Estudio de la diversidad estructural y funcional de las comunidades microbianas de los suelos uruguayos con respecto a la fitodisponibilidad del fósforo

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Doctora en Ciencias Agrarias

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RESUMEN

El fósforo (P) es esencial para el crecimiento vegetal. El uso excesivo de fertilizantes fosfatados asociado a la intensificación agrícola ha tenido un fuerte impacto negativo en el ambiente y la economía. Los microorganismos del suelo desempeñan un papel clave en el ciclo del P, mediando su fitodisponibilidad a través de mecanismos

enzimáticos. Los suelos de pastizales del bioma Campos desarrollados sobre diversos materiales parentales se caracterizan por una baja disponibilidad de P. En esta tesis se estudió: a) la diversidad estructural de las comunidades procariotas en cinco unidades de suelo con distintos materiales parentales y estado nutricional; b) los perfiles funcionales de estas comunidades vinculados al ciclo del P y su relación con las propiedades fisico-químicas del suelo y c) la distribución y abundancia de ocho enzimas clave del ciclo del P en pastizales de Uruguay y del mundo. Las comunidades procariotas se estudiaron mediante un abordaje metagenómico (gen 16S rARN y metagenoma total). Los principales resultados fueron: a) la estructura del suelo, el contenido de nutrientes y la capacidad de retención de agua influyen en la composición de las comunidades procariotas, compuestas principalmente por *Archaea*, *Firmicutes*, *Acidobacteria*, *Actinobacteria* y *Verrucomicrobia*, con variaciones en su abundancia según el tipo de suelo. b) Los perfiles funcionales de las comunidades fueron modelados por las mismas propiedades fisico-químicas que la diversidad taxonómica; la diversidad funcional fue menor que la taxonómica, lo que sugiere redundancia funcional. c) La fosfatasa alcalina PhoD fue la enzima más abundante y ampliamente distribuida filogenéticamente, seguida por las fosfatasas ácidas NSAP-A y NSAP-C. Se encontró una fuerte asociación entre la abundancia y diversidad de los genes que codifican estas tres enzimas y el pH, la temperatura máxima y la evapotranspiración. Los resultados indican que la diversidad procariota estructural y funcional se ve influenciada por las propiedades fisico-químicas del suelo y variables ambientales, por lo que su comprensión es esencial para la gestión sostenible del P en agroecosistemas del bioma Campos.

Palabras clave: bioma Campos, ciclo del fósforo, comunidades procariotas, metagenómica, ion ortofosfato

Study of the structural and functional diversity of microbial communities in Uruguayan soils with respect to phosphorus phytoavailability

SUMMARY

Phosphorus (P) is essential for plant growth; its excessive use as a fertilizer in agricultural intensification has negatively impacted the environment and the economy. Soil microorganisms play a key role in P cycling, mediating its phytoavailability through enzymatic mechanisms. In this thesis, we studied: a) the structural diversity of prokaryotic communities in five soil units with different parent materials and nutritional status; b) the functional profiles of these communities linked to P cycling and their relationship with soil physicochemical properties; c) the distribution and abundance of eight key enzymes of P cycling in grasslands of Uruguay and the world. Prokaryotic communities were studied using a metagenomic approach (16S rRNA gene and total metagenome). The main results were: a) Soil structure, nutrient content, and water retention capacity influence the composition of prokaryotic communities, composed mainly of *Archaea*, *Firmicutes*, *Acidobacteria*, *Actinobacteria* and *Verrucomicrobia*, with variations in their abundance according to soil type. b) Functional profiles of the communities were modeled by the same physicochemical properties as taxonomic diversity; functional diversity was lower than taxonomic diversity, suggesting functional redundancy. c) PhoD alkaline phosphatase was the most abundant and phylogenetically widely distributed enzyme, followed by NSAP-A and NSAP-C acid phosphatases. A strong association was found between the abundance and diversity of genes encoding these three enzymes and pH, maximum temperature and evapotranspiration. The results indicate that structural and functional prokaryotic diversity is influenced by soil physicochemical properties and environmental variables, making its understanding essential for the sustainable management of the P cycle in agroecosystems of the Campos biome.

Keywords: Campos biome, phosphorous cycle, prokaryotic communities, metagenomic, orthophosphate ion

1. INTRODUCCIÓN

1.1 Presentación del contexto y antecedentes

El fósforo (P) es el segundo macronutriente requerido para el desarrollo de las plantas luego del nitrógeno (N). Por lo tanto, garantizar la disponibilidad y accesibilidad de P a largo plazo es crucial para la producción mundial de alimentos. La roca fosfórica —principal fuente de P hoy en día— ha sido fundamental para sustentar la alimentación de miles de millones de personas. Sin embargo, el fosfato de roca es un recurso no renovable y requiere de aproximadamente 10-15 millones de años para formarse. A su vez, las reservas de roca fosfórica están cada vez más contaminadas y muy concentradas geográficamente, lo que plantea una vulnerabilidad geopolítica. Con la tasa de explotación actual, se prevé que los depósitos hoy conocidos se agotarán en las próximas décadas y que, en consecuencia, este elemento será un recurso restrictivo (Cordell et al., 2009). La intensificación en el uso de P como fertilizante ha tenido un gran impacto ambiental: ha degradado la calidad del agua en ríos, lagos y océanos costeros, y ha creado floraciones de algas tóxicas y zonas muertas. Las crecientes demandas y la preocupación por disminuir el impacto ambiental han impulsado la evaluación de fuentes alternativas de P, así como la forma en que este es utilizado en el sistema de producción mundial de alimentos (Cordell et al., 2009). En este sentido, en el contexto mundial, regional y nacional, existe una creciente preocupación por el recurso P en los ecosistemas naturales y en los agroecosistemas. En el ámbito mundial se han conformado plataformas de trabajo en red, con atención al uso y manejo sostenible del recurso en los sistemas productivos y, a la vez, a la protección de la salud ambiental de ríos, mares y océanos. Tal es el caso de European Sustainable Phosphorus Platform (ESPP, https://phosphorusplatform.eu/), Sustainable Phosphorus Alliance (https://phosphorusalliance.org/) y Global Phosphorus Research Initiative (GPRI, http://phosphorusfutures.net/).

1.1.1 El fósforo en el suelo

El P se encuentra en los suelos formando diferentes compuestos químicos no solubles y, por lo tanto, no disponibles para las plantas. Esto hace que muchos suelos sean pobres en este elemento esencial. Incluso en suelos considerados como fértiles no se encuentran concentraciones mayores a 10 µM en su forma soluble, los iones ortofosfato (Gyaneshwar et al., 2002). El P se encuentra en el suelo en dos fracciones: fosfatos orgánicos (fosfatos incorporados a compuestos orgánicos) y fosfatos de origen inorgánico. Los bajos niveles de formas solubles de P se deben a la alta reactividad de ambas fracciones con iones de calcio (Ca), hierro (Fe) o aluminio (Al) presentes en el suelo, formando complejas asociaciones que precipitan en éste (Gaiero et al., 2020, Zhou et al., 2017) (figura 1). La conversión de estas dos fracciones, de su estado insoluble a su forma soluble, se lleva a cabo mediante dos mecanismos distintos: la solubilización, que se refiere a la movilización del fósforo inorgánico (PI), y la mineralización, que se refiere a la movilización del fósforo orgánico (PO) (Hinsinger et al., 2015).

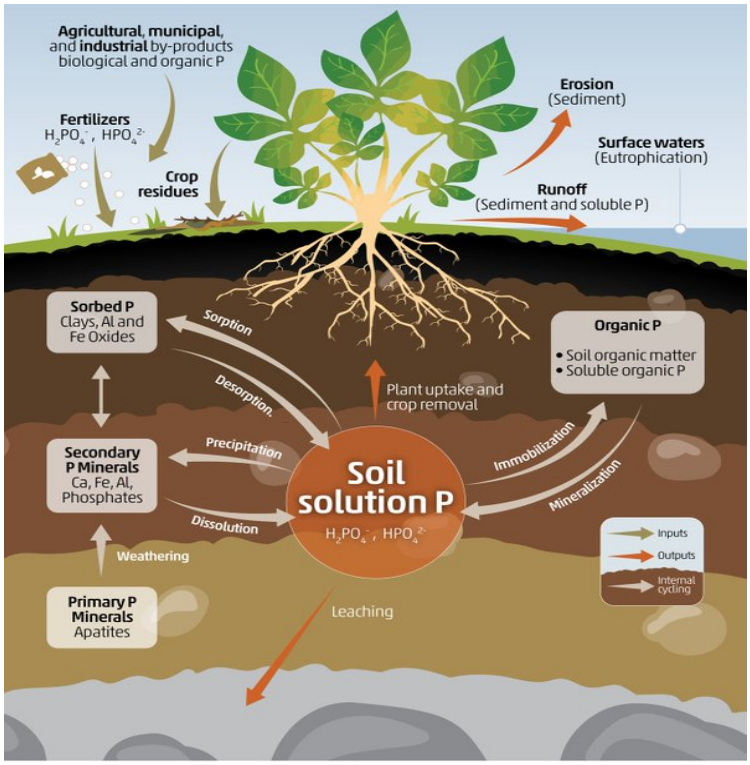

Figura 1: ciclo del fósforo en el suelo. Fuente: FAO (2022).

La proporción de cada fracción varía entre los distintos tipos de suelos. La composición de las moléculas en las que queda retenido el P se ve influenciada, entre otros factores, por el material madre del suelo, el grado de meteorización, el pH y la temperatura. Los principales compuestos de P orgánico identificados son el inositol fosfato, los fosfolípidos y los ácidos nucleicos (Gyaneshwar et al., 2002). El inositol fosfato, presenta seis grupos ortofosfato (InsP6), de nombre químico myo-inositol 1, 2, 3, 4, 5, 6-hexaquis (dihidrógeno fosfato), llamado comúnmente ácido fítico o fitato. Los fitatos son muy estables y tienden a acumularse en suelos vírgenes, pudiendo representar hasta el 80 % o más del PO (Quiquampoix y Mousain, 2005, Turner et al., 2005). Dada su naturaleza química, es una molécula altamente reactiva; se comporta de manera similar a los iones fosfato, reaccionando con los iones presentes en el suelo y formando complejos insolubles. Los fosfolípidos y ácidos

nucleicos forman el pool de P lábil, de fácil acceso para los organismos presentes (tabla 1; figura 1).

Tabla 1: formas de fósforo orgánico en el suelo.

	DENOMINACIÓN	CARACTERÍSTICAS
Inositol fosfato	Inositol hexafosfato	Precipitados no solubles muy estables (50-80 %)
Fosfolípidos		Lábil (1-5 %)
Ácidos nucleicos		Lábil (0,2-2,5%)

El PI se encuentra en minerales primarios como las apatitas, adsorbidos a arcillas (P lábil), ocluido y/o precipitado (no lábil) y en solución en forma de fosfato ácido ($HPO4^{=}$) y fosfato diácido ($H2PO4^{-}$) (Gyaneshwar et al., 2002) (tabla 1).

Tabla 2: formas de fósforo inorgánico en el suelo (adaptado de Tsai y Rosseto, 1992).

	DENOMINACIÓN	COMPOSICIÓN	CARACTERÍSTICAS
	Hidroxiapatita	$3Ca_3(PO_4)_2Ca(OH)_2$	Mayor abundancia
Fosfatos de Calcio	Oxiapatita	$3Ca_3(PO_4)_2CaO$	
	Fluoroapatita	$3Ca_3(PO_4)_2CaF_2$	Mayor abundancia
	Carbonoapatita	$3Ca_3(PO_4)_2CaCO_3$	
	Fosfato tricálcico	$3Ca_3(PO_4)_2$	
	Fosfato bicálcico	$CaHPO_4$	Mayor solubilidad
Fosfatos de hierro	Vivianita	$Fe_3(PO_4)_28H_2O$	
	Estrengita	$FePO_42H_2O$	
Fosfatos de Aluminio	Variscita	$AlPO_42H_2O$	

Los suelos del Uruguay tienen niveles de suministro de P que no son suficientes para el desarrollo de la mayoría de los cultivos y pasturas sembradas. Si bien los niveles de P del suelo pueden ser altos, estos se encuentran en formas no asimilables. En los suelos del Uruguay, la fracción de P ligada al Fe representa la proporción mayor de PI fijado (Hernández y Meurer, 1998, Hernández, 1997), mientras que el PO representa, en promedio, el 51 % del P total (Hernández et al., 1995).

Para superar la limitación de fósforo en el suelo, que puede afectar la producción, se recurre a la aplicación de fertilizantes ricos en fósforo (Cordell et al.,

2009). Sin embargo, más del 70 % del fósforo agregado se vuelve insoluble rápidamente por su alta reactividad, como se describió anteriormente (Morón, 1996, Hernández y Zamalvide, 1998), lo que hace su utilización muy ineficiente. Adicionalmente, Uruguay importa la totalidad del P necesario para la actividad agropecuaria, por lo que la fertilización fosfatada representa un costo de producción alto.

1.1.2 Los microorganismos y el ciclo del fósforo

La participación de los microorganismos en la solubilización de fosfatos inorgánicos se reportó ya en 1903 (Kucey et al., 1989) y, desde entonces, ha sido tema de diversos y extensos estudios (Kucey et al., 1989, Tandon, 1987, Goldstein, 1986, Subba Rao, 1982).

Los microorganismos involucrados en la movilización del P son ubicuos y su número varía según el suelo. Las bacterias y arqueas capaces de movilizar el P constituyen entre el 1-50 % y los hongos entre 0,1-0,5 % de la población total de microorganismos de cada suelo particular (Wang et al., 2021, Gyaneshwar et al., 2002). En general, las bacterias solubilizadoras de P superan en número a los hongos con la misma capacidad en 2-150 veces (Kucey et al., 1989, Kucey, 1983, Banik y Dey, 1982); ambos grupos son capaces de promover la conversión de fosfatos insolubles a iones ortofosfato (Rodríguez et al., 2006).

En la naturaleza existe una amplia gama de mecanismos mediante los cuales los microorganismos pueden solubilizar el P, y gran parte del ciclo de este nutriente en el suelo se atribuye a las bacterias y a los hongos (Khan et al., 2009). Algunas especies bacterianas tienen el potencial de mineralizar y solubilizar el PO y el PI, respectivamente (figura 2).

Los principales mecanismos de solubilización del PI por parte de los microorganismos incluyen:

- Acidificación: el fósforo puede ser liberado en forma de fosfatos por la acidificación del suelo mediante la excreción de protones (Drouillon y Merckx, 2003, Gyaneshwar et al., 2002).
- Producción de ácidos orgánicos: algunos microorganismos, como las bacterias y los hongos, producen ácidos orgánicos que mediante una reacción entre sus grupos hidroxilo y carboxilo forman complejos con el catión unido al fosfato, siendo este último convertido a formas solubles (Karpagam y Nagalakshmi, 2014). .Entre ellos, el ácido glucónico y el ácido 2-cetoglucónico parecen ser los agentes más frecuentes de solubilización del fosfato mineral (Walpola y Yoon, 2012, Song et al., 2008, Welch et al., 2002).
- Disolución de minerales: el P también se puede solubilizar mediante la disolución de minerales que contienen P, como la apatita. Este proceso a menudo se ve favorecido por la acción de microorganismos productores de ácido.

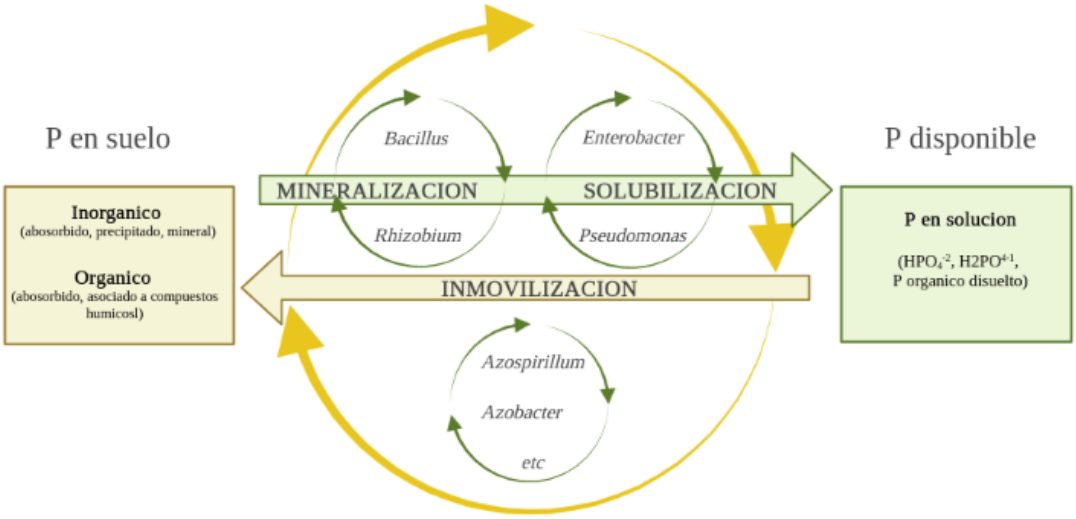

Figura 2: esquema del ciclo del fósforo mediado por microorganismos en el suelo. Fuente: Kim et al. (1998).

La mineralización microbiana de PO está fuertemente influenciada por los parámetros ambientales. De hecho, la alcalinidad moderada favorece la

mineralización de PO (Paul y Clark, 1988). La degradación de los compuestos orgánicos que contienen el P depende principalmente de las propiedades físico-químicas y bioquímicas de sus moléculas; por ejemplo, ácidos nucleicos, fosfolípidos y fosfatos de azúcar se descomponen fácilmente, pero el ácido fítico, los polifosfatos y los fosfonatos se descomponen más lentamente (McGrath, 1995, Ohtake, 1996, McGrath, 1998, citados por Karpagam y Nagalakshmi, 2014).

En condiciones de deficiencia de PI, las bacterias del suelo producen enzimas capaces de reciclar el PI desde compuestos orgánicos para su uso propio y/o para las plantas (Kumar et al., 2017, Fitriatin et al., 2011) . Estas enzimas llamadas fosfatasas catalizan la ruptura hidrolítica de un enlace entre P y otro átomo. Las fosfatasas están involucradas principalmente en la hidrólisis de los enlaces C-P y O-P presentes en la mayoría de los compuestos orgánicos que se encuentran en los suelos. Las fosfomonoesterasas representan la clase más abundante de fosfatasas extracelulares liberadas por bacterias del suelo (Park et al., 2022, Margalef et al., 2017, Sharma et al., 2008). Estas se dividen en tres grandes grupos de enzimas; las fosfatasas alcalinas, las fosfatasas no específicas ácidas y las fitasas (Sharma et al., 2008, Hui et al., 2013). Los dos primeros grupos de enzimas catalizan la hidrólisis de fosfoésteres orgánicos y ambas muestran una amplia especificidad de sustrato (Cai et al., 2021, Thaller et al., 1998). A diferencia, las fitasas específicamente liberan el PI desde moléculas de ácido fítico (Gaiero et al., 2020, Bergkemper et al., 2016, Morrison et al., 2016, Huang et al., 2009, Jorquera et al., 2008, Rossolini et al., 1998).

Entre los microorganismos capaces de solubilizar y/o mineralizar P se han reportado especies de bacterias y de hongos con similares funciones. Dentro de las bacterias se encuentran especies de los géneros *Pseudomonas*, *Agrobacterium*, y *Bacillus* (Babalola y Glick, 2012). Otras bacterias solubilizadoras y mineralizadoras de P incluyen varias especies de *Azotobacter* (Kumar et al., 2014), *Bacillus* (Altier et al., 2020, David et al., 2014, Jahan et al., 2013), *Bradyrhizobium*, *Burkholderia* (Istina et al., 2015, Zhao et al., 2014, Mamta et al., 2010), *Enterobacter*, *Erwinia* (Chakraborty et al., 2009), *Kushneria* (Zhu et al., 2011), *Paenibacillus* (Fernández-

Bidondo et al., 2011) *Ralstonia*, *Rhizobium* (Tajini et al., 2012), *Rhodococcus*, *Salmonella*, *Serratia*, *Sinomonas* y *Thiobacillus* (David et al., 2014, Postma et al., 2010). Los géneros fúngicos reportados han sido *Aspergillus*, *Fusarium*, *Paecilomyces*, *Penicillium*, entre otros (Sharma y Sharma, 2021, Suyamud et al., 2020, Srinivasan et al., 2012, Barnerjee et al., 2010, Sulbarán et al., 2009, Buch et al., 2008, Gulati et al., 2007, Son et al., 2006, Whitelaw, 2000). Los hongos, a diferencia de las bacterias, han demostrado ser más eficientes en la solubilización del PI debido a su capacidad de explorar y colonizar el suelo a grandes distancias (Alori et al., 2017).

El conocimiento generado sobre las interacciones entre las plantas y los microorganismos ha llevado al desarrollo de inoculantes microbianos, que hoy son utilizados como biofertilizantes en diversos sistemas de producción. En el ámbito nacional, la línea de investigación de microorganismos promotores del crecimiento (MPC) tiene una larga trayectoria. Se han caracterizado y aislado cepas nativas de *Pseudomonas fluorescens,* con capacidad de supresión de fitopatógenos y de promoción del crecimiento vegetal (Altier et al., 2013, Yanes et al., 2012, Höfte y Altier 2010, Quagliotto et al., 2009,). Muchas de estas cepas poseen características bioquímicas con potencial para mejorar la disponibilidad de P, tales como producción de sideróforos y enzimas (De La Fuente et al., 2004).

Recientemente, combinando el conocimiento sobre MPC y el rol clave de los microorganismos en el ciclo biogeoquímico del P, se estableció una estrategia para el desarrollo de un biofertilizante-P con base en cepas nativas de *Bacillus* spp. en el marco del proyecto *Desarrollo de inoculantes para la movilización de fósforo como insumo en la producción agrícola*. A partir de este proyecto se conformó una colección de 181 cepas de *Bacillus sensu latu* (*B. pumilus, Priestria aryabhattai, Priestria megaterium, B. cereus sensu lato, B. thuringiensis, B. simplex, Lysinibacillus xylanilyticus, Brevibacterium frigoritolerans, Paenibacillus barcinonensis*), las cuales fueron testadas para diferentes características deseables

para un bioinsumo. Se caracterizaron por la actividad mineralizadora de PO (actividad fitasa y producción de ácidos orgánicos) y solubilizadora de PI (P-Ca, P-Fe, P-Al), en medio sólido y líquido; la producción de auxinas AIA y enzima ACC desaminasa, la capacidad de fijación de N, la producción de biofilm, movilidad y motilidad (características asociadas a la rizocompetencia) e inocuidad. Se identificaron tres cepas con las características deseables y que además presentaban capacidad de promoción del crecimiento vegetal (Altier et al., 2020).

Por otro lado, en el ámbito internacional, el estudio de las interacciones de los rizobios con las plantas no leguminosas ha mostrado que estas bacterias son capaces de promover el crecimiento vegetal a través de mecanismos directos e indirectos. Mehboob et al. (2009) mostraron que existen incrementos en la producción de materia seca (raíces y parte aérea) y/o el rendimiento en girasol, trigo, arroz, lechuga, algodón, sorgo y maíz, debido a la inoculación con rizobios. El género *Rhizobium*, junto con *Pseudomonas* y *Bacillus*, han mostrado la capacidad de solubilizar P (fijado por el suelo y/o aplicado como fertilizante), lo que ha resultado en incrementos en los rendimientos de cultivos (Cerecetto et al., 2021, Gyaneshwar et al., 2002, Abd-Alla, 1994).

1.1.3 Metagenómica como herramienta de estudio

La metagenómica se define como el estudio de las comunidades microbianas ambientales utilizando un conjunto de herramientas genómicas para acceder directamente a su contenido genético (Quince et al., 2017). A partir de esto, se define a un metagenoma como el conjunto de genomas provenientes de una muestra ambiental. Esta disciplina, independiente de cultivo, tiene el potencial de responder preguntas fundamentales de la ecología microbiana. Permite comprender la diversidad genética y estructural de las poblaciones, así como los roles ecológicos de la mayoría de los microorganismos, especialmente en ecosistemas complejos como lo es el suelo.

Existen diferentes enfoques en los trabajos metagenómicos: los trabajos descriptivos que se ocupan de saber quiénes están y en qué proporción (metagenómica estructural) y los trabajos que buscan entender qué funciones están presentes (metagenómica funcional) en el ambiente estudiado.

Los primeros estudios metagenómicos descriptivos utilizaron la secuenciación capilar para el estudio de ambientes de baja diversidad microbiana, como los son los biofilms (Tyson et al., 2004) y las minas ácidas (Edwards et al., 2000). La llegada de las tecnologías de secuenciación masiva ofreció una rápida, relativamente económica y masiva obtención de datos de secuencia, que cambió por completo el alcance de esta clase de estudios metagenómicos. A partir de allí, pudieron ser estudiados ambientes con mayor diversidad de microorganismos, como lo son el suelo (Fierer et al., 2007, Leininger et al., 2006), el cual se estima que contiene la mayor diversidad de microorganismos en la tierra (entre 5000 y 10000 especies de microorganismos por gramo de suelo) (Ghazanfar y Azim, 2009), y el microbioma humano (Palmer et al., 2007, Ley et al., 2005, Gill et al., 2006, Turnbaugh et al., 2006, Backhed et al., 2004) .

Dentro de las técnicas moleculares "no cultivables" empleadas, se destaca el uso de secuencias de ARNr, las cuales ofrecen la ventaja adicional de caracterizar con una resolución superior a los microorganismos presentes en las comunidades. Esto ha permitido reconstruir relaciones filogenéticas entre diferentes especies. Al utilizar información de secuencias de ARNr, es posible diseñar cebadores específicos para grupos particulares de microorganismos, lo que, a su vez, facilita una caracterización más eficiente de los cambios en las comunidades en condiciones naturales. (Hurt et al., 2001).

El gen 16S ARNr es el más utilizado como marcador filogenético de taxones microbianos (Pace et al., 1997). Se encuentra en todos los organismos vivos, con la notable excepción de los virus, y representa más del 80 % del ARN bacteriano total. La estructura del gen del ARNr 16S se compone de regiones conservadas intercaladas con regiones variables. De esta forma, al centrarse en una pequeña parte

del genoma microbiano, hace bajar los costos de secuenciación de forma significativa. Este enfoque ha sido particularmente eficaz para el monitoreo de los cambios de las comunidades microbianas (Techtmann et al., 2016, Fierer et al., 2012, Caporaso et al., 2011). Adicionalmente, la creación de bases de datos como SILVA (https://arb-silva.de/) permitieron la identificación de microorganismos con control de calidad actualizado, alineando secuencias de genes de ARN ribosomal de Bacteria, Archaea y Eukaryota (Yilmaz et al., 2011).

Para el abordaje de la metagenómica funcional, se pueden tomar diferentes caminos: o bien se construyen bibliotecas metagenómicas y se busca identificar nuevas enzimas relacionadas a funciones específicas o es tomado el ADN metagenómico y secuenciado (*shotgun* de ADN). Esta estrategia de secuenciación toma el genoma total de la comunidad, este es fragmentado y luego los fragmentos de ADN son secuenciados y ensamblados. Desde que Venter et al. (2004) utilizaron este abordaje para el estudio de la comunidad microbiana en el Mar de los Sargazos por primera vez y hasta la actualidad, la cantidad de información conocida acerca de los genomas ambientales se ha visto incrementada.

Los estudios iniciales de metagenómica del suelo se basaron en la construcción de bibliotecas (cromosoma artificial bacteriano [BAC], cósmido, fósmido), que luego fueron secuenciadas con la intención de encontrar genes que codificaran para productos de interés, como proteínas con actividad antimicrobiana o enzimas (Daniel, 2005). A través de la metagenómica se ha podido acceder a un gran número de genes, los cuales codifican para nuevas enzimas o para enzimas más eficientes desde el punto de vista biotecnológico. Este abordaje permite la identificación de las vías metabólicas involucradas en el proceso de movilización del P mediante la prospección de enzimas relacionadas a este, así como a la identificación de nuevos microorganismos solubilizadores de P (Prayogo et al., 2020; Chhabra et al., 2013).

1.1.4 El microbioma y las variables ambientales

El microbioma del suelo se ve influenciado por factores bióticos y abióticos (Xue et al. 2017, Griffiths et al. 2011), tales como las propiedades edáficas, la temperatura y la humedad, así como el tipo de vegetación. El pH del suelo y el contenido de carbono orgánico, N y P son algunos de los factores más influyentes que determinan los ensamblajes microbianos (Fierer y Jackson, 2006, Martiny et al., 2006). Estos factores establecen el contexto en el que se producen las interacciones microbianas, lo que da lugar a diferentes ensamblajes y funciones (Fanin y Bertrand, 2016, Kinkel et al., 2011, Garbeva et al., 2004). No existe un microbioma del suelo típico; la abundancia de taxones bacterianos y de arqueas puede variar considerablemente en función del tipo de suelo, el uso de la tierra y las condiciones ambientales (Fierer, 2017). Sin embargo, existen asociaciones entre la abundancia de filos, el tipo de suelo y el uso del suelo. Recientemente, Pino et al. (2023), mediante un estudio del microbioma del suelo a gran escala basado en genes marcadores (16S rRNA e ITS) en Australia, reportaron cambios en la betadiversidad debidos principalmente a la química del suelo —pH y capacidad efectiva de intercambio catiónico (CEIC)— y los ciclos de temperatura del suelo y de temperatura de la superficie terrestre. Identificaron patrones espaciales de las comunidades microbianas coincidentes con los tipos de suelo y su pedogénesis. Asimismo, reportaron una mayor riqueza de microorganismos raros en los suelos cultivados, lo que podría comprometer las funciones del suelo a largo plazo. Otros estudios locales identificaron asociaciones entre ciertos filos, el uso del suelo y el grado de degradación. Neal et al. (2017) identificaron a los filos *Gemmatimonadetes* y *Armatimonadetes* asociados especialmente con suelos degradados. Mientras que en un estudio de las comunidades procariotas del suelo de una rotación de pasturas y arroz, las comunidades bacterianas y de arqueas estaban dominadas por los filos *Firmicutes* y *Proteobacteria* en pasturas, mientras que los filos *Methanocellales* y

Methanosarcinaceae dominaban en suelos bajo cultivo de arroz (Fernández-Scavino et al., 2013).

El mantenimiento de la diversidad taxonómica y funcional de las comunidades microbianas es esencial para el funcionamiento de los ecosistemas (Philippot et al., 2013, Fierer et al., 2006). La diversidad funcional microbiana tiene un rol central en el ciclo de nutrientes y se ve influenciada por las propiedades del suelo, incluyendo el pH, el contenido de materia orgánica, la disponibilidad de nutrientes y la textura (Wang et al., 2019, Guo et al., 2018, Zhang et al., 2018). En particular, las comunidades microbianas desempeñan un papel fundamental en el reciclaje del P del suelo (Richardson y Simpson, 2011), como se mencionó anteriormente. Están involucradas tanto en la solubilización como en la mineralización del P, a través de mecanismos enzimáticos tales como las fosfatasas, los cuales se ven influenciados por variables ambientales y propiedades del suelo, incluyendo el pH del suelo, el N total, la precipitación y la temperatura (Khan et al., 2009). La actividad de dichas enzimas se ve afectada, por ejemplo, por el contenido de nutrientes del suelo. Una proporción equilibrada de nutrientes puede aumentar la actividad de las fosfatasas del suelo, lo que mejora el ciclo del P en el suelo y el crecimiento de las plantas. Por el contrario, una proporción desequilibrada de nutrientes puede disminuir la actividad de las fosfatasas del suelo, lo que limita la disponibilidad de P y reduce el crecimiento de las plantas (Zheng et al., 2018, Margalef et al., 2017). El pH del suelo también tiene un efecto sobre el tipo de enzimas producidas por los microorganismos y en su actividad al cambiar los estados bioquímicos/moleculares de los inhibidores y/o activadores en la solución del suelo y en la concentración de los sustratos (Dick et al., 2011), independientemente de la abundancia de los genes que codifican estas enzimas (Fraser et al., 2017).

