ANOVA was applied to estimate the effect of time and type of carbon source on *As* and *Cz* relative abundance; the least significant difference comparison values (LSD, 95% confidence interval, expressed as %) were calculated among samples. Time: 28.1; sample: 23.4. Mean values were separated using lowercase and capital letters as shown in Table 2S of Supplementary Materials. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1
Viable cell load (in log cfu/mL) of *A. syzygii* LMG 21419 and *C. zemplinina* CBS 9494 during 96 h of aerobic incubation in YP medium supplemented with glucose (YPG) at 110 mM under mono (M) and co-culture (C) conditions.

Time (h)	*A. syzygii* LMG 21419		*C. zemplinina* CBS 9494	
	M	C	M	C
0	3.90 ± 0.17^{A}	3.76 ± 0.14^{A}	3.91 ± 0.17^{A}	3.90 ± 0.13^{A}
8	4.11 ± 0.16^{A}	4.11 ± 0.10^{A}	4.13 ± 0.22^{A}	3.91 ± 0.35^{A}
24	5.69 ± 0.24^{B}	6.57 ± 0.33^{B}	7.03 ± 0.31^{B}	7.54 ± 0.20^{B}
36	6.88 ± 0.21^{C}	7.51 ± 0.23^{C}	8.39 ± 0.34^{C}	7.23 ± 0.23^{B}
48	8.42 ± 0.25^{D}	8.11 ± 0.17^{C}	7.89 ± 0.12^{B}	7.13 ± 0.19^{B}
72	8.41 ± 0.19^{D}	8.19 ± 0.14^{C}	7.39 ± 0.31^{B}	6.40 ± 0.22^{B}
96	8.10 ± 0.23^{D}	8.16 ± 0.29^{C}	7.21 ± 0.21^{B}	3.02 ± 0.08^{C}

One way- ANOVA was applied to estimate the effect of time on *As* and *Cz* viable cell counts; the least significant difference comparison values (LSD, 95% confidence interval, expressed as log cfu/mL) were calculated among samples. *As*: 0.74 (monoculture), 0.74 (co-culture); *Cz*: 0.88 (monoculture), 0.77 (co-culture). Detection limit: 1 log cfu/mL. Mean values with different superscript letters differ significantly ($P \leq 0.05$).

As regards *Cz* monoculture, glucose promoted its growth, reaching the highest cell load (8.4 ± 0.3 log cfu/mL) after 36 h, followed by a slight decrease (ca. one order of magnitude) up to the end of incubation. On the contrary, when *Cz* was co-cultured with *As*, viable cell loads resulted lower than those recorded under monoculture conditions. In particular, a difference of > 3 orders of magnitude was registered in the late stationary phase.

The pH values, in both monocultures, dropped down from 6.9 at 36 h to 4.7–5.2 at 48 h.

The increase of glucose concentration (to 110 mM) induced changes in the relative abundance of these two strains in comparison to that previously described (Fig. 3). In particular, *As* represented about the 50% of total microbial population at 36 h of incubation, resulting the dominant strain starting from 48 h until the end of incubation.

The concentration of glucose added to YP medium and those of microbial metabolites produced during fermentation are reported in Table 2. At 72 h, the reduction in glucose concentration was evident for *As* monoculture in concomitance with gluconic acid accumulation; however, glucose was still detected at 67 mM in *As* monoculture at the end of incubation (96 h). The concentration of gluconic acid produced by *As* from glucose (YPG medium) at the end of incubation (ca. 30 mM) is in accordance with that detected by Sainz et al. (2016) considering several *Acetobacter* species.

Cz was more efficient in glucose consumption; in fact, glucose concentration was reduced already after 36 h, and becoming not detectable after 72 h. This result supports the ability of *C. zemplinina* strains to deploy metabolisms for high rate of glucose consumption in environments characterized by high- (Englezos et al., 2015) as well as low-sugar concentration (Rantsiou et al., 2017). The reduction in glucose concentration was accompanied by the production of ethanol, resulting the main product from glucose metabolism while gluconic acid was not detected.

The ability of *C. zemplinina* CBS 9494 to convert glucose into ethanol under aerobic conditions (Crabtree positive effect) could be considered a result of the colonization of the same ecological niche by NSY and AAB. In fact, it was demonstrated that *Acetobacteriaceae* is the likely donor of alcohol dehydrogenase gene (Adh1) to *Wickerhamiella/Starmerella* yeast clade (Gonçalves et al., 2018) whose deletion resulted in a five-fold decrease in ethanol production from sugar during aerated growth. However, the sour rot microbiota is composed by different microorganisms including filamentous fungi, NSY and AAB species (Loureiro et al., 2012). In addition, different sour rot related microorganisms were identified in rotten berries of red (Barata et al., 2012a; Mateo et al., 2014) and white (Lleixà et al., 2018; Mateo et al., 2014) wine grape varieties as well as in rotten white table grape berries (Pinto et al., 2017).

