Previous legume identity influences wheat protein content through organic matter depolymerization

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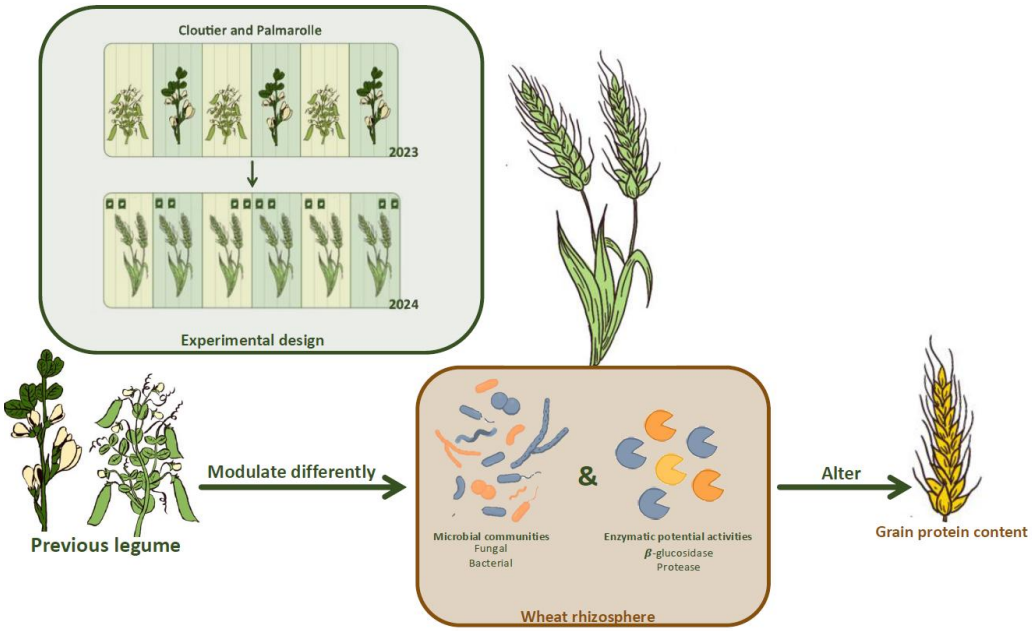
23 **Highlights**

24 - Previous crop identity distinctively shaped wheat rhizosphere microbial
25 communities
26 - Previous legume altered the wheat rhizosphere microbial depolymerization
27 capacity
28 - Wheat grain protein content was higher following faba bean than peas
29

30 Graphical Abstract



31

Abstract

In crop rotation systems, plant-soil feedback (PSF) effects can modulate nutrient cycling in soil, like organic nitrogen cycling. Organic nitrogen (N) is present in plant and microbial necromass, and it must be depolymerized by microbes to become available for crops. Since plant identity can modulate the microbial communities' diversity and composition, we thought that previous crop identity would also change the microbial depolymerization capacity, and thereby impact soil N availability, and the quality and yield of the following crop. To test this, two legumes (*Vicia faba* L., i.e. faba bean, and *Pisum sativum* L., i.e., yellow pea) were grown in two fields in Abitibi-Témiscamingue, Québec, Canada. During the following growing season, spring wheat (*Triticum aestivum* L.) was sown and received, or not poultry manure as an organic fertilizer. We determined the diversity and composition of the microbial communities and their enzymatic depolymerization capacity in the soil and the rhizosphere each growing season. During wheat growth, previous legumes shaped bacterial (p-value = 0.006) and fungal (p-value = 0.001) communities and modulated the enzymatic activity of wheat-associated rhizosphere microbes. Faba bean increased β-glucosidase activities by up to 13% (1.13-fold) in the wheat rhizosphere compared to peas. Faba bean as a previous crop also increased wheat grain protein content at harvest by up to 0.17% as compared to peas. Altogether, our results show that faba beans, through lasting shifts in the microbial communities, can enhance microbial depolymerization activities in the wheat rhizosphere, impacting its N nutrition. Understanding plant-soil feedback in crop rotation systems is crucial to improve our practices and sustainably meet crops' nutritional needs.

Key words

Plant-Soil feedback, Microbial communities, Rhizosphere, Nitrogen, Depolymerization, crop rotation, Grain quality,

57 1. Introduction

58 Agricultural practices have a significant impact on belowground microbial communities,
59 altering their functionality, community structure, and diversity (Calderon et al., 2001; Li
60 et al., 2020; Zhao et al., 2020). Monocropping often leads to lower soil microbial diversity
61 and decreased plant growth and yield compared to crop rotation systems (Bak et al.,
62 2022; Adebayo et al., 2025). Crop rotation systems (i.e., growing different crop species
63 sequentially on the same land) enhance soil nutrient content and nutrient cycling (Wang
64 et al., 2023b), limit the establishment of plant pathogens (Zhou et al., 2023) and enhance
65 soil health and microbial diversity (Yang et al., 2024). Crop rotation systems are therefore
66 fundamental in organic farming to maximize yield without the use of synthetic chemical
67 inputs (Gamage et al., 2023).

68

69 The benefits of crop rotation systems can be explained by positive plant-soil feedback
70 (PSF). The previous crop modulates soil microbial communities and physicochemical
71 properties, impacting the next crop (Mariotte et al., 2018; Wang et al., 2025). Indeed,
72 plants release a myriad of compounds, shaping spatially and temporally distinct microbial
73 communities (Zhalnina et al., 2018). Positive plant-soil feedback includes pathogen
74 reduction and beneficial microbes' enrichment (Ma et al., 2017; Hannula et al., 2021).
75 *Arabidopsis thaliana* changes its exudation pattern in response to the pathogenic
76 bacterium *P. syringae* (Yuan et al., 2018). After six passages in the same soil, these
77 changes created soil microbial communities antagonist to the pathogen, resulting in
78 healthy plants (Yuan et al., 2018). Due to highly diversified plant traits, functions and

79 exudation patterns, PSF effects are species-specific, meaning that we cannot associate a
80 PSF effect with a plant family (Hannula et al., 2020). Plant-soil feedbacks are also not
81 limited to the original plant species and can affect other plants grown in the same soil,
82 creating multiple possibilities for crop rotation systems (Murrell et al., 2020). For instance,
83 winter wheat (*Triticum aestivum* L.) grown continuously had a lower yield compared to
84 winter wheat growing after oilseed rape, because oilseed rape changed the microbial
85 communities and the abundances of functional genes related to inorganic N-cycling
86 (Kaloterakis et al., 2025). This plant-specific rhizospheric effect can persist for many
87 growing seasons (Bay et al., 2021).
88
89 Besides changing the microbial community structure and abundance of specific genes,
90 crops also affect more generally the carbon (C) and nitrogen (N) cycles (Knops et al.,
91 2002). Plant-Soil feedback effects on nutrient cycling can be attributed to both living and
92 dead crops. Plant litter origin (above vs belowground) and chemistry mediate the
93 decomposition rate (Freschet et al., 2013), and microbial decomposer abundance and
94 diversity over time (Bhatnagar et al., 2018). A classic use of PSF in agricultural settings is
95 legume-cereal rotations. Legumes are well known to enhance soil N through biological
96 fixation, and using them in crop rotation systems replenishes soil N without using
97 chemical fertilizers (Gan et al., 2015). But not all legumes are identical. For instance, faba
98 bean (*Vicia faba* L.) fixed 185 kg N ha^{-1} and forage peas (*Pisum sativum* L.) 165 kg ha^{-1},
99 resulting in different amounts of C and N in their residues (Lupwayi and Soon, 2015).
100 Living plants also influence microbial decomposer communities and their functionality

101 through their C-rich root exudates. Since litter and root exudates composition differ
102 among plant species, different crops in rotation systems will not have the same effect on
103 soil nutrient cycling and on the next crop grown (Huo et al., 2017). Therefore, the choice
104 of rotation systems design, especially the choice of crop species, is crucial to optimize PSF
105 effects associated with nutrient cycling. The mechanistic link between shifts in microbial
106 communities caused by the crop's identity, soil nutrients availability, and the following
107 crop's growth and nutrition is, however, not clear, especially in organic agroecosystems.
108 One possible explanation could be changes in organic N depolymerization.
109
110 Organic N depolymerization is a limiting step of the enzymatic cascade leading to
111 mineralization and nitrification, producing inorganic N available for crops (Jan et al.,
112 2009). The input of C-rich compounds, from plant litter and/or root exudates, steers up
113 the C: N ratio, forcing microbes to mine for nitrogen (Jones et al., 2009; Kuzyakov and Xu,
114 2013) by depolymerizing soil organic matter (SOM) containing organic N, such as protein,
115 chitin and amino acid (Zhu et al., 2014). When their elemental demand is met, microbes
116 will mineralize the excess N into inorganic form (ammonium and nitrate), usable by
117 plants. Not to mention that small organic N forms (e.g. amino acids) are also usable by
118 plants, highlighting the importance of N depolymerization for crop nutrition (Näsholm et
119 al., 1998). Extracellular enzymes produced by microbial decomposers mediate soil N
120 depolymerization. Microbes will release extracellular enzymes in soil, and their enzymatic
121 activities will be induced by the presence of their substrate (Geisseler and Horwath,
122 2008). Nitrogen release from depolymerization depends on the microbial decomposer

