Temporal shifts in endophyte bacterial community composition of sessile oak (*Quercus petraea*) are linked to foliar nitrogen, stomatal length, and herbivory

Luigimaria Borruso [1] , **Camilla Wellstein** [1] , **Alessia Bani** [1] , **Sara Casagrande Bacchiocchi** [1] , **Ania Margoni** [1] , **Rita Tonin** [1] , **Stefan Zerbe** [1] , **Lorenzo Brusetti** [Corresp. 1]

[1] Faculty of Science and Technology, Free University of Bozen/Bolzano, Bolzano, Italy

Corresponding Author: Lorenzo Brusetti
Email address: lorenzo.brusetti@unibz.it

We studied the relationship between plant functional foliar traits and the endophytic bacterial communities associated in trees, taking the example of sessile oak (*Quercus petraea* (Matt.) Liebl). Forty-five samples with replicates of eight leaves per sample were collected in spring, summer and autumn. Bacterial community diversity was analyzed via Automated Ribosomal Intergenic Spacer Analysis (ARISA). The leaf traits specific leaf area, level of herbivory, stomatal number, stomatal length, carbon and nitrogen concentration were measured for the leaves of each sample. For statistical analysis, linear mixed effect models, the Canonical Correlation Analysis (CCA) and Non-Parametric Multivariate Analysis of Variance (NPMANOVA) were applied. Herbivory, nitrogen and carbon concentration were significantly different in autumn compared to spring and summer (p value < 0.05), while stomatal length was differentiated between spring and the other two seasons (p value < 0.01). The seasonal differentiation of the bacterial community structure was explained by the first and second axes (29.7 % and 25.3 %, respectively) in the CCA. The bacterial community structure significantly correlated with herbivory, nitrogen concentration and stomatal length. . We conclude that herbivory, nitrogen content, and size of stomatal aperture at the leaf level are important for endophyte colonization in oaks growth in alpine forest environments.

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4 Luigimaria Borruso[*], Camilla Wellstein[*], Alessia Bani, Sara Casagrande Bacchiocchi, Ania
5 Margoni, Rita Tonin, Stefan Zerbe and Lorenzo Brusetti
6
7 Faculty of Science and Technology, Free University of Bozen-Bolzano, Piazza Università 5, 39100
8 Bolzano, Italy.
9
10 * These authors contributed equally to this work.
11
12 Corresponding author
13 Lorenzo Brusetti, lorenzo.brusetti@unibz.it

15
16 Short title: Endophytes and foliar traits in oak

18   **ABSTRACT**

19   We studied the relationship between plant functional foliar traits and the endophytic bacterial

20   communities associated in trees, taking the example of sessile oak (*Quercus petraea* (Matt.) Liebl).

21   Forty-five samples with replicates of eight leaves per sample were collected in spring, summer and

22   autumn. Bacterial community diversity was analyzed via Automated Ribosomal Intergenic Spacer

23   Analysis (ARISA). The leaf traits specific leaf area, level of herbivory, stomatal number, stomatal

24   length, carbon and nitrogen concentration were measured for the leaves of each sample. For

25   statistical analysis, linear mixed effect models, the Canonical Correlation Analysis (CCA) and

26   Non-Parametric Multivariate Analysis of Variance (NPMANOVA) were applied. Herbivory,

27   nitrogen and carbon concentration were significantly different in autumn compared to spring and

28   summer (p value < 0.05), while stomatal length was differentiated between spring and the other

29   two seasons (p value < 0.01). The seasonal differentiation of the bacterial community structure

30   was explained by the first and second axes (29.7 % and 25.3 %, respectively) in the CCA. The

31   bacterial community structure significantly correlated with herbivory, nitrogen concentration and

32   stomatal length. . We conclude that herbivory, nitrogen content, and size of stomatal aperture at

33   the leaf level are important for endophyte colonization in oaks growth in alpine forest

34   environments.

35

36

37   **INTRODUCTION**

38   One of the major interfaces of biological interaction is between microbiota and plants. While many

39   integrative studies exist, regarding description of bacterial taxa related to host plants and linking

40   bacterial and plant communities across different spatial scales (e.g. symbiosis), temporal scales

41   remain less unexplored. Plant functional traits are increasingly used in ecological research and are

42   a promising avenue to link plant characteristics to environmental factor in interdisciplinary

43   researches (*Cornwell et al., 2008; Wellstein et al., 2011*). Leaf environment is characterized by

44   foliar functional traits that are hypothesized to affect the hosted microbiome. In the context of the

45   leaf environment of deciduous trees and inhabiting endophytic bacteria, intra-annual dynamics are

46   of special interest.

47   Endophytic bacteria are ubiquitous inhabitants that colonize the inner parts of most

48   terrestrial plant species beyond the epidermal cell layers (*Lodewyckx et al., 2015; Santoyo et al.,*

49   *2016*). Inside the plant, there are diverse ecological niches in which endophytic bacteria can

50   survive and grow, i.e. within cells, in the intercellular space and in the vascular systems (*Jacobs,*

51   *Bugbee & Gabrielson, 1985; Bell et al., 1995*). Endophytic bacteria are very important to the host

52   plant as they can contribute to the maintenance of its growth and health by, e.g. promoting nutrient

53   acquisition and defense against pathogens *(Hirano et al. 1982; Afzal, Khan & Sessitsch, 2014*).

