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(Article begins on next page)



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**Bacterial species associated with sound and *Botrytis*-infected grapes
from a Greek vineyard**

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Running title: Bacteria in botrytised grapes

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ABSTRACT

Grape bacterial microbiota plays central roles in the quality of grapes and wine, yet its diversity remains poorly described. In the present study, bacterial species associated with sound and *Botrytis*-infected grapes of two cultivars originating from the same vineyard were assessed. Isolates were identified by PCR–Denaturing Gradient Gel Electrophoresis (PCR-DGGE) and sequence analysis of partial 16S rRNA gene. Comparable counts were recorded between *Botrytis*-infected and sound grape samples. In all cases, the majority of isolates belonged to different species of *Enterobacteriaceae*. The dominant species in the vineyard was *Klebsiella oxytoca* that was found in different combinations with *Citrobacter freundii*, *Enterobacter* spp., *Erwinia* sp., *Pantoea dispersa*, *Tatumella ptiseos* or other species. In fermenting musts, those populations declined while other species evolved, like *Lactobacillus plantarum* and *Enterobacter ludwigii*. Populations in botrytised samples persisted longer during spontaneous fermentations. Present study suggests that bacterial diversity on grapes may be wider than previously described.

Keywords: grape must fermentation, bacteria, *Botrytis*, grapes, wine.

1. Introduction

Grapes represent a natural reservoir of bacterial species that may influence wine production and storage. Different bacteria may be transferred from grapes into the winery and perturb the vinification process. For instance, grapes are considered the primary source for lactic acid bacteria (LAB), which conduct the malolactic fermentation, a process that improves wine flavor and stability (Lonvaud-Funel 1999). Spontaneous malolactic fermentations are commonly conducted in several wineries by native bacteria that are possibly carried over by grapes. On the other hand, spoilage of wines caused by bacteria is a major concern for the wine industry. Despite that, little is yet known about the origin of those species. It seems, however, likely that they originate from both the grapes and the winery (Gonzalez et al., 2005; Renouf et al., 2005).

While in the case of yeast grape microbiota, it has been well-documented that *Hanseniaspora uvarum* dominates in vineyards worldwide, there is not such a clear picture for bacteria. Research hitherto points to significant structural differences among bacterial communities on sound grapes from diverse geographical regions. For instance, Subden et al. (2003) found that *Pantoea agglomerans* and *Pseudomonas corrugate* dominate in ice-wine musts from Ontario, while several LAB and non-LAB species like *Gluconobacter oxydans*, *Serratia rubidaea* and *Enterobacter gergoviae* were encountered on grapes from the Bordeaux area (Renouf et al., 2005; 2007). In another study, the community on grapes from New South Wales was composed of several LAB and/or non-LAB populations, such as *Sporolactobacillus inulinus* and *Asaia siamensis* (Bae et al., 2006).

Accumulating data suggest that the microbial ecology of damaged or botrytised berries may differ from that of sound grapes (Fleet, 1999; Gadoury et al., 2007). For instance, increased populations of acetic acid bacteria were observed in *Botrytis*-infected grapes compared to sound samples (Barbe et al., 2001). Damaged grapes are often transferred accidentally into the winery, while botrytised grapes are exploited by the wine industry in the production of superior sweet wines. Thus, it is important to investigate the magnitude of bacterial diversity in *Botrytis*-infected grapes and its evolution during fermentation. In this case study, *Botrytis*-infected grapes from a Greek vineyard were surveyed and compared with their sound counterparts. Common species and also some unexpected diversity was unveiled that was further assessed during subsequent spontaneous alcoholic fermentations.

2. Materials and methods

2.1 Isolation procedure

Healthy (1) and *Botrytis*-infected (2) grape samples from two red grapevine cultivars, ‘Mavroliatis’ (M) and ‘Sefka’ (S), were collected at the time of harvest from the experimental vineyard of the Agricultural University of Athens, Greece. Vines of each variety were cultivated in parallel single rows that were 20 m long and 40 m apart. Sound or botrytised grapes were randomly and aseptically collected throughout each row–cultivar and pooled to form a single homogenous, representative sample for each sanitary status. Thus, a total of four grape samples (M-1, M-2, S-1 and S-2) were analysed.