123 communities, soil physicochemical properties, and the organic substrate availability
124 (Séneca et al., 2021), all of which are influenced by the identity of the plant and the crop
125 rotation system. Increased N depolymerization may be one of the mechanisms
126 responsible for the benefits of rotation systems. Yet, to our knowledge, few studies have
127 tested that hypothesis under organic production conditions.
128
129 Here, we hypothesize (H1) that peas and faba beans, two legumes, have different lasting
130 influences on the microbial communities' diversity and composition, changing the
131 microbial depolymerization capacity, and thereby impacting soil inorganic N availability
132 and quality and yield of wheat grown the following season. We also hypothesize (H2) that
133 the addition of fertilizer would lessen the previous crop's impact on wheat. To test these
134 hypotheses, we grew wheat with and without organic fertilizer in plots where peas or
135 faba beans had been previously grown, under production conditions in two different
136 fields in the Abitibi-Témiscamingue region (Québec Canada).
137
138

2. Materials and methods

2.1 Experimental design

We conducted a two-year field experiment in Abitibi-Témiscamingue (Québec Canada) on two farms under actual farm operating conditions, one in Palmarolle (48.665°N, 79.201°W) and in Cloutier (48.00297°N, 79.15°W). Both sites were grassland previously cropped to perennial forages for at least three years before our experiment. Palmarolle soil texture is clay with 80, 355 and 565 g kg^{-1} of sand (2000–50 µm), silt (50-20 µm) and clay (<2 µm) particles at 0-15 cm depth, respectively. It belongs to the Gleysolic order according to the World Reference Base for Soil Resource system (WRB, 2007) and the Canadian System of Soil Classification (Group et al., 1998). Cloutier soil texture is a silty clay with 38, 481 and 520 g kg^{-1} of sand, silt and clay particles at 0-15 cm depth, respectively, and also belongs to the Gleysolic order (Group et al., 1998) (WRB, 2007).

In fall 2022, both sites were moldboard plowed to a depth of 17 cm. In spring 2023, successive passes with a disk harrow and a field cultivator were carried out to prepare the seedbed. Faba beans and peas were sown in 2023, using the producer's equipment (Table 1). Three blocks were split into two, allowing four plots of the same legume to be next to each other, facilitating seeding. Thus, eight plots per block were sown, for a total of 24 plots in each field (3 blocks with each 4 faba bean plots and 4 pea plots). Plots in Palmarolle were 11 m wide by 100 m long, while those in Cloutier were 4 m wide by 120 m long. In Palmarolle, faba bean was buried at the end of the growing season, while pea was harvested. In Cloutier, both crops were harvested at the end of the season (Table 1).

160 The following year (2024), the same plots were divided into two to test the effect of
161 organic fertilization on wheat. Twelve plots (4 plots/block; two plots that received faba
162 beans and two plots that received peas in 2023) received the equivalent of 0.5 Mg ha^{-1}
163 and 1 Mg ha^{-1} of granulated chicken manure (N:5-P:3-K:2) for Palmarolle and Cloutier
164 fields, respectively. Wheat was sown on the same day as the fertilization occurred (i.e.,
165 May 30th at Palmarolle and June 04 at Cloutier). At the end of the growing season, wheat
166 was sampled and harvested (Table 1).

167 Table 1: Sowing, Harvesting and sampling dates for Cloutier et Palmarolle in 2023 and 2024

Field	Crops	Sowing date	Harvesting date	Beginning of the season soil sampling date	Mid-growing season soil sampling date
Cloutier	Faba beans	2023-06-07	2023-10-17	2023-06-06	2023-08-01
	Peas		2023-09-27		
	Wheat	2024-06-04	2024-09-12	2024-05-23	2024-07-31
Palmarolle	Faba beans	2023-05-21	Burried	2023-05-21	2023-07-31
	Peas		2023-09-18		
	Wheat	2024-05-30	2024-09-04	2024-05-21	2024-07-30

168

169 2.2 Soil sampling

170 Every year, we sampled twice during the season, in the beginning and mid-growing season
171 (Table 1). Wheat was approximately in the tillering phase when we sampled. We sampled
172 five points per plot (one in the center and four in the corners, at approximately 0.5 m
173 from the plot borders), and we combined all samples into a composite, creating one
174 sample for every 24 plots. From the five points, we sampled the bulk soil by collecting soil
175 (0-15cm) between rows. When present (mid-growing season), we sampled the
176 rhizosphere of the main crops (faba bean, peas or wheat) by gently removing plants from

177 the same five points, shaking the roots to remove the excess soil and considering the
178 remaining soil attached to the rhizosphere (i.e., the soil influenced by plant roots). Soil
179 samples were kept in a cooler until they were brought back to the laboratory. Samples
180 were kept at 4 °C before sieving and aliquoting. We separated the rhizospheric soil from
181 the roots before sieving. We sieved (4mm) rhizospheric and bulk soil, and we prepared
182 two aliquots for each sample: one stored at 4 °C for enzymatic assays and
183 ammonium/nitrate quantification, and another stored at -20 °C for DNA extraction.
184
185 2.3 Wheat sampling
186 At the end of the growing season, we sampled wheat to determine yield and protein
187 content. Each plot was subsampled at four randomly distributed locations within the plot.
188 At each location, we sampled a quadrat of wheat by placing two 76 cm long half-frames
189 on the soil at about 45 cm width from each other, including 5 wheat rows (approximately
190 0.342 m^2). At each subsample point, we measured the internal width between half-frames
191 to calculate precisely the sampling area, and we harvested wheat. We stored wheat in
192 paper bags and brought it back to the laboratory. Wheat was then dried at 35 °C for
193 several days and kept for further analysis.
194
195 2.4 DNA extraction and sequencing
196 We extracted soil DNA from 0.25g of bulk and rhizosphere soil (previously sieved at 4mm)
197 samples using the DNeasy PowerLyzer power soil kit (Qiagen) following the
198 manufacturer's protocol. DNA was eluted in 50 μL of elution buffer and was stored at

199 −20°C until further analyses. We prepared libraries for 16S and ITS amplicon sequencing
200 according to Illumina's "16S Metagenomic Sequencing Library Preparation" guide
201 (Part#15044223Rev.B). We prepared libraries for the bacterial 16 S rRNA gene using
202 primers 16S: 515Y and 926R, and for the fungal ITS1 region, we used the primers ITS1F
203 and 58A2R. NextSeq (Illumina) sequencing (PE300 10M reads) was performed at the
204 *Centre d'expertise et de service Génome Québec* (CESGQ, Montréal, Canada).
205
206 2.5 Enzymatic potential activities quantification
207 We quantified the potential activity of three exoenzymes involved in SOM
208 depolymerization (deaminases, β-glucosidases and proteases) using colorimetric
209 methods. Every protocol follows the same principle: soil samples were incubated with an
210 excess of substrate under optimal conditions. Therefore, the result is the potential
211 activity, meaning that they do not reflect real-time activity in the soil.
212
213 2.5.1 Protease activity
214 To quantify the potential activity of soil protease, we used a protocol adapted from Jesmin
215 et al. (2022) (Jesmin et al., 2022). Briefly, we incubated 1g of dried sieved (4mm) soil with
216 2.5 mL sodium caseinate (2%) and 2.5 mL Tris buffer for two hours at 50°C and 100 rpm.
217 After incubation, we added 5mL Trichloroacetic acid (1 g L^{-1}) to stop the enzymatic
218 reaction, and we used 0.75mL Na_2CO_3 and 0.25 mL Folin & Ciocalteu's phenol (1:3) to
219 quantify tyrosine, the product that produces a blue colour. We used 500 μL of every
220 sample to measure the absorbance at 650-700 nm using a TECAN Infinite M plate reader.