54   This is particularly true for long-lived plant species such as trees and consequently they could

55   affects forest ecosystems (*Griffin & Carson, 2015; Griffin et al., 2016; Griffin et al., 2017; Tashi-*

56   *Oshnoei, Harighi & Abdollahzadeh, 2017*). Endophytic bacteria have often long-term ecological

57   interactions with the host plants including symbiosis, mutualism and commensalism. They can

58   either be obligate or facultative endophytes. Obligate endophytes are strictly associated with the

59   host plant and they are eventually transferred vertically through plant generations (*Santoyo et al.,*

60   *2016*). Facultative endophytes originate from the surrounding environment and they are often

61   included within epidermal cell layers (*Hardoim, van Overbeek & van Elsas, 2008*). More

62   complicate is the definition of endophytic pathogens, since historically endophytes have been

63   defined as non-harmful microorganisms (*Hallmann et al., 1997)*. Recently the advances of

64   molecular microbiology have shown the complex dynamics of pathogenesis possibly related to the

65   physiologic behavior of entire microbial communities, rather than of a single strain (*Fürnkranz et*

66   *al., 2012*; *Erlacher et al., 2014*). In this respect, harmful and beneficial endophytes could have in

67   common several mechanisms to colonize and diffuse into plant tissues (*Berg, Eberl & Hartmann,*

68   *2005*).

69   Different plant organs have diverse ways of colonization. Free-living soil microorganisms

70   colonize roots (*Bulgarelli et al., 2012; Edwards et al., 2015*). Leaf endophytic bacteria, especially

71   in case of tall trees, can be acquired from the leaf surface via stomata that represent apertures in

72   the foliar tissue connected to the intercellular space (*Ou et al., 2014; Carrell, Carper & Frank,*

73   *2016; Griffin & Carson 2015*). It has been hypothesized that leaves are a suitable surface for

74   exchange with bacteria inhabiting the atmosphere (*Bowers et al., 2009*). Microbial communities

75   inhabiting leaves, including endophytic bacteria, appear to be rather specialized, given that they

76   share less than 1% of the bacterial species with soil (*Kim et al., 2012*).

77   While numerous publications are focused on leaf epiphytes (*Hirano et al., 1982; Balint-*

78   *Kurti et al., 2010; Lopez-Velasco et al., 2011*), leaf endophytic bacteria remain largely unexplored.

79   Moreover, the possible role of plant functional traits for bacterial community dynamics represents

80 a research gap. In detail, there are a few studies regarding the temporal dynamic and the
81 environmental factors driving the endophytic bacterial communities associated with forest tree
82 species. Previous works revealed that endophytes are subject to leaf age and leaf developmental
83 stage in grapevine and in elm (*Mocali et al., 2003; Bulgari et al., 2014*). However, the potential
84 influencers of the endophytic bacteria composition behind leaf aging are still not well understood,
85 especially in forest plants. To the best of our knowledge, no papers have been published so far
86 about a possible linkage between endophytic community assemblages, seasonality and leaf plant
87 traits. Since it well known that stomata represents the main door for bacterial leaf colonization
88 (*Underwood, Melotto & He, 2007*), it is reasonable hypothesize that any changes of stomata
89 morphology due to leaf ageing could provoke consequences on the final endophytic community
90 composition. This could reflect the previous results of Mocali et al. (*2003*) and of Bulgari et al.
91 (*2014*).

92   In our study, we aimed to test (i) if there is a temporal gradient associating leaf aging with
93 bacterial turnover and (ii) if foliar plant traits are linked to bacterial community dynamics across
94 time. For this reason, we tested two hypotheses: (i) seasonality affects endophytic bacterial
95 community structure due to changes in foliar chemical composition, and (ii) there is a strict link
96 between endophytic bacterial community structure and foliar traits because some traits, such as
97 stomatal length, could favor the entering of bacterial cells into leaves. To assess the validity of our
98 hypotheses, we investigated a sessile oak forest located in the on Alps in the Northern Italy through
99 an entire growing season.

100

101 **MATERIALS AND METHODS**
102 **Study site and sampling**
103 The study area is located in the Monticolo nature reserve on the hillslopes of the Mitterberg at 550
104 m a.s.l. in South Tyrol, Italy. The selected study site is representative of the present oak forest,
105 dominated by sessile oak (*Quercus petraea* (Matt.) Liebl.) with few specimens of Scots pine (*Pinus*
106 *sylvestris* L.) in the tree layer as well as of Sweet chestnut (*Castanea sativa* Mill.) and Manna ash
107 (*Fraxinus ornus* L.) in the understory. The forest grows on acidic shallow soil above porphyry
108 bedrock on a west-south-west oriented slope. We selected five individuals of sessile oak within
109 the study site, i.e. a circular plot of 15 m radius (706 m²) representing relatively homogeneous site
110 conditions within the slope. From each tree, we selected three branches taking eight leaves from

111 the same branch, which were used to assess the endophytic bacteria as well as the foliar functional

112 traits and herbivory. From a previous study, it was clear which types of herbivory were generally

113 present in the study species (*Q. petraea*) in this forest. Based on Labandeira et al. (2007) seven

114 types of herbivory were found across oaks of the forest. In the present study, we considered only

115 the most frequent herbivory types that would not affect the functional traits' analysis, i.e. margin

116 and hole feeding and sucking Labandeira et al. (2007).

