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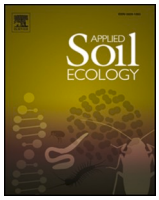

Cattle manure application triggers short-term dominance of *Acinetobacter* in soil microbial communities

Eduardo Pérez-Valera *, Wesley de Melo Rangel, Dana Elhottová

Biology Centre of the Czech Academy of Sciences, Institute of Soil Biology, Na Sádkách 7, 370 05 České Budějovice, Czech Republic

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ABSTRACT

Manure application improves soil productivity but also spreads microorganisms, some of which can be of clinical relevance. The ability of manure to spread common human pathogens has been widely studied but we lack understanding on whether it also disseminates opportunistic pathogens like *Acinetobacter* and other non-fermenting Gram-negative bacteria (NFGNB). We designed a microcosm experiment simulating the application of fresh manure to soil to analyse the effects on soil microbial communities (and vice versa), focusing on *Acinetobacter* and other NFGNB. We conducted two independent experiments with fresh cattle manure from a dairy farm and two pasture soils from different organic farms. We sampled the microcosms on days 2, 7, 14, 28 and 84, and characterized the microbial communities through sequencing of 16S rRNA amplicons from i) total communities and ii) those cultured on CHROMagar Acinetobacter (i.e., selective for NFGNB) after 24-h growth. Manure altered the community composition of soil microorganisms whereas the reverse effects were weaker, showing a transition to an environmentally structured community. *Acinetobacter* species increased their relative abundance in manure and soil under manure on day 2, especially in soils previously exposed to γ-irradiation to reduce the load of native microorganisms. Although manure spread most *Acinetobacter* phylotypes in the soil, it also stimulated a few from the soil that became occasionally abundant in manure. This study demonstrates that *Acinetobacter* species may dominate in soil and manure for a short time after deposition, and highlights their high responsiveness and competitiveness to changes likely associated with an increase in labile resources.

1. Introduction

The growing demand for animal husbandry to meet global food requirements generates tons of manure that applied to soil can improve its productivity while helping to recycle the residues (Thangarajan et al., 2013). Meanwhile, manure is also an important source of mammalian intestinal microorganisms (hereinafter 'manure microorganisms'), some of them potential pathogens that could enter the soil and spread into the environment (Chee-Sanford et al., 2009). This raises important ecological concerns and threads to human and animal health that, therefore, demand a better understanding of both the taxa involved and the circumstances that allow them to spread and persist in the environment.

Manure microorganisms can rapidly colonise the soil, although their ability to survive and eventually dominate may depend on the soil properties as well as the new conditions in the soil after deposition. Firstly, the soil is a nutrient-poor environment as compared with manure, with strong abiotic constraints (e.g., water content, pO_2 or pH) that may limit their survival (Chee-Sanford et al., 2009; Cools et al., 2001; Jechalke et al., 2014). However, although most manure microorganisms do not survive long in soil, improved abiotic conditions such as higher water content or carbon substrates could prolong their survival (Unc and Goss, 2004), but also stimulate certain native soil taxa that are not normally competitive under resource-limiting conditions (Goldfarb et al., 2011). Previous studies have found short-term growth bursts of manure microorganisms in soil (Leclercq et al., 2016; Udikovic-Kolic et al., 2014), suggesting that immigration, probably associated with nutrient enrichment, could be a more relevant process than mere stimulation of native microorganisms. Secondly, native microorganisms in the soil could be key to preventing the introduction of foreign taxa. Indeed, more diverse native microbial assemblages may hinder the soil invasion by manure-borne microorganisms, a relationship likely mediated by resource availability and utilization (Chen et al., 2017; van Elsas et al., 2012). The simultaneous study of microbial changes in both soil and manure after application, either in the presence or absence of native microorganisms, may help to understand the potential role of both biotic and abiotic components in the spread of manure microorganisms.

* Corresponding author.
E-mail address: eduardo.perez.valera@upb.cas.cz (E. Pérez-Valera).

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The potential of animal husbandry manure to transfer microorganisms of clinical concern (e.g., risky pathogens) may be elevated, as it has been widely studied in crop soils receiving artificial amendments of manure (e.g., Liu et al., 2017; Sukhum et al., 2021), in pasture soils sustaining livestock (Elhottová et al., 2012) or under experimental conditions (Chen et al., 2017; Pérez-Valera et al., 2019; Wang et al., 2018). The spread into the environment of genera such as *Escherichia*, *Salmonella*, *Campylobacter* or *Bacteroides* following manure has received much attention in the literature due to both their presence in animal husbandry manure and their potential pathogenicity (Bicudo and Goyal, 2003; Cools et al., 2001; Pachepsky et al., 2006; Rieke et al., 2018). Interestingly, manure can also spread other Gram-negative bacteria that cannot catabolise glucose (i.e., non-fermenters), such as *Acinetobacter* or *Pseudomonas* (Heuer et al., 2009; Leclercq et al., 2016; Macedo et al., 2021), which are among the most problematic pathogens worldwide (Rice, 2008). Despite the potential risk posed by non-fermenting Gram-negative bacteria (hereafter 'NFGNB') to public health, little is known about the role of manure and its interaction with soil in the spread of these potentially opportunistic pathogens.

The genus *Acinetobacter* comprises both environmental species and strains with the potential to cause severe nosocomial infections due to their ability to develop resistance to antimicrobials, transformability, and persistence in the environment for a very long time (Doughari et al., 2011). Although all members of the genus *Acinetobacter* are known to be strict aerobes (Towner, 2006), it has been shown that some individuals can survive under anaerobic conditions (Hrenovic et al., 2019; Pulami et al., 2020). The way *Acinetobacter* species survive anaerobiosis remains unclear, although they may use polyphosphate reserves as an energy source in the absence of oxygen (Kortstee et al., 1994; Pulami et al., 2020). Most *Acinetobacter* species are ubiquitous organisms that can be found in different environments like soil, water, and sewage (Vallenet et al., 2008). Although the natural habitat for most clinically relevant *Acinetobacter* strains (e.g., *A. baumannii*) is not known, it has been found that animal husbandry manure, either raw or after anaerobic digestion, can be an important source of risky strains (Fernando et al., 2016; Hrenovic et al., 2019; Kyselková et al., 2016; Pulami et al., 2020). Whether these strains can actually be transmitted into the environment remains unclear. Previous studies have shown that manure can rapidly introduce risky *Acinetobacter* species into the soil, benefiting greatly from fresh nutrients to become shortly abundant (Leclercq et al., 2016). Their high tolerance to different environmental conditions coupled with the elevated likelihood that they carry or develop resistance to antimicrobials in manure-soil environments make the study of these taxa of central interest. To date, there is limited evidence of the spread of these strains in soil following the application of cattle manure.

In this study we aimed at experimentally investigating i) the effects of fresh cattle manure on the community composition of soil microorganisms, and vice versa, i.e., soil impacts on microorganisms in manure, ii) the manure-soil exchanges and further fluctuations of *Acinetobacter* spp. and other NFGNB and iii) the role of native microbial communities in preventing or favouring microbial exchanges and spread. We hypothesise that fresh manure might transfer and locally favour the dominance of *Acinetobacter* spp. and other potentially risky Gram-negative bacteria in the soil. We set up two 84-days microcosm experiments using manure from a dairy cattle farm and two pasture soils from different organic farms as detailed in Fig. S1. The experimental microcosms, differently combining fresh manure and soil, were sampled at five different times (i.e., 2, 7, 14, 28, and 84 days). Trends in total communities and NFGNB were evaluated via 16S rRNA amplicon sequencing of i) DNA directly extracted from soil and fresh manure samples and ii) DNA of bacterial cultures grown in a selective medium for *Acinetobacter* and other NFGNB.