Under co-culture conditions, ethanol was released already after 24 h by reaching a concentration higher than that found in *Cz* monoculture; glucose depletion was achieved starting from 72 h while gluconic acid was not found until the end of incubation. Starting from 36 h acetic acid was found only in *As-Cz* co-cultures reaching ca. 45 mM at the end of incubation; pH decreased from 4.3 at 24 h to 3.3 at 96 h of incubation. In *Cz* monoculture, ethanol accumulated whereas in *As-Cz* co-culture ethanol concentration was reduced after 48 h of incubation, in concomitance with the increase of acetic acid concentration. Under co-culture condition, glucose was virtually consumed at 48 h, finding an incomplete ethanol oxidation to acetic acid at 96 h. The low concentration of acetic acid produced from ethanol could be explained by other metabolic pathways performed by *Cz*. Acetate and ethanol can be converted, via acetaldehyde (García et al., 2016), into Acetyl-CoA that is involved in the synthesis of fatty acids. Both Acetyl-CoA and fatty acids are precursors of volatile compounds such as acetate esters and ethyl fatty acid esters (Englezos et al., 2018).

Table 2
Mean concentrations (mM) of glucose, organic acids and alcohols in YPG (110 mM) inoculated with *A. syzygii* LMG 21419 (*As*), *C. zemplinina* CBS 9494 (*Cz*), or both microorganisms (As-Cz) during 96 h at 25 °C under aerobic conditions.

Time (h)	Sample	Glucose (mM)	Gluconic acid (mM)	Glycerol (mM)	Ethanol (mM)	Acetic acid (mM)
0	–	93.8 ± 12.2^{E}				
24	As	89.1 ± 8.3^{Eb}	nd	nd	nd	nd
	Cz	110.4 ± 6.6^{Fc}	nd	nd	nd	nd
	As-Cz	60.7 ± 14.6^{Da}	nd	nd	13.7 ± 1.7^{A}	nd
36	As	91.7 ± 8.3^{Ec}	nd	nd	nd	nd
	Cz	63.3 ± 7.2^{Db}	nd	nd	45.6 ± 3.4^{Ba}	nd
	As-Cz	24.7 ± 0.4^{Ca}	nd	2.9 ± 0.1^{A}	63.8 ± 2.6^{Cb}	3.9 ± 0.1^{A}
48	As	91.5 ± 8.8^{Ec}	13.8 ± 2.0^{A}	nd	nd	nd
	Cz	16.4 ± 1.6^{Bb}	nd	12.0 ± 0.9^{Cb}	95.9 ± 10.2^{Ea}	nd
	As-Cz	0.9 ± 0.1^{Aa}	nd	3.1 ± 0.1^{Ba}	125.2 ± 4.2^{Gb}	16.2 ± 1.1^{B}
72	As	72.9 ± 9.4^{D}	33.2 ± 4.5^{B}	nd	nd	nd
	Cz	nd	nd	10.9 ± 0.9^{Cb}	79.3 ± 7.7^{Db}	nd
	As-Cz	nd	nd	2.7 ± 0.3^{Aa}	17.9 ± 4.3^{Aa}	29.4 ± 3.2^{C}
96	As	67.0 ± 5.3^{D}	27.3 ± 4.3^{B}	nd	nd	nd
	Cz	nd	nd	14.7 ± 1.1^{Db}	106.9 ± 8.9^{Fb}	nd
	As-Cz	nd	nd	2.1 ± 0.2^{Aa}	18.6 ± 1.6^{Aa}	44.9 ± 5.2^{D}

Nd: not detected. One or Two way- ANOVA was applied to estimate the effect of time and sample on metabolite concentration; the least significant difference comparison values (LSD, 95% confidence interval, expressed as mM) were calculated among samples for each metabolite. Glucose: 14.9 (time), 10.5 (sample); Ethanol: 8.6 (time), 6.7 (sample); Glycerol: 0.9 (time); 0.7 (sample); Gluconic acid: 6.2 (time); Acetic acid: 4.1 (time). Means with different superscript letters differ significantly ($P \leq 0.05$); capital letters separated mean values based on incubation time, whereas lowercase letters separated mean values based on sample.

It is possible to guess that acetic acid was the result of the bacterial oxidation of ethanol, highlighting the ability of *As* to growth in YP supplemented with ethanol, as reported in Fig. 2. After 48 h of incubation, *As-Cz* co-cultures were also characterized for low concentrations of propionic acid (2.5–3 mM). This organic acid could be produced by AAB as a sugar degradation product (Barata et al., 2011) and it was demonstrated to support the growth of *D. melanogaster* larvae (Depetris-Chauvin et al., 2017). As recently reported by Matsushita and Matsutani (2016), the strategy of sugar utilization played by acetic acid bacteria colonizing sugar-rich niches, and usually defined as "oxidative fermentation", is based on the oxidation of sugars and alcohols, their released into natural environment and their further utilization.

Under co-culture conditions in YPG, *As* did not produce gluconic acid, probably because glucose metabolism performed by *Cz* was faster than "oxidative fermentation" of glucose carried out by the bacterium; it seems reasonable to suppose that *As* changed its metabolic behaviour from "oxidative fermentation" of glucose in monoculture to ethanol oxidation when co-cultured with *Cz*. In fact, acetic acid, produced by *As* from ethanol, started to accumulate after 36 h of co-culture incubation.

The growth of sour rot related microorganisms in rotten berries is promoted by multiple carbon sources.