221 The potential protease activity is expressed in µmol tyrosine release g^{-1} dry soil h^{-1}
222 incubation and was quantified using a tyrosine standard curve (2-fold serial dilution from
223 1.0 to 0.005 mM).
224
225 2.5.2 β-glucosidases activity
226 To quantify the potential activity of soil β-glucosidase, we used a protocol adapted from
227 Eivazi and Tabatabai (1988) (Eivazi and Tabatabai, 1988). Briefly, we incubated 1g of dried
228 sieved (4mm) soil with a buffer solution containing p-nitrophenyl glycoside (25mM) at
229 37°C for 1 h. After incubation, we added calcium chloride (0.5M) and Tris buffer (0.1M,
230 pH 12) to extract p-nitrophenol, resulting from the degradation of the substrate.
231 Extraction of the product produces a yellow colour, and we measured absorbance at 415
232 nm using a TECAN Infinite M plate reader. The potential activity is expressed in µmol p-
233 nitrophenol released g^{-1} dry soil h^{-1} incubation and was quantified using a p-nitrophenol
234 standard curve (2-fold serial dilution from 0.5 to 0.0078 g L^{-1}).
235
236 2.5.3 Deaminase activity
237 To quantify the potential activity of soil deaminases, we used a protocol adapted from
238 Killham and Rashid (1986) (Killham and Rashid, 1986). Briefly, we incubated 1g of dried
239 sieved (4mm) soil with 1.2-diamino-4-nitrobenzene (0.06g L^{-1}) for 20h at 25°C, 100 rpm.
240 The substrate is red, and the colour intensity decreases with the deamination of the
241 benzene ring. After incubation, we extracted twice the remaining substrate using
242 methanol 100% and filtered (Whatman no1) the solution to remove any impurities. We

243 measured the absorbance at 405 nm using a TECAN Infinite M plate reader. The potential
244 activity is expressed in and was quantified using a 1.2-diamino-4-itrobenzene standard
245 curve (0/0.008/0.011/0.014/0.019/0.025/0.034/0.045/0.06 g L^{-1}).
246
247 2.6 Available inorganic nitrogen quantification
248 To quantify ammonium and nitrate, we first needed to extract nitrogen from the soil,
249 using a protocol adapted from Maynard et al. (1993) (Maynard et al., 1993) .
250 To do so, we incubated 1 g of dried sieved (4mm) soil with 10 mL of KCl (2M) for 1h at 25
251 °C at 100 rpm. After incubation, we filtered (Whatman no. 42) the soil slurry to remove
252 any soil and debris. Soil extracts were kept at 4°C until further analyses.
253
254 2.6.1 Ammonium quantification
255 To quantify ammonium in soil extracts, we adapted a protocol from Rhine et al. (1998)
256 (Rhine et al., 1998). Briefly, we added 100 µL of the sample (standard and/or soil extract)
257 to a 96-well plate. Using a multi-channel pipette, we added 50 µL of citrate reagent, 50
258 µL of phenylphenol nitroprusside, 25 µL of buffered hypochlorite reagent and 50 µL of
259 deionized water to each well and incubated 45 minutes at room temperature. Based on
260 the Berthelot method, ammonium in soil extracts reacts with the buffered hypochlorite
261 solution and the phenylphenol nitroprusside, producing a blue product. After incubation,
262 we measured the absorbance at 660 nm using a TECAN Infinite M plate reader and the
263 concentration of ammonium is expressed in mg g^{-1} of air-dried soil and quantified using a
264 $(NH_4)_2SO_4$ standard curve (2-fold serial dilution from 20 to 0.0195 mg L^{-1}).

265

266 2.6.2 Nitrate quantification

267 To quantify nitrate in soil extracts, we adapted a protocol from Hood-Nowotny et al.
268 (2010) (Hood-Nowotny et al., 2010). Briefly, we added 100 µL of the sample (standard
269 and/or soil extract) to a 96-well plate. Using a multi-channel pipette, we added 100 µL of
270 VCl_3 solution and 100 µL of the Griess reagent mix (a solution containing 1:1 Griess
271 solution 1 and Griess solution 2) to each well, and we incubated the plate for 1h at 37°C.
272 Based on the VCL_3/Griess method, nitrate in soil extracts is converted to nitrite with VCL_3,
273 and nitrite reacts with both Griess solutions to produce a pink color. After incubation, we
274 measured absorbance at 540 nm using a TECAN Infinite M plate reader and the
275 concentration of nitrate is expressed in mg g^{-1} of soil and quantified with a standard curve
276 of KNO_3 (2-fold serial dilution from 10 to 0.0195 mg L^{-1}).

277

278 2.7 Wheat grain yield and protein content

279 At the end of the 2024 growing season, wheat was hand harvested (see section 2.3) to be
280 threshed using a laboratory thresher (DL-350, Wintersteiger, Ried im Innkreis,
281 Austria)..Grains were then cleaned with a sample cleaner (MNL Pfeuffer, Kitzingen,
282 Germany). Grain yield (Y_{FH}, kg ha^{-1}) was determined at the subsample level by weighing
283 clean grain harvested for the given surface area sampled at each subsampling point (see
284 section 2.3). Grain protein content (%) was analyzed using a Near-infrared spectrometer
285 (Infratec 1241, FOSS, Hillerød, Denmark), and wheat yield was reported on a 13.5%
286 relative humidity content ($Y_{13.5}$, kg ha^{-1}) as follows: $Y_{13.5} = (Y_{FH} \times (100 - W_{FH})) / (100 - 15)$.

287 We used $Y_{13.5}$ for our statistical analyses. We determined yield and protein content for
288 each subsample harvested in the field (four subsamples), and we used the mean of the
289 subsamples to perform our statistical analyses.
290
291 2.8 Bioinformatic analyses
292 We analyzed amplicon sequencing data (16S rRNA gene and ITS region) using the DADA2
293 pipeline in R to process raw reads. For 16S, we used truncLen (280,250) and maxEE (2,2)
294 to filter and trim forward and reverse reads. We used the pooled method for removing
295 chimeras, and we assigned taxonomy with the SILVA reference database. After the
296 assignation, we removed chloroplasts and mitochondria from our dataset before further
297 analysis. For ITS, we used truncLen (270,250) and maxEE (2,2) to filter and trim forward
298 and reverse reads. We used the pooled method for removing chimeras, and we assigned
299 taxonomy with the UNITE reference database.
300
301 2.9 Statistical analysis and data visualization
302 We performed Statistical analyses in R (version 4.5.1, *The R Foundation for Statistical*
303 *Computing),* and we created figures using the package ggplot2. We divided and
304 performed statistical analyses for each field (i.e., Cloutier and Palmarolle) separately. For
305 testing the effect of the previous legume crop (i.e., pea and faba bean), organic
306 fertilization (i.e., with or without manure), soil compartment (i.e., rhizosphere and bulk
307 soil) and block on potential enzymatic activities, soil ammonium and nitrate content and,
308 wheat yield ($Y_{13.5}$, kg ha^{-1}) and protein content, we performed analysis of variance

309 (ANOVA) when the assumptions were met. If they were not met, we performed a Kruskal-
310 Wallis non-parametric test, testing each factor independently. To validate ANOVA
311 assumptions, we performed Shapiro-Wilk tests to check the normality of the residuals
312 and Bartlett tests to check the homogeneity of variances.
313 To find out if there was any significant correlation between enzymatic activities, soil N
314 content and wheat yield and protein content, we performed Spearman correlations for
315 every sampling time (see supplementary material) and soil compartment.
316 We performed Kruskal-Wallis non-parametric tests to determine the effect of previous
317 crop, organic fertilization, soil compartment and block on Alpha diversity and relative
318 abundance data. To analyze the bacterial community structure, we first transformed our
319 dataset using the Hellinger transformation. We then used Euclidean distance matrices
320 based on our transformed data to visualize the community structure using a principal
321 coordinate analysis (PCoA). We tested the effect of the previous crop, organic fertilization
322 and soil compartment on the community using PERMANOVA (1000 permutations).
323
324 2.10 Data availability
325 The R code used to analyze the data and generate the figures is available on GitHub
326 (https://github.com/le-labo-vergeau/). Raw sequencing reads were deposited in the NCBI
327 SRA repository under BioProject accession PRJNA1390138.
328

329 3. Results

330 We focused our analysis on the mid-growing season samples (2024) to assess previous
331 legume crops' PSF effects, except when it is mentioned.

332

333 3.1 Previous crop PSF on microbial communities' composition

334 3.1.1 Cloutier 2024

335 The bacterial and fungal community compositions of wheat plots differed significantly
336 depending on the previous legume sown in 2023 and the addition of organic fertilization
337 (i.e. manure) in 2024 (Fig. 1.A and 1.C ; Table S.1 and S.2). For the bacterial community
338 (Fig. 1.A), there was also a significant interaction between the previous crop and the
339 fertilization, meaning that depending on the previous crop sown, fertilization's effect
340 varied. In Figure 1.A, we can see that the addition of manure increases the variability of
341 the bacterial community composition, especially when the previous crop was peas. For
342 the fungal community (Fig. 1.B), there was no interaction between the effect of the
343 previous crop and the organic fertilization. The soil compartment (rhizosphere and bulk
344 soil) modulated the fungal communities' composition (Fig. 1.B), but not the bacterial
345 communities.