2. Materials and methods

2.1. Microcosms set-up and sampling of source material

We designed a microcosm experiment to mimic the deposition of fresh manure on the soil, by delimiting the materials with a sterile plastic mesh (1.4 mm) as in Pérez-Valera et al. (2019). Briefly, experimental plastic pots (approximately 300 mL) were delimited horizontally by bottom, intermediate and top layers as schematised in Fig. S1. Fresh manure was applied on the top of both natural and γ-irradiated soils (Fig. S1). Both the top layer (i.e., fresh manure) and the intermediate layer (i.e., soil) were sampled for further downstream analyses. The bottom layer (i.e., soil) was not sampled. Experimental treatments included i) manure over natural soil (M + S) and ii) manure over γ-irradiated soil (M + γS), iii) natural soil underneath manure (S + M) and iv) γ-irradiated soil underneath manure (γS + M). Microcosms containing solely either manure (M) or natural soil (S) were established as controls. The soil layer below the fresh manure (i.e., intermediate layer; approximately 120 g soil, 1 cm) was delineated downward so that soil samples could be sampled at a constant distance from the manure.

The soils used for the microcosm experiments were taken from a grazing site (pasture) of two cattle organic farms (S and B) located in the Czech Republic (ca. 48°North, 14°East). We have previously used soil from these farms to study the effects of manure on the soil resistome under laboratory conditions (Pérez-Valera et al., 2019). Soil samples were collected in September 2018. At each farm (S and B), a soil sample mixture of ten subsamples (5–15 cm) was collected from 1 × 1 m plots along a linear transect (200 m). Both soils were transported to the laboratory in an icebox and stored at 4 °C until setting up the microcosm experiment. Four days before the start of the experiment, the soil samples were pre-incubated at 20 °C in the dark. The abiotic properties of the soils are given in Table S1.

To evaluate the interactive effects of fresh manure on soils with reduced biological viability, soils from both farms (S and B) were γ-irradiated. Gamma radiation produces low soil disruption compared to other methodologies while effectively reducing viable microorganisms (Berns et al., 2008; McNamara et al., 2003). Soils were treated by 76.8 kGy γ-radiation (1.6 kGy h^{-1}: two 24 h-cycles, 3-days delay between cycles) from a ^{60}Co source (Research Centre Řež, Czech Republic), a dose shown to be effective according to our previous experiments (Pérez-Valera et al., 2019). No bacterial colonies were detected in TSA plates inoculated with γ-irradiated soil (5 g in 45 mL sterile 0.9% NaCl) incubated during 2 weeks at 28 °C.

Fresh livestock excrement, hereafter referred to as "fresh manure", was collected from a private dairy farm (different from farms S and B) in the Czech Republic (ca. 48°North, 14°East). The information on farm management, cow gut bacterial community and resistome has been described previously (Kyselková et al., 2015). Fresh manure from 20 adult animals (3–7 years old) was collected aseptically, as described elsewhere (Kyselková et al., 2015; Pérez-Valera et al., 2019), and pooled into one composite sample. Several aliquots were separated for subsequent chemical, bacteriological and genetic analyses. Fresh manure was sampled on the same day of microcosm establishment and taken to the laboratory for immediate use. The abiotic properties of the fresh manure are given in Table S1.

2.2. Experimental design and microcosms sampling

Two independent experiments with fresh manure and soil from farms S and B were conducted in September (experiment S) and October 2018 (experiment B), respectively. For each experiment, three microcosm replicates per treatment were destructively sampled on days 2, 7, 14, 28 and 84. During sampling, top and intermediate layers were thoroughly separated and subdivided into aliquots for downstream analyses. Microcosms solely containing fresh manure (M) or soil (S) were also set up in triplicate and sampled similarly. In total, there were six control

microcosms comprising six samples (i.e., 3 × M, 3 × S), and six experimental microcosms comprising 12 samples (i.e., 3 × M + S, 3 × M + γS, 3 × S + M and 3 × γS + M) per experiment and time point (i.e., 18 samples × 5-time points × 2 experiments = 180 samples). Microcosms were covered with a perforated lid allowing aeration and incubated at 20 °C in the dark. Water was not replenished throughout the experiment. After sampling, several aliquots were taken and stored for soil physical and chemical analyses (ca. 10 g at 4 °C), DNA extraction (ca. 2 g at −20 °C), and cultivation on CHROMagar (see below).

2.3. Abiotic properties of soil and manure

Humidity was calculated as the weight loss after oven-drying the samples (105 °C). pH was measured in a KCl suspension (1:5 w/v). Total C and N levels were determined by dry combustion on elemental analyser (vario MICRO cube, Elementar GmbH, Germany). Total P was measured colourimetrically by the ammonium molybdate-ascorbic acid method on a flow injection analyser (FIA, Lachat QC8500, Lachat Instruments, USA) after perchloric acid digestion (Kopáček and Hejzlar, 1995).

2.4. Cultivation in plates with CHROMagar Acinetobacter

The reciprocal effects of fresh manure and soil on cultivable *Acinetobacter* and other NFGNB were evaluated via cultivation on CHROMagar Acinetobacter (CHROMagar, Paris, France). This medium is highly selective for *Acinetobacter* and other non-fermenting Gram-negative bacteria (Gordon and Wareham, 2009). Immediately after microcosm sampling, CHROMagar Acinetobacter plates were inoculated with soil or manure material as follows: 5 g of either soil or manure from every treatment were deposited in flasks containing 45 mL of sterile 0.9% NaCl. Flasks were exposed to sonication for 2 min preceded by 30 s vortex. One hundred microlitres of serial 1/10 dilutions were plated on CHROMagar plates (up to 10^{-5}) in duplicate. Colony-forming units (CFUs) were estimated by visually counting the plate after 24-h incubation. Plates inoculated with the control soil in experiment B were incubated for up to 48-h due to the low incidence of the studied group on day 2.

Biomass from solid medium plates (ca. 0.1 g) was harvested after counting, by re-suspending the microbial colonies from two plates in sterile 0.9% NaCl. The dilutions were chosen as those that accounted for ca. 1000–5000 CFUs for treatments containing fresh manure and ca. 100–1000 CFUs for those containing soil. The microbial biomass was thoroughly homogenised across the plates and evenly distributed into four 1.5 mL plastic tubes. Microbial cells were then pelleted by centrifugation at 11,000 RPM for 5 min. After removal of the supernatant, tubes were stored at −20 °C for downstream analyses. Biomass harvesting and further analyses were not performed for control soils in experiment B on day 2 due to the low incidence of the studied group.

2.5. DNA extraction

DNA from the pelleted culture was extracted with the Fast DNA Spin Kit (MP Biomedicals, Santa Ana, CA, USA) following the manufacturer's recommendations. Environmental DNA was extracted from ca. 0.5 g soil or manure using Fast DNA Spin Kit for Soil (MP Biomedicals, Santa Ana, CA, USA) according to the manufacturer's recommendations with an additional wash step with guanidine thiocyanate. The quality of the DNA used for downstream PCR analyses was evaluated by electrophoresis in 1% agarose gel run in 0.5 × TAE buffer and quantified using Qubit v3.

2.6. DNA amplicon sequencing

DNA sequencing of 16S rRNA PCR-amplified gene fragments was performed from DNA samples isolated from i) soil and manure samples and ii) bacterial biomass after cultivation with the Illumina platform and primers 515F (5′-GTGCCAGCMGCCGCGGTAA-3′) and 806R (5′-GGACTACVSGGGTATCTAAT-3′) (Caporaso et al., 2012). Each sample contained a unique 4–5 bp barcode and a two-base linker (GT or CC) before the primer. PCR amplifications were performed in triplicate using the Q5® High-Fidelity PCR Kit (New England Biolabs, MA, USA) under the following conditions: 4 min at 94 °C, 25 cycles of 30 s at 94 °C, 1 min at 50 °C and 75 s at 72 °C, followed by 10 min at 72 °C. PCR products were pooled per sample and later purified with the MinElute PCR Purification kit (Qiagen, Hilden, Germany). Afterward, PCR products were pooled in equimolar amounts. Sequencing was performed at the Institute of Microbiology (Prague, Czech Republic) using Illumina MiSeq with v3 chemistry.