The presence/absence of *Botrytis* in the respective samples was confirmed by ELISA, using antibody BC-12.CA4 (Meyer and Dewey, 2000) as described by Dewey et al. (2000). About 3 kg of grapes were sequentially crushed in a Stomacher (Lab Blender 400). Skins and seeds were removed by using a sieve and 1.5 l of juice was then transferred into a 2 l flask and allowed to ferment spontaneously without any additives. D-Glucose/D-fructose and ethanol contents were determined by using appropriate enzymatic kits (Boehringer Mannheim/R-Biopharm, Darmstadt, Germany). Samples were taken immediately after crushing and at two time points during spontaneous alcoholic fermentation at 20 °C., i.e. at the middle (day 8-11) when about 50% of sugars were consumed, and final (day 28) stages of fermentations when sugars were depleted. Samples were successively diluted in Ringer's solution and appropriate dilutions were plated on the following media: (i) MRS Agar (pH 5.5) overlaid with the same medium and incubated at 30 °C for 3-5 days and (ii) GYC Agar (glucose 5 %, yeast extract 1 %, CaCO₃ and agar 2 %, pH 6.3) incubated at 30 °C for 3-5 days under aerobic conditions. Media were supplemented with 100 mg/l cycloheximide to suppress yeast growth. For species identification, 20-25 colonies per agar plate were randomly collected. Isolates were further purified by repeated streaking and stored at -80 °C with glycerol (30% final concentration) before subjected to molecular analysis.

2.2 PCR amplification

Bacterial isolates were subjected to genomic DNA extraction according to Manzano et al. (2003). The V1 region of 16S rRNA gene was amplified using the primers P₁V₁-GC (5'-GCG GGC CGC GCG ACC GCC GGG ACG CGC GAG CCG GCG GCG GGC GGC GTG CCT AAT ACA TGC-3') (the GC clamp is underlined) and P₂V₁ (5'-

TTC CCC ACG CGT TAC TCA CC-3') (Cocolin et al., 2001). The V1-V3 region was amplified using the primers P₁V₁ (without the GC clamp) and P₄V₃ (5'-ATC TAC GCA TTT CAC CGC TAC-3') (Klijn et al., 1991). PCRs were performed in a total volume of 50 µl containing 10 ng of template DNA, 20 pmol of each primer, 100 µM of each dNTP and 1U of DyNAzyme™ EXT DNA Polymerase (Finnzymes, Oy, Finland) in the incubation buffer provided by the manufacturer of the enzyme. Amplifications were achieved in a PTC-200 Peltier thermal cycler (MJ Research, Waltham, Mass., USA) as previously described (Cocolin et al., 2001). PCR products were separated by gel electrophoresis in 1.0 % (w/v) agarose gel, detected by ethidium bromide staining and photographed under UV light using the GelDoc system (Bio-Rad, Hercules, Calif.). Sizes of fragments were determined using a standard molecular weight marker (100 bp ladder, Fermentas).

2.3. DGGE analysis

Sequence dependent separation of bacterial PCR amplicons was performed with the DCode universal mutation detection system (Bio-Rad) in 8 % polyacralamide gels (bisacrylamide 37.5:1) and a denaturing gradient from 40 to 60 % with 100 % corresponding to 7 M urea and 40 % (wt/vol) formamide, increasing in the direction of the electrophoretic run at 130 V and 60 °C, for 4 h as described by Rantsiou et al. (2008).