En el ciclo del fósforo intervienen numerosos genes que pueden agruparse según su participación en las distintas etapas. Las proteínas transportadoras de fosfatos, codificadas por los genes *pstA*, *phoU* y *ugpQ*, facilitan la captación de iones fosfato por microorganismos y plantas, y los transportan a través de las

membranas celulares. Por otro lado, cuando los microorganismos experimentan escasez de P, los genes reguladores (por ejemplo, *phoB*, *phoR*) se activan y ayudan a conservar y reciclar el P dentro de las células (Santos-Beneit 2015). Estos genes son parte del sistema regulador de dos componentes (PhoBR), denominado regulón Pho, el cual regula la transcripción de genes que codifican enzimas involucradas en la movilización del P en condiciones de bajo PI (Park et al., 2022, Lidbury et al., 2017). Los transportadores de fosfato (por ejemplo *pstA*, *phoU*, *ugpQ*) codifican proteínas que transportan iones de fosfato a través de las membranas celulares, lo que facilita la absorción de P por plantas y microorganismos (Zeng et al., 2022, Oliverio et al., 2020, Bergkemper et al., 2016). Los genes de mineralización de PO codifican enzimas capaces de liberar P a partir de ésteres orgánicos de fosfato. Las fosfatasas alcalinas y las fosfatasas ácidas no específicas (del inglés non-specific acid phosphatases, NSAP) catalizan la hidrólisis entre carbono y fósforo en ésteres orgánicos de fosfato. Las fitasas liberan específicamente PI del ácido fítico (Gaiero et al., 2020, Bergkemper et al., 2016, Morrison et al., 2016, Huang et al., 2009, Jorquera et al., 2008, Rossolini et al., 1998).

Las fosfatasas alcalinas son producidas por una amplia gama de bacterias, arqueas y hongos, que desempeñan un papel importante en el recambio microbiano del P (Li et al., 2021). PhoD, PhoX y PhoA son tres tipos diferentes de fosfatasas alcalinas, siendo PhoD la más abundante y ubicua (Ragot et al., 2015). Tanto PhoD como PhoX fueron identificadas como enzimas extracelulares dependientes de Ca^{2+} y PhoA como una enzima intracelular dependiente de Zn^{2+} (Neal et al., 2018). Las fosfatasas alcalinas muestran una amplia especificidad de sustrato y una alta eficiencia catalítica (Cai et al., 2021, Rodríguez et al., 2014). Estas características permiten a los microorganismos que albergan los genes que codifican para estas enzimas utilizar fuentes alternativas de P en condiciones de P limitado, lo que les confiere una ventaja sobre las plantas (Li et al., 2021).

Las fosfatasas ácidas se dividen en tres grupos, NSAP clase A, NSAP clase B y NSAP clase C; ninguna de ellas exhibe una fuerte especificidad de sustrato, de ahí

sus nombres (Thaller et al., 1998). Estas enzimas son producidas tanto por microorganismos como por plantas, mostrando su mayor actividad enzimática en suelos ácidos (Gaiero et al., 2017). Diferentes estudios metagénomicos han estudiado esas enzimas, observando cómo varían en abundancia y diversidad en diferentes ambientes (Neal et al., 2018, Bergkemper et al., 2016). Neal et al. (2018) mostraron que NSAP clase C era más abundante en suelos ácidos en condiciones limitantes de P en comparación con NSAP clase A. Además, se ha informado que estas enzimas muestran una mayor actividad y una mayor abundancia de los genes que las codifican en la rizosfera en comparación con el suelo (Fraser et al., 2017, Spohn y Kuzyakov, 2013).

Las fitasas son producidas por bacterias, hongos, plantas y cierto grupo de animales capaces de catalizar la mineralización de P orgánico de fitato a P inorgánico (Ariza et al., 2013, Tu et al., 2011, Jorquera et al., 2008). Las familias de fitasas más comunes en microorganismos son la fitasa beta-propulsora (BPP), la fitasa cisteína —similar a la proteína tirosina fosfatasa (CPHY)— y la fitasa ácida histidina (HAPhy) (Singh y Satyanarayana, 2011). Las principales diferencias entre las familias de fitasas son estructurales, relacionadas principalmente con diferencias en el sitio activo que determina qué grupo fosfato del fitato se desfosforila, y los requisitos de cofactores. A pesar de ello, todas las fitasas pueden liberar las seis moléculas de fosfato contenidas en el fitato (Misset, 2002). Las fitasas muestran diferentes pH y temperaturas óptimas para su actividad en condiciones de laboratorio (Caffaro et al., 2020) y también dependen de las especies de microorganismos del suelo (Amadou et al., 2021). Además, la actividad enzimática se ve afectada por el tipo de suelo, la textura y la mineralogía al variar la capacidad de retener una enzima activa (Azeem et al., 2014, Tang et al., 2006, Rao et al., 1994).

1.1.5 Bioma de pastizales

Los pastizales son uno de los biomas más extensos y ampliamente distribuidos en la superficie de la Tierra. Estos biomas están definidos por diversos factores, tales como las condiciones climáticas, el pastoreo y el fuego (Zhou et al., 2017, White et al., 2000). Se desarrollan en zonas áridas y semiáridas, con períodos fríos y secos estacionales y presentan altas tasas de evapotranspiración (Barnett y Facey, 2016, Lenhart et al., 2015, Knapp et al., 2002). La comunidad vegetal característica de los pastizales está dominada por gramíneas y especies relacionadas, junto con otras especies arbustivas que tienen diferentes estilos de vida. Los ensamblajes de la comunidad vegetal dependen en gran medida de las variables climáticas. Además, la mayor parte de la biomasa aérea de los pastizales, junto con las bajas tasas de descomposición, genera importantes acumulaciones de materia orgánica en los perfiles del suelo (Blair et al., 2014).

El bioma Campos, uno de los biomas de pastizales, está ubicado en Sudamérica, se extiende desde el centro-este de Argentina hasta Uruguay y el sur de Brasil. Es un ecosistema único cuyo paisaje está influenciado por las características edafotopográficas de la región, lo que lo convierte en un *hotspot* de biodiversidad con más de 4000 especies de plantas templadas y subtropicales (Camargo et al., 2019, Andrade et al., 2018, Modernel et al., 2016). El bioma Campos presta servicios ecosistémicos vitales, incluyendo el almacenamiento de carbono, la regulación del agua, el control de la erosión del suelo y el ciclo de los nutrientes. Sin embargo, enfrenta retos debido a la creciente demanda de producción de alimentos y las prácticas ganaderas extensivas (Baeza y Paruelo, 2020, Tiscornia et al., 2019). Por ello, para la preservación de este bioma, es necesario lograr un equilibrio entre la producción de alimentos y los esfuerzos de conservación en la región (Weyland et al., 2017, Pillar et al., 2012, Altesor et al., 2005).

El bioma Campos se desarrolla sobre una gran diversidad de tipos de suelos, que varían según las condiciones geográficas y climáticas de la región. Estos suelos

suelen ser profundos y bien drenados, lo que permite el crecimiento de su vegetación característica. Sin embargo, a pesar de su aparente fertilidad, los suelos de este bioma suelen tener bajos niveles de nutrientes, especialmente N y P, lo que puede limitar el crecimiento de la vegetación. La interacción entre el suelo, el clima y la vegetación crea un equilibrio delicado pero único que contribuye a la biodiversidad y a la mantención de los servicios ecosistémicos de esta importante región (Jaurena et al., 2021, Camargo et al., 2019, Modernel et al., 2016, Royo Pallarés et al., 2005).

En entornos con escasa intervención humana, como lo es el bioma Campos, el ciclo de la materia orgánica, la disponibilidad de nutrientes y la formación de agregados son resultados directos de la actividad microbiana (Vargas et al., 2015). Dicha actividad es capaz de despolimerizar y mineralizar N, P y azufre (S), típicamente ligados a moléculas orgánicas, modulando la disponibilidad de formas inorgánicas de estos nutrientes en el suelo, incluyendo especies iónicas como amonio, nitrato, fosfato y sulfato, las formas nutritivas preferidas por las plantas (Richardson y Simpson, 2011, Van Der Heijden et al., 2008).

En Uruguay, los ecosistemas de pastizales son fundamentales para la producción ganadera extensiva y representan un importante recurso natural del país. Se extienden sobre una superficie cercana al 60 % del territorio nacional y se han desarrollado principalmente sobre suelos con basamento cristalino, basalto y sedimentario de limos terciarios (Paruelo y Altesor, 2023, Perez Rocha, 2020, Lezama et al., 2019). Además, el campo natural es un ecosistema altamente resiliente que ha demostrado su capacidad para resistir perturbaciones ambientales y climáticas. Sin embargo, la intensificación de la producción ganadera y la expansión de la agricultura representan una amenaza para la sostenibilidad de estos ecosistemas, lo que destaca la importancia de adoptar prácticas de manejo sostenible y de conservación de la biodiversidad.

Los suelos uruguayos están particularmente bien descritos: su evolución y propiedades fisico-químicas muestran fuertes asociaciones con el material parental subyacente (Durán et al., 1999). Sin embargo, aún queda mucho por conocer en

relación con las comunidades microbianas que habitan en estos suelos y su papel en los ciclos biogeoquímicos. En particular, la comprensión de las interacciones microbianas que participan en el ciclado de los nutrientes como el C, el N y el P es esencial para el diseño de estrategias de manejo de suelos que promuevan la productividad y la sostenibilidad de los sistemas agropecuarios con menor impacto ambiental. Por lo tanto, es necesario llevar a cabo estudios que permitan caracterizar la diversidad y la función de las comunidades microbianas en los suelos del bioma Campos de Uruguay

1.2 Hipótesis y objetivos

1.2.1 Hipótesis

Las comunidades microbianas de los suelos de Uruguay presentan diferencias estructurales y funcionales con respecto al ciclo del fósforo, determinadas por el material madre y las características físico-químicas de estos. Mediante un abordaje metagenómico es posible identificar los genes clave involucrados en la solubilización y mineralización del P.

1.2.2 Objetivo general

El propósito de este trabajo es contribuir a la comprensión del rol de las comunidades microbianas en la dinámica del P de los suelos bajo campo natural de Uruguay y pastizales del mundo. Para ello, se plantea como objetivo general evaluar la diversidad microbiana existente en suelos formados sobre materiales madre contrastantes y representativos de las regiones de Basalto, Litoral, Cristalino y Noreste, y prospectar genes funcionales asociados a la movilización del P en dichos suelos.

1.2.3 Objetivos específicos

OE1) Caracterizar la diversidad estructural de las comunidades microbianas asociadas a la dinámica del P en suelos de Uruguay con distintas formas de retención y contenidos de P mediante un abordaje metagenómico.

OE2) Identificar genes de origen microbiano involucrados en la movilización del P orgánico en suelos de Uruguay.

OE3) Explorar la relación entre las propiedades físico-químicas de los suelos en estudio y:

a) La diversidad y estructura de las comunidades de microorganismos.

b) Los genes funcionales al ciclo del P.

1.3. ESTRUCTURA GENERAL DE LA TESIS

Esta tesis consiste en un capítulo inicial de introducción, tres artículos científicos que constituyen la estructura central de la tesis y un capítulo final de discusión general y conclusiones globales.

El artículo titulado *Soil structure, nutrient status and water holding capacity shape Uruguayan grassland prokaryotic communities* se publicó en la revista *FEMS Microbiology Ecology* [https://doi.org/10.1093/femsec/fiaa207] y sus autores fueron Silvia Garaycochea, Héctor Romero, Elena Beyhaut, Andrew L. Neal y Nora Altier. Los resultados obtenidos en este trabajo abordan el primer objetivo específico y el objetivo específico 3a, y constituye el segundo capítulo de esta tesis. El objetivo de este trabajo fue describir las comunidades procariotas asociadas a cinco suelos uruguayos con diferente material parental y estado nutricional, bajo pasturas naturales. Asimismo se analizó la relación entre estas comunidades y las propiedades físico-químicas características de los distintos suelos. La estructura y diversidad de las comunidades procariotas se caracterizaron mediante la secuenciación masiva de amplicones del gen 16S rRNA.

El segundo artículo titulado *Functional gene and enzyme profiling of prokaryotic soil communities in the Campos biome of Uruguay: Insights into phosphorous cycling* tiene por autores a Silvia Garaycochea, Héctor Romero, Olagoke F. K, Cordula Vogel y Nora Altier. Será enviado a la brevedad a revista a seleccionar. En este manuscrito se aborda en parte el objetivo específico 2 y el objetivo específico 3b y constituye el capítulo 3 de la tesis. Este trabajo tuvo como objetivo estudiar los perfiles funcionales de cuatro unidades de suelo del bioma Campos de Uruguay con diferente material parental y estado nutricional. Estas unidades son representativas de pastizales naturales (ITA, SPO, TBO) y suelo agrícola (YNG). Las enzimas y genes involucrados en el ciclo del P se predijeron con PICRUSt2 y se determinó la actividad enzimática de fosfatasa ácida (ACP), alcalina (ALP) y fitasa.

El tercer artículo titulado *Abundance and phylogenetic distribution of eight key enzymes of the phosphorus biogeochemical cycle in grassland soils* fue aceptado en la revista *Environmental Microbiology Reports* [https://doi.org/10.1111/1758-2229.13159] y sus autores fueron Silvia Garaycochea, Nora Altier, Carolina Leoni, Andrew L. Neal y Héctor Romero. En este estudio se analizaron 74 metagenomas del suelo de 17 biomas de pasturas distribuidos alrededor del mundo para evaluar la distribución y abundancia de los genes que codifican para ocho enzimas clave del ciclo del P (PhoD, PhoX, PhoA, NSAP-A, NSAP-B, NSAP-C, BPP y CPhy) y su relación con los factores ambientales. En este trabajo se abordó el objetivo específico 3b y constituye el capítulo 4 de la tesis.

2. <u>Soil structure, nutrient status and water holding capacity shape Uruguayan grassland prokaryotic communities</u>

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RESEARCH ARTICLE

Soil structure, nutrient status and water holding capacity shape Uruguayan grassland prokaryotic communities

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One sentence summary: This study shows the influence of the soil nutrient status and water holding capacity on the structure of soil prokaryotic communities of natural grassland ecosystem of Campos biome.

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ABSTRACT

Soil microbial communities play critical roles in maintaining natural ecosystems such as the Campos biome grasslands of southern South America. These grasslands are characterized by a high diversity of soils, low available phosphorus (P) and limited water holding capacity. This work aimed to describe prokaryotic communities associated with different soil types and to examine the relationship among these soil communities, the parent material and the soil nutrient status. Five Uruguayan soils with different parent material and nutrient status, under natural grasslands, were compared. The structure and diversity of prokaryotic communities were characterized by sequencing 16S rRNA gene amplicons. *Proteobacteria*, *Actinobacteria*, *Firmicutes*, *Verrucomicrobia*, *Acidobacteria*, *Planctomycetes* and *Chloroflexi* were the predominant phyla. Ordination based on several distance measures was able to discriminate clearly between communities associated with different soil types. Edge-PCA phylogeny-sensitive ordination and differential relative abundance analyses identified *Archaea* and the bacterial phyla *Firmicutes*, *Acidobacteria*, *Actinobacteria* and *Verrucomicrobia* as those with significant differences among soil types. Canonical analysis of principal coordinates identified porosity, clay content, available P, soil organic carbon and water holding capacity as the main variables contributing to determine the characteristic prokaryotic communities of each soil type.

Keywords: Campos biome; natural grasslands; prokaryotic communities; soil nutrients; soil physicochemical variables; soil structure; soil

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INTRODUCTION

Soil microbial communities play a critical role in the functioning of ecosystems since they influence several important ecosystem processes, including nutrient acquisition (Andreote, Pereira and Silva 2017; Fierer 2017), carbon, phosphorus (P) and nitrogen (N) cycling, and soil formation (Van Der Heijden, Bardgett and Van Straalen 2008). Bacteria and Archaea account for a large proportion of soil microbiome biodiversity and are closely associated with biogeochemical cycles, energy flow and degradation of pollutants (Bardgett and Van der Putten 2014; Bodelier 2011). Soil microbiomes are influenced by both biotic and abiotic factors (Griffiths *et al.* 2011; Xue *et al.* 2018), such as edaphic properties, temperature and moisture, as well as vegetation type. Soil pH and content of organic carbon, N and P are some of the most influential factors that determine microbial assemblages (Fierer and Jackson 2006; Martiny *et al.* 2006). These factors set the context for microbial interactions to occur, which leads to different assemblages and functions (Garbeva, Van Veen and Van Elsas 2004; Kinkel, Bakker and Schlatter 2011; Fanin and Bertrand 2016).

There is no typical soil microbiome; the abundance of bacterial and archaeal taxa may vary considerably depending on soil type, land use and environmental conditions, as described above (Fierer 2017). However, there are apparent associations between phyla abundance, soil type and land use. For example, Neal *et al.* (2017) compared soil microbiome assemblages from three different land uses (arable, bare fallow and grassland) and found *Gemmatimonadetes* and *Armatimonadetes* associated particularly with degraded soil. Furthermore, in a study of soil prokaryotic communities of a pasture–rice rotation, bacterial and archaeal soil communities were dominated by *Firmicutes* and *Proteobacteria* under pasture, but *Methanocellales* and *Methanosarcinaceae* dominated under rice (Fernández Scavino *et al.* 2013).

The Campos biome is a natural grassland ecosystem of southern South America with a landscape heterogeneity that is reflected in subregions defined by vegetation communities associated with edaphotopographic characteristics (Modernel *et al.* 2016; Camargo *et al.* 2019). This natural ecosystem provides important environmental services (Pillar, Tornquist and Bayer 2012), and is a hotspot of biodiversity with over 3000 species of temperate and subtropical plants. These natural grasslands mainly used for animal production in extensive grazing systems (Modernel *et al.* 2016) are facing contradictory pressures and the concern to be preserved (Carvalho *et al.* 2009); Pillar, Tornquist and Bayer 2012). Due to the increasing food production demand, the natural grassland biomes are endangered (Baeza and Paruelo 2020). There are changes in the land use and they are being displaced by the expansion of agricultural practices and intensive livestock production (Modernel *et al.* 2016; Oliveira *et al.* 2017).

Uruguayan Campos grasslands are characterized by a high diversity of soil types, low phosphorus (P) availability and limited water holding capacity (WHC; Allen *et al.* 2011). The low levels of dissolved inorganic P found in soils (typically <10 mg kg^{-1}) result from the high reactivity of the orthophosphate (PO_4^{3-}) ion with calcium (Ca) in alkaline soils, and iron (Fe) and aluminum (Al) in acidic soils (Gyaneshwar *et al.* 2002). The organic P fraction is unavailable for plants, and in both cases, enzymes are required to release orthophosphate for plant uptake. Organic P represents a large part of the total P (50–75%) (Hernández, Otegui and Zamalvide 1995). In environments with little human intervention, such as the Campos biome, the cycling of organic matter, nutrient availability and aggregate formation are direct results of microbial activity (Vargas *et al.* 2015). Such activity is capable of depolymerizing and mineralizing N, P and sulfur (S), typically bound to organic molecules, modulating the availability of inorganic forms of these nutrients in the soil, including ionic species such as ammonium, nitrate, phosphate and sulfate, the preferred nutrient forms for plants (Van Der Heijden, Bardgett and Van Straalen 2008; Richardson and Simpson 2011). Uruguayan soils are particularly well described: their evolution and physicochemical properties show strong associations with underlying parent material (Durán, Califra and Molfino 1999). However, little is known about the resident soil microbial communities of Campos soils and how those communities are influenced by the different soil types, nutrient availability and land use. This study aimed to characterize prokaryotic communities in the different soil types and explore relationships between the communities and soil parent material and the nutrient status. Five soils typical of the Campos biome were selected, based upon their differential parent material and nutrient status, particularly P form retention and ratio P inorganic/P organic.

MATERIALS AND METHODS

Soil collection

Five Uruguayan soil units were selected as representative of different agroecological regions (Hernández, Otegui and Zamalvide 1995; Hernández and Zamalvide 1998). The principal criterion for soil unit classification was parent materials: basalt for Itapebí Tres Árboles (ITA), crystalline basement for Sierra de Polanco (SPO), sandstone for Tacuarembó soils (TBO) and tertiary silt for both Tala Rodríguez (TRO) and Young (YNG). The selected soils have different ratios of organic P to inorganic P, as well as different mechanisms for inorganic P retention, associated with Fe, Al or Ca. Four soil units consisted of natural grassland ecosystems (ITA, SPO, TBO, TRO), whereas YNG was close to an agriculturally managed area. A description of the five soils is presented in Table 1.

Sampling methodology

For each of the five selected soil units, two locations were chosen. Five geo-referenced replicates of each soil were collected during Autumn 2015 from each location, complemented by environmental variables (Altier and Zerbino 2012). Each replicate represented aggregated soil from 15 samples taken with a 3 cm diameter core to a depth of 10 cm (effectively the A Horizon). Replicates were spaced 3 m apart. Soil samples were transported to the laboratory at 4°C where they were sieved through a 2-mm mesh to remove roots and plant detritus (within three days of sampling). Sieved soils were stored at −20°C until nucleic acid extraction. As an exploratory study, the ITA, TBO, SPO, YNG soil units were sampled following the above protocol in Autumn 2014. Three replicates of each soil type were collected and geo-referenced in the eight locations (two by soil type) without environmental variables measures.

Soil properties

The soil samples were characterized by their physicochemical properties. Soil total nitrogen (N) was determined by combustion at 900°C and subsequent N_2 thermal conductivity detection; available phosphorus (APR) was determined by the resin membrane technique (Sharpley, Sims and Pierzynski 1994) and citric acid extraction followed by colorimetric estimation (APC)

Table 1. Soil characteristics of 10 sampled locations corresponding to soil units of the Uruguayan Campos biome: Itapebí Tres Árboles (ITA), Sierra de Polanco (SPO), Tacuarembó (TBO), Tala Rodríguez (TRO) and Young (YNG).

Soil unit	Code	Parental material	Soil type (USDA)	Land use
Itapebí Tres Árboles	ITA	Basalt	Argiudoll Pachic, smectitic, fine, thermic.	Natural grassland
Sierra de Polanco	SPO	Crystalline	Argiudoll Typic (shallow), Fine-loamy, superactive, mixed, thermic	Natural grassland
Tacuarembó	TBO	Sandstone	Hapludalf Typic, Fine-loamy (coarse), siliceous, active, thermic	Natural grassland
Tala-Rodriguez	TRO	Tertiary silt	Natraquolls Typic, superactive, mixed, fine, thermic.	Natural grassland
Young	YNG	Tertiary silt	Argiudoll Pachic, fine, superactive, mixed, thermic	Agricultural ecosystem

(Murphy and Riley 1962); available potassium (K) and available sodium (Na) were determined by ammonium acetate (pH 7) extraction followed by atomic emission spectrometry; and Ca and Mg were determined by ammonium acetate (pH 7) extraction followed by atomic absorption spectrometry. Soil pH was measured by a potentiometric determination in water. Soil organic carbon (SOC) was determined by combustion at 900°C and subsequent CO_2 infrared detection. The cation exchange capacity (CEC) was determined by acid–base titration. Soil bulk density (BD) was used as an indicator of soil porosity and measured for oven-dried (24 h, 105°C) undisturbed soil cores using a 100 cm^3 metal sampling cylinder (Lienhard *et al.* 2013). Soil granulometric composition was determined and physical parameters were calculated, including aeration (Po), permanent wilt point (PWP) and WHC. Clay content (CC) was determined by the hydrometric method (Gee and Bauder 1986). Analysis of variance (one-factor ANOVA) ($P < 0.05$) and *post hoc* Tukey's HSD test were applied to the pairwise comparison among group means of soil units with a confidence level of 95%. All basic statistical procedures were performed using R-base (R core Team 2018).

DNA extraction and marker gene amplicon sequencing

Soil Deoxyribonucleic Acid (DNA) was extracted from 0.25 g aliquots of soil using the Power Soil DNA Isolation kit (Qiagen, Valencia, CA, USA) following the manufacturer's protocols. The V3–V4 region of the 16S ribosomic Rinonucleic Acid (rRNA) gene was amplified by PCR with the following primers: forward 5′-CCTACGGGNGGCWGCAG and reverse 5′-GACTACHVGGGTATCTAATCC, selected from Klindworth *et al.* (2013) in a 25 μL reaction volume containing 12.5 μL KAPA HiFi HotStart ReadyMix 2× (Roche, Penzberg, Germany), KAPA HiFi HotStart DNA Polymerase (0.5 U per 25 μL reaction) in a proprietary reaction buffer containing deoxiribose nucleotide triphosphate (dNTPs) (0.3 mM of each dNTP at 1×), $MgCl_2$ (2.5 mM at 1×) and stabilizers, 0.2 μM of each primer and 0.5 ng μL^{-1} of target DNA. The following temperature steps were applied: 3 min at 95°C, 25 cycles of 30 s at 95°C, 30 s at 55°C, 30 s at 72°C, followed by a final elongation for 5 min at 72°C. The PCR product size was verified with Bioanalyzer DNA 1000 chip, to the V3 and V4 primer pairs; the amplicon expected size is 550 bp. The PCR products were purified with AMPure XP beads (Beckman Coulter, Brea, CA). In the second PCR, the dual indices and Illumina sequencing adapters were attached to amplicons using the Nextera XT index kit according to the manufacturer's instructions (Illumina, San Diego, CA, USA) in a 50 μL reaction volume containing 25 μL KAPA HiFi HotStart ReadyMix 2× (Roche, Penzberg, Germany), KAPA HiFi HotStart DNA Polymerase (0.5 U per 25 μL reaction) in a proprietary reaction buffer containing dNTPs (0.3 mM of each dNTP at 1×), $MgCl_2$ (2.5 mM at 1×) and stabilizers, and 5 μL of resuspended PCR product DNA. The following temperature steps were applied: 3 min at 95°C, 8 cycles of 30 s at 95°C, 30 s at 55°C, 30 s at 72°C, followed by a final elongation for 5 min at 72°C. The library was cleaned with AMPure XP (Beckman Coulter, Brea, CA) before quantification. A 1:50 dilution of final library size was verified with Bioanalyzer DNA 1000 chip (Agilent, Santa Clara, CA, USA); the final library size was ~630 bp. Amplicon sequencing of 16S rRNA genes was carried out on an Illumina MiSeq platform (Illumina, San Diego, CA; 2 × 300 bp, paired-end) following the manufacturer's instructions. Sequences were de-multiplexed by using MiSeq Controller Software. Raw sequences are available at NCBI Sequence Read Archive (SRA, https://www.ncbi.nlm.nih.gov/sra) under the accession number PRJNA643923.

Sequence and statistical analysis

Raw Illumina sequences data were pre-processed with the Microbiome Helper pipeline (Comeau, Douglas and Langille 2017). Paired-end reads were joined with PEAR software (Zhang *et al.* 2014) and quality-filtered, and primer and adapter sequence regions were removed with the *read_filter.py* script from Microbiome Helper pipeline, generating an average read length of 590 bases. Paired reads shorter than 400 bases were removed. The sequences included in subsequent analyses were those with a minimum quality score of 30 across at least 90% of the sequence length, containing no ambiguous bases, and with no more than 10 consecutive low-quality base pairs and one base mismatch. Chimeric sequences were removed with *chimera_filter.pl* from Microbiome Helper pipeline. The remaining quality filtered reads were used in subsequent analyses. Operational taxonomic unit (OTU) identification and taxonomic assignation were performed using the Quantitative Insights into Microbial Ecology pipeline (QIIME version 1.9.0) (Caporaso *et al.* 2010b). OTUs were assigned using the open-reference method (Navas-Molina *et al.* 2013). Sequences were clustered into OTUs using a 97% sequence similarity based on the UCLUST classifier (Edgar 2010) and each representative sequence was aligned to the Greengenes 13-8 reference database (DeSantis *et al.* 2006) with PyNAST (Caporaso *et al.* 2010a). A maximum-likelihood 16S rRNA gene phylogenetic tree was constructed with RAxML version 7.0.4 software using default settings and the GTR model (Stamatakis 2006). This was manually edited and plotted with iTOL (Interactive Tree of Life; Letunic and Bork 2007). Taxonomy was assigned to each OTU using USEARCH version 7.0 (Edgar

2013) based upon a 90% confidence threshold and the Greengenes phylogeny. The resulting OTU table was filtered using a minimum cluster size of 0.1% of the total reads (Bokulich *et al.* 2013).

Rarefaction curves and observed species were calculated using QIIME (Caporaso *et al.* 2010b). The Chao1 lower-bound estimator of species richness (S_{Chao1}), relative abundance-based coverage estimator (ACE) and Shannon entropy (H') were calculated with the function *estimate* from the Vegan R package (Oksanen *et al.* 2019). Phylogenetic diversity (PD) was calculated using the *pd* function of the Picante R package (Kembel 2010). One-way ANOVA and *post hoc* Tukey's HSD tests were carried out for each diversity index to identify significant differences among group means ($P < 0.01$) in alpha-diversity estimates between soil units (tested factors).

Different beta-diversity measures were computed for comparison. First, based on the identified OTUs, the abundance-sensitive Bray–Curtis distance, and abundance- and phylogeny-sensitive weighted UniFrac (Lozupone *et al.* 2007) and Kantorovich-Rubinstein (here named KR-o) (Evans and Matsen 2012) distance metrics were computed. Also, edge-PCA (Matsen and Evans 2013) ordination was performed on these results. KR-o distances and edge-PCA were performed using the *guppy* binary implemented in *pplacer* version 1.1. (Matsen, Kodner and Armbrust 2010).

Comparisons of community assemblages using the different distance metrics were first tested for heteroscedasticity using PERMDISP ($P_{perm} < 0.05$) (Anderson 2006). Permutational multivariate analysis of variance (PERMANOVA) was used to test assemblage differences between different soils and *post hoc* pairwise comparisons were performed in those cases where a significant treatment effect was identified, with significance levels of 95%. Non-metric multi-dimensional scaling (NMDS) was performed based on the Bray–Curtis metric as implemented in the Vegan R package (Oksanen *et al.* 2019). Principal coordinates analysis (PCoA) was performed using weighted-UniFrac and KR-o metrics. Canonical analysis of principal coordinates (CAP) was performed using weighted-UniFrac to calculate the correlation between physicochemical properties and prokaryotic communities. PERMDISP ($P_{perm} < 0.05$), PERMANOVA ($P_{perm} < 0.05$), PCoA and CAP were performed using PRIMER PERMANOVA+ version 7.0.13 (PRIMER-*e*, Auckland, New Zealand) and 99 999 permutations. The tested factors were the different soil units. Graphics were produced with the R package ggplot2 (Wickham 2016) and tree and domain composition diagrams were drawn using Archaeopteryx (https://sites.google.com/site/cmzmasek/home/software/forester).

Estimation of differentially abundant OTUs among soil units was performed with DESeq2 (Love, Huber and Anders 2014) on a reduced set of OTUs (>200 sequence across the whole set), using a two-factor model, WHC and APR, without an interaction term. These two factors were selected based on CAP analyses. Differential OTUs were classified using the SILVA 132 16S rRNA gene database (Quast *et al.* 2013). R-base (R core Team 2018) was used to determine the correlation between WHC, APR and differential OTUs relative abundances. All basic statistical procedures were performed using R-base (R core Team 2018).

RESULTS AND DISCUSSION

Soil properties

General soil physicochemical characteristics of each soil unit are summarized in Tables 2 and 3. The 50 samples collected from different soil units differed significantly in Ca, APC, APR, CC, Po, BD and WHC. Ca ranged from 0.95 to 27.76 milliequivalents (meq) (100 g of soil)$^{-1}$, APR ranged from 4.20 to 61.95 μg P g^{-1} (Table 2), CC varied from 12.02% (sandstone soil) to 40.28% (basalt soil) and WHC ranged from 61.33 (crystalline soils) to 184.90 mm (basalt soils) (Table 3). As expected, based on the sampling criteria used in this study, it was possible to identify specific soil properties characteristic of each soil unit.