2.7. DNA sequence processing

Microbial 16S rRNA amplification and sequencing produced 8,041,862 reads, 24,401 ± 989 (mean ± SE) reads per sample from environmental DNA (n = 186, including 2 experiments × 3 manure samples at time 0) and 19,793 ± 563 reads per sample from the cultures (n = 177). After removing low-quality sequences (i.e., average Phred <30; short sequences <200 pb; those containing Ns), sequences were demultiplexed in SEED 2 (Větrovský et al., 2018) with the fastq-join tool (Aronesty, 2011). Primers were later trimmed from both forward and reverse sequences. DNA sequences were further denoised and dereplicated in QIIME2 2019.7 (Bolyen et al., 2019) using DADA2 (Callahan et al., 2016). The taxonomy was assigned with the "classify-sklearn" algorithm of QIIME2 against SILVA 132 (Quast et al., 2013). After taxonomy assignment, sequences whose taxonomy identification referred to chloroplast, mitochondria or Eukaryota, or remained unassigned were purged from downstream analyses. DNA sequences that did not properly align against the SILVA v132 template in mothur (Schloss et al., 2009) and whose identification was not possible at the level of phylum were also eliminated. We inferred amplicon sequence variants (ASVs), which provide better resolution and accuracy than OTU-based methods (Porter and Hajibabaei, 2018). A total of 10,810 ASVs were generated, of which 10,650 ASVs were identified from environmental DNA samples (including ASVs from both Bacteria and Archaea domains) and 477 ASVs from bacteria harvested from CHROMagar Acinetobacter. Raw sequence data were deposited in the European Nucleotide Archive (http://www.ebi.ac.uk/ena/data/view/PRJEB47082 and http://www.ebi.ac.uk/ena/data/view/PRJEB47093).

2.8. Statistical analysis

We analysed the microbial composition and the reciprocal effects of fresh manure and soil on total communities and NFGNB using non-metric multidimensional scaling (NMDS) generated using Bray–Curtis dissimilarities with "phyloseq" version 1.36 (McMurdie and Holmes, 2013) in R statistical software version 4.1.1 (R Core Team, 2019). We further evaluated compositional shifts using permutational analysis of variance (PERMANOVA), performed using the *adonis* function of the "vegan" R package (Oksanen et al., 2015). We included a Bray–Curtis dissimilarity matrix as the dependent variable, and treatment, time and their interaction as independent variable factors. We also tested the influence of soil properties on microbial composition by performing similar PERMANOVAs with the abiotic properties as additive variables. The dispersion was tested for all conditions using the *betadisper* function in "vegan".

We tested the reciprocal effects of manure and soil on *Acinetobacter* from total communities and culturable NFGNB by pairwise comparisons of relative abundances at the genus level (after collapsing with the *taxa_glom* function of "phyloseq") using separated linear models in R, with post-hoc comparisons using the *glht* function of "multcomp" R package (Hothorn et al., 2008). Computed *p* values were FDR-adjusted using Benjamini–Hochberg. Heatmaps at the ASV level were also

generated using phyloseq and treated-control pairwise comparisons evaluated via a negative binomial distribution (Gamma-Poisson) using the *deseq* function from "DESeq2" R package (Love et al., 2014). These models used *Wald* tests and pairwise comparisons once dispersion was accounted for using DESeq2's pairwise comparison capabilities. To improve sensitivity, we previously filtered out rare genera and ASVs, i.e., with fewer than 20 counts for total communities and 50 counts for NFGNB in at least 10% of samples.

We evaluated the effects of native soil communities by estimating the difference (Δ) in the relative abundance of main phyla and genera between each sample (from M + S, M + γS, S + M or γS + M treatments) and the average of its respective control, either soil (S) or manure (M). Next, we used individual linear models for each taxon (within each time point and experiment) to evaluate whether taxa were differentially abundant when comparing the delta of natural and γ-irradiated soils, using the *glht* function in "multcomp" for R. Similar models were used to test the differences in lognormal-transformed CFUs and soil abiotic variables in every treatment i) with respect to the control and ii) by comparing natural and γ-irradiated soil after computing the difference (Δ) with the control. All *p* values were FDR-adjusted for multiple testing.

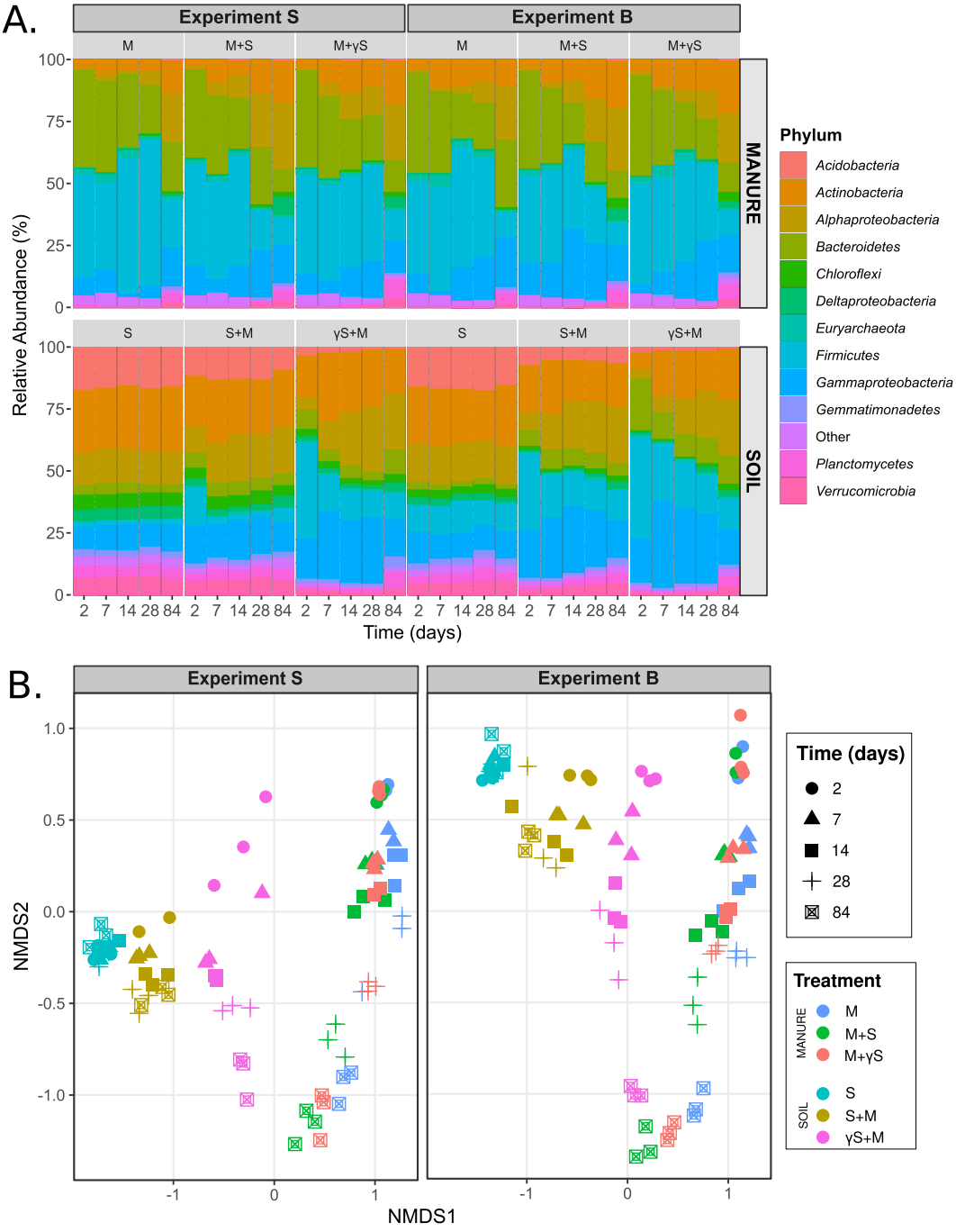

Fig. 1. Relative abundance of main microbial phyla (A) and unconstrained ordination analysis (NMDS) based on Bray–Curtis dissimilarities (B) of microbial communities in manure and soil samples over two 84-d experiments (S and B). Treatments included control manure (M), manure in contact with soil (M + S), manure in contact with γ-irradiated soil (M + γS), control soil (S), natural soil in contact with manure (S + M) and γ-irradiated soil in contact with manure (γS + M). A single NMDS analysis (stress = 0.13) was performed for both experiments.