In some AAB species, glucose is used to produce exopolysaccharides (EPS) such as acetan and cellulose (Gullo et al., 2016; Velasco-Bedrán and López-Isunza, 2007) and gluconate that can accumulate in the medium or feed the pentose phosphate pathway (Velasco-Bedrán and López-Isunza, 2007). *Acetobacter syzygii* LMG 21419 is able to produce cellulose in the biofilm matrix (Valera et al., 2015) that could increase the severity of sour rot. However, the accumulation of gluconic acid can inhibit the production of cellulose, as found in *A. xylinum* culture media (Cheng et al., 2002). The cellulose production is under a quorum sensing (QS) regulation in *A. syzygii* LMG 21419 (Valera et al., 2016); therefore, further studies on this strain should be addressed to understand the role of QS on oxidative fermentation and cellulose production during grape berry colonization.

As concerns the ethanol catabolism, this carbon source can be used by AAB species to produce acetic acid and microbial biomass. In addition, ethanol, through the conversion in Acetyl-CoA, feeds the glyoxilate shunt of the TCA cycle (Velasco-Bedrán and López-Isunza, 2007).

Our data showed that in YPG co-culture media, the first metabolite produced from *As-Cz* was ethanol at 24 h, presumably from *Cz*; acetic acid was detected at 36 h of incubation in presence of both ethanol and residual glucose. Although in *A. pasteurianus*, the release of both gluconic and acetic acid from multiple carbon sources (ethanol, acetic acid, and glucose) was reported (Mounir et al., 2016), this simultaneous production was not detected in *As* co-cultured with *Cz*.

After 96 h of incubation the viable cell load of *Cz* under co-culture conditions was reduced > 3 orders of magnitude (Table 1) while acetic acid concentration increased to 44.9 mM (Table 2). In order to verify a possible adverse effect of high acetic acid concentration on *Cz* viability, growth kinetics of *Cz* monoculture in YPG (110 mM glucose) supplemented with acetic acid at 36.6 and 55 mM (YPG_AA) were monitored spectrophotometrically for 48 h at 25 °C (Fig. 2S showed in Supplementary Materials, corresponding to the step 3 of Fig. 1).

Cz did not grow in YP exclusively supplemented with acetic acid (YP_AA1 and YP_AA2 media) and in YPG_AA_55 mM. In YPG_AA_36.6 mM *Cz* started to grow with a delay of > 10 h in comparison with YPG. After 48 h of incubation, plate counting confirmed the absence of viable cells in YP_AA (both concentrations) and YPG_AA_55 mM, while *Cz* reached 6.5 ± 0.1 log cfu/mL in YPG_AA_36.6 mM, the same viable load found in YPG.

Our results are in agreement with those recently reported by Fischer et al. (2017) related to some distinctive traits of a *Saccharomyces-Acetobacter* co-cultures grown in sugar based medium, in which, yeast population decline was concurrent with acetic acid accumulation.

Results of experiments carried out in this work clearly indicate the negative effect of acetic acid on *Cz* viability, only partially reduced in presence of glucose.

3.3. Influence of As-Cz co-cultivation in YPG supplemented with gluconic acid

In order to shed light on the effect of gluconic acid on growth kinetic of both strains, further experiments were set up (step 3 of Fig. 1). It was previously demonstrated that gluconic acid increases during sour rot, especially in the later phase (Barata et al., 2012b), when simple sugars are still released from damaged berry tissues (Pinto et al., 2017); under these conditions, gluconic acid does not seem to have a particular selective pressure against both AAB and NSY populations that usually occur at viable load higher than 7 log cfu/g (Pinto et al., 2017).

The influence of increasing gluconic acid concentrations in YPG (110 mM) on the growth of *As* and *Cz* monocultures was preliminary evaluated.

The growth curves allowed the calculation of the Inhibition Index turbidity ratio (II_{TR}) values classifying the inhibition activity recorded as high (1–0.75), medium (0.7–0.45), low (0.4–0.2) or absent (0.2–0) (de Candia et al., 2017).

The addition of sodium gluconate at 55 mM in YPG did not affect viability of both strains (II_{TR} values closed to 0) showing a low inhibition also when the concentration was increased to 110 mM.

On the contrary, a complete inhibition (II_{TR} value of 1) was found for both strains for all sodium gluconate concentrations from 250 to 550 mM.

Antimicrobial activity played by gluconic acid against both strains and for all concentrations tested was additionally determined enumerating surviving cells at the end of incubation; these viable cell count values allowed the calculation of the Inhibition Index (II) (Baruzzi et al., 2015), as shown in Table 1S reported in Supplementary Materials.

As concerns *A. syzygii* LMG 21419, gluconic acid concentrations of 250 and 350 mM showed bacteriostatic activity. Gluconic acid at 450 and 550 mM did not allow the development of any bacterial or yeast culturable cell.

The negative effect of gluconic acid on cell viability of acetic acid bacteria was suggested by Nieto-Peñalver et al. (2014) against two strains of *Gluconacetobacter diazotrophicus* by using cell free supernatant at unknown concentration of gluconic acid. Recently, Shafiei et al. (2017) demonstrated that high rate of glucose oxidation displayed by some *Acetobacter* strains under monoculture conditions, and consequent accumulation of gluconic acid, resulted in cellular injuries and non-viable cells. Since 55 mM gluconic acid concentration in YPG seemed do not affect *As* and *Cz* monoculture growth kinetics, its effect was evaluated under co-culture conditions (Fig. 4).