2.4. Restriction enzyme analysis

For restriction reactions of the V1-V3 region of 16S rRNA gene, approximately 500 ng of the respective PCR products were incubated for 1 h at 65 °C with 10 U of *TaqI* restriction endonuclease (Takara, Japan). Restriction fragments were separated by gel

electrophoresis in 3% (w/v) agarose gel, detected by ethidium bromide staining, and photographed. Sizes of fragments were estimated using a low molecular weight marker (New England Biolabs).

2.5. Sequence analysis

PCR products were purified using the QIAquick[®] PCR Purification Kit (Qiagen, Germany) according to the manufacturer's instructions. By using forward (P₁V₁) and reverse (P₄V₃ or P₂V₁) primers both DNA strands were sequenced with an ABI 3730 XL automatic DNA sequencer by Macrogen (<http://www.macrogen.com>). Blast searches of sequences were performed at the National Centre for Biotechnology Information (NCBI) GenBank data library. Clustal X (1.83) software (<http://www-igbmc.u-strasbg.fr/BioInfo>) was used to perform sequence alignments among the isolates and other homologous sequences available in the GenBank.

2.6. Nucleotide sequence accession numbers

Nucleotide sequences have been deposited in the NCBI GenBank data library under the accession numbers FJ599766-FJ599769 and FJ599771-FJ599779.

3. Results

3.1. Bacterial abundance in grape berries and fermenting musts

In grape samples no bacterial growth was detected on MRS agar, while in GYC medium relatively high and comparable numbers were encountered between sound and *Botrytis*-

infected samples of the two grapevine cultivars. Most of the colonies did not produce halos and only isolates identified later as *Gluconobacter cerinus* were surrounded by a clear zone on GYC agar. Counts ranged between 5.0 and 5.3 log CFU/ml for ‘Mavroliatis’ sound (M-1) and ‘Mavroliatis’ botrytised (M-2) samples, and 5.9 versus 6.3 log CFU/ml for ‘Sefka’ sound (S-1) and ‘Sefka’ botrytised (S-2) samples, respectively. Relatively high pH values were recorded for grape samples, i.e., 3.6 for M-1 and S-2, 3.8 for S-1, and 4.0 for M-2. The sugar content was 180 for M-1, 288 for M-2, 182 for S-1, and 163 g/l for S-2.

During the fermentation course, bacterial counts on GYC medium sharply declined to undetectable levels by days 5-8 (Table 1). As opposed, in MRS medium bacterial populations of all samples rapidly increased during the first 5 days reaching relatively high levels (6.6-7.2 log CFU/ml). Thereafter, remarkable differences were observed between musts originating from botrytised and sound samples. While in healthy samples counts gradually declined till the end of fermentations (day 28), in botrytised samples high bacteria counts persisted in both samples throughout the course. At the mid-stage of fermentation, counts ranged between 5.4-6.1 log CFU/ml for sound samples (day 11) or 6.7-6.8 log CFU/ml for botrytised samples (day 8). By day 28 counts further decreased to 1.9-4.0 log CFU/ml in healthy but persisted (6.3-7.7 log CFU/ml) in botrytised samples. Similar pH values (3.7-3.8) were recorded for different samples by the end of the course. The ethanol content was 11.5 for M-1, 11.0 for M-2, 11.3 for S-1, and 10.8 % v/v for S-2.

3.2. *Species identification*

A total of 253 bacterial isolates from grapes, the middle and the final stages of fermentations were analyzed by PCR-DGGE of the V1 16S rDNA region. Based on different DGGE profiles isolates were clustered into 12 respective groups. Representative isolates from each group (groups I to XII; Fig. 1) were identified at the species level by sequence analysis of the V1–V3 region within the 16S rRNA gene. Sequencing and phylogenetic analyses assigned bacteria to 13 species, namely *Bacillus subtilis*, *Citrobacter freundii*, *Enterobacter* sp., *Enterobacter ludwigii*, *Erwinia* sp., *Gluconobacter cerinus*, *Klebsiella oxytoca*, *Lactobacillus plantarum*, *Pantoea dispersa*, *Providencia rettgeri*, *Serratia marcescens*, *Staphylococcus epidermidis* and *Tatumella tyseos*.