3. Results

3.1. Total community

3.1.1. Composition of microbial communities in soil and manure

Fresh manure altered the community composition of soil microorganisms, as shown in both natural (S + M) and γ-irradiated (γS + M) soil treatments as compared with unamended control soils (S) (Fig. 1). Specifically, fresh manure induced a peak in *Firmicutes* (up to 42% relative abundance) in the soil as well as increases in *Gammaproteobacteria* and *Bacteroidetes*, and decreases in *Actinobacteria*, *Acidobacteria* and *Alphaproteobacteria*, the latter being the dominant phyla in the control soil (S) (Figs. 1A and S2). Manure-induced shifts in microbial communities depended on the presence of native soil microorganisms, with the impacts being greater in γ-irradiated soil (γS + M) treatments (Figs. 1A and S2). Most phyla showed significant differences between S + M and γS + M treatments with respect to the control (S) at one or more time points (Fig. S2). Soil microorganisms showed consistent responses between experiments (i.e., experiment S vs experiment B), with changes mainly dependent on the treatment (39–41% variance), time (13–16% variance) and the treatment × time interaction (20% variance) (PERMANOVA, Table 1). Unconstrained ordination analysis (NMDS) based on Bray–Curtis dissimilarities highlighted the impacts of fresh manure on the microbial community composition as summarised by the first axis of the ordination analysis (NMDS1), with little variation during incubation in the control soils (Fig. 1B).

In contrast, the soil had a limited impact on the community composition of manure microorganisms, which was dominated by *Bacteroidetes* (ca. 40%) and *Firmicutes* (ca. 40%) on day 2 (Fig. 1A). Shifts in microbial phyla caused by soil were more pronounced over time, especially on days 28 and 84, with lowered dominance of *Firmicutes*, *Bacteroidetes* and *Gammaproteobacteria*, and increases in *Actinobacteria*, *Deltaproteobacteria* and *Alphaproteobacteria* (Figs. 1 and S2). By using γ-irradiated soil, we found that native soil microorganisms influenced the composition of manure microorganisms, especially at longer incubation times, as shown by the increased levels of *Alphaproteobacteria* and *Verrucomicrobia* in M + S as compared to M + γS treatments on days 28 and 84 (Fig. S2). Differences in the composition of manure microorganisms were roughly consistent between experiments, with shifts mainly driven by incubation time (50–52% variance) as shown in the NMDS2 axis (Fig. 1B), but also by treatment (5–7% variance) and the interaction between them (15–18% variance) (PERMANOVA, Table 1).

3.1.2. Dominance of Acinetobacter and other potentially risky taxa

The genus *Acinetobacter* did represent a significant fraction (ca. 3–10% relative abundance) of the total microbial community in both manure and manure-amended soil treatments (Fig. 2A) on day 2, despite being rare in fresh manure (<0.01% relative abundance, data not shown) or undetected in control soils at any time point. Specifically, two taxa (i.e., ASV_21 and ASV_25), most likely from manure origin, were responsible for the peak in the genus *Acinetobacter* in both manure and soil communities (Fig. 2B). The pulse in *Acinetobacter* species lowered rapidly, particularly in natural soils (S + M) and manure treatments from the experiment S (Figs. 2A and S3), while other bacterial genera such as *Pseudomonas*, *Stenotrophomonas* or *Burkholderia* became more abundant in both experiments over time (Fig. S3). Some taxa such as *Achromobacter*, *Stenotrophomonas* and *Sphingobacterium* showed higher relative abundance in γS + M than in S + M treatments, especially at longer incubation times (Fig. S3).

3.2. Cultured non-fermenting Gram-negative bacteria (NFGNB)

3.2.1. Microbial colony-forming units (CFUs)

We also evaluated the reciprocal effects of fresh manure and soil on NFGNB by growing them on a selective medium (Fig. S4). Fresh manure increased microbial CFUs in treatments containing either natural (S + M) or γ-irradiated soil (γS + M) on day 2. The increase, of about two orders of magnitude compared to the control soil (S), fluctuated but remained significantly elevated throughout the incubation period. The presence of native soil microorganisms impacted the microbial CFU counts, i.e., natural soils amended with fresh manure (S + M) showed initially (day 2) higher microbial CFUs than those exposed to γ-irradiation (γS + M); however, the trend reversed after 1-week incubation (Figs. S4 and S5). Compared with the control, the increase in soil microbial CFU due to manure tended to be higher in experiment B, regardless of treatment or time (Fig. S5).

Soil also increased the microbial CFUs in treatments containing manure, although its effect was generally weaker than the reverse (Fig. S4). The increase in microbial CFUs in manure on soil did not depend on the treatment (i.e., in contact with natural or γ-irradiated soil) except for experiment S on day 7 (Fig. S5). In the long term, on day 84, microbial CFUs showed a stabilization trend in soils from both experiments while those in control manure showed a remarkable increase, significantly surpassing those soil that received fresh manure.

3.2.2. Composition of NFGNB communities in soil and manure

Sequencing of 16S rRNA amplicons from bacterial cultures selected with CHROMagar Acinetobacter revealed an increase in the relative abundance of the genus *Acinetobacter* (> 52% relative abundance) in almost all experimental conditions after two days of incubation, followed by the genera *Pseudomonas*, *Achromobacter* or *Stenotrophomonas* depending on the treatment and experiment (Fig. 3A). In contrast to the total community (characterized by amplicon sequences from environmental DNA), the bacterial culture composition showed greater within-treatment variation and different community composition between experiments, possibly due to the source material (Fig. 3B). While *Acinetobacter* was the most abundant genus in the control soils of experiment S, it was scarcely present in the soils of experiment B, which were mainly dominated by *Cupriavidus* spp. (Fig. 3A). The initial pulse in the genus *Acinetobacter* became more acute in soil treatments with reduced viability of native microorganisms (i.e., >82% relative abundance in γS + M as compared with >63% in S + M treatments) (Fig. 4A). The relative abundance of the genus *Acinetobacter* declined rapidly in all experimental conditions containing or in contact with manure (on day 14), being replaced by other *Gammaproteobacteria* such as the genera *Achromobacter*, *Pseudomonas* or *Stenotrophomonas* (Figs. 3A and 4).

Table 1
Permutational analysis of variance (PERMANOVA, 999 permutations) to evaluate the significance of treatment, time and the treatment × time interaction on microbial community composition over two 84-d experiments (S and B). All conditions tested were significant (all $p < 0.05$).