Prokaryotic community analysis

Sequencing of 16S rRNA gene (V3–V4) amplicons resulted in a total of 6 733 323 sequences with an average length of 442 bases. High-quality reads from each soil sample were subsampled to 12 496 sequences (the number of sequences associated with the smallest sample). A total of 4547 OTUs were obtained using a 97% identity threshold across the whole sample set. This set was reduced to 1160 when considering OTUs with >200 sequences across the whole set. A total of 27 phyla were identified across all sites. *Proteobacteria* (26.6%), *Actinobacteria* (18.1%), *Firmicutes* (17%), *Verrucomicrobia* (14.2%), *Acidobacteria* (11.3%), *Planctomycetes* (1.9%) and *Chloroflexi* (1.5%) were the predominant phyla with a combined prevalence over 90% (Figure S1, Supporting Information).

Alpha diversity

Rarefaction curves showed a similar pattern for all samples from all sites, suggesting that sequencing had captured similar levels of diversity values of each soil unit (Figure S2, Supporting Information). The highest differences of alpha diversity were observed for ITA soil (PD: 56.23; S_{Chao1}: 3222.13; H': 6.86). The lowest H' values were found in the samples of SPO soil. However, PD associated with SPO were similar to those of ITA. This is indicative of a prokaryotic community with relatively divergent taxa. Different behavior was observed in TRO samples, with high H' values but low PD, indicating that the community of TRO is formed by phylogenetically closer taxa. On the other hand, the low values in YNG (PD: 38.03; S_{Chao1}: 2633.78; H': 6.2) and TBO (PD: 39.36; S_{Chao1}: 2274.2; H': 6.2) were consistent in the three alpha-diversity indices. The one-factor ANOVA and *post hoc* Tukey's HSD test showed significant differences ($\alpha = 0.01$) among the diversity values of each soil unit. The highest differences in the pairwise comparisons were recorded between ITA and both TRO and SPO soil units (Fig. 1; Data S1, Supporting Information).

Beta diversity

The downstream analysis was performed using the 2015 dataset with phylogeny-sensitive weighted-UniFrac distance (Fig. 2), phylogeny-insensitive Bray–Curtis dissimilarities (Figure S3A, Supporting Information) and KR-o distance (Figure S3B, Supporting Information) to compare their power in recovering biologically meaningful patterns. PCoA based on weighted-UniFrac distances accounted for 60.6% of total phylogenetic variability on the first two axes (Fig. 2). The analysis showed a clear separation of prokaryotic communities according to soil unit. There was no significant heterogeneity of multivariate dispersion between the soils (pseudo-F = 2.3, $p_{perm} = 0.195$) (Fig. 2). PERMANOVA indicated a significant effect of soil unit upon the OTU assemblages (pseudo-F = 24.5, $p_{perm} < 0.0001$) and *post hoc* pairwise comparisons indicated that all assemblages were significantly different from the others (smallest pseudo-t = 2.7, $p_{perm} < 0.0001$).

Table 2. Chemical properties of Campos soils. Soil unit (SU): Itapebí Tres Árboles (ITA), Sierra Polanco (SPO), Tacuarembó (TBO), Tala Rodriguez (TRO), Young (YNG); titratable acid (TA), cation exchange capacity (CEC), % base saturation (% BS), soil organic carbon (SOC), organic matter (OM), available P by resin method (APR) and available P by citric acid method (APC). Superscript letters show the statistical differences among adjusted means of the soil units; different letters mean statistical differences [one-factor ANOVA and *post hoc* Tukey's HSD test ($P < 0.05$)].

SU	Ca (meq/100 g)	Mg (meq/100 g)	K (meq/100 g)	Na (meq/100 g)	TA (meq/100 g)	CEC (meq/100 g)	% BS	pH	% N	SOC (%)	OM (%Cx1,72)	APR (µg P/g)	APC (µg P/g)
ITA	14.7^{a}	5.86^{a}	0.37^{a}	0.62^{ab}	7.86^{a}	29.43^{a}	73.30^{ab}	5.43^{a}	0.40^{a}	5.05^{ab}	8.68^{ab}	4.20^{a}	3.35^{a}
SPO	2.96^{b}	1.96^{b}	0.39^{a}	0.42^{ab}	6.00^{ab}	11.26^{b}	52.06^{cd}	5.86^{a}	0.26^{a}	2.88^{ab}	4.95^{ab}	8.94^{ab}	13.23^{a}
TBO	0.95^{b}	0.30^{b}	0.08^{a}	0.27^{a}	2.95^{b}	2.65^{b}	36.20^{c}	5.20^{a}	0.10^{b}	0.69^{a}	1.19^{a}	61.95^{c}	33.68^{ab}
TRO	12.30^{a}	4.90^{ac}	1.00^{a}	1.29^{b}	7.76^{ab}	27.33^{a}	71.60^{ad}	6.06^{a}	0.33^{a}	3.11^{ab}	5.36^{ab}	4.76^{ab}	3.75^{a}
YNG	27.76^{c}	2.90^{bc}	0.95^{a}	0.40^{ab}	4.15^{ab}	34.83^{a}	91.86^{b}	6.53^{a}	0.53^{c}	6.09^{b}	10.47^{b}	35.39^{bc}	65.31^{b}

Table 3. Physical properties of Campos soils. Soil unit (SU): Itapebí Tres Árboles (ITA), Sierra Polanco (SPO), Tacuarembó (TBO), Tala Rodriguez (TRO), Young (YNG); bulk density (BD), clay content (CC), permanent wilt point (PWP), porosity (Po) and water holding capacity (WHC). Superscript letters show the statistical differences among adjusted means of the soil units; different letters mean statistical differences [one-factor ANOVA and *post hoc* Tukey's HSD test ($P < 0.05$)].

SU	% CC	BD (g/cc)	Po	PWP (mm/10 cm)	WHC (mm)
ITA	40.28^{a}	0.98^{a}	63.00^{a}	16.90^{ab}	184.90^{a}
SPO	27.99^{ab}	1.31^{b}	51.00^{b}	10.1^{c}	61.33^{b}
TBO	12.02^{b}	1.42^{c}	46.00^{c}	4.30^{d}	122.70^{c}
TRO	14.37^{b}	1.23^{d}	53.66^{bd}	15.10^{a}	117.03^{c}
YNG	31.66^{ab}	1.18^{d}	56.00^{d}	18.70^{b}	178.10^{a}

NMDS ordination of phylogenetically insensitive Bray–Curtis dissimilarities showed a similar distribution as PCoA, with a stress value of 0.009 (Figure S3, Supporting Information). In this sense, all respective axes of PCoA-WU, PCoA-KR and NMDS-BC provided very similar ordinations (Data S2, Supporting Information). However, NMDS-BC detected some level of dispersion within samples, not evident in PCoA-WU or PCoA-KR (Figure S3, Supporting Information). When data from 2014 and 2015 were amalgamated, the results were very similar, clustering samples from the same sampling points together. Nevertheless, it is interesting to note that a slight, albeit significant, difference between years was observed, suggesting the potential of future longitudinal intra-soil studies (Figure S4, Supporting Information).

Up to 68% of total 16S rRNA gene assemblage phylogenetic variation was explained by the first two edge-PCA axes (Figure S5, Supporting Information). The prokaryotic community from TRO did not show high association with both edge-PCA axes. The microorganism assemblage variation associated with the first axis separated soil types. Following the criteria of analysis from Matsen and Evans (2013), the differences observed in the prokaryotic communities between TBO sandy soils and clay soils (ITA, SPO, TRO, YNG) were primarily related with a higher contribution of OTUs classified as *Archaea* and bacterial phyla *Firmicutes*, *Acidobacteria*, *Actinobacteria* and *Verrucomicrobia* (Figure S5, Supporting Information).

YNG and ITA soil units have very similar prokaryotic communities; both are dominated by *Verrucomicrobia* and *Actinobacteria* phyla. The ITA community is more phylogenetically diverse, as indicated by the higher PD value. The edge-PCA and its phylogenetic interpretation indicate that the phyla *Firmicutes* and *Acidobacteria* differed in their relative abundance between these two sites. *Firmicutes*, represented principally by the genus *Bacillus*, had a higher relative abundance in YNG soils with their higher SOC and APC than ITA soils (Figure S5, Supporting Information). In contrast, *Acidobacteria* were more abundant in ITA soil samples: *Koribacteraceae* and *Solibacteraceae* families were characteristic of this soil prokaryotic community. This result was confirmed by UPGMA clustering of Bray–Curtis dissimilarities (data not shown). The difference in relative abundance in *Firmicutes* and *Acidobacteria* phyla in both soils could be associated with the sensitivity of these phyla to changes in nutrient content (Hermans *et al.* 2017; Karimi *et al.* 2018).

The microorganism assemblage variation associated with the second edge-PCA axis also separated soil types. This axis was related to a higher relative abundance of *Proteobacteria*, *Chloroflexi*, *Planctomycetes* and a second *Verrucomicrobia* lineage (Figure S5, Supporting Information). SPO soils were associated with the highest *Planctomycetes* relative abundance, but the lowest *Firmicutes* relative abundance. The high relative abundance of *Planctomycetes* is associated with the low nutrient availability and WHC/PWP of crystalline basement soils. This phylum has been reported to show negative associations with soil nutrient content (Lauber *et al.* 2008; Hermans *et al.* 2017). *Actinobacteria* also had relatively low relative abundance in SPO soils. The *Actinobacteria* phylum is involved in soil functions such as nutrient cycling and organic matter turnover (Nasrabadi *et al.* 2013; Lewin *et al.* 2017), and changes in its community composition have been reported to be associated with nutrient and water availability (Kopecky *et al.* 2011). The aforementioned supports the hypothesis that the structure of soil prokaryotic communities is strongly influenced by soil characteristics (Lauber *et al.* 2008).

The second edge-PCA axis also reveals other differential phyla between the YNG and ITA communities. The *Thermogemmatisporaceae* family of the *Chloroflexi* phylum and *Hyphomicrobiaceae* and *Bradyrhizobiaceae* of the *Proteobacteria* phylum were characteristic of the ITA prokaryotic community, together with the *Acidobacteria* families detected by the first axis.

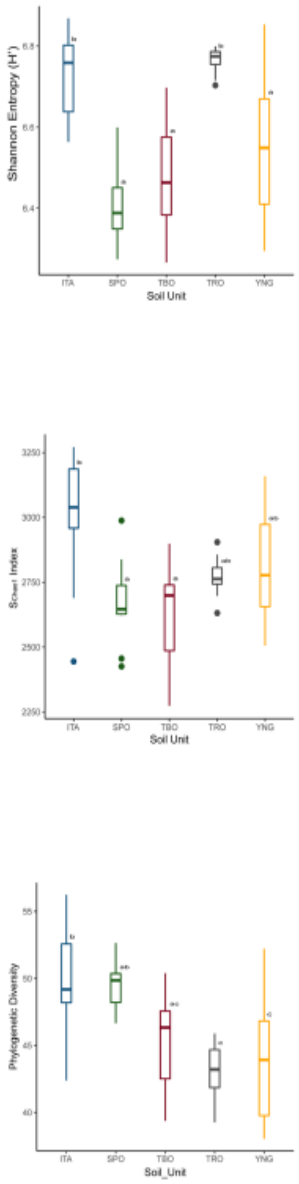

Figure 1. Boxplot of alpha diversity indices associated with each soil unit: (A) Shannon entropy (H'); (B) S_{Chao1} lower bound estimate of species richness; (C) phylogenetic diversity (PD). The boxes denote interquartile ranges (IQR) with the median as a horizontal line inside the box. Whiskers extending up to the most extreme points within 1.5-times the IQR from the first and third quartiles, respectively. The points marked beyond the horizontal box limits are outliers of the distribution. One-factor ANOVA and *post hoc* Tukey's HSD tests were carried out for each diversity index to identify significant differences ($P < 0.01$) in alpha-diversity estimates between soil units (tested factor). The letters a, b and c are used to clarify whether the difference between any pair of soil units calculated by Tukey's HSD test was statistically significant ($P < 0.01$), and there was a significant difference in the prokaryotic diversity of soils among the soil units sharing no common letter markers. There was a significant effect of soil unit ($P < 0.01$) for the three alpha indices. Each sample is represented by a point; each soil unit is identified by color: ITA (blue), SPO (green), TBO (red), TRO (gray) and YNG (orange).

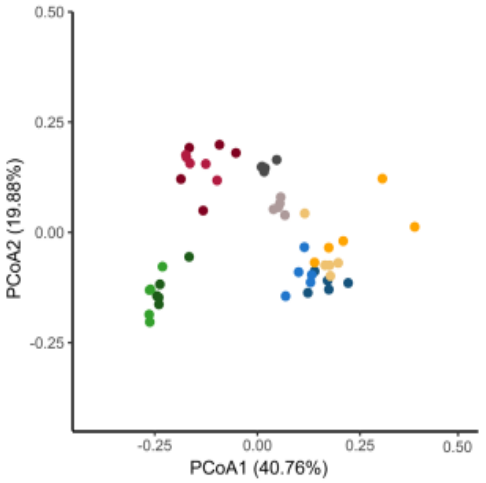

Figure 2. PCoA ordination based on weighted-UniFrac distances between prokaryotic communities of each soil unit, incorporating both OTU relative abundance and phylogeny. Each point represents replicates from ITA (blue and light blue), SPO (green and light green), TBO (red and light red), TRO (gray and light gray) and YNG (orange and light orange). The percent variation explained by each principal coordinate is indicated on the axes. There was no significant difference in assemblage dispersion (PERMDISP; pseudo-$F = 2.3$; $p_{perm} = 0.195$), but a significant difference in OTU assemblages (PERMANOVA; pseudo-$F = 24.5$; $p_{perm} < 0.0001$) between soils.

Relationship between prokaryotic community phylogenetic structure and soil properties

CAP based on weighted-UniFrac distance between prokaryotic assemblages was chosen to test the relationship between phylogenetic composition and soil properties, given the widespread use of this metric and the similar results provided by the three β-diversity metrics (see above). Initially, all five soil units were included, regardless of their history and management (Fig. 3; Data S3, Supporting Information). Eight of the environmental variables had correlation coefficients (r) > |0.20| with at least one of the first two CAP axes. CAP axis 1 (canonical correlation [δ^2] = 0.999) was characterized by associations with CC ($r = 0.521$), available P as APC ($r = 0.534$) and APR ($r = 0.362$), SOC ($r = 0.354$), PWP ($r = -0.251$) and Po ($r = -0.207$). TRO soils were distinct from the other sites and associated with particularly low CC, APC and SOC. The highest environmental variables associated most strongly with the CAP axis 2 ($\delta^2 = 0.994$) were WHC ($r = -0.858$), PWP ($r = -0.341$) and Ca ($r = -0.205$) The axis separated the wetter soils (YNG and ITA) from the others (Fig. 3). These results reveal that the WHC, soil texture and nutrient status are associated with differences in prokaryotic community assemblages as previously reported (Brockett, Prescott and Grayston 2012; Delgado-Baquerizo *et al.* 2018; Karimi *et al.* 2018).

CAP analysis also indicated that the composition of the TBO soil community was strongly associated with low CC and SOC. The prokaryotic community in this soil were dominated by *Verrucomicrobia* and *Archaea*. The properties of the TBO sandy textured soil—low CC and low nutrient content—may contribute to the proliferation of taxa that can adapt to restrictive conditions of growth, such as *Verrucomicrobia*, which has a highly flexible metabolism (Balmonte *et al.* 2016).

As it was discussed above, YNG and ITA soils present the highest WHC and PWP among the soil units studied (Table 3).

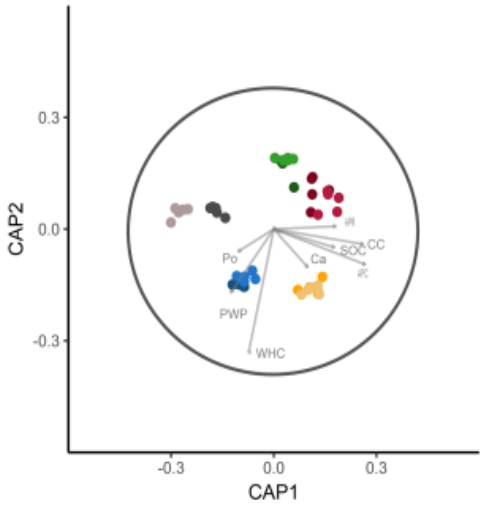

Figure 3. CAP based on weighted-UniFrac distance for prokaryotic communities in the different soils. PERMANOVA analysis with 99 999 permutations was performed to determine the significance between prokaryotic communities of five soil units with two locations in each one ($n = 10$) and soil physicochemical properties. ITA (blue and light blue), SPO (green and light green), TBO (red and light red), TRO (gray and light gray) and YNG (orange and light orange). Vector labels are APC (available P by Citric Acid extraction), ARP (available P by resin membrane), Ca, CC (clay content), CEC (cation exchange capacity), Po (porosity), PWP (permanent wilt point), SOC (soil organic carbon), TA (titratable acid) and WHC (water holding capacity). The variables' vector length is relative to the circle radius and represents the correlation between each variable and the axes.

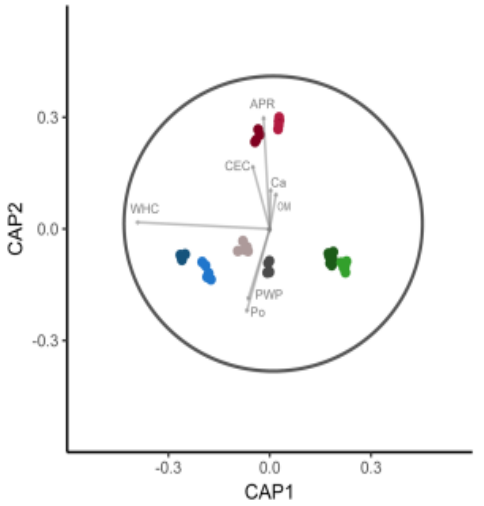

Figure 4. CAP based on weighted-UniFrac distance for prokaryotic communities in the different soils excluding YNG. PERMANOVA analysis with 99 999 permutations was performed to determine the significance between prokaryotic communities of the four soil units consisting of natural grassland ecosystems in its prokaryotic communities. For each soil were included two locations ($n = 10$) and soil physicochemical properties ITA (blue and light blue), SPO (green and light green), TBO (red and light red), TRO (gray and light gray). Vector labels are ARP (available P by resin membrane), BD (bulk density), Ca, CC (clay content), CEC (cation exchange capacity), Mg, Po (porosity), PWP (permanent wilt point), SOC (soil organic carbon) and WHC (water holding capacity). The variables' vector length is relative to the circle radius and represents the correlation between each variable and the axes.

These environmental conditions could favor the proliferation of *Firmicutes* in this soil. Land adjacent to the YNG soil unit has been managed predominantly as agricultural over the last century. The sampling sites were not in a field with frequent mineral fertilization; however, the vicinity of intensive agriculture and fertilizer drift may explain the shifts in the soil prokaryotic community. The ITA prokaryotic community is characterized by a higher relative abundance of *Acidobacteria* compared with YNG (Figure S5, Supporting Information). *Acidobacteria* is a ubiquitous soil phylum, but little is known about its ecophysiology. They have a low complement of SSU rRNA genes, suggesting that they are a relatively slow growing group. Genes associated with a wide range of carbohydrate and polysaccharide metabolic pathways have been identified in representative organisms of this phylum (Kielak *et al.* 2016). This suggests that the group plays an important role in organic carbon turnover in soils. Studies indicate that pH and nutrient availability influence *Acidobacteria* abundance in soils (Ward *et al.* 2009; Kielak *et al.* 2016; Eichorst *et al.* 2018; Ivanova *et al.* 2020). There was very little variability in pH between the Campos soils studied here (Table 2), but they varied significantly in nutrient content. This may explain the differences in *Acidobacteria* relative abundance.

Variation in WHC between the soils was associated with CAP axis 2, and the phylogenetic separation of the ITA and YNG prokaryotic communities from the other soils, particularly SPO, was associated most strongly with this edaphic variable. In addition, ITA soils have high Po, suggesting that these soils have more pore space associated with air and water that facilitates nutrient diffusion/advection and cell-cell communication. At the other extreme, SPO soils are associated with the low Po and WHC. The community of SPO soil was characterized by *Planctomycetes* phylum (Figure S5, Supporting Information). Borer, Tecon and Or (2018) showed through a mathematical modelling how the pore network may influence the spatial organization of soil microbes by considering nutrient and oxygen counter-gradients and cell motility. They showed that total bacterial relative abundance decreased with a reduction of pore network connectivity. The dynamics, composition and distribution of soil microbes are shaped by heterogeneous water and resource distribution, and by their ability to rapidly adapt to dynamic changes in local conditions (Tecon and Or 2017). However, deeper analyses are necessary to understand how porosity, pore size distribution and pore connectivity influence environmental prokaryotic community assemblages (Rabbi *et al.* 2016; Borer, Tecon and Or 2018; Neal *et al.* 2020).

A second CAP analysis using weighted-UniFrac distance was performed excluding YNG. Three physical properties (WHC, Po, PWP) and four chemical soil properties (APR, CEC, Ca, OM) were identified with $r > |0.20|$. Most of these variables were consistent with the previous analysis including YNG, showing the same trend of correlation (Data S4, Supporting Information). WHC was again associated with the highest correlation coefficient with CAP axis 1 ($r = -0.967$). In this analysis, we could reveal a higher negative relation of SPO crystalline basement soil with WHC, compared with the previous analysis. CAP Axis 2 showed correlations with APR ($r = 0.602$), Po ($r = -0.446$), PWP ($r = -0.380$) and CEC ($r = 0.339$) (Fig. 4). A clear-cut separation was observed between sites differing in the type of soil. TBO soil communities, developed in soil over sandstone parent material, are particularly different from the communities belonging to other soils.

We could also reveal a higher negative relation of TBO sandy soil with Po and PWP compared with the previous analysis.

OTUs with different relative abundances in sites under natural grassland (ITA, SPO, TBO and TRO, but excluding YNG) were identified using WHC and APR as factors in the DESeq2 routine (Love, Huber and Anders 2014). Twenty-nine OTUs show small but significant different relative abundances across the different soil units ($p_{adj} < 0.05$) (Data S5, Supporting Information). Three OTUs associated with *Actinobacteria*, *Chloroflexi* and *Planctomycetes* were enriched in sites with either high WHC or APR (Tables 2 and 3; Data S5, Supporting Information). The remaining twenty-six tend to be higher (i.e. negatively associated) in soils with lower WHC and/or APR (Tables 2 and 3; Data S5, Supporting Information). OTUs were classified taxonomically using SILVA (Data S5, Supporting Information); most were also detected by edge-PCA analysis as the phyla with a differential relative abundance among soil units (Figure S5, Supporting Information), namely *Verrucomicrobia*, *Firmicutes*, *Chloroflexi*, *Actinobacteria* and *Planctomycetes*. The OTUs of three orders of *Actinobacteria* are of interest: *Acidimicrobiales*, *Gaiellales* and *Solirubrobacteriales*. Free-living *Actinobacteria* are especially abundant in alkaline and organic matter-rich soil (Barka *et al.* 2016). They play key roles in the turnover of organic carbon. Some members of this phylum can degrade organic compounds from a wide range of sources, including decaying plant material, chitin and hydrocarbons (Sharma, Dangi and Choudhary 2014; Lewin *et al.* 2017). Some *Actinobacteria* have variable responses in the production of acid and alkaline phosphatases, which release P from organic sources (Nasrabadi *et al.* 2013). It is interesting to note that these OTUs are not detected as differentially abundant when including P-enriched YNG samples (data not shown). In this sense, it has been shown how fertilization impacts on the composition of soil microbial communities (Jangid *et al.* 2008; Wang *et al.* 2019). These results were consistent with reports of how chemical fertilization alters the structure and function of prokaryotic communities by affecting nutrient balance, organic matter content and other edaphic properties such as pH (Jangid *et al.* 2008; Lauber *et al.* 2008; Kopecky *et al.* 2011; Wang *et al.* 2019).

In summary, we report the effect of soil type on the soil prokaryotic communities providing insights in the ecological processes shaping them in natural ecosystems under similar climate conditions and land use (Fierer and Jackson 2006; Fierer 2017). Our data suggest that soil structure, nutrient status and WHC significantly modulate prokaryotic community assemblages in this subtropical Campos natural grassland biome. Excluding YNG samples from the analysis revealed the impact of agriculture practices on these communities. *Actinobacteria*, *Chloroflexi*, *Proteobacteria* and *Verrucomicrobia* were the main responsive phyla identified in the four soil types by a variety of different statistical approaches. In particular, the removal of agricultural soil (YNG) allowed the identification of differences in the orders *Solirubrobacteriales* and *Gaiellales*.

Recently, Zhang *et al.* (2019) showed that the type of vegetation ecosystem has a high influence in actinobacterial community structure. Such differences may be linked to differential sets of metabolic functions from each community responding to the different conditions of nutrients, water and porosity related to CO_2 and O_2 concentration. Additionally, differences observed between ITA and SPO soil communities warrant further analysis to generate an understanding of the functional diversity associated with water dynamic and nutrient cycling in greater detail.

The Campos Biome is one of the few in the world that still conserves developed soils under natural grasslands. Understanding how the anthropogenic practices affect the belowground communities in their diversity and functional ecology is an essential step in the pursuit of a more sustainable land management.

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SUPPLEMENTARY DATA

Supplementary data are available at FEMSEC online.

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Conflict of interest. None declared.

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3. Functional gene and enzyme profiling of prokaryotic soil communities in the Campos biome of Uruguay: Insights into phosphorous cycling

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Abstract

Soil microorganisms play a critical role in efficient phosphorus (P) cycling through enzyme-mediated mechanisms. Soil properties, including pH, organic matter content, nutrient availability and texture influence the abundance and activity of enzyme coding genes. In Uruguayan Campos Biome soils, P availability is limited to less than five ppm and the organic fraction accounts for more than half of the total soil P. This work aimed to study the prokaryotic functional profiles regarding P mobilization of four Uruguayan Campos Biome soils with different parent material and nutrient status, representative of natural grasslands (ITA, SPO, TBO) and agricultural soil (YNG). The prokaryotic enzymes and genes involved in P cycling were analyzed using the PICRUSt2 pipeline and enzyme activities (acid and alkaline phosphatases and phytase). Clustered by their P-cycling genes using non-metric multidimentional scaling (NMDS), different soil units showed similar gene abundance and taxonomic contribution in the cases of ITA and SPO and TBO and YNG soil units, respectively. Enzyme activity in all the four soil units from the Uruguayan Campos biome showed high levels of acid phosphatase activity compared to alkaline phosphatase and phytase. The canonical analysis of the principal coordinates (CAP) showed that nutrient status (nitrogen, P, organic carbon, and cation exchange.

Key words: prokaryotic communities, phosphorous cycle, phosphatase activity, functional profiles

Introduction

The Campos biome in South America is a unique ecosystem that consists mainly of temperate and subtropical grasslands. Its landscape is influenced by the edaphotopographic characteristics of the region, making it a biodiversity hotspot with over 4,000 species of temperate and subtropical plants (Camargo et al., 2019, Andrade et al., 2018, Modernel et al., 2016). However, this ecosystem faces challenges due to increasing demand for food production and extensive livestock practices (Baeza and Paruelo, 2020, Tiscornia et al., 2019). However, despite its challenges, the Campos biome is critical in providing vital ecosystem services, including carbon storage, water regulation, soil erosion control and nutrient cycling. In order to maintain the sustainability of these services, it is imperative to strike a balance between food production and conservation efforts in the region. (Weyland et al., 2017, Pillar et al., 2012, Altesor et al., 2005).

Soil microorganisms play a vital role in preserving the health and functionality of ecosystems. They perform critical functions such as nutrient cycling, carbon sequestration, and disease suppression (Chen *et al*., 2020; Delgado-Baquerizo *et al.*, 2016). Given the limited availability of high-quality P sources and the high cost of fertilizer, the use of microorganisms to increase P availability could be an efficient and sustainable strategy to address the problem of low P availability in Uruguayan soils. However, little is known about the P functional profiles of the microbial communities prevalent in Uruguayan soils.

Phosphorus (P) is the second limiting element for plant growth after nitrogen (N) and is a crucial factor that can affect productivity. The available soil P in the Uruguayan Campos grasslands is usually less than five ppm on average (Jaurena et al., 2021, Morón, 1996, Hernández et al., 1995). This deficiency is attributed to the reactivity of orthophosphate with calcium, iron and aluminum in the acidic and alkaline soils found in these grasslands (Gyaneshwar et al., 2002). In addition, a significant proportion of total P is present in the organic fraction, which plants cannot utilize without the intervention of enzymes. Microorganisms are crucial for

breaking down organic matter, releasing essential nutrients, such as P and N, maintaining soil structure, preventing erosion and promoting water infiltration (Cavicchioli et al., 2019, Jacoby et al., 2017, Ingham, 2009).

The soil microorganisms play a fundamental role in recycling soil P (Richardson and Simpson, 2011). They can solubilize and mineralize P through enzyme-mediated mechanisms involving diverse enzymes such as phosphatases (Khan et al., 2009). A balanced nutrient ratio can increase soil phosphatase activity, improving soil P cycling and plant growth. In contrast, an unbalanced nutrient ratio can decrease soil phosphatase activity, limiting P availability and reducing plant growth (Zheng et al., 2018, Margalef et al., 2017). Soil pH also affects the enzymes produced by microorganisms and their activity by influencing the inhibitors or activators in the soil solution and the substrate concentration (Dick et al., 2011), independently of the abundance of the genes encoding these enzymes (Fraser et al., 2017).

The phosphorus cycle involves a large number of genes, which are categorized according to their role in different stages of the cycle. Genes such as *pstA*, *phoU*, and *ugpQ* encode phosphate transporter proteins that enable the uptake of phosphate ions by microorganisms and plants by facilitating their transport across cell membranes. Conversely, when microorganisms experience phosphorus starvation, phosphate starvation regulatory genes, such as phoB and phoR, are activated to conserve and recycle phosphorus within cells. These genes encode proteins responsible for phosphate transport (e.g., *pstA*, *phoU*, *ugpQ*), further enhancing phosphorus uptake by both plants and microorganisms. P mineralization genes (e.g., *phoD*, *phy*, *phoC*) encode enzymes that catalyze the hydrolysis of organic phosphate esters, releasing phosphate and organic molecules (i.e., non-specific acid phosphatases - NSAP, alkaline phosphatase (PhoD, PhoA, PhoX and phytase such as BPP, among others) and Pi-solubilizing genes (e.g., *gdc*) (Zeng et al., 2022, Oliverio et al., 2020, Bergkemper et al., 2016).

The understanding of the P functional profiles of microbial communities in Uruguayan soils remains limited. However, the acquisition of the metagenomic data and the study of their associations with soil physicochemical properties provides a comprehensive perspective on the microbial functions within this ecosystem and could make a significant contribution to the conservation of the health and functionality of ecosystem services in the Campos biome. This knowledge not only enables us to develop more effective ecosystem management strategies, but also contributes to the long-term maintenance and conservation of the valuable ecosystem services provided by the Campos biome.

This study aimed to characterize the P functional profiles of four soil units from the Uruguayan Campos biome. The functional profiles were obtained by inferring the genes and enzymes involved in P cycling using the PICRUSt2 (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States 2) pipeline, enzyme activities, and their variability over soil properties. As a complementary technique, we used a metagenome shotgun sequencing approach to better understand microbial functions in the P cycle. The selected soil units had different parent material and nutrient status and represented two land uses: three were under natural grasslands, mainly used for livestock grazing, and one was agricultural soil.