The supplementation of gluconic acid to YPG did not change growth kinetic of *Cz* neither in mono nor in co-culture. It can be concluded that the inhibitory activity, not fungicidal, previously displayed by gluconic acid against *Cz* (Fig. 2B), is lost supplementing a suitable carbon source.

Given the ability of *Cz* to quickly metabolize glucose, as already shown in Section 3.2, the occurrence of gluconic acid, could negatively affect *As* viability more in co-culture than in monoculture in which there is no competition for glucose.

In fact, growth kinetic of *As* monoculture in YPG_GA was quite similar to that found in YPG (Table 1), both substrates at 110 mM glucose. On the contrary, during co-culture, gluconic acid at 55 mM in YPG_GA, showed inhibitory activity against *As* within 48 h of incubation as already found in YPGA monoculture during the entire incubation period, in which gluconic acid was supplemented at 36.6 mM (Section 3.1, Fig. 2).

In the medium containing both glucose and gluconic acid, *As* consumed glucose after 48 h (Table 3). It is interesting to note that, at 72 h, gluconic acid concentration increased to about 86 mM from a baseline (T0 values) of 55 mM. The gluconic acid resulting from glucose

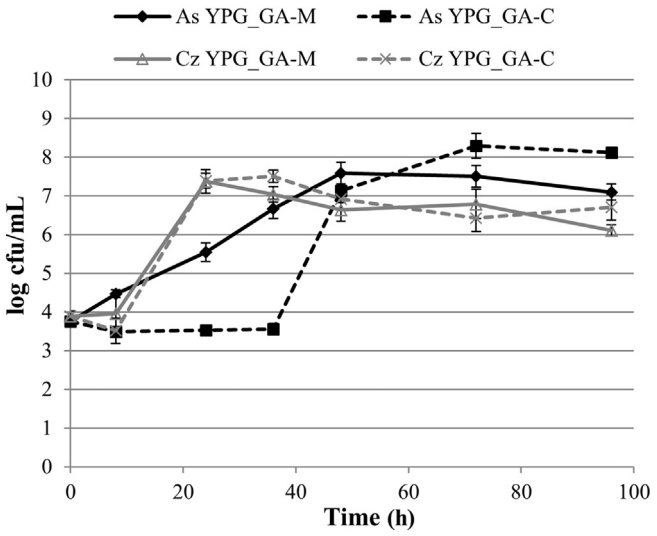

Fig. 4. Microbial growth kinetic of *A. syzygii* LMG 21419 (*As*) and *C. zemplinina* CBS 9494 (*Cz*) during 96 h of aerobic incubation in YP medium supplemented with both glucose (110 mM) and gluconic acid (55 mM) under mono (M) or co-culture (C) conditions. One way- ANOVA was applied to estimate the effect of time on *As* and *Cz* viable cell counts; the least significant difference comparison values (LSD, 95% confidence interval, expressed as log cfu/mL) were calculated among samples for each strain. *As*: 0.74 (monoculture), 0.63 (co-culture); *Cz*: 1.00 (monoculture); 0.86 (co-culture). Mean values were separated using capital letters as shown in Table 3S of Supplementary Materials.

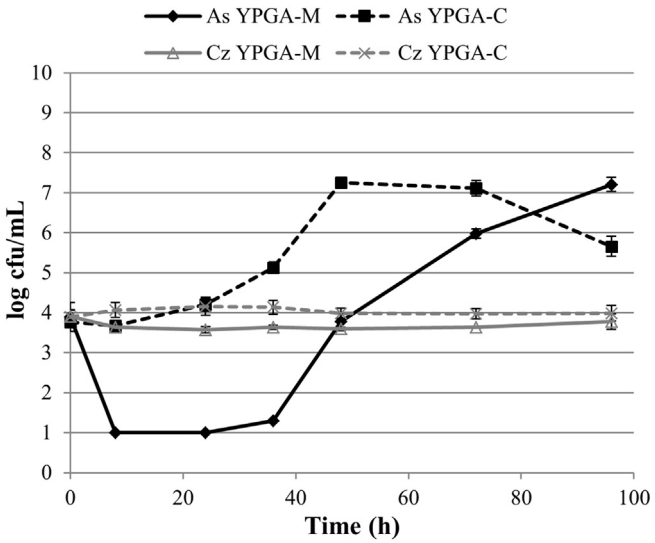

Fig. 5. Microbial growth kinetic of *A. syzygii* LMG 21419 (*As*) and *C. zemplinina* CBS 9494 (*Cz*) during 96 h of aerobic incubation in YP medium supplemented with gluconic acid at 110 mM under mono (M) or co-culture (C) conditions. One way- ANOVA was applied to estimate the effect of time on *As* and *Cz* viable cell counts; the least significant difference comparison values (LSD, 95% confidence interval, expressed as log cfu/mL) were calculated among samples. *As*: 0.73 (monoculture), 0.54 (co-culture); *Cz*: 0.45 (monoculture), 0.74 (co-culture). Detection limit: 1 log cfu/mL. Mean values were separated using capital letters as shown in Table 4S of Supplementary Materials.

metabolism (about 30 mM) was close to that produced in YPG not supplemented with gluconic acid, as reported for *As* monoculture in YPG at 110 mM (Table 2). Results here reported suggest that *As* did not oxidize neither gluconic acid supplemented in the YP medium nor that produced from glucose oxidation released in the YPG medium, confirming that gluconic acid production from glucose and its oxidation to 2-keto-d-gluconate, 5-keto-d-gluconate, and 2,5-diketo-d-gluconate is strain dependant (Sainz et al., 2016).