		Experiment S				Experiment B		
		df	F	R^2	p	F	R^2	p
Manure	Treatment	2	3.7	0.07	0.001	3.26	0.05	0.001
	Time	4	13.0	0.50	0.001	15.5	0.52	0.001
	Treatment × time	8	1.9	0.15	0.002	2.67	0.18	0.001
Soil	Treatment	2	24.6	0.42	0.001	23.46	0.39	0.001
	Time	4	3.9	0.13	0.001	4.70	0.16	0.001
	Treatment × time	8	2.9	0.20	0.001	2.99	0.20	0.001

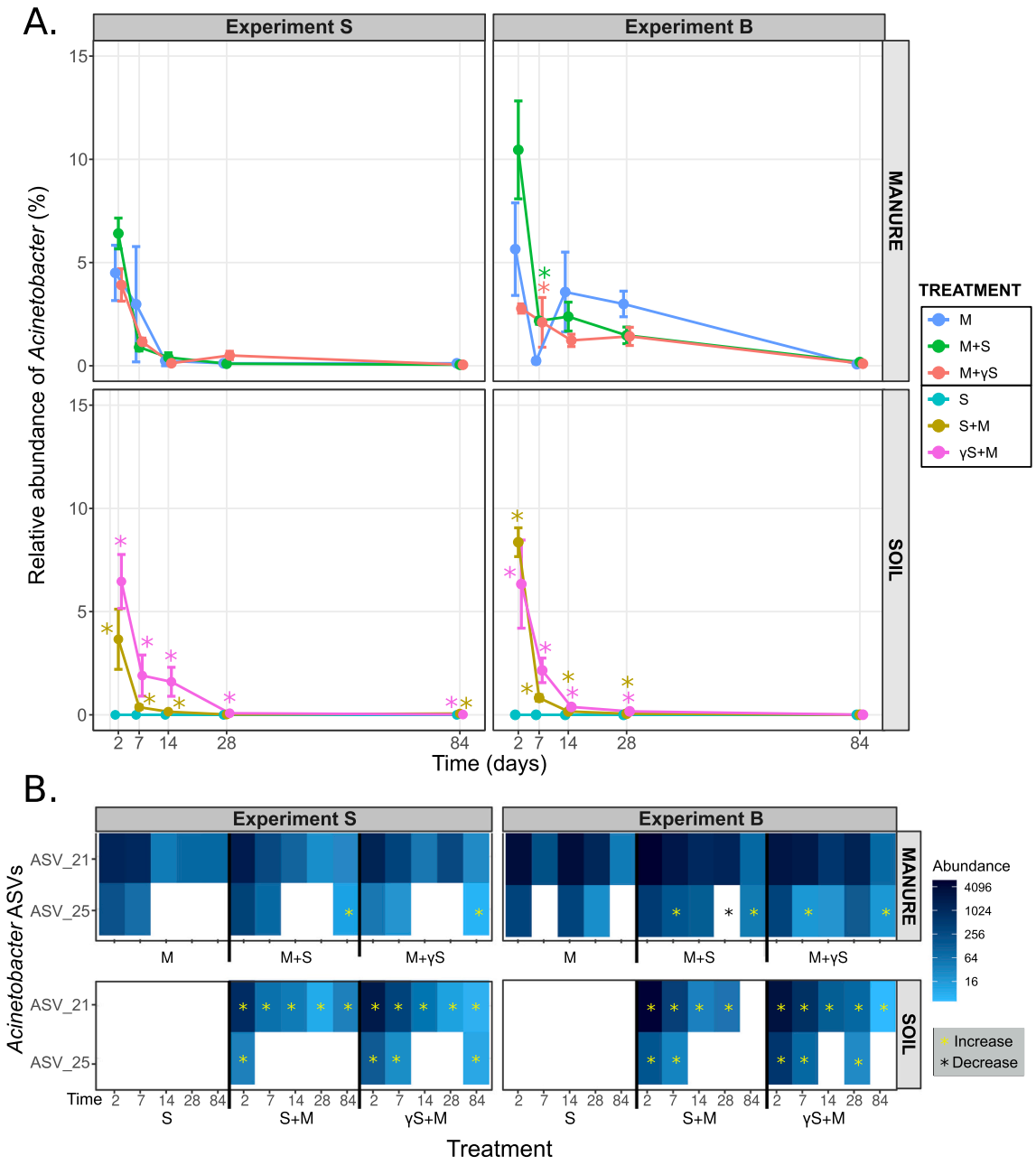

Fig. 2. Relative abundance (%) of the genus *Acinetobacter* (16S rRNA amplicon sequences) in manure and soil samples (A) and heatmaps showing the abundance of the most relevant *Acinetobacter* ASVs (B) over two 84-d experiments (S and B). Average values (n = 3) per treatment, time and experiment are shown. Error bars indicate standard errors. Asterisks indicate significant differences with the control at each time point ($p < 0.05$). Abundances (in B) were log-transformed for visualization. ASVs are ordered by their summed abundance.

The increase in *Acinetobacter* species also occurred in manure, regardless of the experimental conditions, with little difference among treatments or experiments (Fig. 4A). As in soil treatments, *Acinetobacter* spp. declined rapidly in manure during incubation, being replaced by the genera *Achromobacter*, *Pseudomonas*, *Stenotrophomonas* or *Sphingobacterium* (Fig. 4A).

A more specific analysis at the amplicon sequence variant (ASV) level revealed occurrence patterns that could be related to the origin of each *Acinetobacter* ASV (Fig. 4B). In particular, ASV_2 was most likely of manure origin, as shown by its no detection in the control soils and its dominance in all experimental conditions containing or in contact with manure (e.g., up to 41% relative abundance in control manure (M) and up to 77% in γS + M in experiment S on day 2). However, ASV_4 probably originated from the soil, as it dominated in the control soil (A) and showed a mutual exclusion pattern in control manure (M) (Fig. 4B). ASV_4 became the most relevant *Acinetobacter* ASV in manure in contact with the soil (M + S), as well as in natural soil underneath manure (S + M) in experiment S on day 2, remaining generally undetected in treatments with γ-irradiated soil (M + γS and γS + M). Both ASV_2 and ASV_4 showed differential abundance patterns in natural soil (S + M) as compared with γ-irradiated soil (γS + M) (Fig. S6). Other *Acinetobacter* ASVs with lower relative abundance such as ASV_10, ASV_25 or ASV_26, were also generally associated with manure, with more scattered distributions and no or little presence in the control soils.