Materials and Methods

Soil sample collection

We selected four soil units to represent different Uruguayan Campos biome agroecological regions (Hernández and Zamalvide, 1998, Hernández et al., 1995). The principal criterion used for this selection was the soil parent material: basalt for Itapebí Tres Árboles (ITA), crystalline basement for Sierra de Polanco (SPO), sandstone for Tacuarembó soils (TBO) and tertiary silt for Young (YNG). The selected soils have different ratios of organic P to inorganic P and different

mechanisms for inorganic P retention associated with Fe, Al, or Ca. Three soil units comprised natural grassland ecosystems (ITA, SPO, TBO), whereas YNG was close to an agriculturally managed area. During Autumn 2015, we collected four geo-referenced replicates from each site. These collections were supplemented with environmental variables, as documented by Altier and Zerbino in 2012. Each replicate comprised aggregated soil derived from 15 samples, extracted using a 3 cm diameter core to a depth of 10 cm, effectively capturing the A Horizon. Replicates were spaced 3 meters apart to ensure representation across the sampling area. Soil samples were transported to the laboratory at 4ºC where they were sieved through a 2-mm mesh to remove roots and plant detritus (within three days of sampling). Sieved soils were stored at −20◦ C until nucleic acid extraction.

Table 3: Soil characteristics of sampled locations corresponding to four soil units of the Uruguayan Campos biome: Itapebí Tres Árboles (ITA), Sierra de Polanco (SPO), Tacuarembó (TBO) and Young (YNG).

Soil Unit	Code	Parental material	Soil type (USDA)	Land use
Itapebí Tres Árboles	ITA	Basalt	Argiudoll Pachic, smectitic, fine, thermic.	Natural grassland
Sierra de Polanco	SPO	Crystalline	Argiudoll Typic (shallow), Fine-loamy, superactive, mixed, thermic	Natural grassland
Tacuarembó	TBO	Sandstone	Hapludalf Typic, Fine-loamy (coarse), siliceous, active, thermic	Natural grassland
Young	YNG	Tertiary silt	Argiudoll Pachic, fine, superactive, mixed, thermic	Agricultural ecosystem

Soil properties

The soil samples were characterized by their physicochemical properties as shown in Table 4. The soil total nitrogen (N) was determined by combustion at 900 ◦C and subsequent N_2 thermal conductivity detection (LECO Truespec; Wright and Bailey, 2001). The available phosphorus (APR) was determined by the resin membrane technique according to Sharpley *et al.*, 1994. To determine the available potassium (K) and available sodium (Na) were determined by ammonium acetate (pH 7) extraction followed by atomic emission spectrometry; and Ca and Mg were determined by ammonium acetate (pH 7) extraction followed by atomic absorption

spectrometry. The soil pH was determined in water potentiometrically (1:2.5 soil/distilled water suspension; Beretta et al., 2014). To determine the soil organic carbon (SOC), we used the combustion at 900 ºC and subsequent CO_2 infrared detection (LECO Truespec; Wright and Bailey, 2001). The cation exchange capacity (CEC) was determined by acid–base titration. Soil bulk density (BD) was used as an indicator of soil porosity and measured for oven-dried (24 h, 105 ºC) undisturbed soil cores using a 100 cm^3 metal sampling cylinder (Lienhard *et al*., 2013). Soil granulometric composition was determined, and physical parameters were calculated, including porosity (Po) and Water Holding Capacity (WHC). The hydrometric method was used to determine the clay content (CC) (Gee and Bauder, 1986). Analysis of variance (one-factor ANOVA) ($P < 0.05$) and post hoc Tukey's HSD test was applied for pairwise comparison among group means of soil units with a confidence level of 95%. All basic statistical procedures were performed using R-base (R core Team 2018).

Table 4: Physicochemical properties of soils from the Uruguayan Campos biome. Soil Unit (SU): Itapebí Tres Árboles (ITA), Sierra de Polanco (SPO), Tacuarembó (TBO), Young (YNG); cation exchange capacity (CEC), soil organic carbon (SOC), available P by resin method (APR), clay content (CC), porosity (Po) and Water Holding Capacity (WHC). Superscript letters show the statistical differences among adjusted means of the soil units; different letters mean statistical differences [one-factor ANOVA and post hoc Tukey's HSD test ($P < 0.05$)].

SU	Ca (meq/100 g)	Mg (meq/100 g)	K (meq/100 g)	Na (meq/100 g)	CEC (meq/100 g)	pH	%N	SOC (%)	APR (µg P/g)	% CC	Po	BD (g/cc)	WHC (mm)
ITA	14.7^{a}	5.86^{a}	0.37^{a}	0.62^{ab}	29.43^{a}	5.43^{a}	0.40^{a}	5.05^{ab}	4.20^{a}	40.28^{a}	63.00^{a}	0.98^{a}	184.90^{a}
SPO	2.96^{b}	1.96^{b}	0.39^{a}	0.42^{ab}	11.26^{b}	5.86^{a}	0.26^{a}	2.88^{ab}	8.94^{ab}	27.99^{ab}	51.00^{b}	1.31^{b}	61.33^{b}
TBO	0.95^{b}	0.30^{b}	0.08^{a}	0.27^{a}	2.65^{b}	5.20^{a}	0.10^{b}	0.69^{a}	61.95^{c}	12.02^{b}	46.00^{c}	1.42^{c}	122.70^{c}
YNG	27.76^{c}	2.90^{bc}	0.95^{a}	0.40^{ab}	34.83^{a}	6.53^{a}	0.53^{c}	6.09^{b}	35.39^{bc}	31.66^{ab}	56.00^{d}	1.18^{d}	178.10^{a}

Metagenome functional predictions and data analysis

We used the OTU table Garaycochea et al. (2020) obtained as an input to predict the function using PICRUSt2, which integrates existing open-source tools to predict genomes of environmentally sampled 16S rRNA gene sequences (Douglas et al., 2020). The PICRUSt2 pipeline was run in Python using the rarefied bacterial 16S rRNA feature table and the default NSTI (nearest sequenced taxon index) cutoff of 2.0.

We selected a subset of KEGG Orthologs (KOs) and enzymes (ECs) involved in P mobilization for further statistical analyses (Gaiero et al., 2021) (Tables 5 and 6). Mainly, we focused on a KOs and ECs subset representative of genes and enzymes involved in the P mobilization, from here pKOs and pECs. Further, PICRUSt2 also provides the OTUs contribution to each predicted function, allowing us to obtain taxonomy-informed analyses.

Table 5: KEGG Orthologs (pKOs) were selected as key phosphorous (P) mobilization functions.

KO (KEGG Orthologs)	Description	P related function
K00117	Quinoprotein glucose dehydrogenase (*gcd* PQQGDH)	P - Solubilization
K07048	Phosphotriesterase	P - Mineralization
K05306	phosphonoacetaldehyde hydrolase (*phnX*)	P - Mineralization
K02041	phosphonate transport system ATP-binding protein (*phnC*)	P – uptake and transport
K02038	phosphate transport system permease protein (*pstA*)	P – uptake and transport
K02039	phosphate transport system protein (*phoU*)	P – uptake and transport
K07636	phosphate regulon sensor histidine kinase (*phoR*)	P- scarcity regulation
K07657	phosphate regulon response regulator (*phoB*)	P- scarcity regulation
K11929	outer membrane pore protein (*phoE*)	P – uptake and transport
K03306	Inorganic phosphate transporter, PiT family (*pit*)	P – uptake and transport
K01126	glycerophosphoryl diester phosphodiesterase	P - Mineralization

	(GDP)	
K05814	sn-glycerol 3-phosphate transport system permease protein (*ugpA*)	P – uptake and transport
K06167	phosphoribosyl 1,2-cyclic phosphate phosphodiesterase (*phnP*)	P - Mineralization
K01113	Alkaline phosphatase D (*phoD*)	P - Mineralization
K01077	Alkaline phosphatase A (*phoA*)	P - Mineralization
K01078	Acid Phosphatase	P - Mineralization
K09474	acid phosphatase (class A) (*phoN*)	P - Mineralization
K01093	4-phytase / acid phosphatase (*appA*)	P - Mineralization

Table 6: Enzymes (pECs) selected as key phosphorous (P) mobilization functions.

EC (Enzyme Commission)	Description	P related function
EC:3.1.4.55	phosphate phosphodiesterase (PDE)	P - Mineralization
EC:3.11.1.2	phosphonoacetate hydrolase	P - Mineralization
EC:3.11.1.1	phosphonoacetaldehyde hydrolase	P - Mineralization
EC:3.6.3.28	phosphonate-transporting ATPase	P – uptake and transport
EC:3.6.3.27	phosphate-transporting ATPase	P – uptake and transport
EC:3.1.3.25	inositol-phosphate phosphatase.	P - Mineralization
EC:4.7.1.1	C-P lyase	P - Mineralization
EC:3.1.3.1	Alkaline phosphatase (ALP)	P - Mineralization
EC:3.1.3.2	Acid phosphatase (ACP)	P - Mineralization
EC:3.1.3.26	Phytase (Phy)	P - Mineralization

Determination of enzyme activity

We determined the activity of the enzymes associated with P organic mineralization, acid phosphatase (ACP), alkaline phosphatase (ALP) and phytase (Phy) in soil suspension prepared in a buffer by ultrasonication with 60 J ml-1 (Marx et al., 2001). For acid and alkaline phosphatase, 0.5g of soil were suspended in 25 ml buffer and sonicated. We used the sodium acetate buffer (50 mM, pH 5) for acid phosphatase, while the modified universal buffer (pH 9.8) was used for alkaline phosphatase. After sonication, we added another 25 ml of buffer and made a soil-solution ratio of 1:100 (w/v). The mixture was stirred on a magnetic stirrer for homogenization shortly before the measurement. Activities of the phosphatases were determined using fluorogenic, methylumbelliferyl-linked substrates (MUF) called 4-methylumbelliferyl-phosphate (M8883, Sigma Aldrich, Munich, Germany). We prepared each enzyme's substrate (800 µM) in various buffers. The soil solutions were incubated at 30 °C. After 60 min incubation, microplates were measured fluorometrically (excitation 360 nm, and emission 450 nm) using a microplate reader (Multi-Mode Microplate Reader SynergyTM HTX, Bio-Tek Instruments, Inc., USA). In the case of phytase, activities were determined in soil suspension using 10 % phytic acid, w/v (P-8810, Sigma Aldrich) as substrate. The soil suspension was prepared with sodium acetate buffer (200 mM, pH 5.5), soil to solution ratio was 1:5 (w/v). Enzyme analyses were carried out with 0.5 ml of the soil suspension followed by the addition of 0.5 ml of sodium phytate solution (Boyce et al., 2004). The mixture was incubated at 37 °C and centrifuged after 60 min incubation at 3800 × g for 5 min. Phosphate release in the supernatant was determined using a malachite green reagent (MAK307, Sigma Aldrich, USA). The absorbance was determined at 620 nm. According to German et al. (2011), all enzyme activities were calculated and presented as µmol min-1 g-1 soil-1 dry weight.

Statistics and data analysis

We conducted non-metric multidimensional scaling (NMDS) based on the Bray–Curtis metric, utilizing the Phyloseq R package (McMurdie and Holmes, 2013), with relative abundance matrices for both pKOs and pECs.

Differential analysis of predicted KEGG Orthologs (pKOs) and Enzymes (pECs) in the relative abundance data (centered log-ratio (clr)) was performed using the 'ALDEx2' package (Fernandes et al., 2013) in R v. 4.1.2 (R Core Team 2021). Significance was assessed using the Wilcoxon Rank Sum test with Benjamini–Hochberg false discovery rate (FDR) correction (Benjamini and Hochberg, 1995).

To evaluate the relationship between the abundance of pKOs and pECs and the soil physicochemical properties of the four soil units, we conducted a Canonical Analysis of Principal Coordinates (CAP) (Anderson and Willis, 2003) using the Vegan R Package version 2.6.2 (Oksanen et al., 2019). CAP analysis was performed using the Bray-Curtis distance, and model significance was determined through permutational multivariate analysis of variance (PERMANOVA) with 999 permutations.

Furthermore, we performed the CAP analysis using the same parameters as described for the pKOs and pECs CAP analysis to assess the correlation between enzyme activity and the soil physicochemical properties across the four soil units.

The Pearson correlation analyses were performed between the relative abundance of pKOs and pECs and the enzyme activity values of ACP, ALP, and Phy using the 'corrplot' package in R (Wei et al., 2017).

Metagenome sequencing and P-enzyme gene analysis

We selected two soil units, ITA and SPO, to perform a complement analysis of the functions involved in PO mineralization of the soil metagenome. These two soil units represent 40 % of the soils of Uruguay, most of which are under natural grasslands for the production of beef cattle.

Soil metagenomic sequencing from ITA and SPO soil units was carried out on a HiSeq Illumina platform (Service CD Genomics, NY, USA; pair-end read 150

bp). Raw sequence quality was analyzed with FastQC software version 0.11.2. Raw sequence data are publicly available on the MG-RAST repository under the project ID ITA: mgp 91922 and SPO: mgp 93346.

We used the reference databases of the P-enzyme involved in the P organic mineralization built by Neal et al. (2017). The P-enzymes included are listed in Table 7.

Table 7: List of P-enzymes involved in the P organic mineralization.

P-Enzyme	Gene	Class	Predicted Cellular Localization	Number of protein sequences in the reference database
PhoA	*phoA*	Alkaline phosphatase	Periplasmic/ Cytoplasmic	293
PhoD	*phoD*	Alkaline phosphatase	Outer membrane/ extracellular	833
PhoX	*phoX*	Alkaline phosphatase	Outer membrane/ extracellular	424
NSAP class A (NSAP-A)	*phoC*	Acid phosphatase	Periplasmic/ Cytoplasmic	750
NSAP class B (NSAP-B)	*aphA*	Acid phosphatase	Periplasmic/ Cytoplasmic	388
NSAP class C (NSAP-C)	*olpA*	Acid phosphatase	Outer membrane/ extracellular	1123
β-propeller phytase (BPP)	*phyL*, *phyS*	Phytase	Outer membrane/ extracellular	108
Cysteine phytase (Cphy)	*phyA*	Phytase	Outer membrane/ extracellular	122

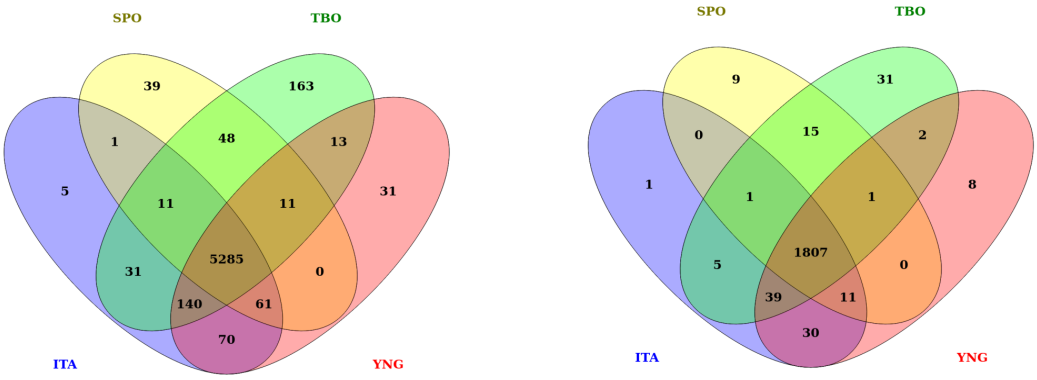
Protein sequence alignments of the respective reference database were performed using MAFFT version 7.4.60 (Katoh et al., 2002) under default parameters. Reference protein phylograms were inferred with IQTree 2 version 1.6.12 (Minh et al., 2020), and the evolutionary models were evaluated with RAxML-NG (Kozlov et al., 2019). Phylograms were plotted with iTOL (Interactive Tree of Life; Letunic and Bork, 2007).

To determine the abundance and diversity of the P-enzymes in the metagenomes, we queried each metagenomic sample against each P-enzyme reference database. First, each metagenomic sample's predicted protein set was queried against each P-enzyme reference database using HMMER version 3.3.1 (http://hmmer.org). Then, the sequences with positive hits were aligned to the correspondent reference database alignment using MAFFT with add sequence option and default parameters.

Results

Metagenome functional predictions

A total of 5910 KEGG Orthologs (KOs) groups and 1960 Enzyme Commission (ECs) were obtained. The four soil units shared 5285 (89.4 %) KO groups, while TBO soils had the highest number of unique KO groups (163; 2.7 %), and ITA soils had the lower number of unique KO groups (5; 0.08 %) (Figure 3). The number of ECs shared by the four soil units was 1807 (92.2 %), observing the same trend recorded for the KO groups; the soil unit with the highest number of unique ECs was TBO (31; 1.6 %), and the one with the lowest number of unique ECs was ITA (1; 0.05 %).



KO EC

Figure 3: Shared KOs and ECs groups among the four soil units. Venn diagram showing the KOs and ECs predicted groups overlapping among ITA, SPO, TBO, and YNG.

The nearest sequenced taxon index (NSTI) values for 99.9 % of the OTUs were < 2 (average NSTI = 0.36). These low NSTI values indicate more accurate predictions from PICRUSt. These results were close to the NSTI values of soil samples studied by Langille et al. (2013) (average NSTI = 0.17), indicating good accuracy in predicting soil microbiome function using PICRUSt.

Phosphorous genes and enzymes predicted

The selected subset of KOs and ECs (Table 5 and 6), representative of genes and enzymes involved in the P mobilization, was used for the following analyses.

We explored the proportions of taxonomic contribution estimated from the pKO and pEC PICRUSt2 data (Figure 4). We observed a predominance of specific phylum related to each specific gene (KO) and enzyme (EC). There was a general predominance of the phylum *Proteobacteria*, *Firmicutes* and *Actinobacteria* associated with almost all pKOs, while certain pKOs have a lesser contribution from other phyla (Figure 4a). For example, the contributing phyla to K01113 (*phoD*) were *Proteobacteria*, *Actinobacteria* and, to a lesser extent, *Acidobacterias*,

Planctomycetes and *Chloroflexi* in all sites; K02038 and K02039 also showed similar contribution patterns. However, the K01078, K02041 and K09474 contribution pattern was dominated by *Proteobacteria* (Figure 4a).

We observed similar contribution taxonomic profiles in ITA and SPO for K01077, K01125, K02038, K02039, K03306, K05306, K05814 and K07048, where the main contributing phyla were *Actinobacteria* and *Proteobacteria*. The contribution profiles for those pKOs in TBO and YNG were dominated by *Firmicutes*.

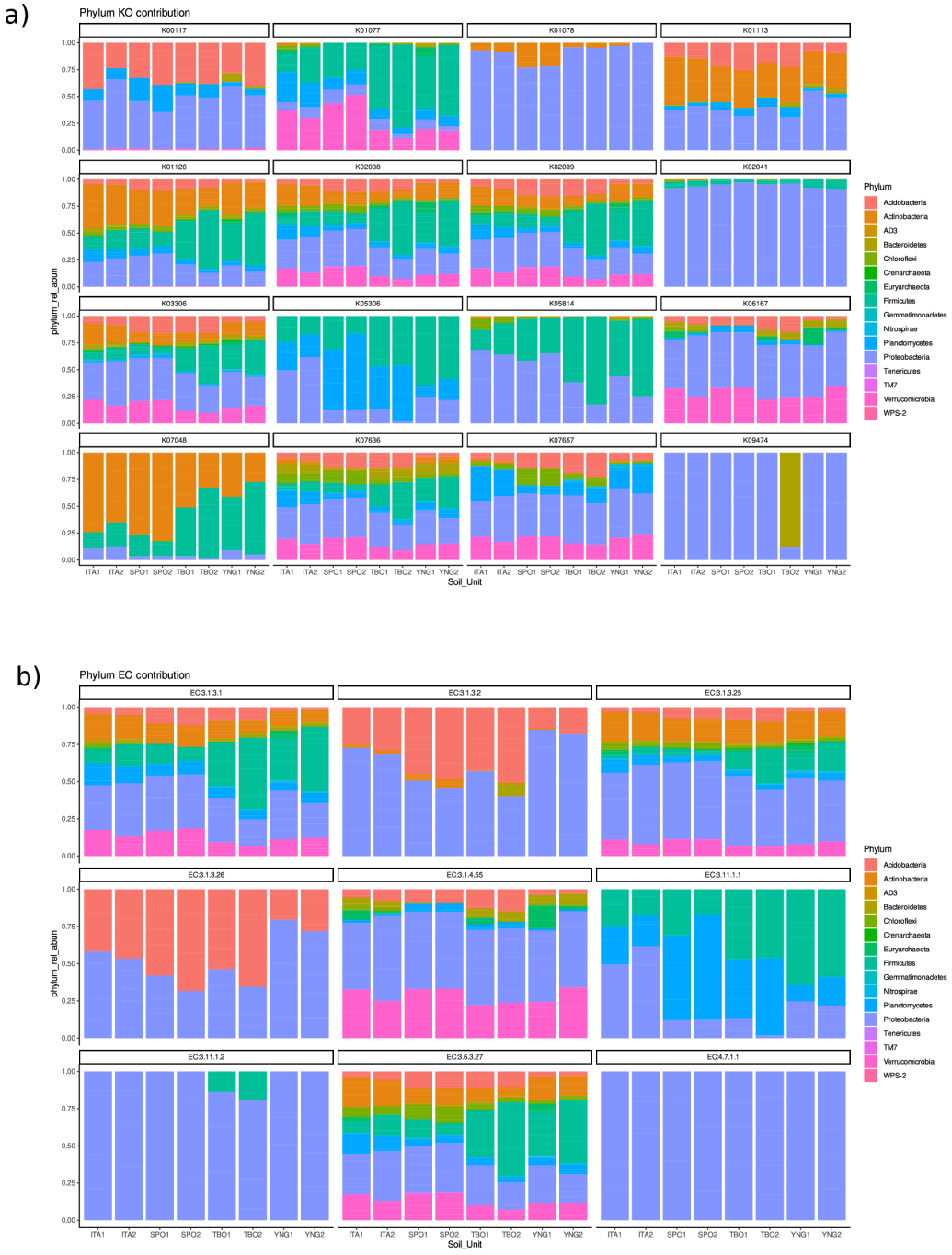


Figure 4: Taxonomic contribution at phylum level to pKOs and pECs predicted by PICRUSt2

Taxonomic contribution analyses of the pECs showed similar results to pKOs, with the phyla *Acidobacteria*, *Firmicutes* and *Proteobacteria* dominating almost all pECs. The taxonomic profiles of EC3.1.3.1, EC3.1.3.25, EC3.1.4.55 and EC6.3.27 showed the contribution of multiple phyla, while EC3.1.1.2 and EC4.7.1.1 were dominated by *Proteobacteria* contributions.

As we observed in the pKOs contribution profiles, the pECs profiles also showed a similarity between ITA and SPO with a predominance of *Proteobacteria* contribution. In contrast, the contribution profiles of TBO and YNG were dominated by *Firmicutes*.

NMDS analysis showed that pKOs and pECs sorted samples according to soil units. Mainly, we observed the assemblage in two clusters: ITA and SPO soils and TBO and YNG soils. PERMANOVA analysis showed a significant effect of soil unit on pKOs and pECs ($F = 16.369$, $p_{perm} < 0.001$ and $F = 11.494$, $p_{perm} < 0.001$, respectively) (Figure 5). This result is consistent with the taxonomic contribution patterns observed for some pKOs and pECs, which clustered ITA and SPO on one side and TBO and YNG on the other along of NMDS1 axis. Both analyses showed that the functional profiles of ITA and SPO soil units revealed similar gene abundance and taxonomic contribution. We found the same trends when analyzing TBO and YNG soil units.

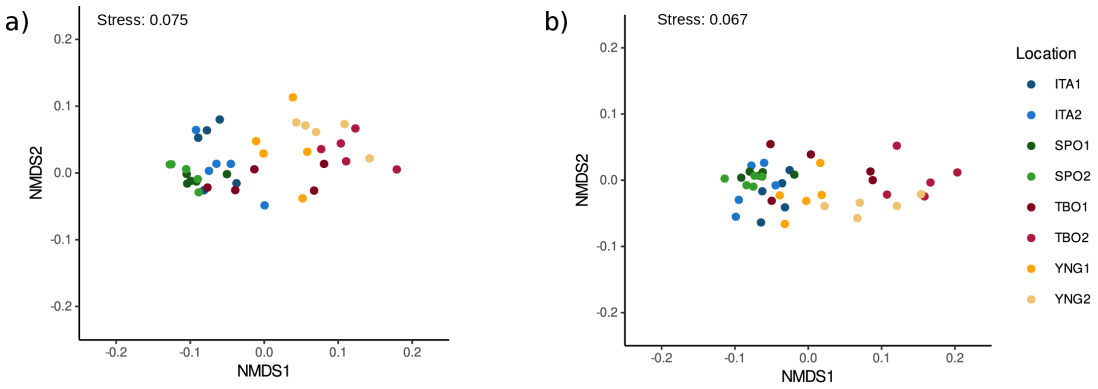


Figure 5: NMDS ordination based on Bray-Curtis distances between the relative abundance; a) pKOs and b) pECs of four soil units. Each point represents replicates from ITA, SPO, TBO, and YNG. There was a significant difference in pKOs and pECs relative abundance ($F = 16.369$, $p_{perm} < 0.001$ and $F = 11.494$, $p_{perm} < 0.001$, respectively) between soil units.

The predicted enzymes that showed significant differences in the relative abundance between ITA and SPO soil units were EC3.1.3.26 (Phytase) and EC3.1.3.2 (acid phosphatase), showing the highest abundance values in ITA (Figure 6).

On the other hand, when we compared the relative abundance of pKOs and pECs within the other cluster (TBO and YNG), we did not find significant differences.

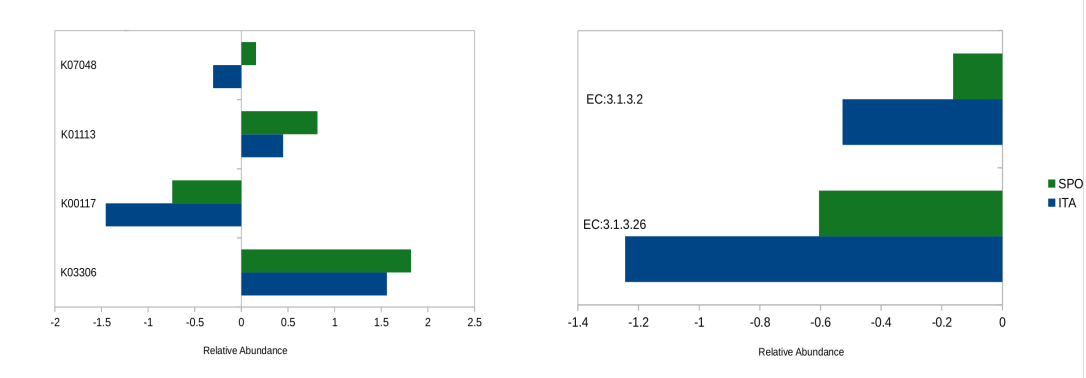


Figure 6: The predicted abundance of pKOs (KEGG orthologs) and pECs (enzyme commission) obtained using PICRUSt2. Relative abundances (centered log-ratio, clr) were compared among Soil Units ITA and SPO to identify the statistical differences ($p < 0.05$). We performed statistical comparisons using the Wilcoxon rank-sum test with Benjamini–Hochberg FDR correction ('ALDEx2' in R).

Relationship between P genes (pKOs) and P enzymes (pECs) and soil physicochemical properties

We used CAP analysis to explore the relationship between pKOs and pEC normalized abundance and soil physicochemical properties (Figures 7 and 8). The constrained model of pKOs based on the Bray-Curtis distance explained 55 % of the variance ($p < 0.001$). We identified seven pKOs (KO7657; KO7636; KO3306; KO2038; KO2039; KO1077; KO1126) responsible for the observed variance (Figure 7). The CAP 1 axis was associated ($r >= 0.20$) with pH, Na, BD, Po, CC and WHC, which separated ITA soil from the others. CAP 2 axis was associated with pH, N, APR, SOC, CEC, Mg, Ca, K, and WHC (Table 8) and separated SPO soil from TBO and YNG.

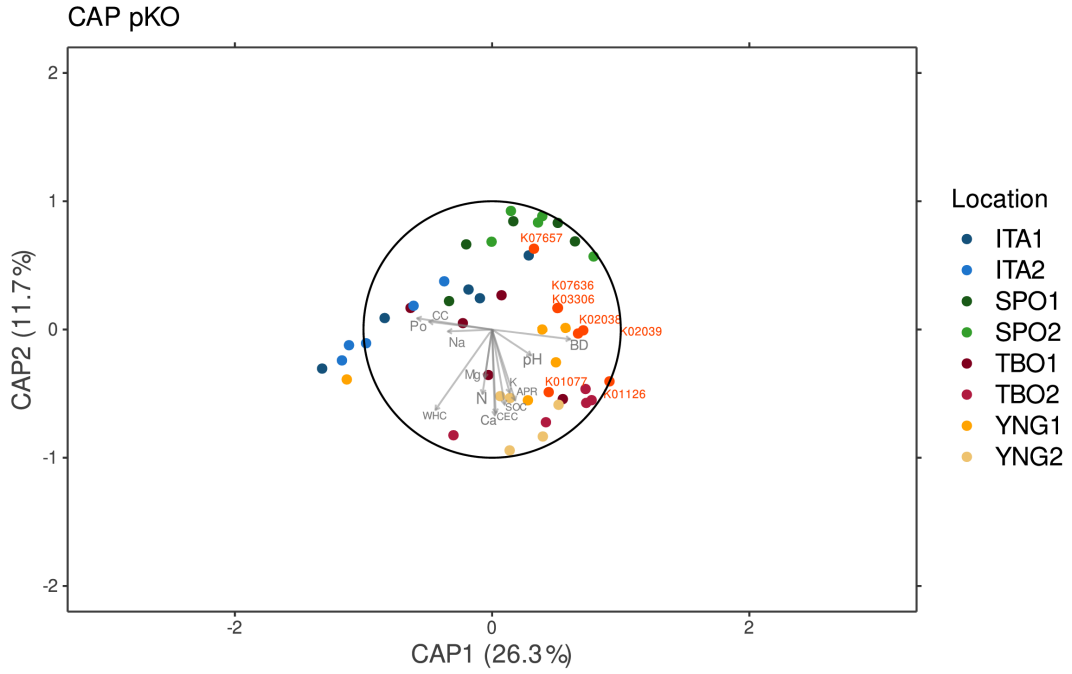


Figure 7: CAP based on Bray-Curtis distance for pKOs. PERMANOVA analysis with 999 permutations was performed to determine the significance among soil units. Two locations were included for each soil unit and soil physicochemical properties (n = 8): ITA (blue and light blue), SPO (green and light green) and TBO (red and light red). Vector labels are pH, Total nitrogen (N), Soil Organic Carbon (SOC), available P by Resin method (APR), Cation Exchange Capacity (CEC), Ca, K, Mg, Na, Bulk Density (BD), Clay Content (CC), Porosity (Po), Water Holding Capacity (WHC). The variable's vector length is relative to the circle radius and represents the correlation between each variable and the axes. CAP analysis was performed with the Vegan R package, and graphics were produced with the R package ggplot2.

The constrained model of the CAP analysis between pECs and the environmental variables accounted for 53.4 % of the observed variance, explained by EC3.1.3.25, EC3.6.3.27 and EC3.1.3 (Figure 8). The CAP 1 axis was associated (r

>= 0.20) with pH, N, CEC, Ca, Mg, Na, BD, CC, Po and WHC (Table 8). This axis separated ITA from SPO and YNG soils. CAP 2 axis was associated with N, APR, SOC, CEC, Ca, K, Mg, BD, CC, Po WHC, separating ITA and SPO soil from TBO and YNG soils.