346

347 3.1.2 Palmarolle 2024

348 The bacterial and fungal community composition of wheat plots is significantly different
349 depending on the previous crop sown in 2023 and the soil compartment (Fig. 1.C and
350 1.D; Table S.1 and S.2). There is also a significant effect ($p = 0.043$) of the interaction

351 between the previous crop and the fertilization on the fungal communities' composition
352 in Palmarolle (Fig. 1.D). Like the bacterial community composition in Cloutier,
353 fertilization makes the fungal community composition more variable (Fig. 1.C).
354
355 [Insert Figure 1 here]
356
357 3.2 Potential Enzymatic activities associated with depolymerization
358 3.2.1 Cloutier 2024
359 During wheat growth, there was significantly more β-glucosidase and deaminase
360 potential activities in the wheat rhizosphere than in the bulk soil (Fig. 2.B and 2.C; Table
361 2). The potential β-glucosidase activity in wheat plots was higher after faba bean than
362 after peas. The addition of organic fertilizer at the beginning of the season did not affect
363 enzymatic activities during wheat growth.
364
365 3.2.2 Palmarolle 2024
366 During wheat growth, there was significantly more protease and β-glucosidase potential
367 activities in the wheat rhizosphere than in the bulk soil (Fig. 2.D and 2.E; Table 2). After
368 faba bean, wheat plots had more protease and β-glucosidase activities than after peas.
369 The addition of organic fertilization at the beginning of the season enhanced the
370 deaminase activity mid-growing season.
371
372 [Insert Figure 2 here]

Table 2: Effects of previous crop, organic fertilization and soil compartments (rhizospheric vs. bulk soil) on proteases, β -glucosidases and deaminases potential activities at 0-15 cm depth in Cloutier and Palmarolle during the wheat mid-season (2024).

	Test	Response variable	Factor/Term	Statistic	df	p-value	
Cloutier	Kruskal	Protease activity	Previous crop	0.409	1	0.5227	
			Compartment	2.929	1	0.0870	
			Fertilization	1.582	1	0.2085	
			Block	2.089	2	0.3518	
	Kruskal	Deaminase activity	Previous crop	0.823	1	0.3643	
			Compartment	**4.423**	**1**	**0.0354**	*
			Fertilization	0.786	1	0.3753	
			Block	4.353	2	0.1135	
	ANOVA	β - glucosidase activity	**Previous crop**	**3.616**	**1**	**0.0648**	.
			Compartment	**78.663**	**1**	**8.59E-11**	***
			Fertilization	0.104	1	0.7486	
			Block	1.438	2	0.2499	
			Previous crop :Compartment	1.344	1	0.5610	
			Previous crop :Fertilization	1.173	1	0.2855	
			Compartment :Fertilization	0.291	1	0.5929	
			Previous crop :Compartment :Fertilization	0.084	1	0.7729	
	ANOVA	Protease activity	**Previous crop**	**8.802**	**1**	**0.00518**	**
			Compartment	**26.272**	**1**	**8.96E-06**	***
			Fertilization	1.064	1	0.30872	
			Block	**3.040**	**2**	**0.05963**	.
			Previous crop :Compartment	0.048	1	0.82787	
			Previous crop :Fertilization	1.247	1	0.27105	
			Compartment :Fertilization	0.007	1	0.93608	
			Previouscrop :Compartment :Fertilization	1.811	1	0.18639	
Palmarolle	ANOVA	β - glucosidase activity	**Previous crop**	**4.105**	**1**	**0.0498**	*
			Compartment	**7.023**	**1**	**0.0117**	*
			Fertilization	0.252	1	0.6185	
			Block	**3.943**	**2**	**0.0278**	*
			Previous crop :Compartment	0.778	1	0.3833	
			Previous crop :Fertilization	0.399	1	0.5313	
			Compartment :Fertilization	0.004	1	0.9516	
			Previouscrop :Compartment :Fertilization	1.316	1	0.2585	
	ANOVA	Deaminase activity	Previous crop	1.661	1	0.2057	
			Compartment	0.033	1	0.8565	
			Fertilization	**4.814**	**1**	**0.0348**	*
			Block	**4.001**	**2**	**0.0270**	*
			Previous crop :Compartment	0.000	1	0.9903	
			Previous crop:Fertilization	0.151	1	0.7001	
			Compartment :Fertilization	0.613	1	0.4387	
			Previouscrop :Compartment :Fertilization	1.266	1	0.2680	

.: almost significant; *: p<0.05; **p<0.01; ***:p<0.001

Factor/Term, Statistic, df and p-value in bold are the significant and/or almost significant values

3.3 Soil inorganic N content

380 Figure 3 shows the amount of inorganic N in the bulk soil and the rhizosphere of wheat,
381 at mid-growing season in 2024. In the wheat rhizospheric soil at Cloutier, there is
382 significantly less inorganic N in ammonium and nitrate forms than in the bulk soil (Fig. 3.A
383 and 3.B; Table 3). In the wheat rhizospheric soil at Palmarolle, there is significantly less
384 nitrate, but more ammonium than in the bulk soil (Fig. 3.C and 3.D; Table 3). Also, in
385 Palmarolle, there was less nitrate in wheat plots after faba beans than after peas,
386 regardless of the compartment (Fig. 3.D).
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388 [Insert Figure 3 here]
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Table 3: Effects of previous crop, organic fertilization and soil compartments (rhizospheric vs. bulk soil) on ammonium and nitrate content at 0-15 cm depth in Cloutier and Palmarolle during wheat growth (2024).

Test	Response variable	Factor/Term	Statistic	df	p-value	
Kruskal	Cloutier Nitrate content	Previous crop	2.102	1	0.1471	
		Compartment	**20.576**	**1**	**5.73E-06**	***
		Fertilization	1.345	1	0.2461	
		Block	1.839	2	0.3987	
Kruskal	Cloutier Ammonium content	Previous crop	3.072	1	0.0797	
		Compartment	**6.225**	**1**	**0.0126**	*
		Fertilization	0.000	1	0.9835	
		Block	2.141	2	0.3428	
ANOVA	Palmarolle Nitrate content	**Previous crop**	**6.492**	**1**	**0.0150**	*
		Compartment	**32.354**	**1**	**1.53E-06**	***
		Fertilization	0.053	1	0.8200	
		Block	**2.958**	**2**	**0.0640**	.
		Previous crop :Compartment	0.104	1	0.7480	
		Previous crop :Fertilization	1.053	1	0.3110	
		Compartment :Fertilization	1.152	1	0.2900	
		Previouscrop :Compartment :Fertilization	0.627	1	0.4330	
ANOVA	Palmarolle Ammonium content	Previous crop	0.978	1	0.3290	
		Compartment	**14.457**	**1**	**0.0005**	***
		Fertilization	**4.570**	**1**	**0.0390**	*
		Block	2.442	2	0.1005	
		Previous crop :Compartment	0.023	1	0.8797	
		Previous crop :Fertilization	0.059	1	0.8096	
		Compartment :Fertilization	0.416	1	0.5230	
		Previouscrop :Compartment :Fertilization	0.763	1	0.3877	

.: almost significant; *: p<0.05; **p<0.01; ***:p<0.001

Factor/Term, Statistic, df and p-value in bold are the significant and/or almost significant values

3.4 Wheat yield and grain quality

3.4.1 Cloutier 2024

The addition of organic fertilizer significantly enhanced wheat grain protein content (Fig. 4: Table 4). Also, faba bean, when used as the previous crop, significantly increased wheat

grain protein content as compared to peas (Fig. 4). Figure 5 shows the correlation between wheat yield, protein content, enzymatic activities, and N content in the bulk soil (Fig. 5.A) and rhizospheric soil (Fig. 5.B). We found a significant positive correlation between bulk soil ammonium content at mid-season and wheat yield (Rho = 0.4513, p-value = 0.0269). We also found a significant positive correlation between bulk soil nitrate content at mid-season and grain protein content (Rho = 0.503, p-value = 0.0121), but a significant negative correlation between wheat yield and rhizospheric soil nitrate content (Rho = -0.4487, p-value = 0.0279).

[Insert Figure 4 here]

[Insert Figure 5 here]

Table 4: Kruskal-Wallis results for the effect of organic fertilization and previous crops on grain protein content and yield ($Y_{13.5}$) in Cloutier

Response variable	Factor	Statistic	p-value	
Yield(kg ha^{-1})	Fertilization	0.093	0.7609	
	Previous crop	1.245	0.2645	
	Block	**10.619**	**0.0050**	**
Protein content (%)	**Fertilization**	**14.087**	**0.0002**	***
	Previous crop	**5.927**	**0.0149**	*
	Block	**10.048**	**0.0066**	**

*: p<0.05; **p<0.01; ***:p<0.001

Factor/Term, Statistic, df and p-value in bold are the significant values

3.4.2 Palmarolle 2024

For Palmarolle, we were only able to determine the grain yield at the end of the growing season. We found no significant effect of fertilization and the previous crop on grain yield (Table 5). There was also no significant correlation between the potential enzymatic activities, N content in the rhizosphere and the bulk soil, and wheat yield (Fig. 6). However, when we looked at the enzymatic activities at the beginning of the season, we found a significant positive correlation between the β-glucosidase activity and grain yield (Rho = 0.543, p-value = 0.00606) (Fig. 7).