3.3. Abiotic properties of soil and manure samples

Fresh manure altered the abiotic properties of the soil by increasing

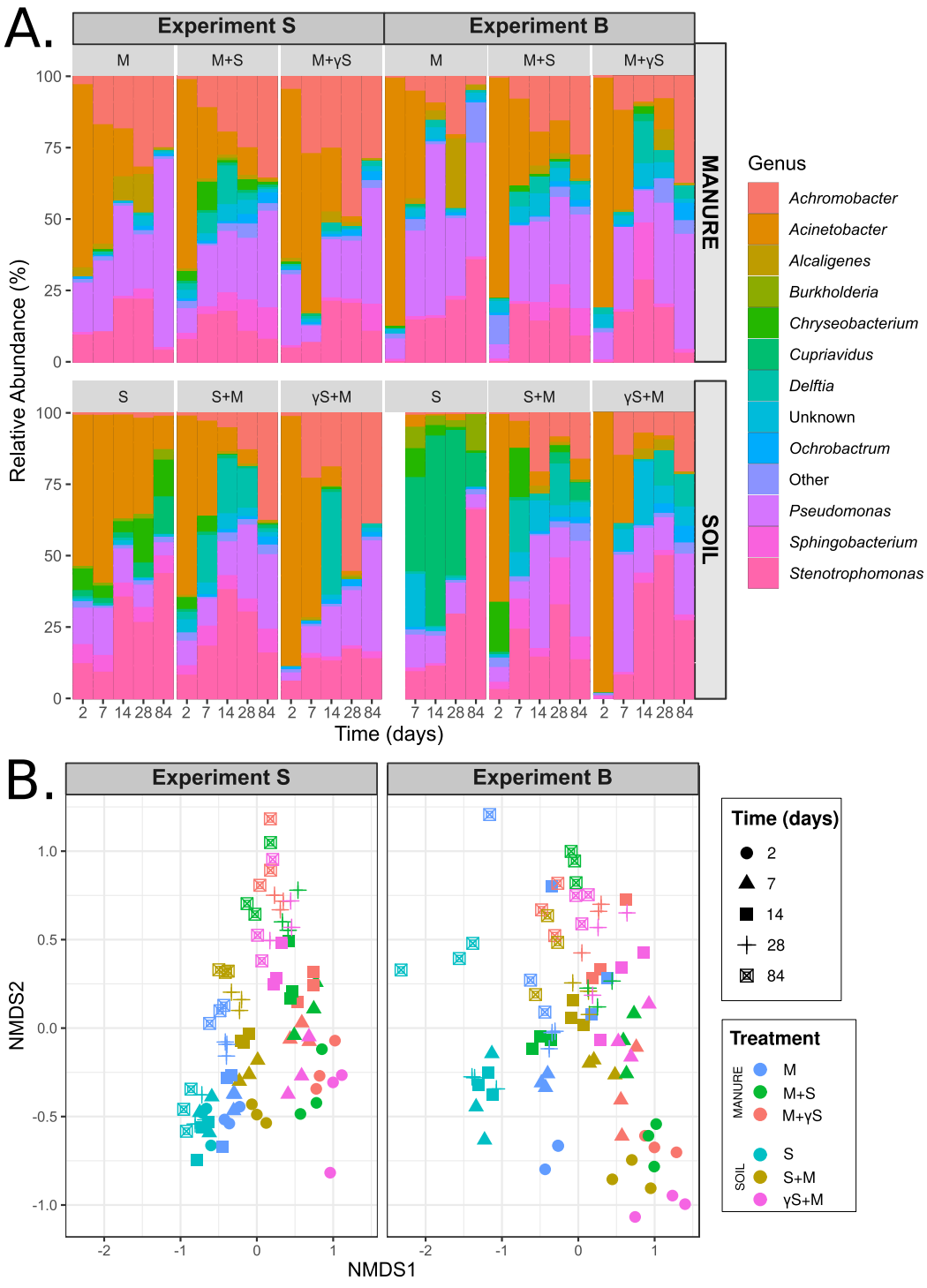

Fig. 3. Relative abundance of main bacterial phyla (A) and unconstrained ordination analysis (NMDS) based on Bray–Curtis dissimilarities (B) of cultured bacterial communities from manure and soil samples over two 84-d experiments (S and B). Treatments included control manure (M), manure in contact with soil (M + S), manure in contact with γ-irradiated soil (M + γS), control soil (S), natural soil in contact with manure (S + M) and γ-irradiated soil in contact with manure (γS + M). A single NMDS analysis (stress = 0.18) was performed for both experiments.

pH, humidity and total C, total N and total P content (Fig. S7). While the increases in soil pH and humidity occurred quickly (i.e., on day 2) and widely across treatments and experiments, those in total C, total N and total P, and the C/N ratio depended on the experiment (i.e., soil). Specifically, the soil in experiment B, which had lower total N and total C content, showed a respective increase with manure on day 2, while the soil in experiment S, which had lower total P content, showed an increase in it on day 14. Levels of total C in experiment S, or total P in experiment B, were not increased in soil treatments at any time point. However, fresh manure did increase total N in both experiments, with the increase occurring faster in the soil with a more limited N content (i.e., experiment B). Shifts in soil properties were roughly consistent between experiments and depended on the native soil community, although the changes were only significant for pH, which increased in γS + M following manure.

Soil effects on manure abiotic properties were generally weaker than

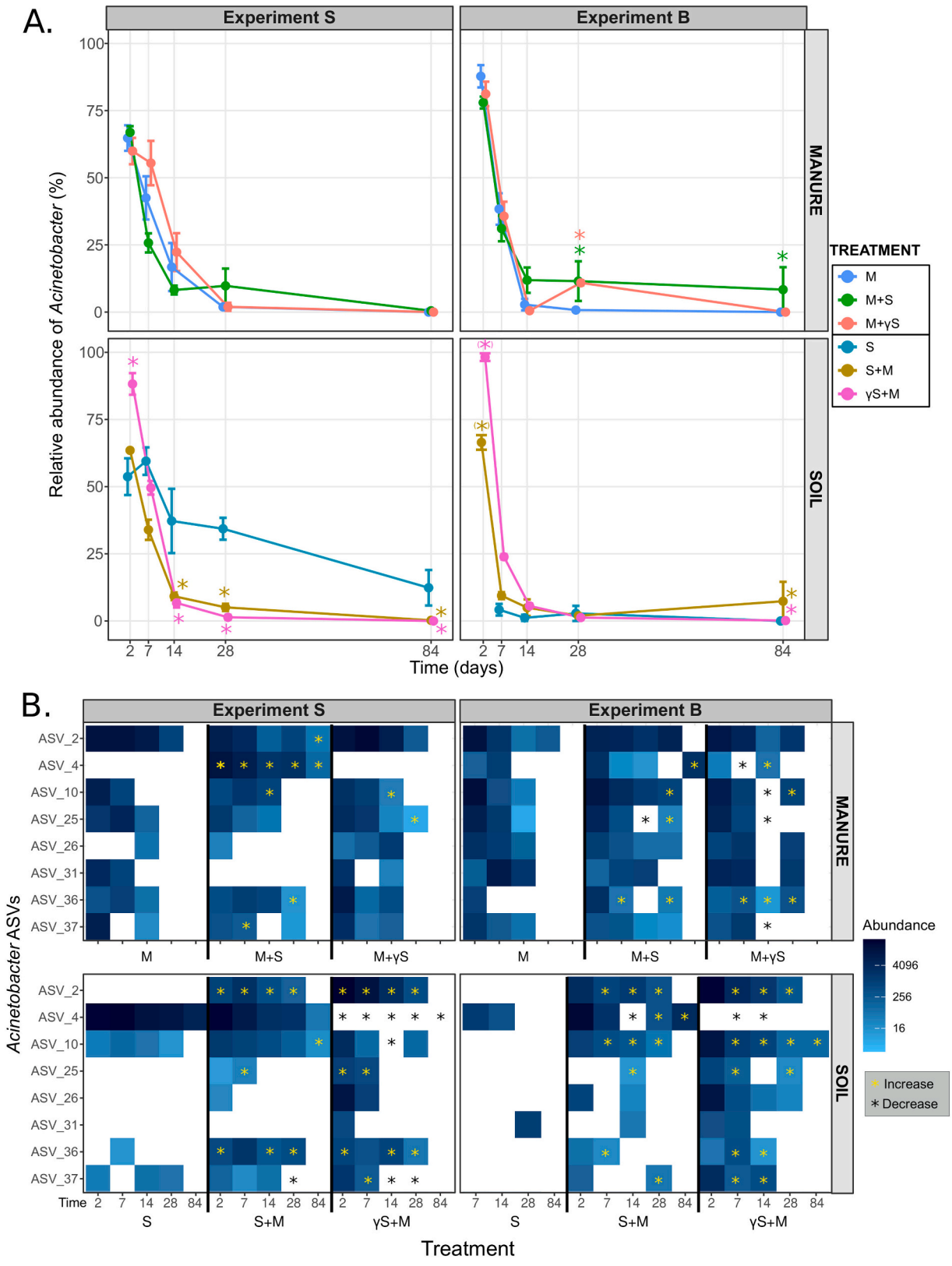

Fig. 4. Relative abundance (%) of the genus *Acinetobacter* (16S rRNA amplicon sequences) in the cultured fraction from manure and soil samples (A) and heatmaps showing the abundance of the most relevant *Acinetobacter* ASVs (B) over two 84-d experiments (S and B). Average values (n = 3) per treatment, time and experiment are shown. Error bars indicate standard errors. Asterisks indicate significant differences with the control at each time point ($p < 0.05$). ASVs shown in the heatmap (B) are those whose relative abundance was higher than 25% in at least two samples and are ordered by their summed abundance. Abundances (in B) were log-transformed for visualization.

vice versa, but increased over time for some variables (Fig. S7). Soil slightly increased manure pH on day 2, but the trend reversed after 7 or 14 days, depending on the experiment, due to changes in control manure pH. Manure in contact with soil showed lower humidity than the control manure, especially in the long term. As in the soil, changes in the abiotic properties of manure were consistent across experiments and depended on the native soil microorganisms, as shown by a decreased pH in M + γS as compared to M + S (Fig. S8).