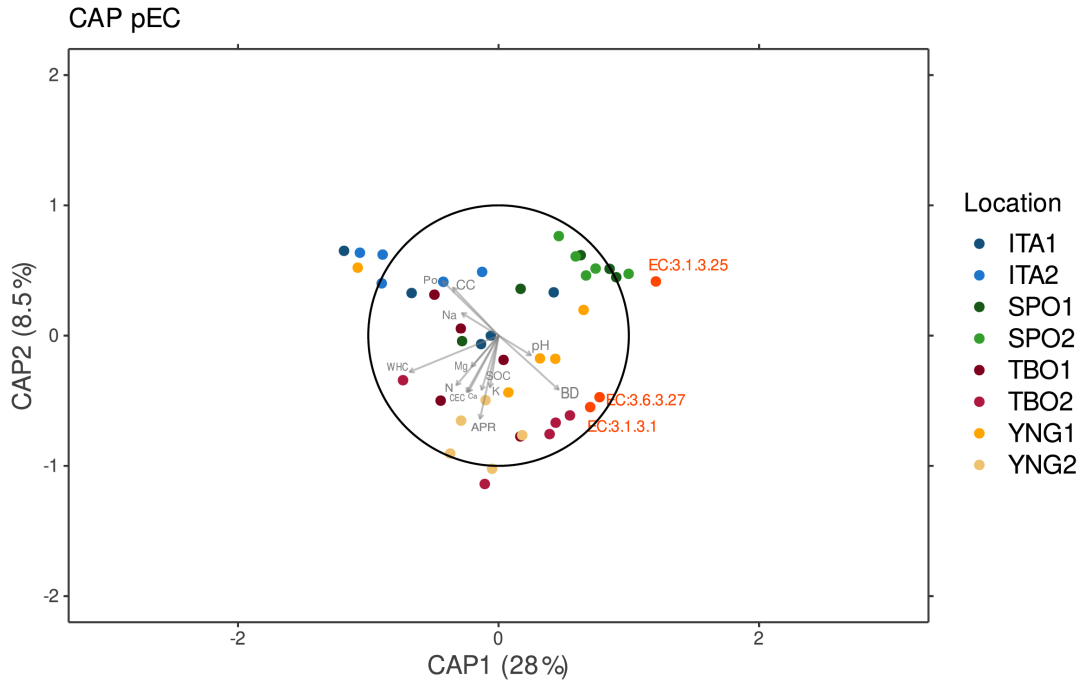


Figure 8: CAP based on Bray-Curtis distance for pECs. PERMANOVA analysis with 999 permutations was performed to determine the significance among soil units. Two locations were included for each soil unit and soil physicochemical properties (n = 8): ITA (blue and light blue), SPO (green and light green) and TBO (red and light red). Vector labels are pH, Total nitrogen (N), Soil Organic Carbon (SOC), available P by Resin method (APR), Cation Exchange Capacity (CEC), Ca, K, Mg, Na, Bulk Density (BD), Clay Content (CC), Porosity (Po), Water Holding Capacity (WHC). The variable's vector length is relative to the circle radius and represents the correlation between each variable and the axes. CAP analysis was performed with the Vegan R package, and graphics were produced with the R package ggplot2.

Table 8: CAP analyses based on Bray-Curtis distance for pKO and pEC profiles, enzymes activities and environmental variables. Environmental variables included: pH, Total nitrogen (N), Soil Organic Carbon (SOC), available P by Resin method (APR), Cation Exchange Capacity (CEC), Ca, K, Mg, Na, Bulk Density (BD), Clay Content (CC), Porosity (Po), Water Holding Capacity (WHC). PERMANOVA analysis with 999 permutations was performed to determine the significance between the soil units.

Variables	pKOs			pEC			Enzyme Activities		
	p-value	CAP1 (r)	CAP2 (r)	p-value	CAP1 (r)	CAP2 (r)	p-value	CAP1 (r)	CAP2 (r)
pH	0.060	0.304	-0.202	0.029	0.247	-0.152	0.001	-0.748	-0.386
N	0.461	-0.074	-0.503	0.453	-0.325	-0.379	0.462	-0.759	0.303
SOC	0.016	0.098	-0.586	0.023	-0.132	-0.416	0.003	-0.487	0.209
APR	0.007	0.180	-0.554	0.007	-0.144	-0.638	0.001	-0.323	-0.139
CEC	0.067	0.028	-0.650	0.023	-0.234	-0.436	0.001	-0.681	-0.008
Ca	0.255	0.021	-0.671	0.219	-0.247	-0.433	0.202	-0.826	-0.239
K	0.433	0.137	-0.495	0.476	-0.066	-0.396	0.163	-0.671	0.076
Mg	0.009	-0.056	-0.379	0.010	-0.209	-0.239	0.031	-0.173	0.336
Na	0.013	-0.347	-0.014	0.007	-0.283	0.174	0.605	-0.201	0.524
BD	0.001	0.608	-0.076	0.001	0.459	-0.414	0.001	0.659	-0.632
CC	0.109	-0.493	0.062	0.082	-0.352	0.369	0.008	-0.787	0.548
Po	0.304	-0.585	0.088	0.247	-0.430	0.418	0.143	-0.683	0.624
WHC	0.635	-0.439	-0.625	0.762	-0.683	-0.279	0.161	-0.714	0.131

	pKOs		pECs		Enzyme Activity	
	p-value	%variance	p-value	% variance	p-value	%variance
CAP model	0.001	55	0.001	53.4	0.001	89.5
CAP1 axis	0.001	26.3	0.001	28	0.001	46.09
CAP2 axis	0.001	11.7	0.01	8.5	0.001	21.06

Enzyme activity

Analysis of enzyme activity for each soil unit showed high values of ACP activity compared to ALP and Phy in all soils (Table 9, Figure 9). The anthropogenically disturbed YNG soils revealed the highest activity values of the ACP and ALP enzymes, with significant differences concerning the other three soil units. Considering the three soil units under natural grasslands, ITA had the highest values of ACP activity, which significantly differed from SPO and TBO soils. The ALP had the lowest activity values in all three soil units representing the natural grasslands and did not differ significantly. In all the studied soils, the Phy activity values were lower than the ACP and ALP enzyme activity values, and we observed the highest enzyme activity in ITA soils.

Table 9: Averages values of enzyme activities per soil unit sites.The significance is denoted by asterisks (*: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$).

Soil Unit	Alkaline phosphatase (ALP) (µmolmin-1 g-1soil-1dry weight)	Acid phosphatase (ACP) (µmolmin-1 g-1 soil-1 dry weight)	Phytase (Phy) (µmolmin-1 g-1 soil-1 dry weight)
ITA 1	1.01	75.18**	7.65
ITA 2	1.66	78.47**	6.86
SPO 1	3.65	42.6**	4.01
SPO 2	2.55	40.11**	8.44*
TBO 1	0.1	27.73*	6.59
TBO 2	0.16	33.21	6.82
YNG 1	33.84**	227.36***	7.23*
YNG 2	26.09**	90.44***	4.96

Relationship between enzyme activity and soil physicochemical properties

We explored the association between enzyme activities and environmental variables using CAP analysis (Figure 10). We identified that the ACP activity was mainly responsible for the explained variance of 89.5 % (Figure 10). The CAP 1 axis was associated with pH, N, APR, SOC, CEC, Ca, K, Na, BD, CC, Po and WHC (Table 8), which separated ITA and YNG from SPO and TBO soils. CAP 2 axis was associated with pH, N, SOC, Ca, Mg, Na, BD, CC and Po and separated ITA from the other soils.

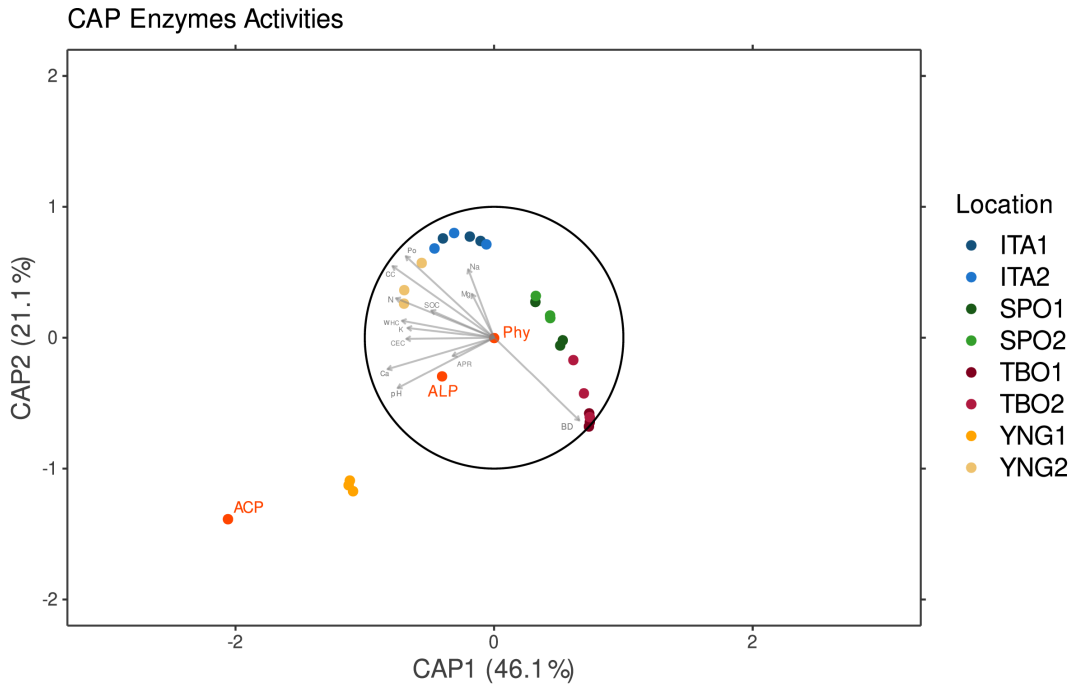


Figure 10: CAP based on Bray-Curtis distance for enzyme activity. PERMANOVA analysis with 999 permutations was performed to determine the significance among soil units. Two locations were included for each soil unit and soil physicochemical properties (n = 8): ITA (blue and light blue), SPO (green and light green) and TBO (red and light red). Vector labels are pH, Total nitrogen (N), Soil Organic Carbon (SOC), available P by Resin method (APR), Cation Exchange Capacity (CEC), Ca, K, Mg, Na, Bulk Density (BD), Clay Content (CC), Porosity (Po), Water Holding Capacity (WHC). The variable's vector length is relative to the circle radius and represents the correlation between each variable and the axes. CAP analysis was performed with the Vegan R package and graphics were produced with the R package ggplot2.

Correlation between P genes (pKOs) and P enzymes (pECs) and enzyme activity

We performed correlation analyses to evaluate the relationship between genes and enzymes predicted by PICRUSt2 and the measured enzymatic activity. The predicted genes represented by the pKO group only showed a significant correlation with K05814 (*ugpA*) (r = 0.43) and K05306 (*phnX*) (r = 0.44) (Figure 11a), genes involved in the P – uptake and transport and mineralization of P organic.

Similar results were obtained when analyzing the correlation with predicted enzymes; there was no significant correlation with the enzyme activity, except for ACP and ALP with EC3.11.1.2 (acid phosphatase) (r = 0.70 and r = 0.62, respectively) (Figure 11b).

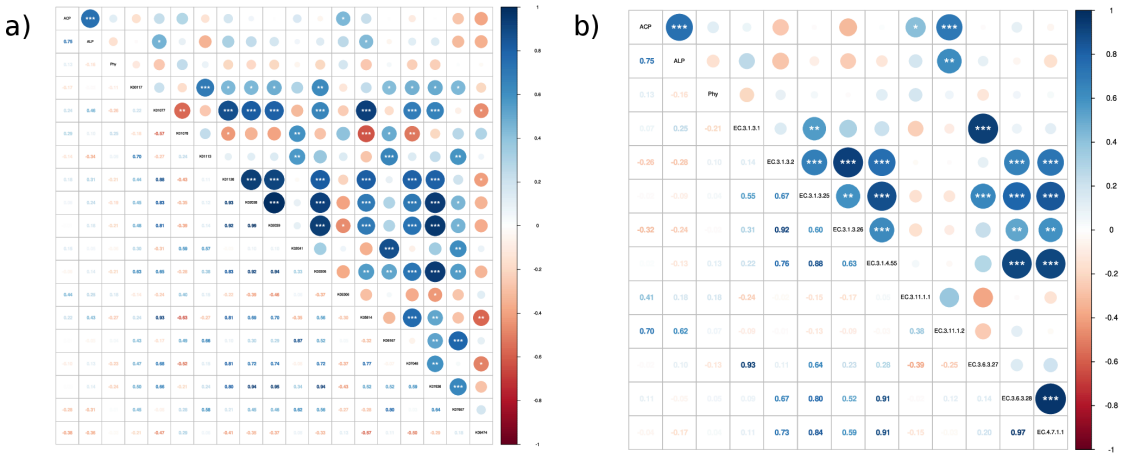


Figure 11: Correlation matrix of enzyme activities (ACP: acid phosphatase; ALP: alkaline phosphatase; Phy: phytase) with a) KO relative abundance (KO: Kegg Orthologs) and b) EC relative abundance (EC: enzyme commission). Correlogram displays the Pearson correlation coefficients that are colored according to their values (blue for the positive values and red for the negative values). The significance is denoted by asterisks (*: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$).

Metagenome sequencing and P-enzyme coding genes analysis

A total of 196 Mb reads were obtained for the ITA soil unit and 207 Mb for the SPO soil unit, with an average length of 150 bp and high quality (Phred score = Q30); 95 % of the reads were used for the following analysis.

To evaluate the diversity and abundance of P-enzyme encoding genes, we compared the ITA and SPO metagenomes with the P-enzyme encoding gene database (Table 7). The abundance and diversity of the alkaline phosphatase encoding genes (*phoD*, *phoX*, *phoA*), non-specific acid phosphatases (NSAP-A, NSAP-C) and phytases (BPP and CPhy) were similar in both soil units, ITA and SPO. We observed a higher relative abundance of all interrogated genes in ITA than in SPO, the *phoD* gene being the most abundant (Figure 12). Regarding the phylogenetic distribution, the alkaline phosphatases predominated at the bacterial family level *Alphaproteobacteria*, *Betaproteobacteria*, and *Actinomycet*es. The acid phosphatase NSAP-A encoding genes harboring bacteria were mainly from *Betaproteobacteria*, *Gammaproteobacteria* and *Actinomycet*es. By comparison, the NSAP-C encoding genes were from *Alphaproteobacteria*, *Nitrospirae* and *Acidobateria* bacterial families. Concerning both phytase genes, the bacteria-harboring them were from the phylum *Proteobacteria* and, for the BPP phytase, also *Cyanobacteria* phylum

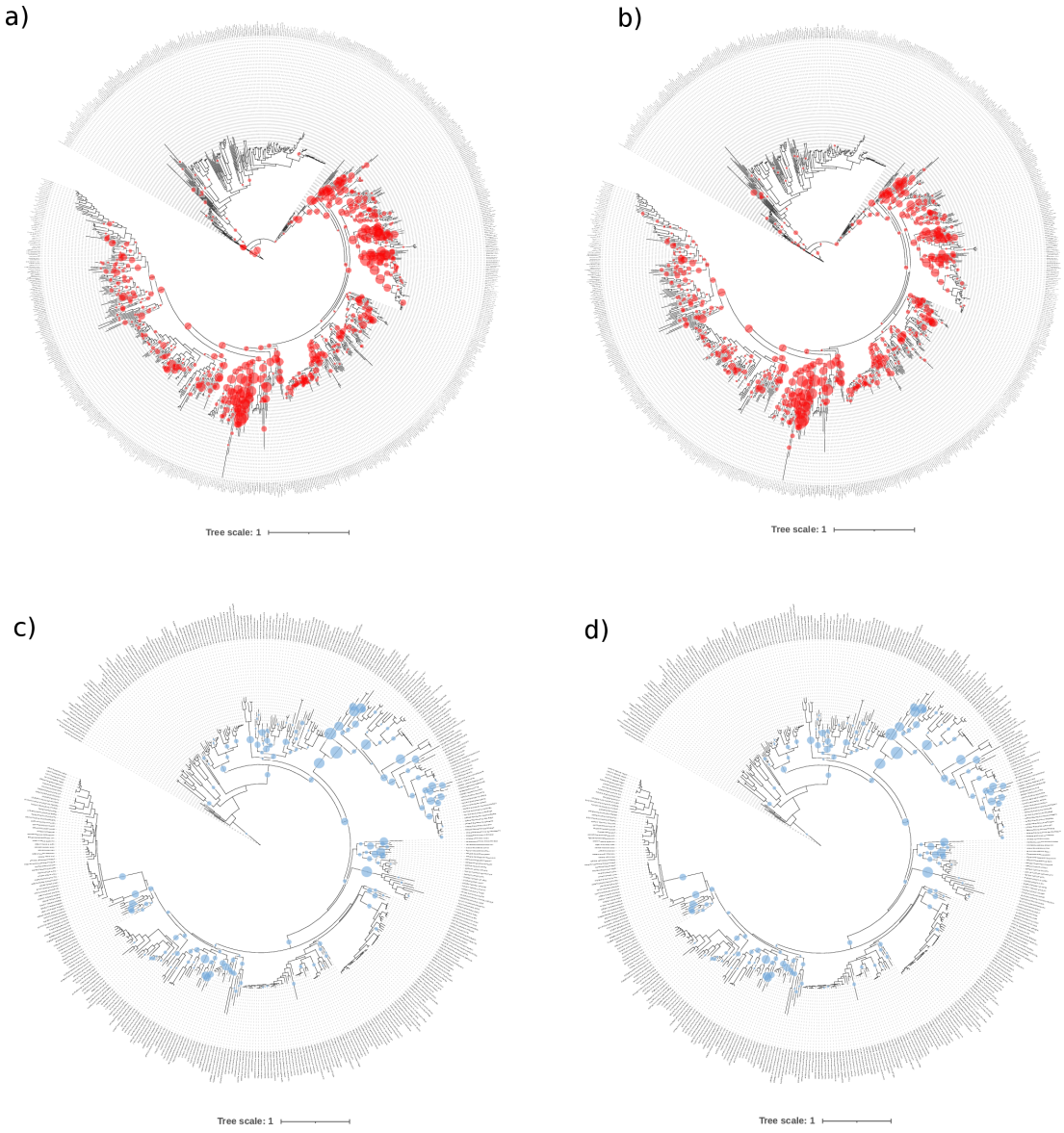


Figure 12: Phylogenetic placements of the predicted proteins of ITA and SPO metagenome to the reference bases of each enzyme: a) ITA - PhoD, b) SPO - PhoD, c) ITA- NSAP-A and d) SPO – NSAP-A. The size of the circle representing placements is proportional to the abundance. Maximum likelihood-based phylogenetic placement of metagenome-derived protein sequences was performed with EPA-ng, and a tree was drawn with iTOL. The circle sizes represent the number of hits per node.

Discussion

The soil physicochemical properties, land use, or climate change effects on microbial communities assemblage are well understood. However, the impact on the microbial function profiles is still limited (Rivett and Bell, 2018). In this work, we proposed to examine the predicted prokaryotic community function of four Uruguayan Campos biome soil units to investigate the relationships between them, soil physicochemical properties and taxonomic structure.

Predicted bacterial function and soil physicochemical properties

The most important factors influencing the functional diversity of prokaryotic soil communities are soil physicochemical properties, such as pH and soil nutrient status (Mocali et al., 2022, Delgado-Baquerizo et al., 2016). Our results showed that the soil units studied here present differences in their functional profiles, which most likely can be explained by their physicochemical properties and particular land use.

The soil units TBO and YNG showed similar functional profiles without significant differences; however, in a previous study, these soil units showed differences in their prokaryotic communities which was not reflected in their functional profiles. Several works have demonstrated that taxonomic and functional diversity are not linearly correlated mainly to functional redundancy (Chen, 2022, Mendes et al., 2015, Lennon and Jones, 2011). Mendes et al. (2017) studied soils with four land uses (Forest, Deforest, Agriculture and Pasture). They showed that some essential functions had many contributing species in soils under agriculture and pasture but did not correlate with the transcription activity for these functions . They found 2- to 4-fold higher transcription rates for these genes in the soil with lower taxonomy diversity (Forest).

We found that the phylum Firmicutes mainly contributed to the certain pKOs and pECs in both soil units. A previous study on the structure of the prokaryotic communities of these soil units showed that *Firmicutes* and *Proteobacteria* were the

phyla that differentiated the YNG soils. In contrast, only *Proteobacteria* differentiated the TBO prokaryotic communities (Garaycochea et al., 2020).

These two soil units are particular cases. The TBO soil unit is developed on sandstone with a sandy texture, and the YNG soil unit represents one of the groups of soils with the best physicochemical properties for agricultural production in the country; hence it supports high human intervention (Alvarez and Cayssials, 1979; Chavez, 2018). In YNG soils, we observed changes in the measured variables, particularly in the nutrient balance with high values of APR and N, which could be attributed to fertilizer use over many years. This soil unit also had the highest values of SOC, is a good soil with high values of CC and Po, and the pH close to neutral. Regarding the enzyme activity in this soil unit, the recorded values for the three enzymes (alkaline phosphatase, acid phosphatase and phytase) were significantly high when compared to the other soil units. This result was not expected for fertilized soils, for which a decrease in phosphatase enzyme activity has been reported in association with high nutrient availability (Dinca et al., 2022, Janes-Bassett et al., 2022, Margalef et al., 2021) . A recent study analyzed the changes that soybean seeds co-inoculated with *Bradyrhizobium elkanii* and *Priestia megaterium* (antes *Bacillus megaterium*) generated in the prokaryotic communities of the soybean rhizosphere under three treatments: no fertilization, 7.7 μg g^{-1}, and 15 μg g^{-1} of available P. The results showed that plant P and yield increased and changes in the relative abundance of different phyla were observed when seed co-inoculation was combined with phosphate fertilization, suggesting that this treatment would improve phosphate nutrition of the soybean crop (Torres et al., pers. Comm. April 2023). We could hypothesize that the high nutrient balance of YNG may produce an effect similar to that observed in the soybean rhizosphere and explain the high enzyme activity in this soil. Despite the experimental design of this work cannot allow us to prove this hypothesis, we could infer that the perturbation, generated by the nearby agricultural practices, may change the soil basal nutrient balance and, as a consequence, the

prokaryotic community assemblage and its functional profile (Dinca et al., 2022, Garaycochea et al., 2020).

The sandy soil TBO did not show clear associations between the functional profiles and soil physicochemical properties. As mentioned above, despite the high P values recorded in this work, this is a nutrient-poor soil with a low relative amount of water and air due to its low porosity (Po, BD) and inability to retain water (WHC). These soil properties affected the prokaryotic community assemblage (Graça et al., 2021, Garaycochea et al., 2020), but could not confirm the effect on its P functional profile.

The ITA and SPO had similarities in their P functional profiles; however, we could identify differences mainly in the P mineralization function. The ITA soil unit showed a higher abundance of predicted enzymes involved in organic P mineralization (ACP and Phy). The CAP analyses showed a high correlation between nutrient content (SOC, N, APR and CEC), soil structure (BD, CC, Po) and WHC, which were the main variables responsible for the ordination of the samples based on functional profiles (pKOs and pECs). The effect of the nutrient balance on the enzymes that mineralize organic P is known, as shown by Margalef et al. (2017), who demonstrated that SOC and N, together with climatic variables, allowed the estimate of the mineralization potential of the soil. Likewise, clay content (CC) has been reported for different soil types as a determinant of enzyme activity due to its stabilizing role (Margalef et al., 2017), as well as a determinant of the abundance of genes encoding P enzymes, especially with the alkaline phosphatase genes (Neal et al., 2017). Considering the ITA and SPO prokaryotic communities and the characterized functional diversity, we found differences in both profiles, which could be a result of the sum of the effects of the physicochemical properties and the contributing prokaryotic communities (Garaycochea et al., 2020, Karimi et al., 2020, Delgado-Baquerizo et al., 2018). Despite the differences identified between the functional profiles of the two soil units, they were similar, as shown by the overlap

between the samples in the NMDS analysis and the CAPs analyses performed, compared to their taxonomic profiles.

ITA and SPO had different prokaryotic community compositions; ITA had high Shannon (H') and Chao1 (S) values compared to SPO, but both soil units had had phylogenetic diversity (PD) values with no significant differences (Garaycochea et al., 2020). These values suggest that the SPO prokaryotic community is composed of more of more divergent taxa, although fewer species are present. . The similarity in P-related functional profiles between these two soil units could be attributed to the functional redundancy exhibited by the different communities. Numerous studies have shown that taxonomic composition shows remarkable variation in response to soil properties, while the functional capabilities of these communities, as inferred from gene abundance, tend to show redundancy (Louca et al., 2018, Nelson et al., 2016). There is increasing evidence that taxonomic diversity may only sometimes predict soil carbon (C) and nitrogen (N) processes. This fact occurs because taxonomic diversity does not necessarily reflect functional diversity, which better indicates ecosystem processes. For example, a study by Delgado-Baquerizo et al. (2016) found that taxonomic diversity was a weak predictor of soil C and N processes in 78 global dryland ecosystems.

In contrast, functional diversity was a much stronger predictor of these processes. Similarly, a study by Zhang et al. (2018) found that microbial functional diversity better-predicted soil C and N processes than taxonomic diversity in grassland soils. Functional diversity may better reflect soil microbial communities functional redundancy and complementarity, which may influence ecosystem processes. Our results may indicate that taxonomic composition may also be a weak predictor of soil P processes. The above reinforces the idea that functional redundancy is essential in maintaining ecosystem functioning, acting as a buffer against changes in taxonomic composition (Jurburg and Falcão Salles, 2015).

We observed a similar trend regarding enzyme activity in ITA and SPO. We identified clay content (CC), porosity (Po), nutrient balance (SOC, N, P, CEC) and

pH as the soil properties with a strong association with ACP and ALP enzyme activity, which clustered the four soil units separately. These results agreed with those reported by Mencel et al. (2022), who stated that enzyme activity is not only affected by soil chemical properties such as pH and nutrient content but also by water and air content in the soil. In addition, acid and alkaline phosphatases sometimes coexist but dominate in different ranges of soil pH (Margalef et al., 2017), which is consistent with our results.

Metagenome sequencing and P-enzyme gene analysis

Metagenomic shotgun sequencing and PICRUSt2 (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States 2) (Douglas et al., 2020) are widely used approaches to study the functional potential of microbial communities. While PICRUSt2 predicts the functional content of the metagenome based on the phylogenetic marker gene 16S rRNA to provide information on the correlation between phylogeny and function (Dube et al., 2019), shotgun metagenomics directly sequences all DNA in a sample, providing a more comprehensive view of the microbial community and its functional potential (Sun et al., 2020).

The comparison we made between PICRUSt2 predicted enzymes and ACP activity showed a high rate of positive correlation, indicating a strong relationship. Although the comparison between predicted genes and enzyme activities showed lower rates of positive correlation, there is still potential for further investigation and discovery. Our results are consistent with those reported in previous studies, where the abundance of genes involved in P mineralization and enzyme activities in soil nutrient cycling processes increased together (Ma et al., 2021, Fierer et al., 2012; Leff et al., 2012). The increase in genes encoding mineralization enzymes and their activity suggests that the soil microbial community is more efficient at the degrading organic matter, thereby increasing nutrient cycling rates (Séneca et al., 2021). The PICRUSt2 is a valuable tool for estimating the organic P mineralization potential of the soil microbiome.

To understand P functions comprehensively, we used metagenomic shotgun sequencing to investigate the P functional potential of microbial communities in two soil units of the Uruguayan natural grasslands. We analyzed seven P-enzyme-coding genes that release P from organic compounds. Our results were consistent with previous findings (Neal et al., 2017, Bergkemper et al., 2016), with the *phoD* gene being the most abundant and phylogenetically diverse in both soils. Although there were differences in gene abundance between the two soil units, we did not identify distinct taxonomic groups associated with the studied P-enzyme encoding genes. Despite the diverse prokaryotic communities at the sites (Garaycochea et al., 2020), the functional profiles of both soils were similar, as predicted by PICRUSt2 and supported by the metagenomic approach. These results suggest that even highly taxonomically diverse microbial communities may exhibit low diversity in functional profiles, as coexisting microorganisms may be taxonomically distinct but encode the same function (Louca et al., 2018).

The natural grasslands are preserved in Uruguay, occupying more than 60 % of the country (Pérez Rocha, 2020, Lezama et al., 2019;), an important part of which is developed on soils with basalt and crystalline parent material (Dirección General de Recursos Naturales: Coneat, carta de suelo y campo natural, http://dgrn.mgap.gub.uy/js/visores/dgrn/). These soil units have vegetation dominated by grasses and, to a lesser extent, legumes (data not shown), with a minimum of human intervention. The vegetation communities, the climatic variables, the soil properties and the microbial communities and their interactions are involved in the ecosystem services. In this work, we have studied the genes and enzymes responsible for the transport, uptake and mineralization of organic P, as well as the activity of three key enzymes of the P cycle, in order to establish a baseline that can contribute to more efficient use of fertilizers, resulting in a reduced impact on the environment. We concluded that nutrient content (N, P, SOC and CEC), soil structure (BD, Po), water content (WHC) and pH were mainly responsible for the differences in the P functional profiles of the four different soil units. We can also

observe less functional diversity than the taxonomic in the soils studied, which could indicate functional redundancy.

Our results indicate that certain soil physical and chemical variables are responsible for shaping the functional profiles of microbial communities in the Campos biome. It would be interesting to know whether the patterns observed here are due to a local effect or whether there could be a global effect of these variables that could influence the functional microbial profiles of grasslands on different soil types, physical and chemical variables, and under other climatic conditions.

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Abundance and phylogenetic distribution of eight key enzymes of the phosphorus biogeochemical cycle in grassland soils

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Abstract

Grassland biomes provide valuable ecosystem services, including nutrient cycling. Organic phosphorus (Po) represents more than half of the total P in soils. Soil microorganisms release organic P through enzymatic processes, with alkaline phosphatases, acid phosphatases and phytases being the key P enzymes involved in the cycling of organic P. This study analysed 74 soil metagenomes from 17 different grassland biomes worldwide to evaluate the distribution and abundance of eight key P enzymes (PhoD, PhoX, PhoA, Nsap-A, Nsap-B, Nsap-C, BPP and CPhy) and their relationship with environmental factors. Our analyses showed that alkaline phosphatase phoD was the dataset's most abundant P-enzyme encoding genes, with a wide phylogenetic distribution. Followed by the acid phosphatases Nsap-A and Nsap-C showed similar abundance but a different distribution in their respective phylogenetic trees. Multivariate analyses revealed that pH, T_{max}, SOC and soil moisture were associated with the abundance and diversity of all genes studied. PhoD and phoX genes strongly correlated with SOC and clay, and the phoX gene was more common in soils with low to medium SOC and neutral pH. In particular, P-enzyme genes tended to respond in a positively correlated manner among them, suggesting a complex relationship of abundance and diversity among them.

INTRODUCTION

Grasslands are one of the most numerous and widely distributed biomes on the Earth's surface. Factors defining grassland biomes are climatic conditions, grazing and fire (White et al., 2000; Zhou et al., 2017). They develop in arid and semi-arid areas, with seasonal cold and dry periods, and high rates of evapotranspiration (Barnett & Facey, 2016; Knapp et al., 2002; Lenhart et al., 2015). The plant community is dominated by grasses and grass-like species, as well as other shrubby species with different lifestyles. Plant community assemblages depend largely on climatic variables. Most of the grassland biomass above-ground, together with the low rates of decomposition, generates significant accumulations of organic matter in soil profiles (Blair et al., 2014). Grasslands also provide several key ecosystem services, such as food, fibre and forage production, water and nutrient cycling, and erosion control. Grassland biomes are habitats for a high diversity of

plants, animals and microorganisms (Blair et al., 2014; Le Roux et al., 2011).