Cz began to consume glucose after 24 h of incubation, as already demonstrated in YPG; the complete glucose consumption was found at 48 h. Ethanol and glycerol, and traces of acetic acid were detected after 48 and 72 h, respectively (Table 3). It can be concluded that the main metabolic pathway active in *Cz* monoculture is the ethanol production from glucose, also when gluconic acid is present.

In co-culture, the complete consumption of glucose (presumably performed by *Cz*) was recorded after 48 h. Ethanol and glycerol were detected starting from 36 h, whereas acetic acid from 48 h to the end of incubation. This result differs from that previously showed for co-culture in YPG at 110 mM (Table 2) when acetic acid was detected already after 36 h. Thus, the delay in the growth of *As*, registered in presence of gluconic acid (Fig. 4), could be also responsible for a delay in the oxidation of ethanol into acetic acid. In fact, acetic acid reached a concentration of 16–18 mM in YPG and YPG_GA at 48 h and 96 h, respectively.

Table 3
Mean concentrations (mM) of glucose, organic acids and alcohols in YPG (110 mM) supplemented with gluconic acid (55 mM), and inoculated with *A. syzygii* LMG 21419 (*As*), *C. zemplinina*CBS 9494 (*Cz*), or both microorganisms (*As-Cz*) during 96 h at 25 °C under aerobic conditions.

Time (h)	Sample	Glucose (mM)	Gluconic acid (mM)	Glycerol (mM)	Ethanol (mM)	Acetic acid (mM)
0	–	96.5 ± 20.0^{C}	54.2 ± 1.2^{B}			
24	As	90.2 ± 18.6^{Ca}	52.1 ± 1.8^{Aa}	nd	nd	nd
	Cz	82.4 ± 23.0^{Ca}	52.2 ± 0.3^{Aa}	nd	nd	nd
	As-Cz	80.8 ± 16.2^{Ca}	51.4 ± 1.1^{Aa}	nd	nd	nd
36	As	100.4 ± 19.4^{Cb}	55.7 ± 1.3^{Bb}	nd	nd	nd
	Cz	19.1 ± 2.5^{Aa}	54.4 ± 2.8^{Bb}	6.4 ± 0.1^{Bb}	41.0 ± 1.7^{Bb}	nd
	As-Cz	31.9 ± 6.4^{Aa}	47.8 ± 1.5^{Aa}	3.0 ± 0.6^{Aa}	35.6 ± 1.4^{Ba}	nd
48	As	97.1 ± 22.7^{C}	59.0 ± 2.0^{Bb}	nd	nd	nd
	Cz	nd	58.4 ± 0.4^{Bb}	17.4 ± 1.9^{Eb}	121.8 ± 5.6^{Fb}	nd
	As-Cz	nd	50.0 ± 2.2^{Aa}	9.4 ± 1.1^{Ca}	66.0 ± 1.1^{Ca}	3.0 ± 0.2^{A}
72	As	37.3 ± 6.4^{A}	71.7 ± 11.2^{Cb}	nd	nd	nd
	Cz	nd	47.0 ± 0.2^{Aa}	14.6 ± 0.5^{Db}	68.9 ± 2.2^{Ca}	1.9 ± 0.2^{Aa}
	As-Cz	nd	50.0 ± 2.2^{Aa}	11.4 ± 1.2^{Ca}	87.8 ± 2.3^{Db}	9.9 ± 1.5^{Cb}
96	As	50.9 ± 4.5^{B}	86.5 ± 0.5^{Db}	nd	nd	nd
	Cz	nd	52.7 ± 0.1^{Aa}	18.5 ± 0.3^{Eb}	111.8 ± 4.2^{Eb}	5.0 ± 0.6^{Ba}
	As-Cz	nd	53.3 ± 4.9^{Ba}	14.5 ± 4.3^{Ca}	9.7 ± 5.6^{Aa}	16.7 ± 1.3^{Db}

Nd: not detected. One or Two way- ANOVA was applied to estimate the effect of time and sample on metabolite concentration; the least significant difference comparison values (LSD, 95% confidence interval, expressed as mM) were calculated among samples for each metabolite. Glucose: 26.1 (time), 18.4 (sample); Ethanol: 5.5 (time), 4.8 (sample); Glycerol: 2.8 (time); 2.5 (sample); Gluconic acid: 6.2 (time); 4.4 (sample); Acetic acid: 1.4 (time); 1.4 (sample). Means with different superscript letters differ significantly ($P \leq 0.05$); capital letters separated mean values based on incubation time, whereas lowercase letters separated mean values based on sample.

In order to verify if the inhibitory effect of gluconic acid under co-culture conditions was masked by glucose, the experiment was repeated in YP at 110 mM of gluconic acid and in absence of glucose. Results of this additional growth kinetics are shown in Fig. 5.