As it is shown in Figure 1, identical DGGE profiles were generated for *K. oxytoca* (lane 2) and *E. ludwigii* (lane 4). To distinguish between those species, an RFLP was detected after sequence inspection. More precisely, Blast search revealed that several isolates of group II (Fig. 1) showed 100 % V1-V3 sequence homology to *E. ludwigii* EN-119^T (Hoffmann et al., 2005), while others to *K. oxytoca* SB2942 (Fevre et al., 2005). In view of the above results, the respective rDNA regions of *E. ludwigii* and *K. oxytoca* were inspected for possible existence of informative RFLP patterns. *In silico* analysis showed that the two species differ by two nucleotides within the V1-V3 region, which provide an additional *TaqI* restriction site for *K. oxytoca* (data not shown). Further experimental verification showed that digestion with *TaqI* generates readily distinguishable banding patterns for clear discrimination of the two species. *K. oxytoca* produces 3 fragments of 420, 160 and 70 bp each, whereas *E. ludwigii* generates two

bands of 580 and 70 bp each. By applying the above restriction enzyme analysis, 41 % of the isolates were identified as *K. oxytoca* and 59 % as *E. ludwigii*.

3.3 Species heterogeneity in grapes and musts

K. oxytoca was the dominant species in all grape samples, irrespective of the sanitary status or cultivar ranging from ca. 4.9 log CFU/ml for both samples M-1 and M-2 and 5.7 versus 6.0 log CFU/ml for samples S-1 and S-2, respectively. Its relative abundance was higher in sound than in *Botrytis*-infected grapes. *Botrytis*-infected grapes also harbored slightly higher bacterial populations than sound grapes of the same cultivar (Fig. 2). Other populations were diversified among different samples. Botrytised grapes of 'Mavroliatis' (M-2) harboured 2 populations of the *Enterobacteriaceae* family (*P. rettgeri* and *T. ptyseos*), and *B. subtilis*. The respective M-1 grapes possessed *G. cerinus*. Botrytised grapes of 'Sefka' (S-2) were resident by *C. freundii*, *Enterobacter* sp., *E. ludwigii* and *S. marcescens*, while S-1 samples harboured *Erwinia* sp., *P. dispersa* and *T. ptyseos*.

Bacterial species richness noticeably declined in grape juices during the spontaneous fermentation courses. Except from *E. ludwigii*, all other species encountered on grapes were not recovered from fermenting samples, either in the middle or the final stage of fermentation. Two other species not detected in grapes evolved, namely *L. plantarum* and *S. epidermidis* (Fig. 2). At mid-stage, *L. plantarum* evolved in all samples, though in somewhat higher percentages in the botrytised samples. *E. ludwigii*, was also detected in different samples, except for M-2. *S. epidermidis* populations also developed sporadically (samples S-1 and M-2) at relatively low percentages, but were not detected

at the final stage of the course. *L. plantarum*, followed by *E. ludwigii*, became the climax bacteria in both *Botrytis*-affected samples. A similar picture was also shown for the M-1 sample, while in S-1 only *E. ludwigii* survived.

4. Discussion

Grapes constitute the primordial source of bacteria in wine production providing must with both beneficial and potentially spoilage species, which may influence the fermentation course, the quality of the final product, and its further preservation. Nevertheless, the range of bacterial diversity on grapes has been poorly addressed, particularly as regards botrytised berries. It has been proposed that botrytised grapes may possess elevated bacterial counts (Barbe et al., 2001). In the present study, comparable counts were recorded on grapes of different sanitary status. In all cases, the native grape ecosystem accommodated mostly phyllospheric species of *Enterobacteriaceae*. The dominant species *Klebsiella oxytoca* was encountered in different combinations with other *Enterobacteriaceae* species, like *Pantoea dispersa*, *Enterobacter* spp. and *Citrobacter freundii*. A similar community structure has been recently encountered in fruit fly-infested fruits (Behar et al., 2008), where different Mediterranean fruit fly-associated *Enterobacteriaceae* were transferred to fly-infested fruits. Among them *K. oxytoca* was extremely stable in rotting fruits, while other members of the community were rather transient. Present results show a similar bacterial community to the one described above, with *K. oxytoca* being the predominant species in grapes, while no clear structure was observed for the other species. The increased population of fruit flies in the