117 Branches were chosen to have the maximum distance between them, i.e. an angle of 120°C

118 between two adjacent branches. In detail, we used three leaves for the functional traits and

119 herbivory measurements and five leaves for the determination of the endophytic bacterial

120 microbial community. We sampled three subsequent seasons in the year 2014, i.e. spring (June

121 5th), summer (August 25th) and autumn (October 20th). A total of 120 leaves per season was

122 collected, 45 leaves were used for the analysis of functional traits while 75 for the analysis of

123 endophytes.

124   We measured six functional traits related to important plant functions, i.e. specific leaf area

125 (SLA), leaf nitrogen content (N), leaf carbon content (C), C:N ratio, stomatal number (STNR) and

126 stomatal length (SL). SL is a measure for the size of stomata (*Taiz & Zeiger, 2002*). For each

127 season, we determined the SLA of the leaves following standard protocols (*Pérez-Harguindeguy*

128 *et al., 2013*). For each leaf, the area was measured the sampling day using a scanner (CanoScan

129 Lide, Canon, Cernusco sul Naviglio, Italy). Subsequently, leaves were oven dried at 70°C for 72

130 hours to obtain their dry weight and the SLA, measured in $mm^2\ mg^{-1}$, was calculated (*Pérez-*

131 *Harguindeguy et al., 2013*). N and C were determined using an elemental analyzer (Flash 2000

132 Organic Elemental Analyzer, Thermo Scientific, Milan, Italy) pooling together the three leaves of

133 each branch.

134   To measure stomatal characteristics, we applied the clear nail polish method described by

135 Hilu & Randall (1984) obtaining epidermal impressions of the abaxial surface of each leaves that

136 were examined under an optical microscope (Leica DMLS, Leica Biosystems, Nussloch,

137 Germany) connected to a digital camera. The images were analyzed through the image processing

138 software DeltaPix InSight, (DeltaPix, Smorum, Denmark). The stomata were counted on three

139 fields of view per leaf on a standard counting area at $400 \times$ magnification to determine the stomatal

140 density as number of stomata (STNR) for each standard counting area. On each counting area, the

141 length of the guard cells of stomata (SL) was measured for 15 randomly selected stomata.

142   For the measurement of herbivory, the percentage of consumed leaf area was measured
143   scanning the leaf surface (CanoScan LiDE 120, Canon) and calculating the lost area using free
144   software ImageJ (https://imagej.net). Because the great majority of the leaves presented a mixture
145   of the investigated herbivory mediated damage types, for each leaf we quantified the total surface
146   damage caused by the summed herbivory damage types.

147

148   **DNA extraction and Automated Ribosomal Intergenic Spacer Analysis (ARISA)**
149   Leaves used for microbiological analysis were processed within 4 hours as follows (Hrynkiewicz
150   K., personal communication): Five leaves for each replicate were disinfected with 70% ethanol
151   twice each for 3 min. Leaves were then washed with sodium hypochlorite (1.5%) and TWEEN®
152   20 for ten minutes, three rinses in sterile, distilled shaking water. Disinfected leaves were grinded
153   to a fine powder under liquid nitrogen using a sterile mortar and pestle. The disinfected samples
154   were stored at -20°C. Triplicates of the water used in the last rise were used as negative for PCR
155   amplification and plated on a LB and TSA medium to verify the disinfection protocol. Absence of
156   PCR amplification products was observed. Furthermore, absence of bacterial colonies was
157   observed in all the plates after 10 days of incubation at 30°C.

158   DNA was extracted using the Qiagen DNeasy PowerPlant Pro Kit (Qiagen, Milan, Italy)
159   accordingly to the user's manual. Extracted DNA was stored at -80°C. The quality and the size of
160   the soil DNA were checked by electrophoresis on 1.2% agarose gel with a marker (Eurogentec
161   Smart Ladder, Belgio). The absorbance (260 nm) of 2 μl of DNA was used to evaluate the
162   concentration of DNA by NanoVue Spectrophotometer (GE Healthcare, Little Chalfont, UK).

163   The 16S-23S rRNA Internal Transcribed Spacer (ITS)-PCR was performed using the
164   primers ITSF and ITSReub labeled with 6-FAM according to the chemical and thermal
165   amplification protocol of Cardinale et al. (*2004*). Capillary electrophoresis was done by STAB
166   Vida Lda. (Caparica, Portugal). Data were investigated via Peak Scanner Software 1.0 (Applied
167   Biosystems, Monza, Italy) and the downstream matrix was normalized and analyzed according to
168   Borruso, Zerbe & Brusetti (*2015*).

169

170   **Data analysis**
171   PAST software (*Hammer, Harper & Ryan, 2001*) was used for the statistical analysis. ANOVA
172   was used to test for differences in the endophytic bacterial richness between seasons. Canonical

173 correspondence analysis (CCA) of the endophytic microbial community structure in dependence

174 of functional leaf traits and season was performed. Non-Parametric Multivariate Analysis of

175 Variance (NPMANOVA) with Bonferroni corrected p-value was applied to investigate differences

176 among the endophytic bacterial communities across the three seasons using Bray-Curtis

177 dissimilarity distance.

178 Linear discriminant analysis (LDA) effect size (LEfSe) algorithm was used to identify taxa

179 preferentially abundant in each season using default parameters. Briefly, the algorithm identifies

180 the indicator bacterial taxa specialized within the 3 seasons (*Segata et al.,* 2011).