A strong bactericidal activity was found within 24 h of incubation against *As* in YPGA monoculture (gluconic acid at 110 mM). After this incubation period, *As* grew by reaching 7.2 ± 0.2 log cfu/mL at the end of incubation. Gluconic acid displayed inhibitory, but not bactericidal, activity against *As* when it was co-inoculated together with *Cz*; however, under these conditions the highest viable cell concentration of *As* was reached 48 h early than that recorded for monoculture.

The negative effect of gluconic acid against *As* was mitigated under co-colture conditions when ethanol was produced by *Cz*. The release of ethanol (by *Cz*) within the same medium supplemented with gluconic acid could play a positive effect towards *As* stressed cells. In fact, the protective effect of ethanol was demonstrated by Shafiei et al. (2017) for the *A. senegalensis* type strain in glucose-yeast extract broth during high-temperature fermentation. Further oxidation of ethanol to acetic acid by *As* could also decrease inhibitory activity of gluconic acid, as registered for the growth of *E. coli* ATCC 35218 in presence of a low concentration of different weak organic acids (Nieto-Peñalver et al., 2014). However, the positive effect of the co-culture on *As* viability in presence of gluconic acid (at 36.6 mM) was also found in absence of glucose (see Fig. 3). Thus, it could also be explained by other mechanisms not investigated here.

Cz viable cell load did not show significant changes throughout the incubation period under both mono- and co-culture conditions (Fig. 5), confirming results of inhibitory, but not lethal, activity of gluconic acid against *Cz* already found at 36.6 mM (Fig. 1). Gluconic acid remained stable at 111.7 ± 10.4 mM, 106.1 ± 10.1 mM and 116.7 ± 6.5 mM, for *As*, *Cz* and *As-Cz* cultures, respectively, as already shown in YPG_GA (Table 3).

As regards the yeast, gluconate can be metabolized through $NADP^+$-linked 6-phosphogluconate dehydrogenase, previously characterized in *Candida* species, to be metabolized in pentose phosphate pathway (Bruinenberg et al., 1983; Kato et al., 1979). However, since both gluconate concentration and viable cell count of *C. zemplinina* CBS 9494 remained stable in YPGA up the end of incubation, it is possible to conclude that this strain did not metabolize gluconate under culture conditions applied.

Our results regarding the glucose metabolism and gluconic acid accumulation, by *As* monoculture in YPG_GA, were in accordance with the gluconic acid accumulation found by Sainz et al. (2016) for several *Acetobacter* species in minimal medium, synthetic must and strawberry puree at high concentration of soluble monosaccharides. Gluconic acid remained unexpectedly stable also in *As* monoculture in YPGA and under short time incubation conditions in which *As* should be forced to metabolize gluconic acid due to the absence of free glucose. It is possible to guess that, after 36 h, survived *As* cells incubation employed other metabolic pathway instead of gluconic acid oxidation.

4. Conclusions

The analysis of data, produced under laboratory conditions, allowed us to depict a possible scenario of microbial population temporal succession and their cross-feeding relationships during sour rot development.

Results demonstrated that *Cz* was always more efficient in glucose utilization with ethanol production suggesting that NSY activity on wounded berries could be the starting step of sour rot.

Under monoculture conditions, the oxidative fermentation of glucose to gluconic acid was the only pathway employed by *As* to grow in YP-glucose, whereas when co-cultured with *Cz* the increase in ethanol concentration could lead *As* to shift its metabolism towards ethanol oxidation with acetic acid release. It is then possible to assume that during sour rot, AAB grow on wounded berries after NSY by oxidising ethanol into acetic acid. We found that acetic acid displayed inhibitory activity against *Cz*. Thus the accumulation of this metabolite during sour rot could reduce NSY population viability, and slowed down the accumulation of ethanol deriving from soluble sugar metabolism.

Therefore, when the spoilage process proceeds, further release of glucose undergoes the oxidative fermentation by AAB, reducing glucose availability for NSY metabolism and leading to accumulation of gluconic acid. In fact, growth kinetics in different media always showed that *Cz* was unable to metabolize gluconic acid without any reduction in its viable cell load.

This scenario agrees with previous reported microbiological studies and with the accumulation of gluconic acid in spoiled berries in late stages of sour rot.

The temporal succession and trophic interaction here reported can be considered a first step towards a fully understanding of AAB-NSY ecological relationship occurring in sour rot that needs to be confirmed by in vivo trials.

Acknowledgements

The Authors thanks the laboratory network project “Biodiversity for the enhancement of the value and safety of typical Apulian food products -BioNet-PTP” (Code 73) funded by POR FESR 2000-2006 for the instrument Varioskan Flash (Thermo Fischer Scientific). Part of this work was supported by Portuguese national funds from Fundação para a Ciência e a Tecnologia (FCT) through the research unit UID/AGR/04129/2013 (LEAF).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijfoodmicro.2019.02.022.

References

Alexandre, H., Charpentier, C., 1998. Biochemical aspects of stuck and sluggish fermentation in grape must. J. Ind. Microbiol. Biotechnol. 20, 20–27.

Barata, A., Pais, A., Malfeito-Ferreira, M., Loureiro, V., 2011. Influence of sour rotten grapes on the chemical composition and quality of grape must and wine. Eur. Food Res. Technol. 233, 183–194.