Greek vineyard may explain, at least partially, the occurrence of fruit fly-associated *Enterobacteriaceae* in this survey. Besides, *Klebsiella* spp. have been recovered from fruits and plants with high content of sugars and acids (Behar et al., 2008; Duncan and Razzell, 1972; Fuentes et al., 1985; Mundt et al., 1978), while *K. oxytoca* strains may also survive at low pH values, i.e. about 4 (Jansen et al., 1984).

Although few studies have focused on the description of bacterial populations on grapes (Bae et al., 2006), it is generally accepted that grape-associated bacterial populations consist mostly of AAB and LAB (Barbe et al., 2001; Fleet, 1999). However, it has been recently shown that grapes, like other phyllospheric habitats, may also harbor several other bacterial species, including *Enterobacteriaceae*, which may become dominant (Subden et al., 2003; Renouf et al., 2005; 2007). There are several factors to shape the grape microbiota, including rainfall, temperature, grape variety, berry maturity, sanitary status, and the application of agrichemicals (Fleet, 1999). Therefore, microbial diversity in grapes may be quite divergent and associated with the particular vineyard. In the present study, the high temperatures during harvest and the presence of botrytised grapes, which promote fruit fly population growth, combined with the relatively increased pH values of grape juices (>3.6) may have enhanced the prevalence of *K. oxytoca* on grape surfaces. However, the dominance of *K. oxytoca* may have negatively affected the growth of minor AAB populations, since AAB are considered “fastidious microorganisms” and are often difficult to cultivate on artificial media (Bartowsky and Henschke, 2008).

Bacterial species richness noticeably declined in all samples during the spontaneous fermentation courses and was restricted to three species, namely *L.*

plantarum, *E. ludwigii* and *S. epidermidis*. A dramatic decrease of species diversity in fermenting musts compared to grapes was also previously recorded (Renouf et al., 2007). The prevalence of *L. plantarum* in wine fermentations has been well described (Beneduce et al., 2004; Lafon-Lafourcade et al., 1984; Spano et al., 2007). It is also likely that its presence is often associated with deterioration of wine quality, through the production of biogenic amine and precursors of ethyl carbamate (Lonvaud-Funel, 1999; Liu, 2002). Therefore, its occurrence in grape surface along with its ability to grow during fermentation needs further consideration. *Oenococcus oeni*, the principal malolactic bacterium frequently isolated from wines, was not detected in this study, suggesting that it was absent or at very low populations. It is likely that the relatively high pH values (>3.6) of fermenting musts facilitated the growth of *L. plantarum*, whilst *O. oeni* is typically associated with wines of lower pH (<3.5) that other species may not withstand (Fleet, 1998; Osborne and Edwards, 2005). The interrelationships among microorganisms may also play crucial roles during the alcoholic fermentation course and determine the pattern of microbial succession. In this context, *L. plantarum* strains have been shown to exhibit antimicrobial activity that strongly inhibits the growth of *O. oeni* (Costantini et al., 2009). Although this possibility could serve as a possible explanation for not detecting *O. oeni*, further studies are required to understand the highly unexplored roles of microbe-microbe interactions in wine fermentations.