181 Given the nested design of the experiment, the variation of leaf functional traits across the

182 three seasons was investigated applying linear mixed effect models in R (R Development Core

183 Team 2014 version 3.1.2), using *nlme* package (*Pinheiro et al., 2017*). For SLA, SL, STNR, N, C,

184 C:N and herbivory we analyzed each trait as response variable, season as fixed variable and, as

185 random factor, we nested the branches from which we sampled the leaves in the respective trees.

186 We log transformed the data that did not satisfied the assumption of variance normality tested with

187 Shapiro test.

188

189 RESULTS

190 Functional leaf traits and insect-mediated damage types

191 Figure 1 and Table 1 shows the results of functional leaf traits investigated in this study across the

192 three sampling seasons. Leaf N and C were significantly lower in autumn than in spring and

193 summer ($p < 0.01$). The C:N ratio and the level of herbivory were significantly higher in autumn

194 than in spring and summer ($p < 0.01$). SL was significantly higher in spring compared to summer

195 and autumn ($p < 0.01$). . STNR and SLA did not show significant differences across the seasons

196 ($p > 0.05$).

197

198 Bacterial community structure

199 An average of 82 ± 15 peaks per sample representing bacterial richness, ranging from 200 bp to

200 1200 bp, were found. No significant results in terms of number of peaks across the three seasons

201 were found (spring 82 ± 9; summer 86 ± 19 and autumn 79 ± 14; ANOVA p-value: n.s.).

202 NPANOVA showed significant differences between the bacterial community structure of spring

203 and of autumn ($p < 0.001$), while the bacterial community structures of summer did not cluster

204   apart representing a bridge between the two seasons (Table 2). Scattered peaks in between the

205   range 550-850 bp were mostly found in spring and autumn, causing the separation between these

206   two seasons. The LDA showed that 13 ARISA peaks were responsible of the discrimination of

207   spring with respect to the other seasons, 14 peaks were typically discriminant for summer, and 9

208   for autumn. Most of those peaks were situated in the 550-880 bp slot, confirming the NPANOVA

209   results (Table 3).

210

211   **Canonical correspondence analysis (CCA)**

212   Canonical correspondence analysis (CCA) was used to investigate the effects of functional leaf

213   traits and the level of herbivory on endophytic bacterial communities across the seasons.

214   Differentiation is illustrated by the first and second axes in the CCA (29.7% and 25.3%,

215   respectively) and the leaf features fitted into the CCA. The CCA ordination diagram (Figure 2)

216   revealed first, that community structure variation appeared along season (temporal sequence) and,

217   second, the existence of relationships between plant foliar traits and endofoliar microbiota across

218   the temporal sequence. In detail, the community variation is related mainly to the level of

219   herbivory, N and SL, and less to STNR, C and SLA (length of vectors in the ordination diagram;

220   Figure 2).

221

222   **DISCUSSION**

223   We explored the seasonal diversity behavior via fingerprinting ARISA of the of leaf endophytic

224   bacterial communities. ARISA is a corroborate technique used to investigate bacterial structure

225   variations and the correlations with environmental parameters (*Esposito et al., 2013; Borruso,*

226   *Zerbe & Brusetti 2015; Pioli et al., 2018*) with a comparable robustness as Next Generation

227   Sequencing (*van Dorst et al., 2014*). Differently by other fingerprinting techniques such as the

228   Length Heterogeneity-PCR or the Denaturing Gradient Gel Electrophoresis of the partial 16S

229   rRNA genes, ARISA can investigate the bacterial community at a deeper taxonomic resolution.

230   Actually, ARISA can reach the subspecies level (*Danovaro et al., 2006*), via detection of the length

231   polymorphisms of the internal 16S-23S ribosomal DNA spacers within the several copies of

232   ribosomal operons in a bacterial cell (typically from 1 to 10 copies; *Gürtler, 1999*). Although the

233   endophytic bacterial communities did not show significant differences in alpha diversity across

234   the three seasons, their beta diversity differed mainly between spring and autumn (Table 2 and

235 Figure 2). These results support the idea of an intimate association between endophytes and the
236 leaf, seen as a dynamic micro-ecosystem that selects for different specific microbial communities
237 along time. Regarding which taxa contribute to the observed difference between seasons, even if
238 the attribution of single ARISA peaks to specific taxa cannot be conclusive due to the real
239 possibility that a peak could be represented by several taxa from different phyla, a putative raw
240 taxon attribution could be done. For instance, according to some authors, many Gram negative
241 bacterial species harbor ITS with tRNA genes (*Triplett et al., 1999; Ranjard et al., 2000*). Their
242 spacers usually range between 500 and 800 bp (*Gürtler & Stanisich, 1996*). On the other side Gram
243 positive bacteria harbor shorter ITS, while rhizobia have very long spacers, often longer than 1000
244 bp (*Gürtler & Stanisich, 1996*). The length range between 500 and 600 bp is what had been
245 observed in our case differentiating spring to autumn. We could hypothesize that seasons have an
246 effect on the diversity of Gram negative bacteria. A different answer of Gram negative bacteria
247 rather than Gram positive bacteria due to different environmental pressures had already been
248 observed in other plant-related compartments, such as rhizosphere (*Ciccazzo et al., 2014*).