[Insert Figure 6 here]

[Insert Figure 7 here]

Table 5: Kruskal-Wallis results for the effect of organic fertilization and previous crops on grain yield ($Y_{13.5}$) in Palmarolle

Response variable	Factor	Statistic	p-value
Yield (kg ha-1)	Fertilization	0.257	0.6120
	Previous crop	0.833	0.3613
	Block	0.247	0.8838

*: p<0.05; **p<0.01; ***:p<0.001

Factor/Term, Statistic, df and p-value in bold are the significant values

4. Discussion

This study aimed to evaluate the impact of the identity of the previous legume crop on microbial communities' composition and on its depolymerization capacity in the following growing season. We hypothesized that a part of the positive legume-cereal rotation effect on wheat yield and grain quality could be mediated by changes in the depolymerization capacity of the microbial community. Lastly, we also hypothesized that the addition of fertilizer would lessen the previous crop's impact. First, we confirmed that legume identity modifies soil microbial communities in the following growing season, and that PSF effects can be transferred to other plant species (in our case, from legumes to wheat). Second, we showed that this multi-year PSF effect can directly alter the depolymerization capacity of the wheat rhizospheric microbial communities, modulating enzymatic capacities associated with depolymerization. Lastly, we found that the identity of previous legumes altered wheat grain protein content, a key parameter of grain baking quality (Nuttall et al., 2017). This suggests that this persistent shift in depolymerization capacity is one of the mechanisms that can explain the beneficial effects of crop rotation systems. Under real farming conditions in fields located 100 km from each other, we confirmed that the previous crops' PSF influenced the microbial communities' composition. In fact, among all factors tested, the previous crop was the only one that consistently shaped microbial communities in both fields.

Faba beans and peas are two legumes used in crop rotation systems to replenish soil N pools. Both crops fix atmospheric N through a symbiosis with rhizobia (Jensen et al.,

466 2010). Their PSF effects on microbial communities' composition were, however, distinct,
467 supporting other findings (Yixuan et al., 2025). It could be explained by the fact that faba
468 beans have a coarser root system (i.e., higher mean root diameter and lower specific root
469 length) and allocate a higher proportion of total plant biomass belowground than pea
470 (Guerrero-Ramírez et al., 2021), therefore adding more organic matter to the soil
471 (Nuruzzaman et al., 2006). Differences in root system sizes can also alter soil physical
472 properties, therefore changing microbial communities. With a root system four times
473 bigger than peas, faba beans promote aggregate formation, creating different
474 microenvironments for microbes in the next growing season (Oliveira et al., 2019).
475
476 Fertilization is known to alter soil properties and microbial communities (Luo et al., 2018).
477 Therefore, variability in microbial communities created by organic fertilization in our
478 study is not surprising. Interestingly, microbial communities resulting from previous crops
479 did not respond the same way to organic fertilization in both fields. It is important to
480 mention that the organic fertilizer amount was not the same in both fields, which could
481 in part explain the difference between the fields (Zhou et al., 2021). Nevertheless, organic
482 fertilization increased the variability of microbial communities coming from peas. It
483 suggests that faba beans have stronger PSF effects, creating microbial communities more
484 resistant to the modification induced by organic fertilization (Shade, 2023). The
485 resistance of the faba bean microbial communities towards organic fertilization input
486 could result from the abovementioned larger addition of root-derived organic matter,
487 priming the microbial community to mineralize organic fertilizer (Aschi et al., 2017).

488

489 We showed that faba bean and peas differently altered the wheat rhizospheric microbial
490 depolymerization capacity in the following growing season, providing a potential
491 mechanism to link the shifts in microbial community to the beneficial effect of crop
492 rotation. This suggests that previous legumes selected differently for microorganisms
493 with a role in the organic N cycle, which persisted among the wheat rhizospheric microbial
494 communities. The PSF effect was not found across both fields for every exoenzyme tested,
495 except for the β-glucosidase potential activity. It can be due to differences in pedoclimatic
496 conditions, which alter the soil physico-chemical properties across both fields (A'Bear et
497 al., 2014). However, in most instances, enzymatic activities are higher in wheat plots after
498 faba beans than after peas. It can be explained, as mentioned above, by the size of the
499 plant itself (above and below ground), since faba beans have a bigger root system than
500 peas (Nuruzzaman et al., 2006), leading to different amounts of litter addition. It is also
501 important to mention that the faba bean was entirely buried in the Palmarolle field at the
502 end of the 2023 growing season. In contrast, faba bean in Cloutier and peas in Cloutier
503 and Palmarolle were harvested. The addition of the totality of aboveground plant material
504 in the soil may have stimulated the microbial community to produce exoenzymes, like
505 proteases, to depolymerize this nutrient source (Freschet et al., 2013). In other words, we
506 found that the identity of the previous legume crops directly modulates enzymatic
507 activities related to depolymerization, providing a mechanistic link between crop rotation
508 system benefits, microbial community composition and functionality.

509

510 Almost every exoenzyme's potential activities were higher in the wheat rhizosphere than
511 in the bulk soil, suggesting that wheat, through its root exudates, stimulate for a
512 community proficient in depolymerization. Our results align with the rhizosphere priming
513 theory, a plant competitive strategy for N (Kuzyakov and Xu, 2013; L'Espérance et al.,
514 2024). Plants change the rhizosphere C: N ratio through their C-rich exudates to force
515 microbes to depolymerize soil organic matter to scavenge for N. When the C-rich
516 exudates are all consumed, microbes will mineralize the N excess, providing inorganic N
517 to plants (Zhu et al., 2014).
518
519 Unlike previous studies (Li et al., 2015; Lupwayi et al., 2019), the addition of organic
520 fertilizer did not have a significant effect on the enzymatic activities, except for the
521 deaminase activity in Palmarolle. This is surprising, since we added a substrate that can
522 stimulate microbes to produce and release exoenzymes. In contrast, Breza et al., (2023)
523 found that the addition of granular urea in more complex rotation systems disturbs the
524 organic N cycle, reducing the depolymerization rate to a similar rate as monocropping. In
525 both cases, fertilization affects exoenzyme activities mediating depolymerization (Breza
526 et al., 2023). In our study, the lack of fertilization effects is perhaps due to the sampling
527 date. We decided to determine enzymatic activities at mid-growing season,
528 approximately two months after the application of organic fertilizer. Organic fertilizer
529 depends on the soil mineralization rate and usually takes longer to be available for plants
530 and microbes (Wang et al., 2023a). Lazicki et al., (2020) showed that poultry manure-
531 based granulated fertilizer was already mineralized after a few weeks of incubation.

532 Therefore, nutrients derived from organic fertilizer were probably already immobilized in
533 microbial or wheat biomass in our study, since we sampled about two months after
534 fertilizer application.
535
536 Without a significant increase in soil inorganic N content, grain protein content was higher
537 after faba bean compared to peas, with and without the addition of organic fertilizer. It
538 was already known that faba bean, through N-fixation, is particularly efficient in
539 increasing soil N for the following crop (Herridge et al., 2008; Peoples et al., 2009). Here,
540 we showed that changes in the microbial community and their depolymerization capacity
541 could also play a role. Indeed, we hypothesized that an enhanced depolymerization
542 capacity would increase the amount of soil ammonium and nitrate. This part of our
543 hypothesis was disproved, since we did not find an increase in soil inorganic N in the faba
544 bean plots, where depolymerization capacity was the highest. It suggests that the
545 enhanced wheat grain quality in the faba bean plots is more likely due to a continuous
546 uptake of the N mineralized by the microbial community or the plant. Alternatively, the
547 N from the faba bean could have already been taken up by the wheat early in the season
548 and remobilized during grain filling. In wheat, the N assimilated by the plant is mostly
549 taken up before and during the anthesis stage (Cox et al., 1985; Sharma et al., 2023). In
550 our case, wheat could have taken up N during and before our mid-season sampling,
551 potentially explaining the better protein content at the end of the season.
552
553

5. Conclusion

In conclusion, we showed that the two legumes tested modulated the microbial community composition differently, resulting in different microbial depolymerization capacity. In comparison to wheat plots after peas, wheat plots after faba bean had a higher microbial depolymerization capacity, resulting in an enhanced wheat grain protein content. Taken together, our results suggest a mechanistic link between shifts in the composition and potential activities of microbial communities and the beneficial effects of crop rotation on wheat nutrition. Understanding plant-soil feedback in crop rotation systems is essential to improve our practices and sustainably meet crops' nutritional needs.

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754 Figure captions

755 Figure 1: Beta diversity of the bacterial (16S) and fungal communities (ITS) from
756 rhizosphere and bulk soil under wheat cropping in Cloutier (A, B) and Palmarolle (C, D) in
757 2024 using a Euclidean distance matrix and principal coordinate analysis. The effect of the
758 previous crop (color) and organic fertilization (form and ellipse) is presented. The R^2 and
759 p-value correspond to the PERMANOVA results for these variables and the compartment
760 (i.e., rhizospheric and bulk soil) variable.