Barata, A., Malfeito-Ferreira, M., Loureiro, V., 2012a. Changes in sour rotten grape berry microbiota during ripening and wine fermentation. Int. J. Food Microbiol. 154, 152–161.

Barata, A., Santos, S.C., Malfeito-Ferreira, M., Loureiro, V., 2012b. New insights into the ecological interaction between grape berry microorganisms and *Drosophila* flies during the development of sour rot. Microb. Ecol. 64, 416–430.

Baruzzi, F., Pinto, L., Quintieri, L., Carito, A., Calabrese, N., Caputo, L., 2015. Efficacy oflactoferricin B in controlling ready-to-eat vegetable spoilage caused by *Pseudomonas* spp. Int. J. Food Microbiol. 215, 179–186. https://doi.org/10.1016/j.ijfoodmicro.2015.09.017.

Bruinenberg, P.M., Van Dijken, J.P., Scheffers, W.A., 1983. An enzymic analysis of NADPH production and consumption in *Candida utilis*. Microbiology 129, 965–971.

Carr, J.G., Whiting, G.C., 1971. Microbiological aspects of production and spoilage of cider. J. Appl. Microbiol. 34, 81–93.

Cheng, H.P., Wang, P.M., Chen, J.W., Wu, W.T., 2002. Cultivation of *Acetobacter xylinum* for bacterial cellulose production in a modified airlift reactor. Biotechnol. Appl. Biochem. 35, 125–132.

de Candia, S., Quintieri, L., Caputo, L., Baruzzi, F., 2017. Antimicrobial activity of processed spices used in traditional southern Italian sausage processing. J. Food Process. Preserv. 41, e13022. https://doi.org/10.1111/jfpp.13022.

De Ley, J., Swings, J., Gosselè, F., Genus, I., 1984. Acetobacter. In: Krieg, N.R., Holt, J.G. (Eds.), Bergey's Manual of Systematic Bacteriology. vol. 1. Williams & Wilkins, Baltimore, pp. 268–274.

Depetris-Chauvin, A., Galagovsky, D., Chevalier, C., Maniere, G., Grosjean, Y., 2017. Olfactory detection of a bacterial short-chain fatty acid acts as an orexigenic signal in *Drosophila melanogaster* larvae. Sci. Rep. 7, 14230.

Drysdale, G.S., Fleet, G.H., 1989. The effect of acetic acid bacteria upon the growth and metabolism of yeasts during the fermentation of grape juice. J. Appl. Bacteriol. 67, 471–481.

Englezos, V., Rantsiou, K., Torchio, F., Rolle, L., Gerbi, V., Cocolin, L., 2015. Exploitation of the non-Saccharomyces yeast *Starmerella bacillaris* (synonym *Candida zemplinina*) in wine fermentation: physiological and molecular characterizations. Int. J. Food Microbiol. 199, 33–40.

Englezos, V., Rantsiou, K., Cravero, F., Torchio, F., Giacosa, S., Ortiz-Julien, A., Gerbi, V., Rolle, L., Cocolin, L., 2018. Volatile profiles and chromatic characteristics of red