The changes in abiotic soil properties drove the changes in the composition of microbial communities, with pH being the most important factor under almost all experimental conditions, as shown in Table 2. In addition, soil humidity and total N content in experiment S and total P content in experiment B were also involved in the changes in the composition of microbial communities (Table 2).

4. Discussion

Our results showed that fresh manure altered the community composition of soil microorganisms, causing an increase in potentially risky *Acinetobacter* species that diminished shortly. A similar increase was detected in manure, with little influence of soil on the community composition of manure microorganisms. Culture-dependent analyses in a selective medium for non-fermenting Gram-negative bacteria (NFGNB) confirmed the dominance of the genus *Acinetobacter* in the short term but also that most *Acinetobacter* ASVs were of manure origin. Interestingly, manure probably stimulated the growth of a few *Acinetobacter* ASVs originating from soil, which became locally abundant in manure. This suggests a bi-directional transfer of microorganisms between manure and soil. Our data showing the initial dominance of the genus *Acinetobacter* suggests an elevated ability of these lineages to thrive and dominate in such contrasting environments (i.e., manure and soil) in the short term, likely in response to a labile resource pulse (Goldfarb et al., 2011). Similarly, the rapid decline in their relative abundance may also suggest low competitive abilities when more labile resources are probably consumed.

4.1. Effects of fresh manure on soil microorganisms

Fresh manure altered the community composition of soil microorganisms by increasing the relative abundances of *Firmicutes*, *Bacteroidetes* and *Gammaproteobacteria*, which are the dominant taxa in cattle manure as widely reported (e.g., Pérez-Valera et al., 2019; Sukhum et al., 2021; Wichmann et al., 2014). The consistency of the shifts between experiments (i.e., using different soils) highlights the capacity of manure-borne microorganisms to rapidly reach down and grow under soil-like conditions (Bech et al., 2014; Chee-Sanford et al., 2009). Physical processes such as water leaching could explain the rapid introduction of microorganisms into soil (Unc and Goss, 2004), as observed with the increased soil humidity following the addition of manure. The fluidic nature of fresh manure is an important factor for spreading microorganisms, especially for those non-motile bacteria, lacking the ability and structures that would allow them to propel themselves. The addition of fresh manure could also favour certain soil microorganisms that benefit from the improved soil abiotic conditions, e.g., nutrients supply (Chee-Sanford et al., 2009; Macedo et al., 2021). Additionally, fresh manure may stimulate the growth of native taxa that are not normally competitive under frequent low-nutrient conditions in soil (Macedo et al., 2021), typically C-limited (Goldfarb et al., 2011). The initial soil increases of *Firmicutes* and *Bacteroidetes*, which were the dominant taxa in fresh manure, and which also do not typically respond or react negatively to C inputs (Goldfarb et al., 2011), highlights the introduction vs nutrient stimulation as the more relevant phenomenon. Likewise, the rapid decline in these groups confirms the low ability to survive under soil-like conditions (Bech et al., 2014; Chee-Sanford et al., 2009; Lopatto et al., 2019). Alternatively, it might suggest that these organisms are less competitive in presence of native soil microorganisms (Pérez-Valera et al., 2019) or when nutrients are more limiting.

The way microorganisms migrate into the soil was further evidenced by including a treatment containing soil with reduced biological viability (i.e., γ-irradiated soil). Specifically, we found consistent decreases in groups that are generally dominant in soils worldwide such as *Alphaproteobacteria*, *Actinobacteria*, *Acidobacteria* and *Verrucomicrobia* (Delgado-Baquerizo et al., 2018), and further increases in *Firmicutes*, *Bacteroidetes* and *Gammaproteobacteria* (dominant taxa in fresh cattle manure) when comparing γ-irradiated with natural soils under manure. This suggests that native soil microorganisms may play an important role in preventing manure microbes from colonizing the soil, as has been documented in the literature (Chen et al., 2017; Goberna et al., 2011; Pérez-Valera et al., 2019; van Elsas et al., 2012). For example, van Elsas et al. (2012) found higher soil colonization by a pathogenic *Escherichia coli* strain when it was introduced into soils with a low diversity of native microorganisms. Chen et al. (2017) found that the transfer of antimicrobial resistance to soil was hindered in experimental microcosms containing mixtures of soil and pig manure and attributed this to the ability of indigenous soil microorganisms to prevent colonization by exogenous microbiomes. In a previous study combining fresh manure and soil in horizontal layers, we also revealed the role of native soil microorganisms not only in preventing soil colonization by exogenous microorganisms but also in hindering the transfer of antimicrobial resistance (Pérez-Valera et al., 2019). Relaxed competition as well as greater niche availability (through less diverse microbiomes) are suggested as mechanisms that may play a role in soil colonization by manure microbes (Chen et al., 2017; Goberna et al., 2011; van Elsas et al., 2012). Indeed, some groups, such as *Gammaproteobacteria*, which are known to respond positively to C inputs (Goldfarb et al., 2011), may take advantage of improved nutrient availability coupled with relaxed competition to become more abundant. Once the labile nutrients are consumed, soil microbial communities may resemble those before manure addition (Sukhum et al., 2021), although they may require more than the three months that this experiment lasted. Meanwhile, our results also showed that microbial communities in manured γ-irradiated soils resembled those from manure over time, indicating a microbial transition based on the incoming microorganisms rather than effects of the abiotic soil environment.

Table 2
Permutational analysis of variance (PERMANOVA, 999 permutations) showing the effects of abiotic properties on the microbial composition of total communities. Significant variables ($p < 0.05$) are shown in bold.

		Experiment S			Experiment B		
		F	R^2	*p*	F	R^2	*p*
Manure	pH	**1.48**	**0.14**	**0.001**	**1.76**	**0.14**	**0.001**
	C	**0.78**	**0.08**	**0.002**	**2.29**	**0.19**	**0.001**
	N	**0.89**	**0.09**	**0.001**	**0.59**	**0.05**	**0.006**
	P	**0.36**	**0.07**	**0.001**	0.29	0.02	0.110
	C/N	**1.02**	**0.08**	**0.001**	0.29	0.02	0.094
	Humidity	0.48	0.03	0.063	0.23	0.02	0.207
Soil	pH	**14.07**	**0.22**	**0.001**	**3.13**	**0.26**	**0.001**
	C	0.17	0.01	0.498	**0.85**	**0.07**	**0.001**
	N	**0.36**	**0.03**	**0.036**	0.38	0.03	0.058
	P	0.26	0.03	0.096	0.14	0.01	0.696
	C/N	0.22	0.02	0.215	0.25	0.02	0.207
	Humidity	**0.85**	**0.08**	**0.002**	0.18	0.01	0.445