761

762 Figure 2: Protease, β-glucosidase and deaminases potential activities in the rhizosphere
763 and bulk soil at 0-15 cm depth under wheat cropping in Cloutier (A, B, C) and Palmarolle
764 (D, E, F) mid-growing season in 2024. The effects of the soil compartment, the previous
765 crop, and fertilization are represented. The organic fertilization is represented by the
766 mean and standard deviation of both compartments and the previous crop. Significant
767 results from Kruskal-Wallis or ANOVA tests for each enzyme's potential activities (see
768 Table 1) are identified with a *($p<0.05$).

769

770 Figure 3: Wheat rhizosphere and bulk soil ammonium and nitrate content mid-growing
771 season in Cloutier (A, B) and Palmarolle (C, D) at 0-15 cm depth. The effects of the
772 compartment, the previous crop, and fertilization are represented. The fertilization is
773 shown by the mean and standard deviation of both compartments and previous crops.
774 Significant results from Kruskal-Wallis or ANOVA tests for ammonium and nitrate content
775 (see Table 2) are identified with a *($p<0.05$).

776

777 Figure 4: Wheat grain protein content of each organic fertilization treatment and previous
778 crops after harvest in Cloutier. The effects of the previous crop and fertilization are
779 represented. Significant results from Kruskal-Wallis tests (see Table 3) are identified with
780 a *($p<0.05$).

781

782 Figure 5: Heatmap of Spearman correlations between wheat grain yield, protein content,
783 and protease, β-glucosidases, deaminases potential activities, ammonium and nitrate
784 content in wheat rhizosphere (A) and bulk soil (B) in Cloutier. The circle's size represents
785 the Spearman correlation coefficient (Rho). Significant results are identified with a
786 *($p<0.05$).

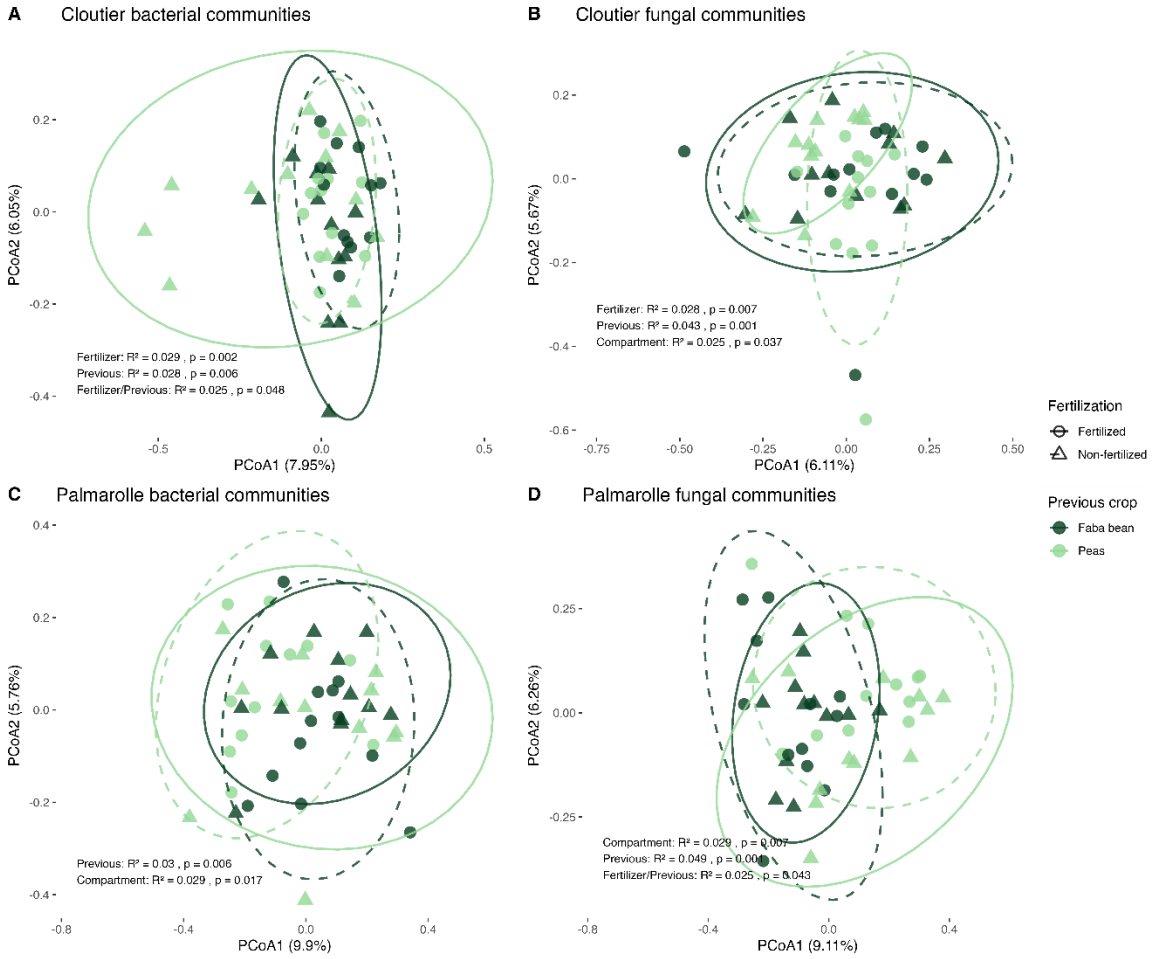
787

788 Figure 6: Heatmap of Spearman correlations between wheat grain yield, and protease, β-
789 glucosidases, deaminases potential activities, ammonium and nitrate content in wheat
790 rhizosphere (A) and bulk soil (B) in Palmarolle. The circle's size represents the Spearman
791 correlation coefficient (Rho). Significant results are identified with a *($p<0.05$).

792

793 Figure 7: Heatmap of Spearman correlations between wheat grain yield, and protease,
794 β-glucosidases, deaminases potential activities, ammonium and nitrate content in and
795 bulk soil in Palmarolle at the beginning of the growing season. The circle's size represents
796 the Spearman correlation coefficient (Rho). Significant results are identified with a
797 *($p<0.05$).

798 Figure 1.



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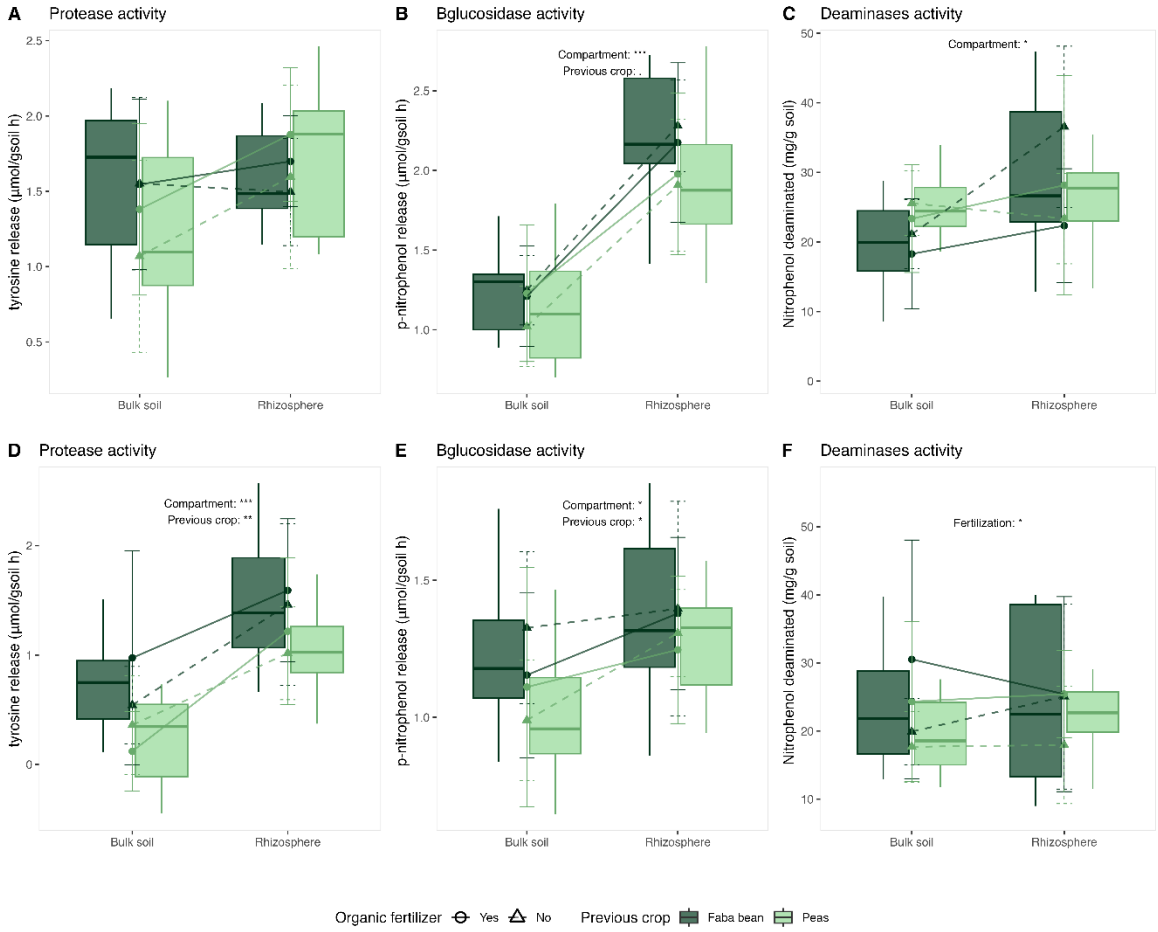
802 Figure 2.