Nutrient cycling, one of the main ecosystem services provided by grasslands, can be defined as the cycling of elements carbon (C), nitrogen (N) and phosphorus (P) between different pools (Dubeux et al., 2007). Soils have low P availability as a result of high reactivity with calcium (Ca), iron (Fe), or aluminium (Al) ions, forming insoluble complexes (Achat et al., 2016). Soil P is present in two fractions, organic (Po) and inorganic phosphates (Pi) whose proportions between soils vary depending mainly on the geological material, pH, temperature and organic matter contributions (Gaiero et al., 2020; Zhou et al., 2017). On average, the organic fraction accounts for over half of total soil P and is a valuable reservoir that could be partially mobilized by microorganisms (Condron et al., 2005; George et al., 2018; Haygarth et al., 2013). The more abundant organic P forms in soils are inositol phosphate, phospholipids, nucleic acids and teichoic acid (Condron et al., 2005; Gyaneshwar et al., 2002). Inositol phosphate (commonly called phytic acid) can account for up to 80% of total organic P (Gerke, 2015; Quiquampoix & Mousain, 2005). Phytic acid reacts with ions present in the soil forming stable and insoluble complexes and so tends to accumulate in natural grasslands soils. On the other hand, phospholipids and nucleic acids are both labile and readily accessible to soil organisms (Gerke, 2015).

The Po mineralization is strongly influenced by several factors, including soil pH, total N, precipitation and temperature, and is mediated by various enzymes with phosphatase activity. These enzymes, which are involved in different stages of the P cycle, are also influenced by such environmental factors (Margalef et al., 2017). Po-cycle genes can be divided into three groups: Po mineralization (e.g. *phoD*, *phy*, *phoC*), transporter genes (e.g. *pstS*, *ugpQ*), and P starvation regulation genes (e.g. *phoB*, *phoR*) (Bergkemper et al., 2016; Oliverio et al., 2020; Zeng et al., 2022). The Po mineralization genes encode enzymes capable of releasing P from organic phosphate esters (henceforth P-enzymes). The alkaline phosphatases and non-specific acid phosphatases (Nsap) catalyse the hydrolysis between carbon and phosphorus in organic phosphate esters. The third group, the phytases, specifically release Pi from phytic acid (Bergkemper et al., 2016; Gaiero et al., 2020; Huang et al., 2009; Jorquera et al., 2008; Morrison et al., 2016; Rossolini et al., 1998). The two-component regulatory system (PhoBR) encoded by phoBR, called the Pho regulon, regulates the transcription of P-enzyme genes under low Pi conditions (Lidbury et al., 2017; Park et al., 2022; Santos-Beneit et al., 2015). Alkaline phosphatases are produced by a broad range of bacteria, archaea and fungi, which play an important role in microbial P turnover (Li et al., 2021). *PhoD*, *PhoX* and *PhoA* are three different types of alkaline phosphatases, with PhoD being the most abundant and ubiquitous (Ragot et al., 2015). Both PhoD and PhoX were identified as Ca2+-dependent extracellular enzymes and PhoA as a Zn2 + -dependent intracellular enzyme (Neal et al., 2018). Alkaline phosphatases show a broad substrate specificity and high catalytic efficiency (Cai et al., 2021; Rodriguez et al., 2014). These characteristics enable microorganisms harbouring these genes to use alternative P sources under P-limited conditions, conferring them an advantage over the plants (Li et al., 2021).

Acid phosphatases are another group of enzymes distributed widely among microorganisms and plants. They are divided into three groups, Nsap class A, Nsap class B and Nsap class C, none of which exhibit strong substrate specificity, hence their names (Thaller et al., 1998). These enzymes are mostly produced by microorganisms and are mostly active in acid soils (Gaiero et al., 2018). To expand the knowledge of these enzymes, metagenomic studies have been carried out to understand how they vary in abundance and diversity in different environments (Bergkemper et al., 2016; Neal et al., 2018). Neal et al. (2018) showed that Nsap class C, a putative extracellular enzyme, was predominant in acid soils under P-limiting conditions compared with Nsap class A a putative intracellular or periplasmic enzyme. These enzyme groups have been observed to have higher activity and gene abundance in the rhizosphere than in the bulk soil (Fraser et al., 2017; Spohn & Kuzyakov, 2013).

Phytases are produced by bacteria, fungi, plants and animals able to catalyse the mineralization of organic P from phytate to inorganic P (Ariza et al., 2013; Jorquera et al., 2008; Tu et al., 2011). Phytase families, more common in microorganisms, are the beta-propeller phytase (BPP), protein tyrosine phosphatase-like cysteine phytase (CPhy) and histidine acid phytase (HAPhy) (Lim et al., 2007). The main differences between the phytase families are structural, mainly related to differences in the active site which determines which phosphate group of the phytate is dephosphorylated, and co-factor requirements. Despite this, all phytases can release the six phosphate molecules contained in the phytate (Misset, 2002). Phytases exhibit different pH and temperature optima in the laboratory (Caffaro et al., 2020) and also are dependent on the soil microorganisms species (Amadou et al., 2021). Moreover, enzymatic activity is affected by soil type, texture and mineralogy by varying the ability to retain an active enzyme (Azeem et al., 2015; Rao et al., 1994; Tang et al., 2006).

Soil microorganisms play an important role in the soil P cycle, mediating P release for plants and other living soil organisms (Awasthi et al., 2011; Richardson & Simpson, 2011). Several prokaryotic phyla have been associated with soil Po mineralization

Acidobacteria, *Actinobacteria*, *Firmicutes* and *Proteobacteria* (Amadou et al., 2021). These Po mineralizing phyla contain a repertoire of genes that allow them to obtain Pi from organic compounds using different strategies. Forest soil study showed *Actinobacteria* and *Proteobacteria* played a dominant role in oxidative phosphorylation, whereas *Firmicutes* contributed to substrate phosphorylation (Ma et al., 2021). The alkaline phosphatase encoded by the *phoD* gene was primarily found in bacteria and was spread across 20 bacterial phyla (Ragot et al., 2015). Grassland microbiome studies showed *Actinobacteria*, *Planctomycetes* and *Proteobacteria* were the dominant bacterial phyla carrying the *phoD* gene, representing over 80% of all sequences (Graça et al., 2021). The *Streptomyces* genomes harbour alkaline phosphatases encoded by *phoA* and *phoD* genes and acid phosphatase class A coding gene (*phoC*) (Tian et al., 2021). Finally, the *Streptococcus* genus has been associated with phytase production and mineralization of phosphate (de Lacerda et al., 2016).

Grasslands are one of the five most important biomes on Earth due to the biodiversity they harbour and their economic importance. This makes it necessary to have a deeper understanding of its functions and dynamics for its preservation. This study aimed, through a global scale analysis of metagenomic data, to assess how eight key prokaryotic P-enzymes involved in P cycling vary in their abundance and diversity in grassland biomes, how are they related between them, how they interact with the general functional profiles, and how is this related to environmental variables. We hypothesized a certain association between the P-enzyme coding genes, and that the different soil properties and climate variables of grassland would affect the profiles of these genes. We then attempted to identify which variables could be drivers of the observed patterns.

EXPERIMENTAL PROCEDURES

Data collection

A total of 376 geo-referenced metagenome samples from 17 projects deposited with MG-RAST were selected through the TerrestrialMetagenomeDB (https://webapp.ufz.de/tmdb/) applying the following filters: Source DB: MG-RAST; seq_technology: Illumina; material: soil; Biome: grasslands, temperate grasslands, savanna and shrubland to assembly the grassland soil metagenomes samples set (Figure S1). All metagenomes included in the dataset were from topsoil samples (depth 10–15 cm). The set of environmental variables was assembled, including soil properties and climatic variables for each sample based on its geographic location. Soil type and physicochemical properties were obtained from SoilGrid 250 m 2.0 – ISRIC World Soil Information. The following properties were included Bulk Density (BD; cg cm^{3-}-1), Clay (g kg-1), Sand (g kg-1), Silt (g kg-1), Cation Exchange Capacity at pH 7 (CEC; mmol(c) kg-1), Total Nitrogen (N; dg kg-1), Soil Organic Carbon (SOC; dg kg-1), pH (water*10). The estimated organic available P (Pav) was estimated based on SOC and N content following a model proposed by Tian et al. (2010) who proposed a C:N:P ratio of 134:9:1 for organic-rich topsoil, we estimated P content in relation to C:P and N:P ratios and took as P value the average between them. Climate variables were obtained from TerraClimate (https://www.climatologylab.org/terraclimate.html), including maximum temperature, (T_{max};°C), Precipitation (ppt; mm), actual evapotranspiration (aet; mm), soil moisture (moisture; mm) and runoff (q; mm) (Table S1). Hereafter they are called environment variables. The collinearity analysis on the environmental variables set was performed with R-base (R core Team 2022), We included variables with $r \leq 0.5$ and meaningful to the study.

The functional annotation based on MG-RAST subsystems level 2 of the 376 selected metagenomes (Table S2) was obtained from the MG-RAST repository (Meyer et al. 2008).

The set of predicted proteins in each metagenome was obtained through the RESTful API of MG-RAST (Wilke et al., 2015). Protein sequences were downloaded using a matR version 0.9.1 package R (Braithwaite & Keegan, 2018).

The set of 376 samples showed imbalances because of the overrepresentation of the same sites, particularly from the northern hemisphere (much more studied) compared with the southern hemisphere. To minimize this bias, subsequent analyses were performed on a balanced reduced subset of 74 grasslands soil metagenomes. This subset included a maximum of three samples per MG-RAST project with the same geo-reference. In addition, soil metagenome data from two Uruguayan sites were generated for this study (Table S3). In the subset, we excluded the samples under high-impact treatments (e.g. fertilization, tillage, etc.). All analyses were performed on this reduced subset of 74 samples from 17 MG-RAST projects (Table S3).

Soil metagenomic sequencing from Uruguay (projects mgp91922 and mgp93346) was carried out on a HiSeq Illumina platform, (Service CD Genomics, NY; pair-end read 150 bp). Raw sequence quality was analysed with FastQC software version 0.11.2. Assembly and functional annotation were performed on the MG-RAST repository. Raw sequence data are publicly available on the MG-RAST repository. Functional annotation based on MG-RAST subsystems level 2 of the 376 selected metagenomes was obtained from the MG-RAST repository (Meyer et al. 2008). The set of

predicted proteins of each metagenome was obtained through the RESTful API of MG-RAST (Wilke et al., 2015). Protein sequences were downloaded using matR version 0.9.1 package R (Braithwaite & Keegan, 2018).

P-enzyme gene identification and phylogenetic analyses

The reference databases of the P-enzyme used in this work were built by Neal et al. (2017). The P-enzymes included are listed in Table 1. It is important to note that the use of any reference database introduces a certain bias in the search space.

Protein sequence alignments of the respective reference database were performed using MAFFT version 7.4.60 (Katoh et al., 2002) under default parameters. Reference protein phylograms were inferred with IQTree 2 version 1.6.12 (Minh et al., 2020) and the evolutionary models were evaluated with RAxML-NG (Kozlov et al., 2019). Phylograms were plotted with iTOL (Interactive Tree of Life; Letunic & Bork, 2007).

To determine the abundance and diversity of the P-enzymes in the metagenomes, we queried each metagenomic sample against each P-enzyme reference database. First, the whole predicted protein set of each metagenomic sample was queried against each P-enzyme reference database using HMMER version 3.3.1 (http://hmmer.org) keeping hits with an e-value below 1e-5. Then, these sequences were aligned to the correspondent reference database alignment using MAFFT—add sequence option and default parameters.

Maximum likelihood-based phylogenetic placement of metagenome-derived protein sequences on the appropriate P-enzyme reference phylogenetic tree was performed with EPA-ng (Barbera et al., 2019). Edge-PCA ordination and Kantorovich-Rubinstein (KR) distance metrics (Evans & Matsen, 2012; Matsen & Evans, 2013) were computed on these results. The edge-PCA and KR distances were performed using gappa (Czech et al., 2020), and tree and domain composition diagrams were drawn using Archaeopteryx (https://sites.google.com/site/cmzmasek/home/software/forester).

Statistical analyses

Canonical analysis of principal coordinates (CAP) (Anderson & Willis, 2003) implemented in Vegan R Package version 2.6.2 (Oksanen et al., 2019) was performed based upon Mahalanobis distance to calculate the relationship between the metagenomes functional profiles (subsystems level 2) and environmental variables. The significance of the model parameters was determined with permutational multivariate analysis of variance (PERMANOVA) with 999 permutations.

The protein/function count matrix (level 4 in the MG-RAST nomenclature), including the eight P-enzymes, for the 74 selected metagenomes was normalized with CPM and TMM methods using the edgeR package (Robinson et al., 2010). This data was used to perform the direct correlations of P-enzymes with environmental variables.

Canonical analysis of principal coordinates (CAP) (Anderson & Willis, 2003) implemented in the Vegan R Package version 2.6.2 (Oksanen et al., 2019) was used to evaluate the relationship between the abundance and diversity of P-specific functions with the environmental variables. CAP analysis associating P-enzyme abundance with environmental variables was performed using Mahalanobis distance. When appropriate, each P-enzyme abundance in each sample was normalized in relation to the sequencing coverage of each P-enzyme. The significance of the model parameters was determined with permutational multivariate analysis of variance (PERMANOVA) with 999 permutations.

The KR distance of each P-enzyme calculated as mentioned above was used to perform the distance-based CAP analyses between the abundance and diversity of each P-enzyme and environmental variables. The significance of the model parameters was

TABLE 1 List of P-enzymes included in the analyses.

P-enzyme	Gene	Predicted cellular localization	Number of protein sequences in the reference database
PhoA	*phoA*	Periplasmic/Cytoplasmic	293
PhoD	*phoD*	Outer membrane/extracellular	833
PhoX	*phoX*	Outer membrane/extracellular	424
Nsap class A (Nsap-A)	*phoC*	Periplasmic/Cytoplasmic	750
Nsap class B (Nsap-B)	*aphA*	Periplasmic/Cytoplasmic	388
Nsap class C (Nsap-C)	*olpA*	Outer membrane/extracellular	1123
β-propeller phytase (BPP)	*phyL*, *phyS*	Outer membrane/extracellular	108
Cysteine phytase (Cphy)	*phyA*	Outer membrane/extracellular	122

also determined with PERMANOVA based on 999 permutations. Graphics were produced with the R package ggplot2 (Wickham, 2016). All basic statistical procedures were performed using R-base (R core Team 2022). All taxonomy names cited are mentioned in italics and agree with Thines et al., 2020.

RESULTS

Metagenome functional profiles and environmental variables

First, we wanted to generate a general perspective of grassland functional landscapes and their relationship with environmental variables. To this aim, we performed a Canonical Analysis of Principal Coordinates (CAP) on a set of 74 grassland soil metagenomes (a reduced data set to correct for imbalances in the sample number per site, see methods). We used as input 168 functional processes (level 2 of the subsystems annotation from MG-RAST, Table S4) and their corresponding environmental variables (Table S3). The constrained model was significant ($p = 0.001$) and explained 24.8% of the total variance observed in the data set. Significant associations ($r > |0.20|$, $p < 0.01$) between the distribution of metagenomes and nine environmental variables were identified. CAP1 axis was correlated with pH ($r = -0.743$), bulk density (BD, $r = -0.521$), soil organic carbon (SOC, $r = 0.564$) and soil moisture ($r = 0.536$). This axis separated samples from low pH soils with average values of 5.65 (e.g. mgp9904, mgp5588, mgp91922 and mgp93346) from those with neutral pH (mgp13948 among others). CAP2 axis was mainly associated with pH ($r = -0.486$), SOC ($r = -0.220$), T_{max} ($r = 0.664$), runoff (q, $r = 0.490$), soil moisture ($r = 0.476$) and Clay ($r = 0.231$). Extreme values of the CAP2 axis corresponded to mgp10450 and mgp10451 (both from Brazil) which were associated with the highest T_{max} (26°C), soil moisture (115.5 mm) and precipitation (ppt) (129 mm) values of the set (Table 3A and Figure S2). To validate the subsampling (74 vs. 376 sample set), we performed CAP analysis in the larger set and examined the correlation between the axes of both analyses. We observed a high positive correlation between the correspondent first and second axes (correlation values >0.60).

Analyses on the abundance of P-enzymes coding genes

We interrogated the predicted protein set of each metagenome against the reference database of PhoD, PhoX, PhoA, Nsap-A, Nsap-B, Nsap-C, BPP and CPhy enzymes to obtain the abundance and phylogenetic distribution of P-enzyme coding genes. Inferred protein relative abundance in each soil metagenome is shown in Table S7 and phylogenetic placements are in Figures 1 and S3.

The alkaline phosphatase genes were the most abundant in the dataset, eight times higher than the acid phosphatase genes and 58 times higher than the phytase genes, independently of the soil properties (Table 2). We also observed differences in the abundance and phylogenetic distribution within each group of P-enzyme genes. The alkaline phosphatase gene *phoD* showed an abundance of five times higher compared with *phoX* and 20 times higher compared with *phoA*. Both genes, *phoD*, and *phoX* had broad phylogenetic distributions and no clear dominant phylotypes (Figures 1 and S3), contrary to the limited phylogenetic distribution observed in *phoA* (Figure S3 and Table S5).

Genes encoding Nsap-A and Nsap-C were the most abundant of the acid phosphatases, with similar abundances (Nsap-C coding gene was 1.3 times higher than Nsap-A one) (Table 2, Table S5), but a different distribution in their corresponding phylogenetic trees. Whilst Nsap-A coding gene showed a broad distribution within its phylogeny (Figure 1c), Nsap-C one was concentrated in the main branches of *Gammaproteobacteria*, *Flavobacteria* and *Sphingobacteria* classes (Figure S3). On the other side, Nsap-B had a low abundance and only *Gammaproteobacteria* variants were found (Figure S3 and Table S5).

BPP coding gene (*phyL* and *phyS*) was the most abundant of the phytases genes and presented a phylogenetic distribution mainly restricted to the *Proteobacteria* phylum (e.g. *Pseudomonas*, *Alteromonas* and *Acinetobacter*) (Figure 1d and Table 2). The CPhy coding gene (*phyA*), with lower abundance, was distributed within *Betaproteobacteria*, *Gammaproteobacteria* and some classes of the *Firmicutes* phylum (Figure S3 and Table 2).

First, we performed simple correlation analyses between normalized genes encoding P-enzyme abundance (by CPM and TMM methods, obtaining equivalent results) and environmental variables showed that *phoD*, *phoX* and *phyL* and *phyS* (BPP) coding gene had a significant correlation ($p < 0.001$) with pH, actual evapotranspiration (aet), precipitation (ppt), runoff (q) and soil moisture. In addition, we observed that *phoD* showed significant correlations ($p < 0.001$) with SOC and estimated organic available P (Pav). Nsap-C coding gene (*olpA*) showed a significant correlation with aet, *q*, ppt and moisture (Table S6). We then move forward to multivariate analyses.

We used CAP analysis to explore the relationship between P-enzyme coding genes normalized abundance and environmental variables (Figure S4). The constrained model based on Mahalanobis distance explained 36.4% of the variance within the data set

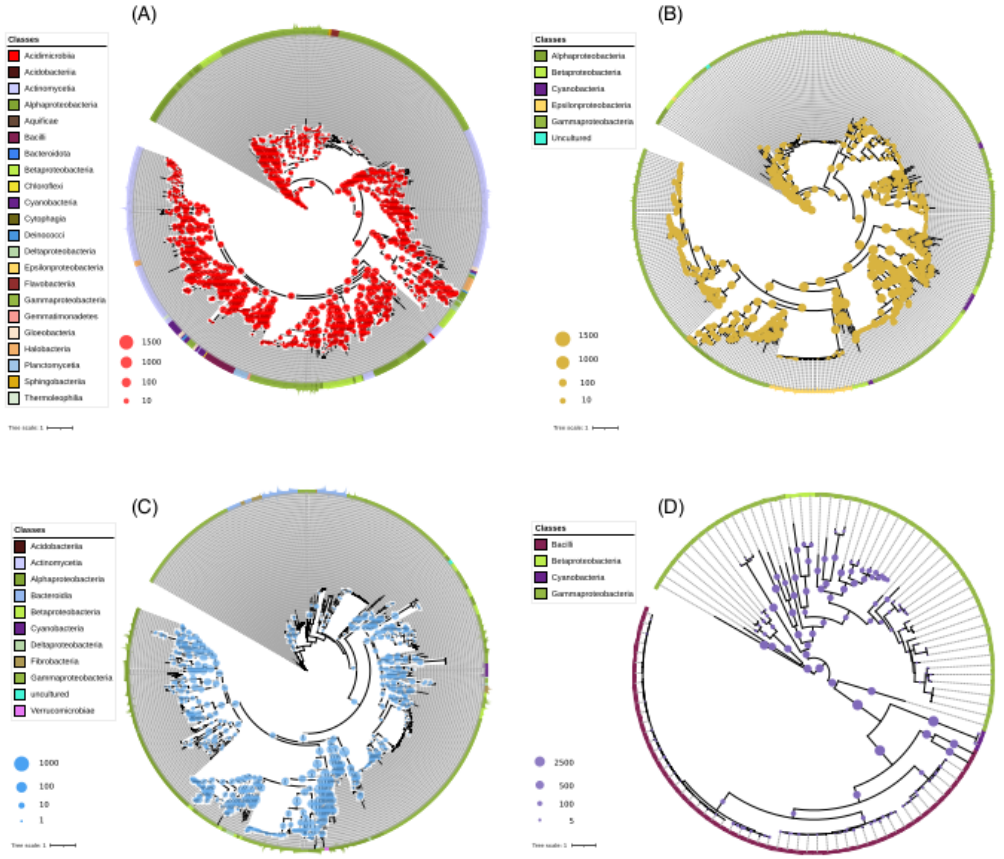

FIGURE 1 Phylogenetic placements of the predicted proteins of each metagenome with respect to the reference bases of each enzyme: (A) PhoD, (B) PhoX, (C) Nsap-A and (D) BPP. The size of the circle representing placements is proportional to the abundance. Maximum likelihood-based phylogenetic placement of metagenome-derived protein sequences was performed with EPA-ng and a tree was drawn with iTOL. The circle sizes represent the number of hits per node. The outer circle shows bacterial classes included in the reference trees.

TABLE 2 Median relative abundance of each P-enzyme.

P-enzyme	PhoD	PhoX	PhoA	Nsap-A	Nsap-B	Nsap-C	BPP	Cphy
Gene	*phoD*	*phoX*	*phoA*	*phoC*	*aphA*	*olpA*	*phyL, phyS*	*phyA*
Median relative abundance (No. of hits)	468.6	96.8	23.3	29.7	0.55	39.9	10.3	0

($p = 0.001$). We identified the alkaline phosphatase genes *phoD*, *phoX* and *phoA* were mainly responsible for the explained variance. CAP1 axis explained 8.9% of the variance ($p = 0.001$) and was associated with pH ($r = -0.70$), BD ($r = -0.48$), Sand ($r = -0.40$), ppt ($r = 0.74$), aet ($r = 0.67$), SOC ($r = 0.52$), Pav ($r = 0.52$) and Silt ($r = 0.49$). We observed that this axis separated samples from metagenomes of clay soils with low pH (5.0–6.8) and high SOC values (Androsols, Cambisols, Ferrasols, Fluvisols, Kastanozem/Luvisol, Luvisol/Kastanozem) from those of neutral or alkaline soils, having lower SOC contents (Chernozem, Luvisol and Kastanozem). CAP2 axis was associated with T_{max} ($r = -0.40$), BD ($r = -0.34$), pH ($r = -0.27$), ppt ($r = 0.41$), actual evapotranspiration (aet, $r = 0.39$) and runoff (q, $r = 0.39$). This axis

TABLE 3 CAP analyses based on Mahalanobis distance for MG-RAST Functional profiles annotations (level 2 Subsystem) and P-Enzyme relative abundance (A) and based on Kantorovich-Rubinstein distance for each of eight enzymes (B)

(A)

	Subsystems	Relative abundance P-enzymes		Subsystems	Relative abundance P-enzymes	
Variables	***p* value**	**CAP1 (*r*)**	**Variables**	***p* value**	**CAP1 (*r*)**	**Variables**
pH	0.001	−0.74	−0.49	0.001	−0.70	−0.27
SOC	0.001	0.56	−0.22	0.009	0.52	0.21
Pav	0.059	0.57	−0.29	0.004	0.52	0.12
CEC	0.057	0.15	−0.16	0.086	0.28	0.04
BD	0.001	−0.52	−0.12	0.001	−0.48	−0.34
Clay	0.001	−0.04	0.23	0.003	0.12	0.18
Sand	0.001	0.12	0.07	0.001	−0.4	−0.02
Silt	0.070	−0.14	−0.31	0.001	0.49	−0.12
aet	0.001	0.65	0.18	0.030	0.67	0.38
q	0.001	0.57	0.49	0.002	0.72	0.39
Mositure	0.002	0.54	0.48	0.079	0.82	0.12

(B)

Variables	Nsap—A			Nsap—B			Nsap—C			PhoA			PhoD			PhoX		
	p value	CAP1 (*r*)	CAP2 (*r*)	*p* value	CAP1 (*r*)	CAP2 (*r*)	*p* value	CAP1 (*r*)	CAP2 (*r*)	*p* value	CAP1 (*r*)	CAP2 (*r*)	*p* value	CAP1 (*r*)	CAP2 (*r*)	*p* value	CAP1 (*r*)	CAP2 (*r*)
pH	0.028	0.06	0.27	0.120	0.47	−0.28	0.012	0.14	0.12	0.074	−0.04	−0.29	0.001	−0.75	0.06	0.008	0.12	−0.08
SOC	0.045	−0.11	−0.18	0.523	−0.64	−0.19	0.016	0.02	−0.26	0.100	−0.18	0.12	0.001	0.22	−0.10	0.007	−0.05	−0.37
Pav	0.197	−0.18	−0.17	0.186	−0.60	−0.27	0.106	−0.02	−0.28	0.325	0.11	−0.35	0.057	0.18	−0.15	0.038	−0.08	0.00
CEC	0.001	−0.38	0.23	0.907	−0.43	−0.07	0.158	0.02	−0.01	0.015	0.05	0.45	0.001	−0.22	−0.17	0.041	−0.08	−0.25
BD	0.026	0.09	0.13	0.002	0.65	0.16	0.034	0.07	0.43	0.433	0.43	−0.05	0.001	−0.39	0.16	0.074	0.03	0.18
Clay	0.061	0.00	−0.35	0.649	−0.30	0.140	0.001	0.18	−0.69	0.070	−0.17	−0.040	0.002	0.16	−0.02	0.010	0.27	0.02
Sand	0.002	0.17	0.39	0.027	0.40	−0.05	0.033	−0.02	0.53	0.571	−0.35	0.38	0.001	−0.28	0.16	0.017	−0.09	−0.05
Silt	0.004	−0.34	−0.26	0.467	−0.34	−0.06	0.101	−0.16	−0.13	0.460	−0.02	0.39	0.001	0.27	−0.22	0.142	−0.10	0.06
aet	0.001	0.01	−0.43	0.848	−0.59	0.32	0.001	0.17	−0.21	0.015	0.11	0.46	0.001	0.78	0.01	0.001	0.28	−0.11
q	0.050	−0.01	−0.46	0.002	−0.80	0.27	0.023	0.00	−0.36	0.582	0.16	0.40	0.050	0.72	−0.07	0.055	0.00	−0.46
mositure	0.071	−0.27	−0.51	0.158	−0.68	0.19	0.044	−0.17	−0.45	0.580	−0.10	0.10	0.033	0.77	−0.20	0.085	−0.18	−0.12
ppt	0.082	0.00	−0.48	0.405	−0.75	0.32	0.009	0.09	−0.30	0.458	0.15	0.45	0.092	0.80	−0.03	0.146	0.16	−0.31
T_{max}	0.001	−0.24	−0.17	0.508	0.09	0.31	0.001	−0.50	−0.04	0.057	−0.18	−0.51	0.001	0.31	−0.41	0.001	−0.61	0.04
	p value	% variance		*p* value	% variance		*p* value	% variance		*p* value	% variance		*p* value	% variance		*p* value	% variance	
CAP model	0.001	33.70		0.050	32.60		0.001	35.70		0.001	13.00		0.001	49.80		0.001	41.40	
CAP 1 axis	0.001	11.54		0.001	11.60		0.001	13.90		0.030	8.70		0.001	19.80		0.001	19.00	
CAP 2 axis	0.01	4.20		0.890	4.10		0.009	4.70		0.570	3.90		0.001	12.90		0.05	4.70	

(Continues)

TABLE 3 (Continued)

(A)

Variables	Subsystems	Relative abundance P-enzymes		Variables	Subsystems		Relative abundance P-enzymes		Variables
	p value	CAP1 (*r*)			*p* value		CAP1 (*r*)		
Variables	BPP				Cphy				
	p value	CAP1 (*r*)		CAP2 (*r*)	*p* value		CAP1 (*r*)	CAP2 (*r*)	
pH	0.001	0.440		0.060	0.010		0.570	0.290	
SOC	0.066	−0.280		−0.480	0.193		−0.260	−0.180	
Pav	0.048	−0.330		−0.530	0.007		−0.340	−0.160	
CEC	0.007	−0.370		−0.620	0.010		−0.320	−0.420	
BD	0.001	0.270		0.360	0.122		0.360	0.100	
Clay	0.022	−0.090		0.050	0.526		0.190	−0.040	
Sand	0.001	0.280		0.260	0.222		−0.110	0.130	
Silt	0.004	−0.350		−0.470	0.302		0.030	−0.160	
aet	0.012	−0.310		−0.370	0.928		−0.490	−0.470	
q	0.01	−0.370		0.060	0.002		−0.330	−0.380	
Moisture	0.169	−0.620		−0.230	0.030		−0.680	−0.400	
ppt	0.164	−0.360		−0.190	0.420		−0.440	−0.460	
T_{max}	0.001	−0.290		0.770	0.508		0.150	0.000	
	p value	% variance			*p* value		% variance		
CAP model	0.001	47.000			0.030		49.500		
CAP 1 axis	0.001	23.000			0.010		10.700		
CAP 2 axis	0.001	5.000			0.100		7.6000		

Note: Environmental variables included: pH, Soil Organic Carbon (SOC), Estimated organic available P (Pav), Cation Exchange Capacity (CEC), Bulk Density (BD), Clay, Sand, silt, actual evapotranspiration (aet), runoff (q), soil moisture (moisture), precipitation (ppt) and maximum Temperature (T_{max}) (Table 3B). PERMANOVA analysis with 999 permutations was performed to determine the significance between the sites/MG-RAST project. The values marked with red are significant at $p < 0.01$.

separated soil metagenomes associated with lower aet values and relatively high T_{max}. All variables were significant with a $p < 0.001$ (Table 3A).

Analyses on abundance and phylogeny of P-enzyme coding genes

To gain deeper insight into the diversity and abundance of P-enzymes coding genes, we performed CAP analyses using Kantorovich–Rubinstein (KR-CAP) distance matrices between samples to include not only abundance but also phylogenetic information.

The *phoD* KR-CAP analysis explained 49.8% of the total variance in the data set ($p < 0.001$). Eleven out of 13 environmental variables were associated with the first two KR-CAP axes. The KR-CAP1 axis was negatively associated with pH ($r = -0.75$), BD ($r = -0.39$), Sand ($r = -0.28$), CEC ($r = -0.22$) and positively with ppt ($r = 0.80$), aet ($r = 0.79$), q ($r = 0.72$), T_{max} ($r = 0.313$), Silt ($r = 0.27$) and SOC ($r = 0.22$). This axis separated soils with low pH, relative high values of SOC and T_{max} (Cambisols, Ferrasols, Mollisols/Phazoem, Luvisol/Kastanozem) from soils with higher pH and lower T_{max}. The KR-CAP2 axis was characterized by a negative association with T_{max} ($r = -0.41$), soil moisture ($r = -0.29$) and Silt ($r = -0.22$). This axis separated soil with neutral pH and relatively high Silt and Sand values from the rest of the samples (Table 3B and Figure 2).