Besides *L. plantarum*, *E. ludwigii*, a member of the *Enterobacteriaceae* family, was also found to persist in fermenting musts. *E. ludwigii* is a species recently delimited from clinical specimens (Hoffmann et al., 2005). Since then, it has been associated with the rhizosphere of different plant species (Pawlicki-Jullian et al., 2010; Shoebitz et al.,

2009). Recently, Ruiz et al. (2010) showed the prevalence of *Enterobacter* sp. at the beginning, mid and final stages of malolactic fermentations in wines from different Spanish wineries. The presence of a *Serratia* sp. was also reported in those wines, suggesting that members of *Enterobacteriaceae* may be introduced into the winery through grapes, survive fermentation and found in the final product. *S. epidermidis* was recovered sporadically at the mid-stage of present fermentations. This is not the first time to show the presence of *Staphylococcus* in the grapevine. Recently, *Staphylococcus* spp. have been recovered from wine grapes of several Australian vineyards (Bae et al., 2006), while *S. epidermidis* has been associated with the phyllosphere of other plants (Berg et al., 2005).

For some time it has been presumed that grapes, particularly the damaged or botrytised berries, constitute rich reservoirs for LAB and AAB species. Accumulating data, without contracting the above fact, suggest that grape bacteria microbiota may be even wider, including species of *Enterobacteriaceae*, *Bacillus*, and *Staphylococcus*. The presence of non-LAB species capable to further survive in fermenting musts, such as *E. ludwigii* in this study, is of considerable importance for the wine industry, underlining the need for further exploration of the native microbiota on grapes.

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Table 1

Bacterial populations in musts at different days of alcoholic fermentation

Sample	Medium	Bacterial populations (log CFU/ml) in fermenting musts						
		3d	5d	8d	11d	14d	22d	28d
M-1	MRS	7.0	7.2	7.0	6.1	6.6	5.7	4.0
M-2		7.2	7.2	6.7	7.7	7.6	7.3	7.7
S-1		7.2	6.6	5.9	5.4	5.6	4.2	1.9
S-2		6.7	6.9	6.8	5.0	4.7	5.1	6.3
M-1	GYC	4.2	<1	<1	<1	<1	<1	<1
M-2		5.6	3.7	<1	<1	<1	<1	<1
S-1		4.7	3.7	<1	<1	<1	<1	<1
S-2		3.7	<1	<1	<1	<1	<1	<1

Figure legends

Fig. 1. DGGE analysis of partial 16S rRNA gene amplicons of bacterial species isolated from grapes or during fermentation. Lanes: 1, *Enterobacter* sp.; 2, *K. oxytoca*; 3, *S. marcescens*; 4, *E. ludwigii*; 5, *C. freundii*; 6, *S. epidermidis*; 7, *P. dispersa*; 8, *T. tyseos*; 9, *Erwinia* sp.; 10, *B. subtilis*; 11, *P. rettgeri*; 12, *L. plantarum*; 13, *G. cerinus*. Latin numbers (I-XII) correspond to different groups of DGGE profiles. Species identification was based on 16S rRNA gene sequence analysis of representative bacterial isolates from each group.

Fig. 2. Bacterial species in sound (M-1, S-1) and *Botrytis*-affected (M-2, S-2) grapes (G) and juices at the mid (MF) and the end (EF) of fermentation courses. The number of bacterial isolates examined per case (n) is indicated above each column.