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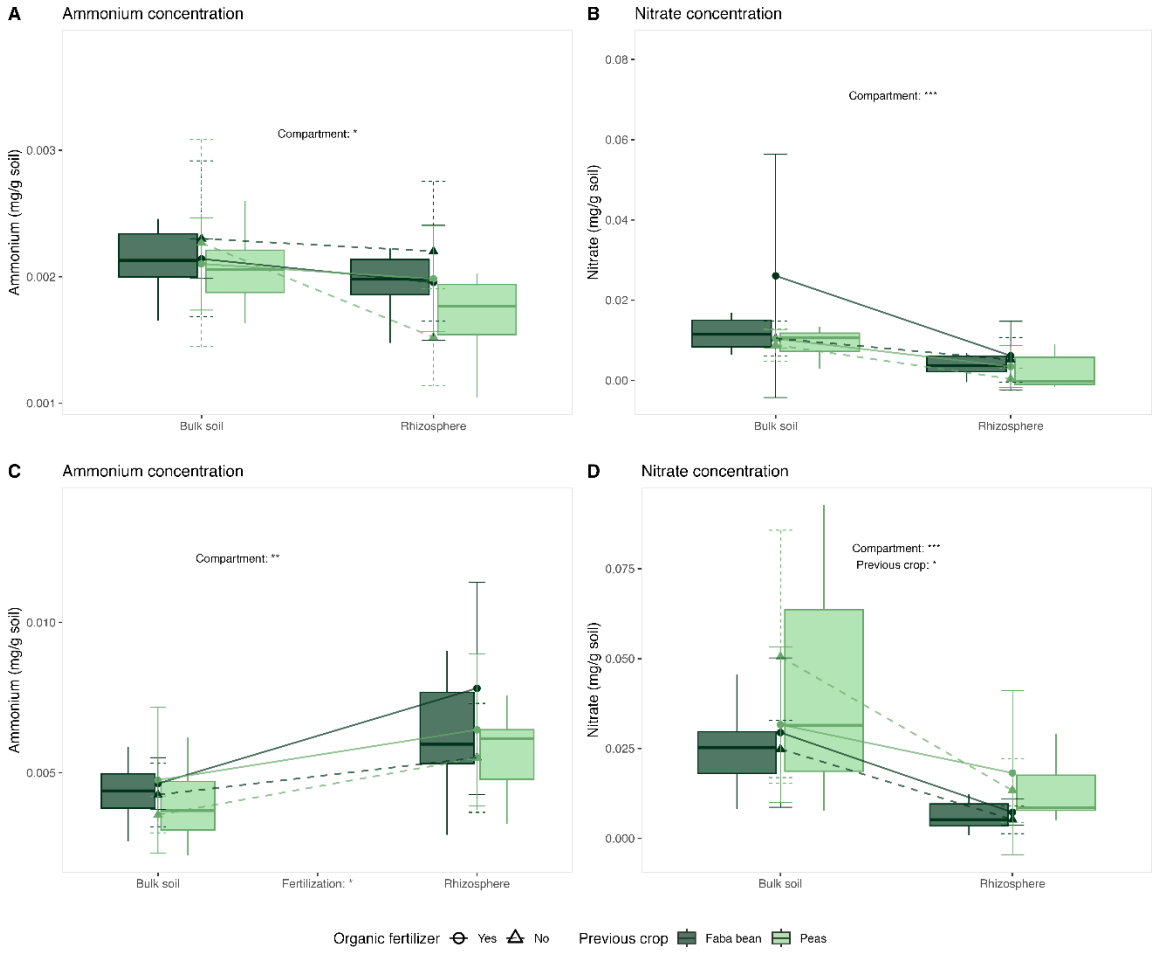
806 Figure 3.



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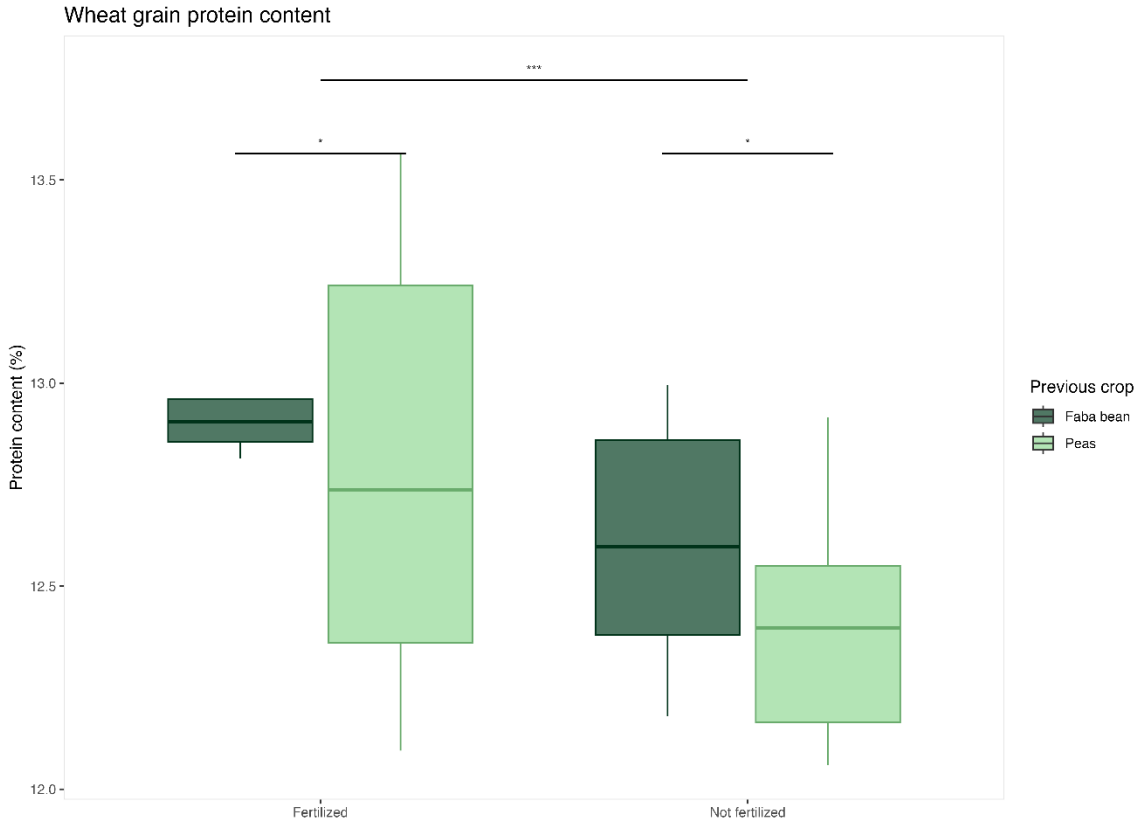
810 Figure 4.



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814 Figure 5.