249   Leaves have been traditionally considered as "short-lived environment", where specialized
250 bacteria can dynamically colonize new niches and leave others according to the leaf continuous
251 modifications over seasons (*Vorholt, 2012*). Bulgari et al. (*2014*) hypothesized that the endophytic
252 communities in *Vitis vinifera* should remain stable across the seasons in absence of bacterial plant
253 pathogens such as phytoplasma. However, this is in contrast with our findings and those of other
254 researchers. Influence of the seasonality on endophytic microbial community composition
255 associated with different tree species (i.e. *Acer negundo*, *Ulmus pumila*, and *U. parvifolia*) were
256 also found by Shen & Fulthorpe (*2015*). Moreover, others observed that the bacterial community
257 composition in the phyllosphere was primarily driven by temporal changes and community
258 succession (*Copeland et al., 2015*). In order to shed light on the processes behind community
259 changes, we explored the role of leaf functional traits in bacterial structure dynamics. In fact,
260 previous studies investigated the effect of migration and community succession in the phyllosphere
261 microbiome and suggested that colonization, persistence, and succession of the community may
262 be key-factors driving the phyllosphere microbiome (*Redford & Fierer, 2009; Shade, McManus*
263 *& Handelsman, 2013; Maignien et al., 2014; Copeland et al., 2015*). The relevance of
264 environmental conditions such as temperature optima of the bacteria or the changing physiology
265 of tree host species have been discussed for their possible effects (*Jansson & Douglas, 2007*).

However, previous studies did not look deeper into possible correlations in terms of plant functional characteristics behind the observed dynamical processes of the microbiome.

Given that we found a clear endophytic seasonal variation, it is highly interesting to understand better the potential leaf-level characteristics possibly associated with bacteria community compositional variation. In fact, based on our results, we suggest that foliar characteristics related to leaf-level herbivory, nutrient contents and stomatal length aperture may affect directly the bacterial community composition over time.

As a matter of fact, herbivory could be an effective way to inoculate microbial insects symbionts, commensals and pathogens into plant tissues. It is the case, for instance, of phytoplasmas inoculated into grapevine by Hemiptera-like leafhoppers (Gonellaet al., 2008; *Alma et al., 2018*). In our forest plots, several herbivory traces have been recorded during the experiments. In general, the majority of insect species causes single distinct damages on leaves (Labandeira et al., 2007). From a survey on *Quercus petraea*'s leaves in the same forest, the herbivory damage types were recorded by using three leaves per individual with a total amount of 180 plant individuals. Seven types of damages were recorded: Margin feeding (detected in the 90% of the observed leaves), surface feeding (61%), hole feeding (45%), sucking (31%), skeletonization (12%), mining (12%) and leaf rolling (9%). This number of herbivory damage types justifies the perception of a relatively high insect diversity since it has been shown that there is a quantitative relation between the richness of damage types and the insect species richness (*Carvalho et al., 2015*). Phloem-feeding insects could act as inoculating vectors of entire bacterial communities between different plant individuals, moving bacterial strains from a tree to another (*Lòpez-Fernàndez et al., 2017*). It is reasonable to hypothesize that, as insect abundance and diversity may change due to season variation (*Grimbacher et al., 2018*), also the endophytic bacterial communities potentially transmissible among tree individuals change, hence contributing to our observed results.

In addition, stomata are the major door for the leaf colonization by foliar bacterial pathogens (*Underwood, Melotto & He, 2007; Melotto,Underwood & He , 2008*). To counteract the entrance of potential pathogens, plants have evolved a number of mechanisms to detect and remove pathogenic bacterial cells from their tissues, regulating to some extent the access of bacteria through stomata (*Gimenez-Ibanez et al., 2017*). This is due to the ability of plants in detecting specific molecular signals such as bacterial lipopolysaccharides, flagellins or elongation

297   factors (*Underwood, Melotto & He, 2007*). Consequently, plants close the majority of their leaf
298   stomata. However, bacteria may overcome this mechanism of defense, entering into the leaf
299   intercellular spaces by taking advantage of those stomatal guard cells that are not able to react to
300   the presence of bacterial signals (*Underwood, Melotto & He, 2007*). We observed that SL could
301   be associated with the shaping of endophytic community structure, especially in spring (Fig. 2).
302   This observation could mirror the entrance of specific as well as unspecific bacterial strains into
303   leaves when the leaves were growing. Actually, Gailing et al (2008) found that in *Quercus robur*
304   the variability of stomatal number is genetically determined. Additionally, Turner & Heichel
305   (1977) demonstrated for *Quercus rubra* that SL reaches it maximum before leaves developed their
306   maximum areas in late spring. It is plausible that bacteria enter into the leaf as soon as it has
307   flashed, and then a sort of successional dynamic is established until reaching an equilibrium once
308   the stomata shape are fixed. The difference in SL between spring leaves from summer and autumn
309   leaves may depend by the contraction of the leaf pool available for measurements caused by the
310   loss of the leaves that occur both naturally during the vegetative season (*Brooke et al., 1996*) and
311   because of the detected herbivory.

312   Finally, leaf nutrient contents and their changes correlate with bacteria dynamics (*Kembel
313   et al.,* 2014). It has been shown that endophytic bacterial taxa able to fix nitrogen occur in oak
314   species (*Tashi-Oshnoei, Harighi & Abdollahzadeh, 2017*), in the wild poplar *Populus trichocarpa*
315   leaves (*Doty et al.,* 2016), and in *Pinus flexilis* needles (*Moyes et al., 2016*), helping plants
316   establishment and growth in N-limited environment. Eventually, a drop in leaf nitrogen content
317   could challenge bacterial shifts towards nitrogen-fixing taxa and in contrast to denitrifying
318   bacteria.