4.2. Effects of soil on manure microorganisms

The design of our experiment also allowed us to investigate the effects of soil on the manure microorganisms as well as the dynamics over time. In contrast to the soil treatments under manure, the soil had little effect on the manure microorganisms, with consistent shifts across treatments during incubation. Microbial assemblages with higher proportions of *Alphaproteobacteria* and *Actinobacteria* replaced those

initially dominated by *Firmicutes* and *Bacteroidetes*. These changes may be compatible with a transition from a gut-structured community to an environment-structured community (Sukhum et al., 2021). The inclusion of a treatment consisting of manure on γ-irradiated soil, where microbial migration from the soil was limited, allowed confirmation that most compositional shifts occurred only through abiotic contact with the soil, regardless of its biotic component. Interestingly, additional shifts in *Firmicutes*, *Gammaproteobacteria* and *Alphaproteobacteria* when comparing manure on natural vs γ-irradiated soil further suggest that migration of microbes into manure may also occur. Whether migration is passive, active or mediated by other soil organisms (Yang and van Elsas, 2018) through the likely water-saturated manure-soil contact surface, remains largely unknown. Soil fauna (<5 mm) could also play a role in the microbial shifts found in natural soil as compared to γ-irradiated soil. For example, *Collembola* and other invertebrates were abundant in our treatments containing natural soil in one of our experiments (Table S2). Moreover, fungi could also help spread bacterial cells from manure (Nazir et al., 2017; Zurek and Ghosh, 2014). The possibility that fungi develop hyphae that connects soil and manure, and that bacteria use the fungal highways (Kohlmeier et al., 2005) to reach the resource-rich manure is appealing but untested. Additional investigations that complement this study, broadening the view to other microbial groups and insects, are needed to provide a more complete view of the ecological processes controlling the manure-soil interactive system.

4.3. Increases in the putative opportunistic pathogen Acinetobacter spp.

We found that the relative abundance of the genus *Acinetobacter* increased under all experimental conditions on day 2 despite being rare in fresh manure and undetected in control soils at any time point. Several studies have found similar growth bursts of *Acinetobacter* species in groundwater (Gao et al., 2020; Rieke et al., 2018) and soil (Leclercq et al., 2016; Wepking et al., 2017) following swine or cattle manure deposition. The increase in *Acinetobacter* species in soil may happen via immigration, via growth-stimulation by labile nutrients or likely both (Goldfarb et al., 2011; Leclercq et al., 2016; Macedo et al., 2021). Leclercq et al. (2016) found that *Acinetobacter* phylotypes thriving in the soil likely migrated from manure, where they were abundant, being later favoured by the addition of nutrients. Despite being strictly aerobic (Towner, 2006), *Acinetobacter* species can survive under anaerobic conditions (Pulami et al., 2020) and are often found in swine and cattle manure (Fernando et al., 2016; Hrenovic et al., 2019; Luo et al., 2021). Strikingly, our study also detected elevated relative abundances of the genus *Acinetobacter* (up to 11%) in dairy cattle manure during incubation, with the same ASVs being abundant in both soil and manure. The dominance of the same *Acinetobacter* ASVs in manure and soil, i.e., in completely different environments in terms of e.g., humidity (~80% vs ~20%) and nutrients (~2-fold differences in total C), emphasises their tolerance and competitive abilities over a period in which the environmental conditions are likely favourable. The fact that members of the genus *Acinetobacter* could be even more relevant when the native soil community was reduced suggests that, in addition to the nutrient pulse, relaxed competition could also promote their dominance in the short term.

Microbial cultivation on a selective medium for *Acinetobacter* and other NFGNB allowed a more sensitive study of manure-soil interactions in the spread of Gram-negative potential opportunistic pathogens. Our analyses showed that fresh manure increased CFU levels in soil by approximately three orders of magnitude, supporting the idea that manure from dairy farms may be an important source of these opportunistic pathogens in soil (Fernando et al., 2016; Leclercq et al., 2016), which are able to persist in the soil for at least three months. Interestingly, lower CFU levels in natural soil underneath manure when compared to γ-irradiated soil suggest that native microbiomes could prevent soil colonization by NFGNB in the long term. The short-term dominance of *Acinetobacter* spp., originally detected in the total community, was also confirmed by using cultivation in a selective media for NFGNB. The higher dominance of *Acinetobacter* species in γ-irradiated soil underneath manure confirmed our previous findings, emphasizing that native microorganisms could be also relevant in preventing the increase in these taxa. A more detailed analysis at the ASV level showed that most *Acinetobacter* taxa originated from fresh manure, as suggested by data from the total community and as found in other studies (Gao et al., 2020; Leclercq et al., 2016; Luo et al., 2021). Surprisingly, we also detected a few soil-unique *Acinetobacter* ASVs that might have been transferred to manure, in a contrary way. This supports our previous findings in the total community at the phylum level, and suggests that the transfer of microorganisms could be bi-directional (i.e., from manure to soil and vice versa) and occur as quickly as on day 2. The dominance of *Acinetobacter* spp. and other NFGNB in soil for some time may have important consequences on soil ecosystem services. On one hand, it might pose public health risks by increasing the levels of antibiotic-resistance genes in the soil, as shown with microorganisms coming from manure sources (Kyselková et al., 2016, 2015; Leclercq et al., 2016; Pérez-Valera et al., 2019; Udikovic-Kolic et al., 2014). This may occur by an increase in the abundance of taxa carrying antibiotic-resistance genes or by horizontal gene transfer to native soil microorganisms (Forsberg et al., 2012). On the other hand, it can also alter the diversity of soil microbial communities and thus soil functionality (Maron et al., 2018), especially in soils with low-diversity microbial assemblages, which are more susceptible to invasion by alien species (van Elsas et al., 2012). Nonetheless, further research is needed to better understand the processes by which fresh manure induces increases in *Acinetobacter* spp. or other genera such as *Pseudomonas*, as well as their potential impact in ecosystem functions and the spread of relevant antibiotic-resistance genes.

The rapid decline of *Acinetobacter* spp. after seven days suggests detrimental conditions for their growth, although the mechanisms explaining both the increase and the cease are unclear. We hypothesise that *Acinetobacter* spp., despite being rare taxa in fresh manure and soil, are strongly competitive taxa in response to labile C pulses under broad environmental conditions (Goldfarb et al., 2011). The fact that rare *Acinetobacter* spp. became dominant in soil and manure for some time under certain conditions suggests that they could be conditionally rare taxa (Shade et al., 2014). This rare-to-dominant phenomenon has been shown as highly relevant in microbial communities exposed to environmental disturbance (Fuentes et al., 2016). Once the conditions allowing the short-term increase are no longer present, likely a decrease in the content of more labile C, *Acinetobacter* spp. become rare again. Shifts in soil and manure pH could be also involved in the cease, as pH tended to increase over time whereas *Acinetobacter* spp. need slightly acidic pH to grow (Towner, 2006). Despite other potentially pathogenic groups, such as the genus *Pseudomonas*, are also known to grow in response to labile C inputs (Goldfarb et al., 2011) and can respond to manure amendments (Leclercq et al., 2016; Macedo et al., 2021), we only detected increases over time in the cultured fraction. In addition to *Acinetobacter* spp., the subsequent increases in the relative abundance of culturable *Pseudomonas* species, as well as in other genera such as *Sphingomonas* and *Stenotrophomonas*, highlight the role that manure may play in the spread of potentially risky opportunistic pathogens in the environment, although further studies are needed to confirm their potential pathogenicity.

5. Conclusions

We conclude that both fresh manure and soil have reciprocal effects on each other's microbial communities, with the effects on soil being stronger in the short term and those on manure more important in the long term. Our experiments demonstrated that fresh dairy cattle manure can spread *Acinetobacter* species in the soil but also stimulate those from the soil through a likely nutrient pulse. The use of γ-irradiated soil

confirms the significant role that native soil communities can play in preventing the spread of putative opportunistic pathogens such as non-fermenting Gram-negative bacteria. Whether these bacteria actually pose a clinical risk due to their resistance to antimicrobials and virulence remains to be elucidated.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.apsoil.2022.104466.

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