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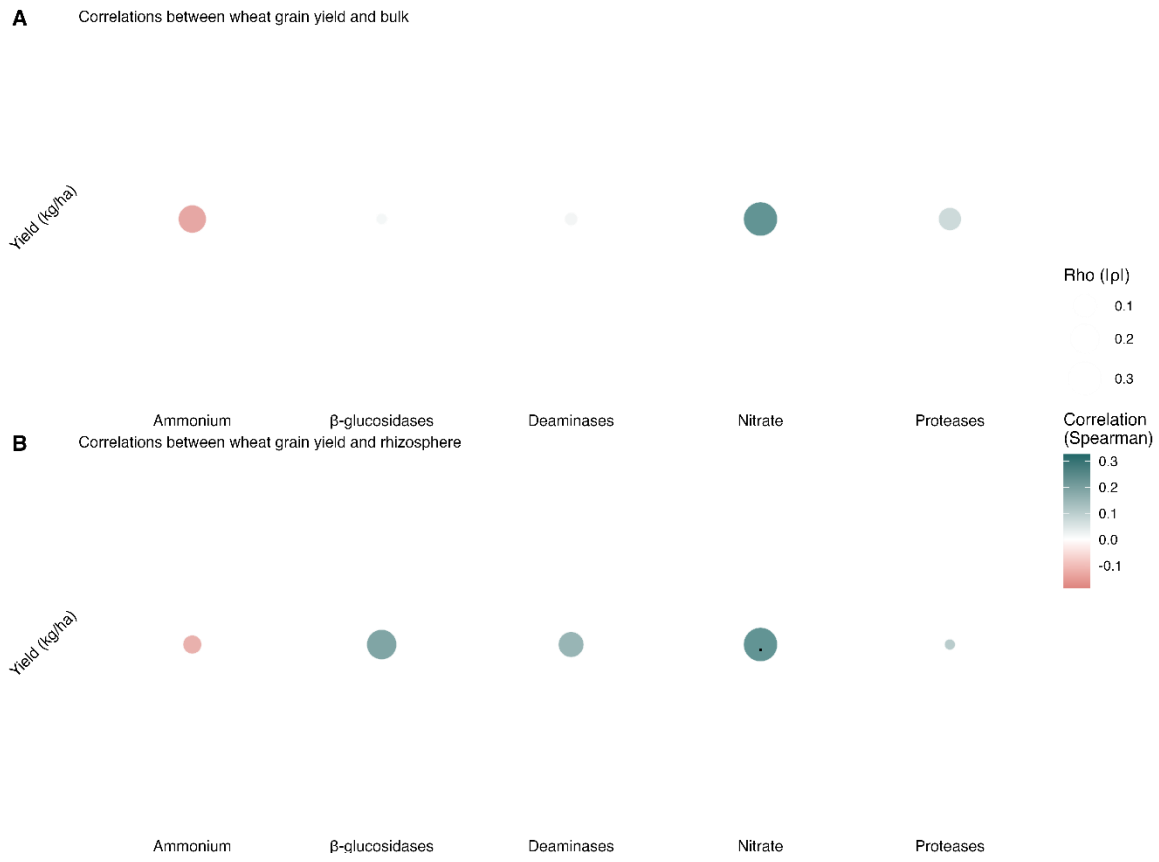
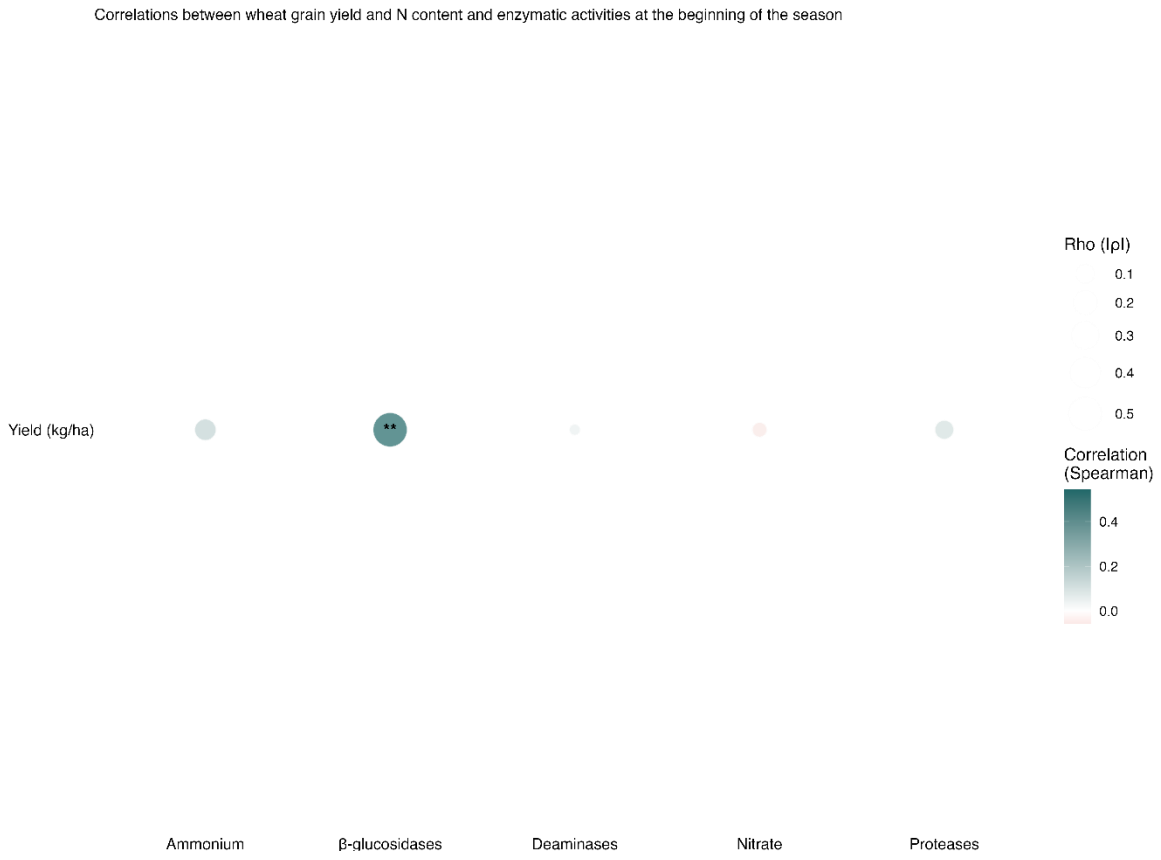
Figure 6.


A
Correlations between wheat grain yield and bulk
Yield (kg/ha)
Ammonium
β-glucosidases
Deaminases
Nitrate
Proteases
Rho (|ρ|)
0.1
0.2
0.3
B
Correlations between wheat grain yield and rhizosphere
Yield (kg/ha)
Ammonium
β-glucosidases
Deaminases
Nitrate
Proteases
Correlation (Spearman)
0.3
0.2
0.1
0.0
-0.1

822 Figure 7.



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825

826 Supplementary results

827 Table S1: PERMANOVA results of Hellinger-transformed Euclidian distances from bacterial communities

828 (16S) data in Cloutier et Palmarolle

	Variable	Df	SumOfSqs	R2	F	p-value	
Cloutier	**Fertilization**	**1**	**0.395**	**0.029**	**1.391**	**0.002**	**
	Previous crop	**1**	**0.378**	**0.028**	**1.333**	**0.006**	**
	Compartment	1	0.271	0.020	0.955	0.481	
	Fertilization: Previous crop	**1**	**0.332**	**0.025**	**1.170**	**0.048**	*
	Fertilization: Compartment	1	0.251	0.019	0.884	0.818	
	Previous crop: Compartment	1	0.267	0.020	0.941	0.55	
	Fertilization: Previous crop: Compartment	1	0.245	0.018	0.865	0.886	
	Residual	40	11.353	0.841			
	Total	47	13.493	1.000			
Palmarolle	Fertilization	1	0.384	0.024	1.148	0.083	
	Previous crop	**1**	**0.477**	**0.030**	**1.427**	**0.006**	**
	Compartment	**1**	**0.454**	**0.029**	**1.360**	**0.017**	*
	Fertilization: Previous crop	1	0.381	0.024	1.140	0.104	
	Fertilization: Compartment	1	0.296	0.019	0.885	0.714	
	Previous crop: Compartment	1	0.304	0.019	0.909	0.633	
	Fertilization: Previous crop: Compartment	1	0.263	0.016	0.786	0.984	
	Residual	40	13.372	0.839			
	Total	47	15.931	1.000			

829 *: p<0.05; **p<0.01; ***:p<0.001

830 Df, SumOfSqs, R2, F and p-value in bold are the significant and/or almost significant values

831

832

833 Table S2: PERMANOVA results on Hellinger-transformed Euclidean distances from fungal communities (ITS)

834 data in Cloutier and Palmarolle

	Variable	Df	SumOfSqs	R2	F	p-value	
Cloutier	**Fertilization**	**1**	**0,471**	**0,028**	**1,350**	**0,007**	*******
	Previous crop	**1**	**0,720**	**0,043**	**2,065**	**0,001**	******
	Compartment	**1**	**0,417**	**0,025**	**1,198**	**0,037**	*****
	Fertilization: Previous crop	1	0,382	0,023	1,095	0,158	
	Fertilization: Compartment	1	0,287	0,017	0,823	0,917	
	Previous crop: Compartment	1	0,285	0,017	0,818	0,934	
	Fertilization: Previous crop: Compartment	1	0,316	0,019	0,908	0,679	
	Residual	40	13,940	0,829			
	Total	47	16,817	1,000			
Palmarolle	Fertilization	1	0,394	0,023	1,116	0,108	
	Previous crop	**1**	**0,844**	**0,049**	**2,391**	**0,001**	*******
	Compartment	**1**	**0,503**	**0,029**	**1,426**	**0,007**	******
	Fertilization: Previous crop	**1**	**0,441**	**0,025**	**1,249**	**0,043**	*****
	Fertilization: Compartment	1	0,296	0,017	0,839	0,824	
	Previous crop: Compartment	1	0,396	0,023	1,123	0,123	
	Fertilization: Previous crop: Compartment	1	0,314	0,018	0,891	0,673	
	Residual	40	14,114	0,816			
	Total	47	17,302	1,000			

835 *: p<0.05; **p<0.01; ***:p<0.001

836 Df, SumOfSqs, R2, F and p-value in bold are the significant and/or almost significant values

837

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