319   Our results of diminishing nutrients such as C and N with leaf aging is in line with other
320   studies (*Li et al., 2017*), while other functional traits related to the stomata, such as STNR and
321   SLA did not vary significantly along season. Another study on leaf traits of seven different woody
322   species grown under experimental conditions shed light on their seasonal variation (*Römermann
323   et al., 2016*). The results of this study highlight that SLA and stomatal size were robust traits across
324   season in terms of small intraspecific variation. In comparison, our species *Q. petraea* also has
325   stable STNR and SLA levels. However, as explained above, the changes in SL most likely reflect
326   changes in the leaves' pool of the forest as the leaves' pool was diminished by herbivory and
327   browsing that led to leaf loss after the spring season. Moreover, herbivory, that increases over

328 season due to elongated exposure time, can have a direct impact on endophytic bacteria as well as
329 an indirect effect by influencing other leaf characteristics. For example, the open structures of the
330 leaf, limited to the size and number of stomata in intact leaves, are largely modified by herbivory
331 that exposes further leaf tissue. Due to the increasing rate of changes that can be assumed with
332 aging (*Suzuki et al.,* 1987; *Chavana-Bryant et al.,* 2017), we expect that leaf characteristics exert
333 a differential impact during aging on the bacterial community.

334

335 **Conclusions**

336 Based on our findings, we suggest that herbivory, nitrogen content, and size of stomatal
337 aperture at the leaf level are important for endophyte colonization in oaks growth in alpine forest
338 environments. We argued that herbivory and stomata length are important doors from where
339 bacteria enter to colonize the leaf. As possible consequence, the endophytic community
340 assemblages switch during the progression of seasons, when the stomatal length increases during
341 the leaf germination and elongation, and when the chemical characteristics of the leaf are different
342 from those in autumn.

343

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347

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561

562 **Figure and Tables captions**

563

564 **Figure 1** Differences of foliar traits (N = nitrogen, C = carbon, C:N = carbon/nitrogen ratio, SLA=

565 specific leaf area, SL = stomatal length, STNR = stomatal number per reference area, HERB =

566 level of herbivory) among three seasons (spring, summer, autumn). Significant differences

567 according to linear mixed effect models followed by post-hoc test are indicated by different lower

568 case letters. Graphics without letters were not significant. Detailed results of linear mixed effect

569 models are given in Table 3.

570

571 **Figure 2** CCA analysis of endophytic communities across a temporal sequence (spring: blue dots;

572 summer: green dots; autumn: red dots) and plant foliar traits. CCA was calculated with the

573 following plant foliar traits: HERB = level of herbivory; STNR = number of stomata; SLA =

574 specific leaf area; SL = length of stomata; N = leaf nitrogen content; C = leaf carbon content.

575

576 **Table 1** Results of linear mixed effect models for each trait . A: leaf nitrogen content; B: leaf

577 carbon content; C: C:N ratio; D: specific leaf area (SLA); E: stomatal length (SL); F: stomatal

578 number /STNR): G: herbivory level. Each single trait was analyzed as response variable, season

579 as fixed variable and branches nested in the respective trees as random variable. The basic level

580 (intercept) corresponds to the spring season.

581

582 **Table 2** *P* value results from Non-Parametric MANOVA (NPMANOVA) with Bonferroni

583 corrected *p* value among endophytic bacterial communities across the three seasons (Bray-Curtis

584 dissimilarity).

585

586 **Table 3** OTU biomarkers characterizing each single season on the basis of the Linear Discriminant

587 Analysis effect size.

Figure 1

Differences of foliar traits among the three analyzed seasons

Differences of foliar traits (N = nitrogen, C = carbon, C:N = carbon/nitrogen ratio, SLA= specific leaf area, SL = stomatal length, STNR = stomatal number per reference area, HERB = level of herbivory) among three seasons (spring, summer, autumn). Significant differences according to linear mixed effect models followed by post-hoc test are indicated by different lower case letters. Graphics without letters were not significant. Detailed results of linear mixed effect models are given in Table 3.