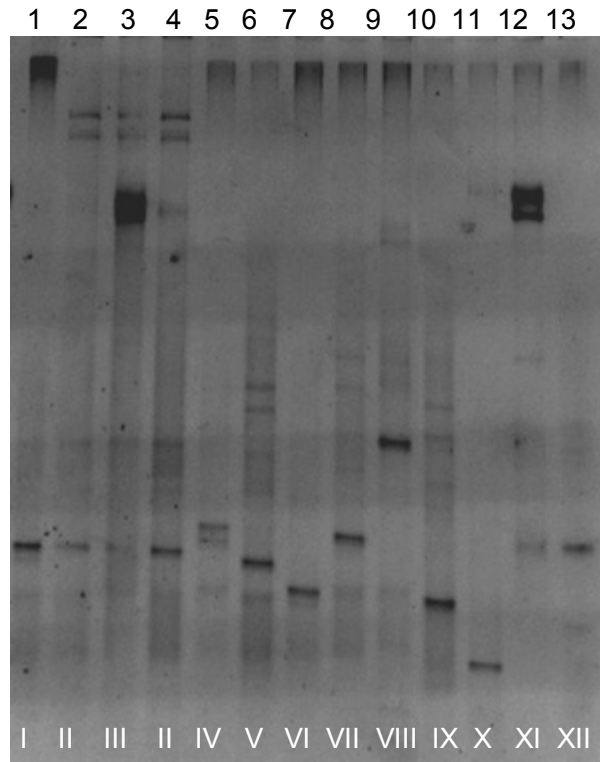


Fig. 1

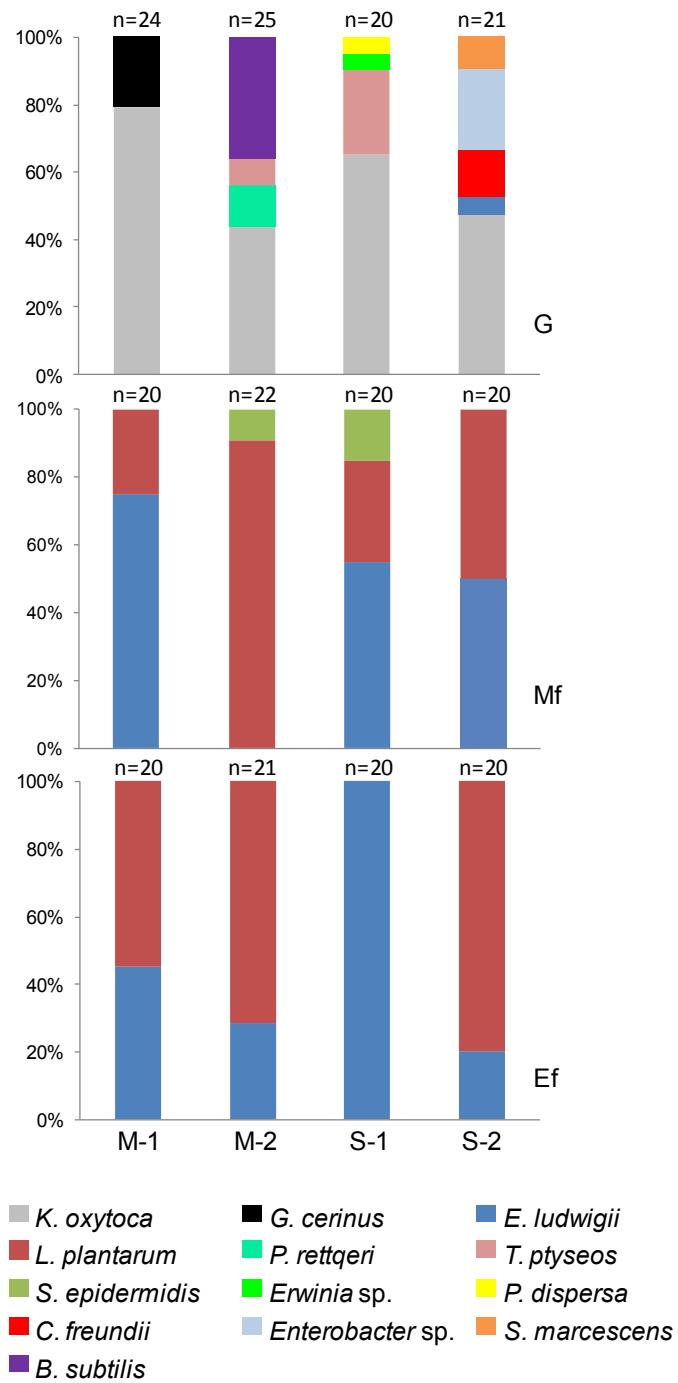


Fig. 2