wines produced with *Starmerella bacillaris* and *Saccharomyces cerevisiae*. Food Res. Int. 109, 298–309.
Fischer, C.N., Trautman, E.P., Crawford, J.M., Stabb, E.V., Handelsman, J., Broderick, N.A., 2017. Metabolite exchange between microbiome members produces compounds that influence *Drosophila* behavior. Elife 6.
García, M., Esteve-Zarzoso, B., Arroyo, T., 2016. Non-Saccharomyces yeasts: Biotechnological role for wine production. In: Morata, A., Loira, I. (Eds.), Grape and Wine Biotechnology. InTechOpen Limited, London, pp. 249–271. https://doi.org/10.5572/64957.
Gonçalves, C., Wisecaver, J.H., Kominek, J., Oom, M.S., Leandro, M.J., Shen, X.X., Opulente, D.A., Zhou, X., Peris, D., Kurtzman, C.P., Hittinger, C.T., Rokas, A., Gonçalves, P., 2018. Evidence for loss and reacquisition of alcoholic fermentation in a fructophilic yeast lineage. eLife 7, e33034.
González, B., Vázquez, J., Cullen, P., Mas, A., Beltran, G., Torija, M.J., 2018. Aromatic amino acid-derived compounds induce morphological changes and modulate the cell growth of wine yeast species. Front. Microbiol. 9, 670.
Gullo, M., Zanichelli, G., Verzelloni, E., Lemmetti, F., Giudici, P., 2016. Feasible acetic acid fermentations of alcoholic and sugary substrates in combined operation mode. Process Biochem. 51, 1129–1139.
Kato, N., Sahm, H., Schütte, H., Wagner, F., 1979. Purification and properties of glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase from a methanol-utilizing yeast, *Candida boidinii*. Biochim. Biophys. Acta, Enzymol. 566, 1–11.
Kregiel, D., James, S.A., Rygala, A., Berlowska, J., Antolak, H., Pawlikowska, E., 2018. Consortia formed by yeasts and acetic acid bacteria *Asaia* spp. in soft drinks. A. Van Leeuw. J. Microb. 111, 373–383.
Lisdiyanti, P., Kawasaki, H., Seki, T., Yamada, Y., Uchimura, T., Komagata, K., 2001. Identification of *Acetobacter* strains isolated from Indonesian sources, and proposals of *Acetobacter syzygii* sp. nov., *Acetobacter cibinongensis* sp. nov., and *Acetobacter orientalis* sp. nov. J. Gen. Appl. Microbiol. 47, 119–131.
Lleixà, J., Kioroglou, D., Mas, A., del Carmen Portillo, M., 2018. Microbiome dynamics during spontaneous fermentations of sound grapes in comparison with sour rot and *Botrytis* infected grapes. Int. J. Food Microbiol. 281, 36–46.
Loureiro, V., Ferreira, M.M., Monteiro, S., Ferreira, R.B., 2012. The microbial communityof grape berry. In: Geros, H., Chaves, M., Delrot, S. (Eds.), The Biochemistry of the Grape Berry. Bentham Science Publishers, Sharjah, United Arab Emirates, pp. 241–268. https://doi.org/10.2174/978160805360511201010241.
Mamlouk, D., Gullo, M., 2013. Acetic acid bacteria: physiology and carbon sources oxidation. Indian J. Microbiol. 53, 377–384.
Marchetti, R., Guerzoni, E., Gentile, M., 1984. Research on the etiology of a new disease of grapes: sour rot. Vitis 23, 55–65.
Mateo, E., Torija, M.J., Mas, A., Bartowsky, E.J., 2014. Acetic acid bacteria isolated from grapes of South Australian vineyards. Int. J. Food Microbiol. 178, 98–106.
Matsushita, K., Matsutani, M., 2016. Distribution, evolution, and physiology of oxidative fermentation. In: Matsushita, K., Toyama, H., Tonouchi, N., Okamoto-Kainuma, A. (Eds.), Acetic Acid Bacteria, Ecology and Physiology. Springer, Tokyo, pp. 159–178. https://doi.org/10.1007/978-4-431-55933-7_7.
Moens, F., Lefeber, T., De Vuyst, L., 2014. Oxidation of metabolites highlights the microbial interactions and role of *Acetobacter pasteurianus* during cocoa bean fermentation. Appl. Environ. Microbiol. 80, 1848–1857.
Mounir, M., Shafiei, R., Zarmehrkhorshid, R., Hamouda, A., Alaoui, M.I., Thonart, P., 2016. Simultaneous production of acetic and gluconic acids by a thermotolerant *Acetobacter* strain during acetous fermentation in a bioreactor. J. Biosci. Bioeng. 121, 166–171.
Nguyen, N.K., Nguyen, P.B., Nguyen, H.T., Le, P.H., 2015. Screening the optimal ratio of symbiosis between isolated yeast and acetic acid bacteria strain from traditional kombucha for high-level production of glucuronic acid. LWT Food Sci. Technol. 64, 1149–1155.
Nieto-Peñalver, C.G., Savino, M.J., Bertini, E.V., Sánchez, L.A., de Figueroa, L.I., 2014. Gluconic acid produced by *Gluconacetobacter diazotrophicus* Pal5 possesses antimicrobial properties. Res. Microbiol. 165, 549–558.
Pinto, L., Caputo, L., Quintieri, L., de Candia, S., Baruzzi, F., 2017. Efficacy of gaseous ozone to counteract postharvest table grape sour rot. Food Microbiol. 66, 190–198.
Rantsiou, K., Englezos, V., Torchio, F., Risse, P.A., Cravero, F., Gerbi, V., Rolle, L., Cocolin, L., 2017. Modeling of the fermentation behavior of *Starmerella bacillaris*. Am. J. Enol. Vitic. 68, 378–385. https://doi.org/10.5344/ajev.2017.16108. (ajev-2017).
Sainz, F., Navarro, D., Mateo, E., Torija, M.J., Mas, A., 2016. Comparison of d-gluconic acid production in selected strains of acetic acid bacteria. Int. J. Food Microbiol. 222, 40–47.
Shafiei, R., Zarmehrkhorshid, R., Mounir, M., Thonart, P., Delvigne, F., 2017. Influence of carbon sources on the viability and resuscitation of *Acetobacter senegalensis* during high-temperature gluconic acid fermentation. Bioprocess Biosyst. Eng. 40, 769–780.
Shiraishi, M., Fujishima, H., Chijiwa, H., 2010. Evaluation of table grape genetic resources for sugar, organic acid, and amino acid composition of berries. Euphytica 174, 1–13.
Valera, M.J., Torija, M.J., Mas, A., Mateo, E., 2015. Cellulose production and cellulose synthase gene detection in acetic acid bacteria. Appl. Microbiol. Biotechnol. 99, 1349–1361.
Valera, M.J., Mas, A., Streit, W.R., Mateo, E., 2016. GqqA, a novel protein in *Komagataeibacter europaeus* involved in bacterial quorum quenching and cellulose formation. Microb. Cell Factories 15, 88.
Velasco-Bedrán, H., López-Isunza, F., 2007. The unified metabolism of *Gluconacetobacter entanii* in continuous and batch processes. Process Biochem. 42, 1180–1190.
Zoecklein, B.W., Williams, J.M., Duncan, S.E., 2001. Effect of sour rot on the composition of White Riesling (*Vitis vinifera* L.) grapes. Small Fruits Rev. 1, 63–77.