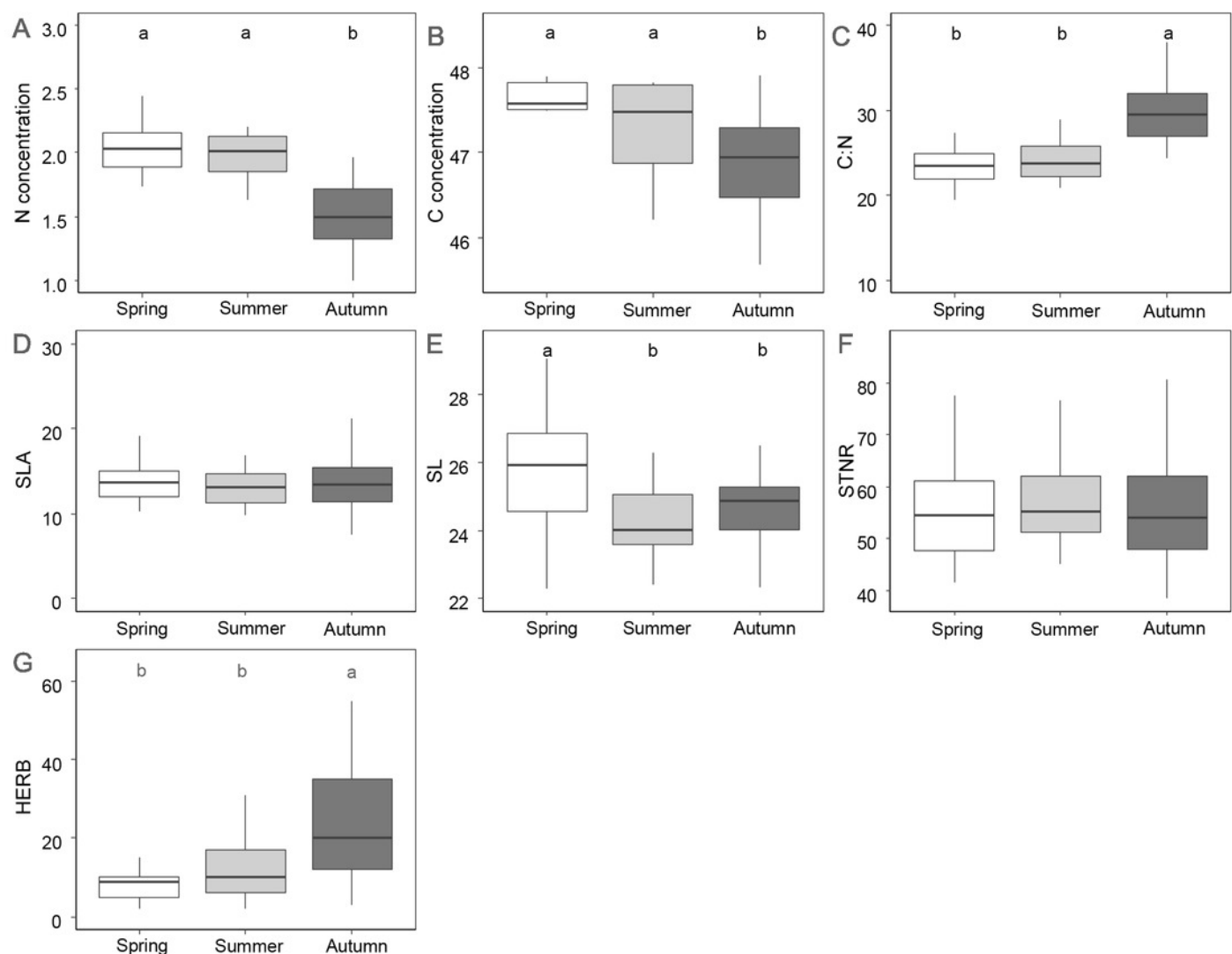
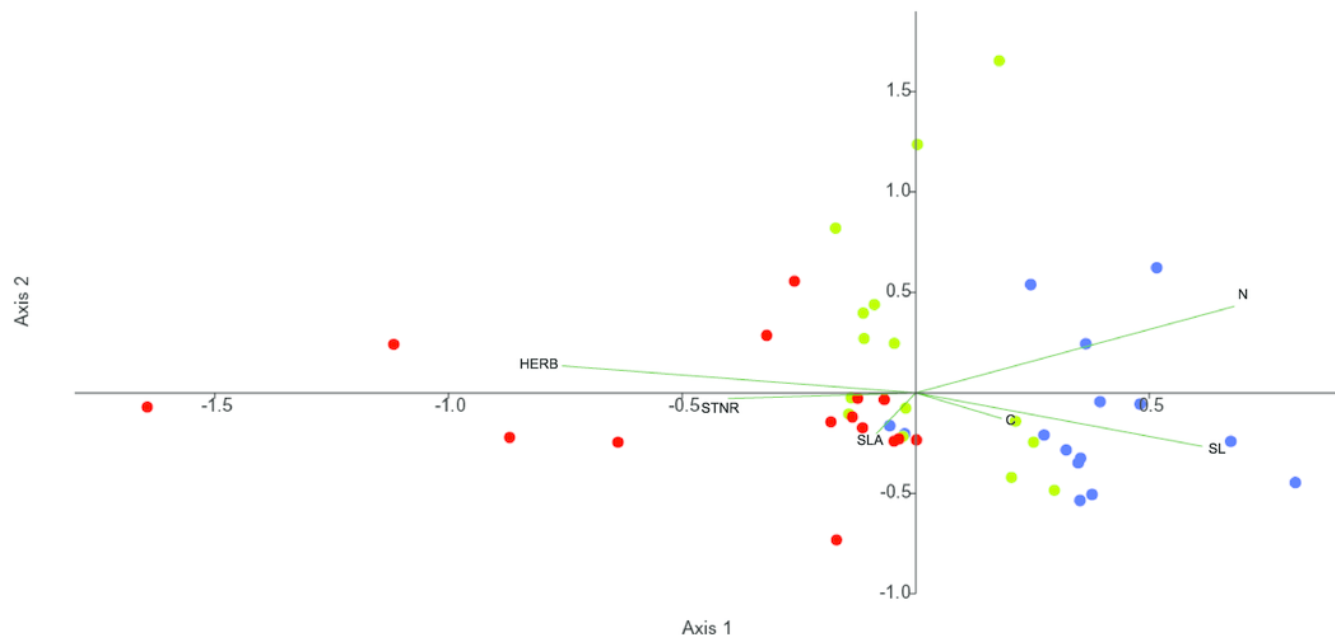



Figure 2

CCA analysis of endophytic communities across the three seasons

CCA analysis of endophytic communities across a temporal sequence (spring: blue dots; summer: green dots; autumn: red dots) and plant foliar traits. CCA was calculated with the following plant foliar traits: HERB = level of herbivory; STNR = number of stomata; SLA = specific leaf area; SL = length of stomata; N = leaf nitrogen content; C = leaf carbon content.