We performed the same analysis for the rest of the P-enzymes coding genes and the results are summarized in Table 3B (Figures S5–S7). Notably, pH, T_{max} and aet were associated with all P-enzyme coding gene distributions. SOC displayed a high correlation with alkaline phosphatases *phoD* and *phoX*, and acid phosphatases Nsap-A and Nsap-C coding genes, and estimated organic available P (Pav) was mainly

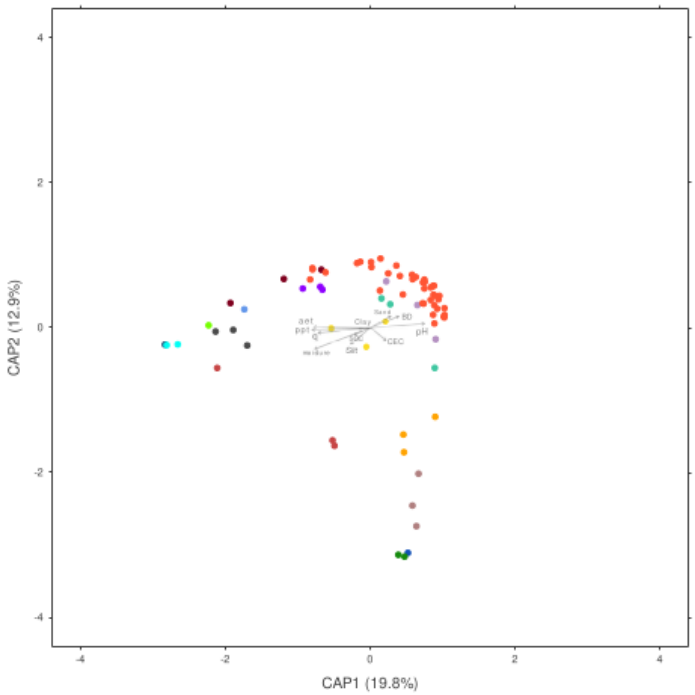

FIGURE 2 CAP based on Kantorovich-Rubinstein distance for *phoD*. PERMANOVA analysis with 999 permutations was performed to determine the significance between the sites/MG-RAST project. For each MG-RAST project, three samples with the same geo-reference were included. Each point represents samples from the project mpg1992 (blue); mpg3520 (green); mpg5588 (dark red); mpg7792 (grey); mpg8624 (mustard); mgp9904 (violet); mgp10450 (dark blue); mgp10523 (stone blue); mgp10541 (turquoise); mgp10956 (yellow); mgp13011 (lilac); mgp13520 (jade); mpg13948(orange); mpg20922 (brown); mgp89409 (brick-red); mgp91922. (light green); mgp93346 (light blue). Vector lengths represent the correlation between each variable and the axes. CAP analysis was performed with the Vegan R package and graphics were produced with the R package ggplot2.

associated with the phytases coding genes. Next, CEC showed a high correlation with alkaline phosphatases and phytase coding genes. Finally, clay content was related mainly to alkaline phosphatase coding genes (Table 3B).

Covariation of P-enzymes genes

To examine the co-variation between P-enzymes we compared their corresponding KR-CAP analyses results. We first limited the analysis to the P-enzymes genes present in at least 50 samples (all but Nsap-B and Cphy coding genes). All first KR-CAP axes showed a highly significant positive correlation between them (Figure 3 and Table S7). The second axes of this analysis showed a different behaviour, *phoA* KR-CAP2 showed no correlation with any other axis, *phoD* and *phoX* KR-CAP2 displayed a similar trend between them and with a rather idiosyncratic relationship with the rest of the genes. Finally, Nsap-A (*phoC*), Nsap-C (*olpA*) and BPP (*phyL* and *phyS*) coding genes displayed a

FIGURE 3 Correlation matrix of KR-CAP axes. (A) Correlogram of the alkaline phosphatases displays the Pearson correlation coefficients between KR-CAP PhoD axes, KR-CAP PhoX, and KR-CAP PhoA; KR-CAP PhoX and KR-CAP PhoA. The correlation coefficients are coloured according to their values; blue is the positive values and red is the negative values. (B) Correlogram of the acid phosphatases displays the Pearson correlation coefficients between KR-CAP Nsap-A axes, KR-CAP Nsap-B and KR-CAP Nsap-C; KR-CAP Nsap-B and KR-CAP Nsap-C. The correlation coefficients are coloured according to their values; blue is the positive values and red is the negative values. (C) Correlogram of the phytases displays the Pearson correlation coefficients between KR-CAP BPP axes and KR-CAP CPhy. The correlation coefficients are coloured according to their values; blue is the positive values and red is the negative values. (D) Correlogram of the most abundance displays the Pearson correlation coefficients. The correlation coefficients are coloured according to their values; blue is the positive values and red is the negative values. Correlation analysis and graphics were performed with the cor R package.

very similar response (Figure 3 and Table S7). No specific trend was observed according to the predicted cellular localization of the proteins.

Nsap-B and Cphy coding genes were present in fewer samples, so we compared them to the other non-specific acid phosphatase and phytase, respectively. For Nsap-B, the KR-CAP1 axis was significantly correlated to the KR-CAP2 axes from the other two Nsap (Figure S8 and Table S7). In addition, the CPhy KR-CAP2 axis was correlated to the BPP KR-CAP1 axis (Figure S8 and Table S7).

Finally, when comparing each P-enzyme CAP analysis with the subsystem level CAP analysis we observed that only *phoD* (CAP1-PhoD vs. CAP2-SS = 0.44), BPP (*phyL* and *phyS*) (CAP1-BPP vs. CAP1-SS = −0.41, CAP2-BPP vs. CAP2-SS = 0.55) and Cphy (*phyA*) (CAP1-Cphy vs. CAP1-SS = −0.59), displayed significant correlations between the axes (Table S8).

Edge-PCA and taxonomic identification of differentially observed P-enzymes coding genes

Edge-PCA analysis was applied to examine the variation in phylogenetic diversity of P-enzyme coding genes among the soil metagenomes; a summary of the results is shown in Table S9. It is important to note that the first and second edgePCA components were highly correlated with the first and second KR-CAP axes (except for the low abundance genes encoding *phoA* and Nsap-B *aphA*), this enables us to connect the environmental variables to specific lineages of each gene.

In the *phoD* analysis, the first edge-PCA axis separated samples by soil type, pH and SOC content. The differences showed that the gene variants of the species *Koribcater versatilis* (class *Acidobacteriia*) and *Rhodanobacter spathiphylli* (class *Gammaproteobacteria*) (Figure 4B) were more abundant in soils classified as Ferrasols, Cambisols, Molisols/Phaeozem and Vertisol/Phaeozem with low pH and relatively high SOC content (left quadrant of Figure 4A). On the other hand, variants associated with *Actinomyces*, *Bacillus* and *Planctomyces* (Figure 4B) were more abundant in Kastanozem, Chernozem, Luvisol and Fluvisols soils with higher pH (ranged to 7.5) and lower SOC content (right quadrant of Figure 4A). The second axis was associated with *phoD* coding genes harboured by *Burkholderiales* and *Acinetobacter* with higher abundance in soils with neutral pH and low clay content (Tables S1 and S9 and Figure 4A).

The alkaline phosphatases *phoX* and *phoA* showed a narrower phylogenetic distribution and *Alphaproteobacteria* (*Rosevivax* and *Agrobacterium* among others) genes were predominant in soils with high SOC values and relatively high T_{max} (23°C) (Table S1 and Table S9). The *Burkholderiales* variants were observed in soil samples with near-neutral pH and average SOC and CEC values (Figure S9). The genes *phoA* of *Pantoea* and *Providencia* together with *Acinetobacter* and *Actinobacter* genera were associated with varying abundance between samples (Figure S9). Again, *Acinetobacter* was differential and more abundant in soils with *circum*-neutral pH and average SOC and CEC values (Figure S9).

We identified the acid phosphatases Nsap-A coding genes harboured by *Pedosphaera*, *Dyella jiangningensis* and *Dyella japonica* as the differentials and the most abundant among soils with average SOC and CEC values and sandy texture (Figure S9). On the other hand, *Sphingomonas* sp., *Phenylobacterium* sp.,

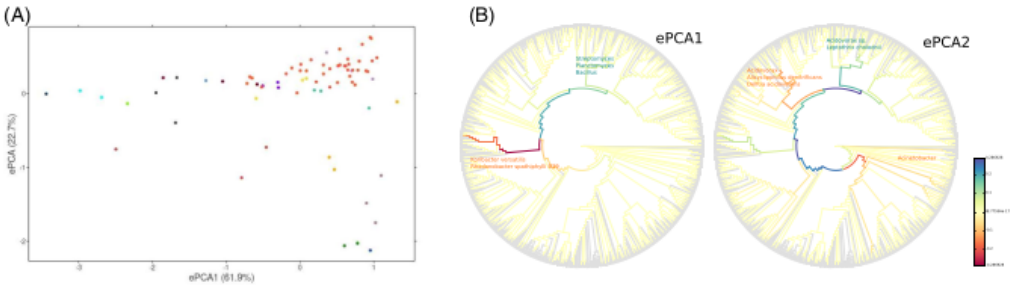

FIGURE 4 (A) Graphic representation of the first two axes of the edge-PCA for *phoD* using samples as observations. Each point represents samples from the project mpg1992 (blue); mpg3520 (green); mpg5588 (dark red); mpg7792 (grey); mpg8624 (mustard); mgp9904 (violet); mgp10450 (dark blue); mgp10523 (stone blue); mgp10541 (turquoise); mgp10956 (yellow); mgp13011 (lilac); mgp13520 (jade); mpg13948 (orange); mpg20922 (brown); mgp89409 (brick-red); mgp91922 (light green); mgp93346 (light blue). (B) The phylogeny distribution of *phoD* hits along the first and second axis of the analysis (proteins with positive coefficients are marked in blue and proteins with negative coefficients are marked in orange). The edge-PCA was performed using gappa software and tree and domain composition diagrams were drawn using Archaeopteryx (https://sites.google.com/site/cmzmasek/home/software/forester).

Rhodanobacter sp. and *Caulobacter* species variants were more abundant in soils with high clay content (Figure S9).

The Nsap-C coding genes harbouring by *Stenotrophomonas* (*Gammaproteobacteria*) and *Pedobacter* genera were differential and the most abundant in soils with low values of soil moisture and actual evapotranspiration (aet). *Enterobacter* Nsap-B coding genes variants predominated in soils classified as Ferralsols, Andosols and Luvisol with acidic pH (range to 5), and high aet and precipitation (ppt) values. Metagenomes from Fluvisol, with a pH = 7, were associated with P-enzymes gene encoding variants of *Photobacter* and *Marinomonas* and were strikingly different from the rest (Figure S9).

We only found the BPP phytase coding genes in soil samples with pH values above 6.6. BPP coding gene variants of the *Acinetobacter*, *Pseudomonas*, *Methylophaga*, *Pseudoalteromonas* and *Alteromonadales* (*Gammaproteobacteria*), and *Shewanella* and *Hylemonella* (*Betaproteobacteria*), dominate in clay soils with high CEC values. BPP genes harboured by *Bacillus* species were most abundant in sandy soils with low nutrient content.

CPhy coding genes from *Beta* and *Deltaproteobacteria*, *Clostridia* and several genera of *Negativicutes* classes varied across the samples but there was no clear signal to reveal associations with environmental variables.

DISCUSSION

Soil ecosystems include complex interrelations among different factors including soil types, plant communities, microbial communities (bacteria, fungi, archaea, viruses and protozoa), macro and micro fauna, environmental variables, etc. (Islam et al., 2020). The present work focused on the Bacterial fraction of the soil microbial community from grassland biomes, in particular the abundance and phylogenetic diversity of P-enzyme coding genes from the grassland biomes, using a metagenomic approach. The analysed samples represent different environmental conditions defined by the physical and chemical soil properties, and climate variables (Amundson, 2013; Islam et al., 2020). We included publicly available data from MG-RAST and other sources for each project/sample. Other interesting data, such as the composition of the plant community or short-term/long-term experiments, was not included, which can constitute an interesting input for the analyses and discussion of our study.

Microbial P enzymes, such as phosphatases (Nannipieri et al., 2011; Rodriguez et al., 2006) and phytases (Tan et al., 2013; Yao et al., 2012), play a crucial role in the phosphorus cycle by participating in the release of Pi from organophosphorus compounds, the last step of the P cycle (Zeng et al., 2022). One valuable result of this study is that it confirms and expands the idea of the large variability in abundance and diversity of P-enzymes coding genes within grassland ecosystems across the planet.

Our analyses showed that the alkaline phosphatases were the most abundant P-enzymes genes in the whole dataset, being the *phoD* gene the most abundant and also with the widest phylogenetic distribution, regardless of the soil properties. This result is in accordance with reports by previous, but more restricted, metagenomic studies where this gene was the most frequently alkaline phosphatase found in different soils (Bergkemper et al., 2016; Park et al., 2022; Tan et al., 2013). The *phoA* was less abundant, and the difference with *phoD* or *phoX* can result from the differences in substrate specificity and co-factor requirements between them. Bacterial cells may possess either *phoX* or *phoA* or both. They are presumed to have similar roles in facilitating access to a diverse array of phosphoester compounds and are more active against organic phosphates and nucleotides. Nonetheless, they may function at varying levels of substrate concentrations (Sebastian & Ammerman, 2011). A study about PhoA activity in marine ecosystems showed that this enzyme has an activity for mono-, di- and triesterase activity (Srivastava et al., 2021). PhoX is essential for utilizing monophosphate esters at low substrate concentrations in *Rhizobium pomeroyi* (Sebastian & Ammerman, 2011). The substrate specificity of PhoD is unknown. Some work has reported phosphodiesterase activity against cell wall teichoic acids and phospholipids (Bergkemper et al., 2016; Rodriguez et al., 2014). However, the contribution to the Pho-regulated phosphatase activity of *Pseudomonas fluorescens* does not seem to be significant (Monds et al., 2006). A new alkaline phosphatase, PafA, has recently been described in plant-associated Bacteroidetes (Lidbury et al., 2021). Unlike PhoD, PhoX and PhoA, this enzyme exhibits constitutive phosphatase activity and is fully functional in the presence of high phosphate concentrations with high monophosphatase activity. PafA plays a critical role in global biogeochemical cycles and has potential applications in sustainable agriculture (Lidbury et al., 2021).

On the other extreme, genes encoding Nspa-B and Cphy were scarce in the whole dataset. These genes tend to show weaker associations with the environmental variables and other P-enzyme coding genes. This could be due to the low numbers in which these genes appear or to genuine biological reasons.

Environmental variables and P-enzyme coding genes abundance and diversity

We showed that several environmental variables are related to the diversity and abundance of P-enzyme coding genes. T_{max}, pH, SOC and soil moisture are

associated with alkaline phosphatase gene abundance. The *phoD* and *phoX* genes showed a high correlation with SOC and clay. Several recent studies report the effect of SOC, N and organic P content on the abundance and diversity of both enzymes and the corresponding bacteria (Li et al., 2021; Ragot et al., 2017; Wei et al., 2021). A local-scale study of three land uses with differential SOC (fallow, arable, grassland) demonstrated there was a positive correlation between alkaline phosphatases gene abundance and soil organic matter contents (Neal et al., 2017). In addition, the predicted extracellular location of both enzymes (Neal et al., 2017) may explain the importance of clay content in relation to its stabilization role, immobilization and maintenance of the enzymatic activity (Margalef et al., 2017). The *phoD* genes are widely distributed among different classes of Bacteria, in this study we found that variants associated with *Koribacter* (*Acidobacteria* class) and *Rhodanobacter* genus (*Gammaproteobacteria* class) were more abundant in soils with relatively high SOC values and low pH. These variants have been identified as a dominant phylotype in arable silty clay loam soil Chromic Luvisol in the United Kingdom (Neal et al., 2017). The second one also has been identified as a dominant phylotype in the rhizospheres of maize and sorghum in a Brazilian Distroferric Red Latosol (Neal et al., 2021). Both bacterial species represent classes that possess a comprehensive set of genes that allow them to use a wide variety of substrates, responding efficiently to environmental changes and conferring their ability to adapt to various ecological niches (Kalam et al., 2020; Kurm et al., 2017). Variants associated with *Bacillus*, *Actinomyces* and *Planctomyces* were prevalent in soils with lower SOC and neutral pH. The last two species have been found dominant in soils with low nutrient content, even the *Planctomyces* showed a negative correlation with this variable (Garaycochea et al., 2020; Hermans et al., 2017; Lewin et al., 2017). Nevertheless, the main driver that explains the difference in species abundances appears to be pH, since all reported species are heterotrophs (Kielak et al., 2016; Saxena et al., 2020). The *phoX* gene represented by the *Burkholderia* genus was preferred in soils with low and medium content of SOC and neutral pH. Bacteria from this genus present a wide repertoire of metabolic pathways making them more competitive in nutrient-restrictive environments, since they are capable to degrade recalcitrant compounds, and unlike most Bacteria, *Burkholderia* species are more competitive in low and moderate pH conditions (Morya et al., 2020; Stopnisek et al., 2014).

Regarding acid phosphatases, the Nsap-A coding genes were found in *Dyella* and *Rhodanobacter* genera. These species use different carbon sources and have been reported to be dominant in acid and neutral soils (Dahal & Kim, 2017; Weon et al., 2009). On the other hand, the Nsap-C coding gene was identified in *Alpha* and *Gammaproteobacteria*, *Flavobacteria* and *Sphingobacteria* classes, consistent with previous evidence (Gaiero et al., 2020; Neal et al., 2017). The proportion of both non-specific acid phosphatases found in the grassland set studied here was similar to that reported for UK grassland soils (Neal et al., 2017). The predominance of acid phosphatases in grassland could be influenced by the interaction between microorganisms and plant communities, as both are capable of producing these enzymes (Mhlongo et al., 2018). The observed proportion of Nsap-B is similar to that reported by Udaondo et al. (2020), who not only found that this enzyme was less abundant in different niches but also that it was restricted to a limited number of microbial families, some of which were pathogens.

In the cases of phytases, BPP coding genes showed an abundance and phylogenetic distribution in accordance with what has been reported. The BPP coding genes are widespread and are distributed among various species of soil bacteria (Huang et al., 2009; Jorquera et al., 2008; Kumar et al., 2017; Lim et al., 2007). However, some studies have observed that the presence of BPP coding genes is rare in *Betaproteobacteria* (Cotta et al., 2016), we found that the BPP coding genes variants were mainly from *Bacillales* and *Beta* and *Gammaproteobacteria*. The BPP coding genes in this study were found restricted to soil with pH above 6.6, which is in accordance with what was reported, particularly in several strains from the *Bacillus* genus, where the BPPs enzymes are optimally active at pH 6.0–7.5 (Cheng & Lim, 2006; Farhat et al., 2008; Huang et al., 2009; Kerovuo et al., 1998; Kumar et al., 2017). On the other hand, the Cphy coding gene was the least abundant enzyme in the grasslands metagenomes, contrary to those found by Neal et al., 2017 where CPhy tended to have a similar abundance that BPP in the studied grasslands from the UK.

The pH appears as an important factor associated with both acid and alkaline phosphatases, as well as phytases, abundance and diversity. Even though, our results show a global trend of an increase in the genes encoding these enzymes (PhoD, PhoX, Nspa-C and BPP) with pH, all enzymes are relatively abundant in the pH range covered in this study, rendering it difficult to test a direct association between the enzyme classification (as acid or alkaline) and the soil pH.

It is important to bear in mind that the taxonomic associations of each gene sequence are dependent on the database, is clear that including different sequences of more taxa might result in the discovery of new variants and/or better assignments of the sequences. Nevertheless, many of the results here obtained will still hold being enriched with the new putative ones.

Co-variation of P-enzyme coding genes

We have shown a strong relationship in the abundance and diversity patterns between the different P-enzymes herein studied. Indeed, KR-CAP analyses show strong correlations between them, somehow weaker in the less abundant genes. These results uncover a somehow intuitive result. We should bear in mind that we are counting the aggregate of each gene in a whole community, thus, variation in abundance and diversity of a given gene is the product of a change at the community level. So the process of selection in the assemblage of each community is a balance between how each organism crafted its genome and the interaction between them and the environment. The high correlation between KR-CAP analyses, which involve abundance, diversity and environmental variables, suggests a tight relationship between the P-enzyme genes. This implies that for each environmental condition, the way each P-enzyme gene contributes to phosphorus cycling and metabolism is connected to the rest of them (in both abundance and diversity). The different taxa that appear associated with each P-enzyme coding gene in the edgePCA analysis (Table S9) are indicative that different organisms are contributing to the P-enzyme gene pool.

Another interesting result was the association of P-enzymes with the general functional profiles (Table S8). Here, the results are somehow at odds with the previous one. The first axes of PhoD, PhoX, PhoA and BPP coding genes were strongly correlated with the CAP2 of the functional profiles. Nevertheless, Cphy and Nsap-X genes, showed no correlation, suggesting that there could be some variability in this respect.

One important question is to understand if the P-enzymes are driven particularly by the change of certain organisms that are carrying them or, in turn, they are following the general major changes in the community structure. One possible hint in this direction is given by the previous comparison, indicating that these P-enzymes genes may be accompanying the general change in the functional structure of the metagenome, whilst there is room for a more idiosyncratic manner. Nevertheless, more studies should be carried out to gain deeper insight into this interesting and complex question.

Concluding remarks

The environmental variables explained a relatively low proportion of the variability in bacterial functional profiles. The use of information from samples from very distant sites determines only the effect on the diversity of the variables with greater differences among the sites. However, T_{max}, soil pH and evapotranspiration were related to the abundance and diversity of almost the eight key enzymes involved in P organic cycling. Likewise, it was possible to identify the effect of other variables with a more localized effect, such as soil texture and soil organic content, as important determinants of microbial community structure and functions. The complexity of the studied system requires a combination of approaches and the generation of local data that allow the understanding of factors affecting the presence of bacteria carrying P-enzymes genes as well as their functionality and to integrate these results into a broader scale to detect global patterns of diversity that could potentially lead to better understanding and management of soil P cycling.

AUTHOR CONTRIBUTIONS

Silvia Garaycochea: Conceptualization (equal); data curation (equal); formal analysis (equal); funding acquisition (supporting); investigation (equal); methodology (equal); resources (equal); writing – original draft (equal); writing – review and editing (equal). **Nora Adriana Altier:** Conceptualization (supporting); funding acquisition (lead); project administration (lead); writing – original draft (supporting). **Carolina Leoni:** Formal analysis (supporting); methodology (supporting); writing – original draft (supporting). **Andrew L. Neal:** Conceptualization (supporting); methodology (supporting); writing – original draft (supporting). **Héctor Romero:** Conceptualization (equal); data curation (equal); formal analysis (equal), methodology (supporting), writing – original draft (equal), writing – review and editing (equal).

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

All data used in this work are publicly available. Additional data, additional results and scripts are available at https://github.com/eletor-uy/Grasslands.

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5.1. DISCUSIÓN GENERAL

En el ámbito mundial, los ecosistemas de pastizales cumplen un importante rol en la conservación de la biodiversidad y proporcionan servicios ecosistémicos tales como el almacenamiento de carbono, la regulación del agua, el control de la erosión del suelo y el ciclo de los nutrientes (Blair et al., 2014, Le Roux et al., 2011). El bioma Campos, al que pertenecen los ecosistemas de pastizales de Uruguay, se desarrolla sobre una gran diversidad de tipos de suelos que varían según las condiciones geográficas y climáticas de la región. En biomas como este, donde la intervención humana es baja, el ciclo de la materia orgánica, la disponibilidad de nutrientes y la formación de agregados son resultados directos de la actividad microbiana (Vargas et al., 2015). No obstante, el uso del suelo en el bioma Campos ha experimentado cambios significativos en las últimas décadas, principalmente debido a la agricultura y la ganadería. En algunas áreas, se ha llevado a cabo la conversión de pastizales naturales en tierras agrícolas y pastizales cultivados, lo que ha modificado la estructura y composición de la vegetación, así como la dinámica de los suelos.

Las comunidades microbianas del suelo son clave en el ciclado de nutrientes como el nitrógeno (N), el fósforo (P) y el carbono (C), que son transformados en formas disponibles para las plantas. De esta manera, las comunidades microbianas contribuyen a mantener el equilibrio de nutrientes en el suelo y se asegura su disponibilidad para la vegetación. Es el caso de la vegetación herbácea de pastizales del bioma Campos, soporte de la producción ganadera de nuestro país, que generalmente se encuentra sobre suelos con bajos niveles de nutrientes.

En este trabajo, se llevó a cabo la caracterización de la diversidad estructural de las comunidades procariotas a través de la secuenciación masiva del gen 16S rRNA y la diversidad funcional mediante la inferencia de las funciones vinculadas al ciclado del P. Se analizaron suelos formados sobre materiales madre contrastantes, teniendo en cuenta el estado de los nutrientes, específicamente la forma de retención

del P y la relación P inorgánico/P orgánico. Basados en los criterios de selección mencionados, se estudiaron cinco unidades de suelo representativas de las regiones de Basalto (Itapebí Tres Árboles-ITA), Litoral (Young-YNG), Cristalino (Sierra de Polanco-SPO), Sur (Tala Rodríguez-TRO) y Noreste (Tacuarembó-TBO). Es importante tener en cuenta que los suelos analizados en la unidad de YNG, una de las zonas más fértiles del país, han sido históricamente utilizados para la agricultura. Por lo tanto, aunque las muestras no fueron tomadas directamente de campos agrícolas, es posible que presenten alteraciones en sus propiedades químicas debido a diversas prácticas agrícolas, entre otras la deriva de la fertilización. Por otro lado, los suelos de la unidad TBO, desarrollados sobre areniscas, representan una zona geográfica reducida del país.

Posteriormente, analizamos la abundancia y diversidad filogenética de los genes codificantes de ocho enzimas claves del ciclo del P del bioma pastizales, a través de un enfoque metagenómico. Para ello, se estudió el microbioma de 17 pastizales distribuidos en distintas partes del mundo, incluyendo dos ubicadas en el bioma Campos de Uruguay (ITA y SPO). Se seleccionaron pastizales que representaban diferentes condiciones ambientales definidas por las propiedades físicas y químicas del suelo, así como por las variables climáticas presentes en cada zona.

5.1.1 Diversidad taxonómica de las comunidades procariotas y su relación con las propiedades fisico-químicas de los suelos del bioma Campos

El P es un elemento esencial para el crecimiento de las plantas y desempeña un papel crucial en la dinámica de los suelos. Los pastizales uruguayos del bioma Campos se caracterizan por una alta diversidad de tipos de suelo, baja disponibilidad de P y limitada capacidad de retención de agua (CRA) (Allen et al., 2011). Los bajos niveles de P inorgánico disuelto encontrados en los suelos (típicamente < 10 mg kg^{-1}) resultan de la alta reactividad del ion ortofosfato ($PO4^{-3}$) con calcio (Ca) en suelos alcalinos, y hierro (Fe) y aluminio (Al) en suelos ácidos (Gyaneshwar et al., 2002). La fracción de P orgánico no está disponible para las plantas y, en ambos casos, se requieren enzimas para liberar el ion ortofosfato para su utilización por las plantas. El P orgánico representa una gran parte del P total (50-75 %) (Hernández et al., 1995). Los suelos uruguayos están particularmente bien descritos: su evolución y propiedades fisico-químicas muestran fuertes asociaciones con el material parental subyacente (Durán et al., 1999). Sin embargo, poco se sabe acerca de las comunidades microbianas residentes en los suelos del bioma Campos y cómo estas comunidades se ven influenciadas por los diferentes tipos de suelo, la disponibilidad de nutrientes y el clima.

En el capítulo dos de esta tesis, se propuso la hipótesis de que las propiedades físicas y químicas de las cinco unidades de suelo seleccionadas para nuestro estudio darían lugar a diferentes comunidades procariotas. Para ello, llevamos a cabo la caracterización de los suelos seleccionados, considerándolos representativos de este bioma, y confirmamos la presencia de diferencias significativas en las propiedades físicas (contenido de arcilla, porosidad, densidad aparente y capacidad de retención de agua) y químicas (Ca, P disponible, N total, C orgánico, capacidad de intercambio catiónico), tal como asumimos al establecer los criterios de muestreo.

El análisis de la diversidad alfa y beta de las comunidades procariotas de estas cinco unidades de suelo también mostró diferencias significativas. Los índices de

diversidad alfa, índices diversidad filogenética de Faith (Faith's PD), Shannon (H´) y Chao1 (SCaho1) indicaron diferencias en la composición de las comunidades. Los suelos de ITA tuvieron los valores más altos de H' y los de SPO, los más bajos de las cinco unidades; sin embargo, los valores de PD fueron semejantes. Esto sugiere que la comunidad procariota de SPO está formada por taxones más divergentes.

Al realizar el análisis de la diversidad beta, obtuvimos resultados que concuerdan con lo que habíamos observado anteriormente; se evidenció una clara separación de las comunidades procariotas según la unidad de suelo, lo que indica que la unidad de suelo tuvo un efecto significativo en la composición de los conjuntos de OTU/taxones. El análisis edge-PCA (Matsen y Evans, 2013) nos permitió identificar los filos que diferenciaban estas comunidades. En nuestro estudio se encontró que las comunidades procariotas presentes en los suelos analizados estaban compuestas principalmente por OTU clasificados como *Archaea* y los filos bacterianos *Firmicutes, Acidobacteria, Actinobacteria y Verrucomicrobia,* aunque con diferentes abundancias en cada suelo. Se observó una gran similitud en las comunidades procariotas de las unidades de suelo YNG e ITA, ya que ambas estuvieron dominadas por los filos *Verrucomicrobia y Actinobacteria.* Sin embargo, la comunidad ITA es filogenéticamente más diversa, como indica el mayor valor de PD. Este análisis indicó que los filos *Firmicutes* y *Acidobacterias* difieren en su abundancia relativa entre estos dos sitios. Los *Firmicutes*, representados principalmente por el género *Bacillus*, registraron una mayor abundancia relativa en los suelos de la unidad YNG, lo que puede estar relacionado al mayor contenido de carbono orgánico en suelo (SOC) y P disponible (APC) respecto a los suelos de la unidad ITA. Por el contrario, las *Acidobacterias* fueron más abundantes en las muestras de suelo de ITA, siendo las familias *Koribacteraceae* y *Solibacteraceae* características de esta comunidad procariota. Las diferencias observadas en la abundancia relativa de los filos *Firmicutes* y *Acidobacteria* en ambos suelos podría estar asociada con la sensibilidad de estos filos a los cambios en el contenido de nutrientes (Karimi et al., 2018, Hermans et al., 2017). Randall et al. (2019) evaluaron

el efecto de distintos manejos de pastoreo y fertilización en la comunidad microbiana de suelos de dos pastizales. Mostraron que en ambos pastizales predominaba el filo *Acidobacteria*, aunque se observaron variaciones en la abundancia de ciertos géneros de este filo en cada suelo, lo que sugiere una posible adaptación de los *Acidobacteria* a las condiciones específicas de cada pastizal. Las diferencias en la comunidad procariota de la unidad de suelo YNG pueden deberse al manejo agrícola de la zona donde se tomaron las muestras. Diversos estudios han evidenciado que las prácticas agrícolas tienen un impacto significativo en la composición y función de las comunidades procariotas del suelo, y en su relación con los parámetros químicos de este. Estas prácticas pueden alterar la estructura y función del suelo, lo que a su vez puede influir en la salud de los cultivos y en la calidad del suelo a largo plazo (Cerecetto et al., 2021, Lee et al., 2020).

En los suelos de SPO se encontró una alta abundancia relativa de *Planctomycetes* y una baja abundancia relativa de *Firmicutes* y *Actinobacterias*, lo que podría estar relacionado con la limitada disponibilidad de nutrientes y la baja capacidad de retención de agua en los suelos de basamento cristalino. Estudios previos han demostrado que el filo *Planctomycetes* tiene asociaciones negativas con el contenido de nutrientes del suelo (Hermans et al., 2017, Lauber et al., 2008), mientras que la baja abundancia relativa del filo *Actinobacteria* podría estar vinculada a la baja disponibilidad de nutrientes y agua (Kopecky et al., 2011).

Para establecer las asociaciones entre las propiedades fisico-químicas y las comunidades procariotas observadas, se realizó un análisis CAP (*Canonical Analysis of Principal Coordinates*). Mediante este análisis fue posible identificar las variables CRA, contenido de arcilla, porosidad y el estado nutricional (P disponible, N total y C orgánico) fuertemente asociadas con la diversidad estructural observada en las cinco comunidades procariotas. Nuestros resultados fueron consistentes con lo reportado por trabajos previos (Delgado-Baquerizo et al., 2018, Karimi et al., 2018, Brockett et al., 2012). Además, observamos que la composición de la comunidad del suelo de TBO estuvo fuertemente asociada a valores de contenido de arcilla (CC) y C

orgánico bajos. La comunidad procariota de este suelo estuvo dominada por *Verrucomicrobia* y *Archaea*. Las propiedades del suelo de textura arenosa de la unidad TBO, que presentan una baja cantidad de nutrientes, pueden favorecer la proliferación de taxones con capacidad de adaptarse a condiciones restrictivas de crecimiento. Un ejemplo de estos taxones son los *Verrucomicrobia*, quienes poseen un metabolismo flexible (Balmonte et al., 2016). En la comunidad procariota de ITA se observó una alta abundancia relativa del filo *Actinobacteria*, el cual es comúnmente encontrado en suelos y conocido por su crecimiento lento. Se ha reportado que organismos representativos de este filo poseen una amplia variedad de genes asociados con diferentes rutas metabólicas de carbohidratos y polisacáridos (Kielak et al., 2016), lo que sugiere que el filo desempeña un papel importante en la renovación del carbono orgánico en los suelos. Varios estudios han demostrado que tanto el pH como la disponibilidad de nutrientes son factores que influyen en la abundancia de *Acidobacteria* en los suelos (Ivanova et al., 2020, Randall et al., 2019, Eichorst et al., 2018, Kielak et al., 2016, Ward et al., 2009). Aunque se observó poca variabilidad en el pH de los suelos del bioma Campos estudiados en este trabajo, sí se encontraron diferencias significativas en el contenido de nutrientes. Estas diferencias podrían explicar las variaciones observadas en la abundancia relativa de *Acidobacterias*.

Posteriormente, se realizó un segundo análisis CAP, pero esta vez excluyendo la información correspondiente a la unidad de suelo YNG, de forma de evaluar el efecto de las propiedades fisico-químicas del suelo sin la posible interferencia generada por el manejo agrícola cercano, como se mencionó anteriormente. Los resultados de este análisis concuerdan con los del anterior, donde se encontró que CRA, porosidad y contenido de nutrientes (P disponible, C orgánico y CIC) presentaron las mayores correlaciones. La mayoría de estas variables coincidieron con los resultados previos, incluyendo el suelo YNG, que mostró una tendencia similar de correlación. Sin embargo, este análisis reveló una mayor separación de las comunidades procariotas según el tipo de suelo. En particular, se observó una

diferencia significativa entre las comunidades del suelo TBO, desarrollado sobre areniscas, en comparación con las de los otros tres suelos.

En resumen, este trabajo reportó los efectos del tipo de suelo y sus propiedades fisico-químicas sobre las el ensamblado de las comunidades procariotas del suelo en ecosistemas naturales bajo condiciones climáticas y usos del suelo similares (Fierer y Jackson, 2006, Fierer, 2017). Nuestros datos sugieren que la estructura del suelo (contenido de arcilla, porosidad), el estado nutricional (P disponible, C orgánico y CIC) y la capacidad de retención de agua (CRA) modulan significativamente los conjuntos de comunidades procariotas de los pastizales del bioma Campos. El resultado de los análisis realizados con o sin las muestras de suelo de YNG permitió evidenciar el impacto de las prácticas agrícolas sobre las comunidades procariotas. Los filos bacterianos *Actinobacteria*, *Chloroflexi*, *Proteobacteria* y *Verrucomicrobia* presentaron los mayores cambios en su abundancia en relación con los diferentes tipos de suelo analizados. En particular, la eliminación del suelo agrícola (YNG) permitió identificar diferencias en los órdenes *Solirubrobacteriales* y *Gaiellales* en los suelos de ITA y TBO, respectivamente, siendo estos suelos los que presentaron los valores más altos de CRA y P disponible.

5.1.2 Diversidad funcional de las comunidades procariotas y su relación con las propiedades fisico-químicas de los suelos del bioma Campos

En el capítulo tres de esta tesis se tuvo como objetivo caracterizar los perfiles funcionales relacionados con el ciclo del P en cuatro unidades de suelo del bioma Campos de Uruguay: ITA, SPO, TBO y YNG. Se utilizó el programa PICRUSt2 (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States 2) para inferir los genes (pKO) y enzimas (pEC) implicados en el ciclo del P, y se evaluaron las actividades enzimáticas y su variabilidad en relación con las propiedades del suelo. Además, se complementó este análisis con la secuenciación del metagenoma completo de dos unidades de suelo (ITA y SPO) con el fin de mejorar la comprensión de las funciones procariotas en el ciclo del P. Las unidades de suelo seleccionadas presentaron diferentes materiales parentales y estados nutricionales, y representaron dos usos de suelo distintos: tres de ellas corresponden a campos de pastizales (ITA, SPO y TBO), principalmente destinados a la producción ganadera extensiva, mientras que YNG es representativa de suelo bajo producción agrícola.

Nuestros resultados mostraron que las unidades de suelo estudiadas aquí presentan diferencias en sus perfiles funcionales. Los perfiles funcionales de las unidades de suelo TBO y YNG fueron similares sin mostrar diferencias significativas, a pesar de tener comunidades procariotas taxonómicamente diferentes. Es importante destacar que varios trabajos han demostrado que la diversidad taxonómica y funcional no se correlacionan linealmente, principalmente debido a la redundancia funcional (Chen, 2022, Mendes et al., 2015, Lennon y Jones, 2011).

Estas dos unidades de suelo son casos particulares. La unidad de suelo TBO se desarrolla sobre areniscas, un tipo de suelo poco frecuente en nuestro país, y la unidad de suelo YNG representa uno de los grupos de suelos con mejores propiedades fisico-químicas para la producción agrícola en el país; de ahí que

soporte una alta intervención humana (Chávez, 2018 , Álvarez y Cayssials, 1979). En los suelos de YNG se observaron cambios en las variables medidas, particularmente en el balance de nutrientes con valores altos de P disponible (APR), N total y C orgánico. En cuanto a la actividad enzimática en esta unidad de suelo, los valores registrados para las tres enzimas (fosfatasa alcalina, fosfatasa ácida y fitasa) fueron significativamente altos en comparación con las otras unidades de suelo. Este resultado no era esperado para suelos fertilizados, para los cuales se ha reportado una disminución en la actividad de la enzima fosfatasa en asociación con una alta disponibilidad de nutrientes (Dinca et al., 2022, Janes-Bassett et al., 2022, Margalef et al., 2021). En un estudio reciente se analizó cómo la coinoculación de semillas de soja con *Bradyrhizobium elkanii* y *Priestia megaterium (*antes *Bacillus megaterium)* afectó a las comunidades procariotas de la rizosfera de soja bajo tres tratamientos con diferentes niveles de P disponible. Se observó que la fertilización fosfatada combinada con la coinoculación de semillas mejoró la nutrición fosfatada del cultivo de soja, lo que aumentó el P de la planta y el rendimiento, así como la abundancia relativa de diferentes filos en la rizosfera de soja (Torres et al., com pers Abril 2023). Se podría plantear la hipótesis de que un fenómeno similar al que se observa en la rizosfera de la soja podría estar ocurriendo en los suelos de YNG con un alto contenido de nutrientes y de materia orgánica. Esto podría explicar explicar en parte la alta actividad enzimática observada en este tipo de suelo.

Aunque el diseño experimental de nuestro estudio no nos permite confirmar esta hipótesis, podemos inferir que las prácticas agrícolas cercanas pueden alterar el equilibrio basal de nutrientes del suelo y, como resultado, su perfil funcional tal como lo observamos en la estructura de la comunidad (Dinca et al., 2022, Garaycochea et al., 2020).

Los perfiles funcionales de P de las unidades de suelo ITA y SPO fueron similares, pero se observaron diferencias en las funciones relacionadas con la mineralización del P orgánico: se encontró una mayor abundancia de enzimas como la fosfatasa ácida y las fitasas en la unidad ITA. Los análisis CAP revelaron una

fuerte asociación entre los perfiles funcionales y el contenido de nutrientes (C orgánico, N total, APR y CIC), la estructura del suelo (contenido de arcilla y porosidad) y la capacidad de retención de agua. De acuerdo con Margalef et al. (2017), el contenido de C orgánico y N total, junto con factores climáticos, pueden ser buenos predictores del potencial de mineralización del P orgánico del suelo. Asimismo, el contenido de arcilla de los suelos es importante para la actividad enzimática por su efecto estabilizador (Neal et al., 2017).

Las diferencias identificadas en los perfiles funcionales de las comunidades procariotas de ITA y SPO podrían ser resultado de la suma de los efectos de las propiedades fisico-químicas y de las comunidades procariotas que contribuyen a las funciones estudiadas. Estos resultados concuerdan con los obtenidos al caracterizar la diversidad estructural de las comunidades en ambas unidades de suelo, así como también con otros trabajos publicados (Garaycochea et al., 2020, Karimi et al., 2020, Delgado-Baquerizo et al., 2018).

Durante la discusión del capítulo dos se mencionó que las comunidades procariotas de ITA y SPO presentaron diferentes composiciones; específicamente, ITA mostró valores más altos índices de diversidad de especies (Shannon (H') y Chao1 (S)) en comparación con SPO. Sin embargo, ambos tipos de suelo presentaron valores de diversidad filogenética (PD) similares y no significativamente diferentes (Garaycochea et al., 2020). La similitud en los perfiles funcionales de P observados en ambas unidades de suelo sugiere que ambas comunidades procariotas podrían realizar la mineralización del P, aunque la función la llevaria a cabo distintas especies. Varios estudios han demostrado que la composición taxonómica varía significativamente con las características del suelo, mientras que el potencial funcional de las comunidades estudiadas basado en la abundancia de genes es redundante (Louca et al., 2018, Nelson et al., 2016).

En cuanto a la actividad enzimática de fosfatasas ácida y alcalina, se observó una tendencia similar en las unidades de suelo ITA y SPO. Se encontró que las propiedades del suelo con mayor asociación a esta actividad enzimática fueron el

contenido de arcilla, la porosidad, el contenido de nutrientes (C orgánico, N total, P disponible y CIC) y el pH. Estas propiedades permitieron agrupar a las cuatro unidades de suelo de manera separada. Estos resultados concuerdan con los reportados por Mencel et al. (2022), quienes afirmaron que la actividad enzimática no sólo se ve afectada por las propiedades químicas del suelo como el pH y el contenido de nutrientes, sino también por el contenido de agua y aire en el suelo. Además, es posible encontrar tanto fosfatasas ácidas y alcalinas en un mismo suelo, pero son más frecuentemente encontradas una u otras en suelos con rangos de pH cercanos a sus óptimos (Margalef et al., 2017).

Asimismo, se evaluó la capacidad de los genes y enzimas inferidos por PICRUSt2 para predecir la actividad enzimática. Se encontró que existía una alta correlación positiva entre la actividad de la fosfatasa ácida y las enzimas inferidas (pEC), lo que indica una fuerte relación entre ellas. Sin embargo, la correlación entre los genes inferidos (pKO) y las actividades enzimáticas fue baja. A pesar de esto, el uso de PICRUSt2 para predecir ciertos genes puede ser una estrategia útil y de bajo costo para futuras investigaciones en este campo. Nuestros resultados son consistentes con estudios previos que han demostrado una relación positiva entre la abundancia de genes implicados en la mineralización del P y las actividades enzimáticas en los procesos del ciclo de nutrientes del suelo (Ma et al., 2021, Leff et al., 2012, Fierer et al., 2012). El aumento de genes que codifican enzimas de mineralización y su actividad sugiere que la comunidad microbiana del suelo es más eficiente en la degradación de materia orgánica, lo que aumenta las tasas de ciclado de los nutrientes (Séneca et al., 2021). Estos resultados respaldan la utilidad del PICRUSt2 como herramienta valiosa para estimar el potencial de mineralización de P orgánico del microbioma del suelo.

Para comprender mejor el potencial funcional de las comunidades microbianas en el ciclo del P en las unidades de suelo de pastizales de Uruguay, se analizó la diversidad y abundancia de ocho genes que codifican enzimas claves en la liberación de P a partir de compuestos orgánicos. Los resultados obtenidos fueron

consistentes con estudios previos (Neal et al., 2017, Bergkemper et al., 2016), destacando el gen *phoD* como el más abundante y filogenéticamente diverso en ambos suelos. Aunque se observaron diferencias en la abundancia de genes entre ambas unidades de suelo, no se identificaron grupos taxonómicos distintos asociados con los genes estudiados. A pesar de la diversidad de las comunidades procariotas en los sitios, los perfiles funcionales de ambos suelos fueron similares, como se predijo con PICRUSt2 y se apoyó con el enfoque metagenómico. Estos resultados sugieren que, incluso en comunidades microbianas con alta diversidad taxonómica, puede haber una menor diversidad funcional debido a que los microorganismos coexistentes pueden ser taxonómicamente distintos, pero codificar la misma función (Louca et al., 2018).

Los pastizales en Uruguay están en gran medida preservados, ocupando más del 60 % del país (Lezama et al., 2019); una parte importante se desarrollan en suelos con material parental basáltico y cristalino (Dirección General de Recursos Naturales: Coneat, carta de suelo y campo natural). En este capítulo, nuestro enfoque se centró en el estudio de los genes y enzimas responsables del transporte, absorción y mineralización del P orgánico, así como en la actividad de tres enzimas clave del ciclo del P. Nuestro objetivo principal fue establecer una línea de base que pudiera contribuir a un uso más eficiente de los fertilizantes y reducir el impacto negativo sobre el medio ambiente. Como resultado de nuestro estudio, concluimos que el contenido de nutrientes (N total, P disponible, C orgánico y CIC), la estructura del suelo (contenido de arcilla, densidad aparente y porosidad), la capacidad de retención de agua y el pH son los principales factores responsables de las diferencias en los perfiles funcionales de P en las cuatro unidades estudiadas. Es importante destacar que, en nuestro estudio, observamos que las variables que determinan la composición taxonómica de las comunidades microbianas también coinciden en gran medida con las que influyen en los perfiles funcionales de P en las cuatro unidades estudiadas. Esto sugiere que la diversidad estructural y funcional de las comunidades microbianas en el suelo están estrechamente relacionadas y pueden ser influenciadas

por factores similares. Esta información es relevante porque puede ser útil para el diseño de estrategias de manejo de suelos que no sólo mejoren la productividad agrícola, sino que también promuevan la salud del suelo y su biodiversidad, lo que garantizaría la provisión de servicios ecosistémicos.

5.1.3 Distribución filogenética y abundancia de ocho enzimas claves del ciclo de fósforo en el bioma de pastizales

En el capítulo cuatro de esta tesis nos enfocamos en el estudio de la fracción procariota de la comunidad microbiana del suelo. Específicamente, analizamos la abundancia y diversidad filogenética de ocho genes codificantes de enzimas del ciclo del P del bioma pastizales, utilizando un enfoque metagenómico. Las muestras analizadas representaron diferentes condiciones ambientales, definidas por las propiedades físicas y químicas del suelo, así como las variables climáticas (Islam et al., 2020, Amundson, 2013). Para la construcción del conjunto de datos, se incluyeron datos públicos disponibles en el repositorio MG-RAST más dos sitios uruguayos (ITA y SPO) y otras fuentes para cada proyecto/muestra.

Una información relevante obtenida en este estudio es la confirmación de la gran variabilidad en la abundancia y diversidad de los genes codificantes de enzimas del ciclo del P, incluyendo las fosfatasas y las fitasas microbianas. Estas desempeñan un papel crucial en el ciclo del P al liberar ion ortofosfato a partir de compuestos organofosforados, interviniendo en el último paso del ciclo (Zeng et al., 2022, Tan et al., 2013, Yao et al., 2012, Nannipieri et al., 2011, Rodríguez et al., 2006).

Se encontró que las fosfatasas alcalinas fueron las enzimas más abundantes en los pastizales estudiados, siendo el gen *phoD* el más abundante y presentando la mayor diversidad filogenética independientemente de las características del suelo, lo cual es consistente con estudios previos (Park et al., 2022, Bergkemper et al., 2016, Tan et al., 2013). Es destacable que la distribución filogenética del gen *phoD* fue similar en los suelos uruguayos estudiados en comparación con otras regiones.

La abundancia del gen *phoA* fue menor en comparación con *phoD* o *phoX*, lo que puede ser el resultado de diferencias en la especificidad del sustrato y los requisitos de cofactores. Es importante destacar que las células bacterianas pueden poseer el gen *phoX*, *phoA* o ambos. Ambas enzimas codificadas por estos genes tienen funciones similares, lo que les brinda una ventaja al facilitar el acceso a una amplia gama de compuestos fosfatados. Sin embargo, pueden funcionar a diferentes niveles de concentración de sustrato (Sebastian y Ammerman, 2011).

La actividad microbiana relacionada con el ciclo del P está influenciada por las propiedades fisico-químicas y climáticas del suelo, como se ha mencionado previamente. Según nuestros resultados, las variables ambientales tienen una relación significativa con la diversidad y abundancia de los genes que codifican las enzimas involucradas en el ciclo del P. Específicamente, la temperatura máxima (Tmax), el pH, el carbono orgánico y la humedad del suelo fueron las variables que mostraron las mayores correlaciones con la abundancia de los genes de fosfatasa alcalina.

Los genes *phoD* y *phoX* presentaron una alta correlación con el contenido de carbono orgánico del suelo y el contenido de arcilla. Varios trabajos recientes han señalado el efecto del contenido de C orgánico, N total y P orgánico en la abundancia y diversidad tanto de las enzimas como de las especies bacterianas correspondientes (Li et al., 2021, Wei et al., 2021, Ragot et al., 2017). Un estudio que consideró tres usos del suelo con distintos contenidos de C orgánico (barbecho, cultivos herbáceos, pastizales) demostró una correlación positiva entre la abundancia de genes de fosfatasas alcalinas y los contenidos de materia orgánica del suelo (Neal et al., 2017). Además, la predicción de la localización extracelular de ambas enzimas (Neal et al., 2017) puede explicar la importancia del contenido de arcilla en relación con su papel en la estabilización, inmovilización y mantenimiento de la actividad enzimática (Margalef et al., 2017). Los genes *phoD* se encuentran ampliamente distribuidos entre diferentes clases de bacterias. En este estudio, se ha observado que las variantes asociadas a los géneros *Koribacter* (clase *Acidobacteria*) y *Rhodanobacter* (clase *Gammaproteobacteria*) fueron más abundantes en suelos con valores relativamente

altos de C orgánico y pH bajo, como en el caso del suelo uruguayo ITA. El género *Koribacter* fue identificado como uno de los diferenciales de la comunidad procariota en este suelo (Garaycochea et al., 2020). Además, estas variantes también han sido identificadas como filotipos dominantes en suelos cultivables y en las rizosferas de maíz y sorgo (Neal et al., 2021, Neal et al., 2017). Ambos géneros poseen un amplio conjunto de genes que les permiten utilizar una gran variedad de sustratos, lo que les permite responder eficientemente a los cambios ambientales y les confiere capacidad de adaptación a diversos nichos ecológicos (Kalam et al., 2020, Kurm et al., 2017). Las variantes asociadas a *Bacillus*, *Actinomycet*es y *Planctomyces* fueron mayoritarias en suelos con menor C orgánico y pH neutro. Los dos últimos filos mencionados se han identificado como dominantes en suelos con bajo contenido de nutrientes, como el suelo de la unidad SPO. Además, se encontró que el filo *Planctomyces* mostró una correlación negativa con esta variable (Garaycochea et al., 2020, Hermans et al., 2017, Lewin et al., 2017). Sin embargo, el principal factor que estaría explicando la diferencia en la abundancia de especies es el pH, ya que todas las especies reportadas son heterótrofas (Saxena et al., 2020, Kielak et al., 2016). El gen *phoX* representado por el género *Burkholderia* fue diferencial en suelos con bajo y medio contenido de C orgánico y pH neutro. Las bacterias de este género presentan un amplio repertorio de rutas metabólicas que las hacen más competitivas en ambientes con restricción de nutrientes, ya que son capaces de degradar compuestos recalcitrantes y, a diferencia de la mayoría de las bacterias, las especies de *Burkholderia* son más competitivas en condiciones de pH bajo y moderado (Morya et al., 2020, Stopnisek et al., 2014).

Los genes que codifican para NSAP-A de fosfatasas ácidas se encontraron en los géneros *Dyella* y *Rhodanobacter*, los cuales se han reportado como dominantes en suelos ácidos y neutros (Dahal y Kim, 2017, Weon et al., 2009). Por otro lado, el gen que codifica para NSAP-C se identificó en varias clases de bacterias, como *Alpha* y *Gammaproteobacteria*, *Flavobacteria* y *Sphingobacteria*, concordando con reportes previos (Gaiero et al., 2020, Neal et al., 2017). La proporción de fosfatasas

ácidas no específicas encontradas en el conjunto de pastizales estudiados en nuestro trabajo fue similar a la reportada para los suelos de pastizales del Reino Unido (Neal et al., 2017). Es importante destacar que estas enzimas fueron encontradas en muy baja abundancia en los dos suelos uruguayos estudiados. La mayor abundancia de las fosfatasas ácidas en los pastizales podría deberse a la interacción entre microorganismos y comunidades vegetales (Mhlongo et al., 2018).

En cuanto a las fitasas, se observó que los genes codificantes de BPP mostraron una abundancia y distribución filogenética consistente con reportes previos. Estos genes están ampliamente distribuidos en la naturaleza y se han encontrado en diversas especies de bacterias del suelo (Kumar et al., 2017, Huang et al., 2009, Jorquera et al., 2008, Lim et al., 2007). En este estudio, se observó que las variantes de genes codificantes de BPP estaban principalmente presentes en *Bacillales* y *Beta* y *Gammaproteobacteria*, y sólo se encontraron en suelos con un pH superior a 6,6. Estos resultados están de acuerdo con lo reportado por diversos autores, en particular para enzimas BPP de cepas del género *Bacillus*, quienes mostraron su actividad óptima en un rango de pH 6,0-7,5 (Kumar et al., 2017, Huang et al., 2009, Farhat et al., 2008, Cheng y Lim, 2006, Kerovuo et al., 1998). Por otro lado, los genes codificantes de Cphy fueron lo que tuvieron la menor abundancia en los metagenomas de los pastizales estudiados, contrariamente a los encontrados por Neal et al. (2017) en los pastizales del Reino Unido, donde CPhy tuvo una abundancia similar a BPP.

En términos generales, el pH es una de las principales variables asociadas a cambios en la abundancia y diversidad de las enzimas fosfatasas ácidas, alcalinas y fitasas. Aunque nuestros resultados indican un incremento global en los genes que codifican estas enzimas (PhoD, PhoX, Nspa-C y BPP) con el pH, todas ellas son relativamente abundantes en el rango de pH que se cubrió en este estudio, lo que dificulta establecer una asociación directa entre la clasificación de la enzima y el pH del suelo.

Nuestros resultados muestran una fuerte relación en los patrones de abundancia y diversidad entre las diferentes enzimas P analizadas. Los análisis CAP indican correlaciones significativas entre ellas, aunque estas correlaciones son más débiles para los genes menos abundantes. Estos resultados sugieren que la selección de cada gen en una comunidad está en equilibrio con la interacción entre los organismos y el ambiente. La alta correlación entre los análisis CAP, que involucran abundancia, diversidad y variables ambientales, sugiere que los diferentes genes de las enzimas P están conectados en términos de su contribución al ciclo del P y al metabolismo. Los diferentes taxones asociados a cada gen codificador de enzimas P indican que diferentes organismos contribuyen al conjunto de genes de las enzimas P.

En resumen, la temperatura máxima, el pH y la evapotranspiración se relacionaron con la abundancia y diversidad de seis de las ocho enzimas clave estudiadas aquí. Asimismo, fue posible identificar el efecto de otras variables con un efecto más localizado, como la contenido de arcilla y el C orgánico, como determinantes importantes de la estructura y funciones de la comunidad microbiana.

5.2. CONCLUSIONES GENERALES

Este trabajo se centró en el estudio de las comunidades procariotas en suelos de pastizales, enfocado en la participación de éstas en la movilización del fósforo orgánico. Los resultados obtenidos ofrecen una base base para comprender el funcionamiento de un sistema natural y contribuir a la formulación de estrategias que aumenten la eficiencia en la utilización del fósforo, promoviendo así sistemas de producción más sostenibles.

En el capítulo dos se describen las comunidades procariotas de cinco unidades de suelo de Uruguay a través del uso del gen 16S rARN. Los taxones diferenciales entre las unidades de suelo fueron *Firmicutes* (YNG), *Acidobacterias* (ITA), *Actinobacteria* (SPO), *Verrucomicrobia* y *Arqueas* (TBO). La estructura del suelo (porosidad, contenido de arcilla), el contenido de nutrientes (P disponible y C orgánico) y la capacidad de retención de agua son las principales variables que intervienen en la modulación de estas comunidades.

En relación con la diversidad funcional, en el capítulo tres se presenta el conocimiento generado sobre los genes y enzimas responsables del transporte, absorción y mineralización del P orgánico, así como la actividad de tres enzimas clave del ciclo del P. Se concluye que la estructura del suelo (densidad aparente, porosidad), el contenido de nutrientes (N total, P disponible, C orgánico y CIC), la capacidad de retención de agua y el pH son los principales factores responsables de las diferencias en los perfiles funcionales de P de las cuatro unidades de suelo estudiadas. Nuestros resultados mostraron que las mismas variables influyen tanto en la diversidad taxonómica como funcional de las comunidades procariotas estudiadas. Se determina una menor diversidad funcional que taxonómica, lo que podría indicar redundancia funcional. El estudio de la diversidad funcional del suelo resulta un mejor predictor del potencial de mineralización del P y proporciona información de mayor relevancia práctica en comparación con el análisis de la diversidad taxonómica.

En el capítulo cuatro se analiza la distribución y abundancia de ocho enzimas clave del ciclo del P (PhoD, PhoX, PhoA, NSAP-A, NSAP-B, NSAP-C, BPP y Cphy) y su relación con los factores ambientales, en un estudio comparativo global. Nuestros resultados mostraron que el gen *phoD*, que codifica la fosfatasa alcalina homónima, es el más abundante y con la distribución filogenética más amplia en todos los pastizales estudiados, incluyendo las del bioma Campos de Uruguay. Además, las fosfatasas ácidas NSAP-A y NSAP-C son las enzimas más abundantes de este grupo, mientras que las fitasas son escasas en el conjunto de datos estudiado.

Las variables estudiadas tuvieron una influencia relativamente baja en la variabilidad de los perfiles funcionales de los microorganismos procariotas. El estudio a escala global muestra una fuerte correlación del pH, la temperatura máxima y la evapotranspiración con la abundancia y diversidad de los genes que codifican para las enzimas más abundantes. Además, se concluye que el contenido de arcilla y el contenido de C orgánico del suelo ejercen un efecto local, resultado que concuerda con en el estudio realizado en los suelos de pastizales de Uruguay, pertenecientes al bioma Campos.

5.3 PERSPECTIVAS

Los resultados de este estudio permitieron establecer una línea de base sobre la diversidad estructural y funcional de las comunidades procariotas asociada al ciclo del P en distintos tipos de suelo pertenecientes al ecosistema de pastizales. Asimismo, los resultados obtenidos nos indican las principales variables (estructura del suelo, contenido de nutrientes y capacidad de retención de agua) y las interacciones que deberían ser exploradas para mejorar la gestión del P en los ecosistemas estudiados.

El abordaje metagenómico y las herramientas utilizadas resultan clave para ampliar las bases del conocimiento sobre el ciclo del P y su fitodisponibilidad, mediada por las comunidades procariotas del suelo. Profundizar en el conocimiento de la diversidad procariota funcional asociada a la movilización del P orgánico tanto en ecosistemas naturales como en aquellos con diverso grado de intervención antrópica (agrícolas, hortícolas y forestales) permitirá su manejo y uso para mantener y mejorar la fertilidad del suelo. Asimismo, podrá contribuir a preservar la oferta de servicios ecosistémicos y, fundamentalmente, a disminuir el impacto negativo sobre la calidad del agua.

Sería necesario priorizar la generación de información y profundizar en el conocimiento sobre:

— los mecanismos microbianos específicos involucrados en la acumulación y movilización del P a partir de los diversos componentes de la materia orgánica del suelo;

— las interacciones específicas del P orgánico con otros nutrientes, considerando el ciclo de P en el contexto de otros ciclos biogeoquímicos;

—el impacto de los cambios en el uso del suelo sobre la diversidad microbiana funcional como predictora del potencial de movilización del P orgánico, teniendo en cuenta las interacciones con el tipo de suelo, el aporte de nutrientes y las comunidades vegetales predominantes;

— el impacto de los exudados radiculares y el efecto rizosfera en la modulación de la diversidad microbiana estructural y la redundancia funcional.

La complejidad del sistema estudiado requiere una combinación de enfoques y la generación de datos locales para detectar patrones globales de diversidad que conduzcan a una mejor comprensión de la dinámica y gestión del P, para contribuir a la conservación del bioma Campos y otros ecosistemas de pastizales del mundo.

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7. ANEXOS

7.1 ANEXOS CORRESPONDIENTES AL CAPÍTULO 2

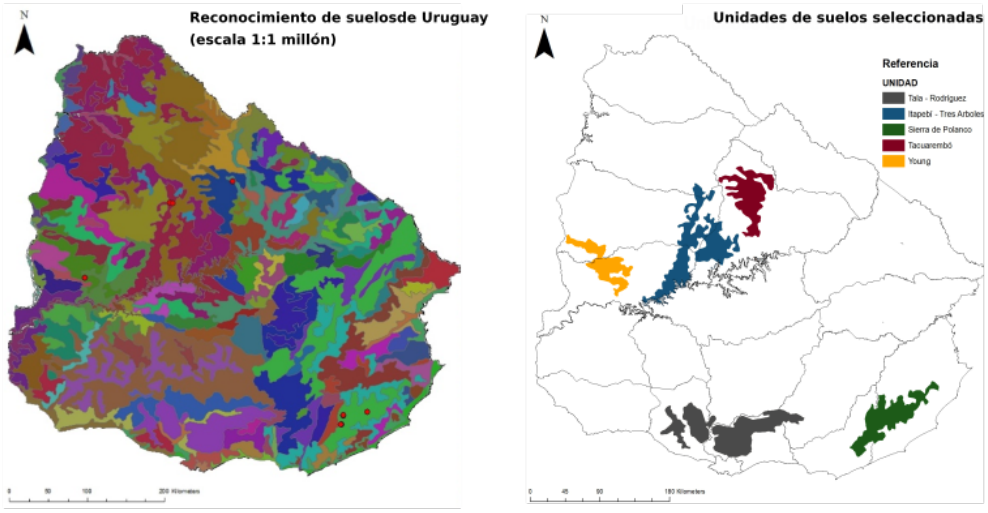

Figura 1: a) Unidades de suelo del Uruguay- Carta de reconocimiento de Suelos del Uruguay - Escala 1:1.000.000, Ministerio de Ganadería Agricultura y Pesca (MGAP), b) Unidades de suelo seleccionadas.