NPMANOVA of the endophytic community structures

P value results from Non-Parametric MANOVA (NPMANOVA) with Bonferroni corrected *p* value among endophytic bacterial communities across the three seasons (Bray-Curtis dissimilarity).

1 **Table 1** Results of linear mixed effect models for each trait (N = leaf nitrogen content, C = leaf

2 carbon content, C:N = C:N ratio, SLA = specific leaf area, SL = stomatal length, STNR = stomatal

3 number, HERB = herbivory level). Each single trait was analyzed as response variable, season as

4 fixed variable and branches nested in the respective trees as random variable. The basic level

5 (intercept) corresponds to the spring season.

Trait	Fixed effect	Value	Std.Error	DF	t-value	p-value
N	(Intercept)	2.04	0.07	28	30.67	0.00
	Summer	-0.07	0.09	28	-0.85	0.41
	Autumn	-0.57	0.09	28	-6.51	0.00
log(C)	(Intercept)	3.86	0.00	28	882.56	0.00
	Summer	-0.01	0.00	28	-1.68	0.10
	Autumn	-0.02	0.00	28	-4.90	0.00
log(C:N)	(Intercept)	3.16	0.05	28	69.09	0.00
	Summer	0.03	0.06	28	0.54	0.59
	Autumn	0.33	0.06	28	5.82	0.00
SLA	(Intercept)	13.98	0.99	28	14.05	0.00
	Summer	-0.84	1.26	28	-0.66	0.51
	Autumn	0.97	1.26	28	0.77	0.45
SL	(Intercept)	25.69	0.58	28	44.00	0.00
	Summer	-1.66	0.31	28	-5.44	0.00
	Autumn	-1.49	0.31	28	-4.87	0.00
STNR	(Intercept)	55.48	3.87	28	14.35	0.00
	Summer	2.37	1.72	28	1.38	0.18
	Autumn	0.81	1.72	28	0.47	0.64
log(HERB)	(Intercept)	2.08	0.13	28	16.29	0.00
	Summer	0.39	0.17	28	2.30	0.03
	Autumn	0.88	0.17	28	5.16	0.00

Linear mixed effect models for each leaf trait

Results of linear mixed effect models for each trait (N = leaf nitrogen content, C = leaf carbon content, C:N = C:N ratio, SLA = specific leaf area, SL = stomatal length, STNR = stomatal number, HERB = herbivory level). Each single trait was analyzed as response variable, season as fixed variable and branches nested in the respective trees as random variable. The basic level (intercept) corresponds to the spring season.

1   **Table 2** *P* value results from Non-Parametric MANOVA (NPMANOVA) with Bonferroni

2   corrected *p* value among endophytic bacterial communities across the three seasons (Bray-Curtis

3   dissimilarity).

Seasons	Summer	Autumn
Spring	0.0804	0.0009
Summer	/	0.2484

LEfSe analysis showing the main OTU biomarkers characterizing each single season.

OTU biomarkers characterizing each single season on the basis of the Linear Discriminant Analysis effect size.

1 **Table 3** OTU biomarkers characterizing each single season on the basis of the Linear Discriminant

2 Analysis effect size.

OTU	Season	LDA-score	p-value
OTU620	Autumn	3.22	0.033
OTU660	Autumn	3.51	0.016
OTU713	Autumn	3.26	0.043
OTU833	Autumn	2.93	0.043
OTU582	Autumn	4.05	0.000
OTU600	Autumn	3.24	0.035
OTU1160	Autumn	2.83	0.039
OTU640	Autumn	3.09	0.034
OTU850	Autumn	3.06	0.035
OTU222	Spring	4.38	0.000
OTU454	Spring	4.00	0.000
OTU671	Spring	3.07	0.043
OTU530	Spring	3.15	0.004
OTU286	Spring	3.18	0.007
OTU361	Spring	3.90	0.000
OTU213	Spring	2.95	0.014
OTU547	Spring	3.65	0.043
OTU238	Spring	3.68	0.050
OTU383	Spring	3.23	0.043
OTU474	Spring	3.68	0.043
OTU654	Spring	4.36	0.004
OTU519	Spring	3.79	0.024
OTU350	Summer	3.08	0.008
OTU575	Summer	3.27	0.042
OTU573	Summer	3.54	0.030
OTU316	Summer	2.98	0.014
OTU842	Summer	2.67	0.043
OTU598	Summer	3.13	0.015
OTU662	Summer	3.16	0.030
OTU666	Summer	2.87	0.014
OTU529	Summer	3.17	0.004
OTU995	Summer	2.91	0.014
OTU727	Summer	2.79	0.043
OTU1119	Summer	2.70	0.014
OTU564	Summer	2.79	0.043
OTU634	Summer	2.82	0.043