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# Effect of overabundance over the gut microbiome and feeding behavior in a large wild herbivore

**Irene Torres Blas**

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# Effect of overabundance over the gut microbiome and feeding behavior in a large wild herbivore

Irene Torres Blas

**Supervisors**

Dr. Emmanuel Serrano Ferron

Dr. Thomas Sharpton

Dr. Santiago Lavín

**Tutor**

Dr. Santiago Lavín

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Els doctor **Emmanuel Serrano Ferron**, investigador principal del grup de recerca *Wildlife Ecology & Health group,* membre del Servei d'Ecopatologia de Fauna Salvatge (SEFaS) i professor titular del Departament de Medicina i Cirurgia Animals de la Universitat Autònoma de Barcelona; el doctor **Santiago Lavín González,** fundador del SEFaS i professor catedràtic del Departament de Medicina i Cirurgia Animals de la Universitat Autònoma de Barcelona; i el doctor **Thomas Sharpton**, professor titular del Department of Microbiology and Department of Statistics, Oregon State University.

Informen:

Que la memòria titulada "**Effect of overabundance over the gut microbiome and feeding behavior in a large wild herbivore**", presentada per **Irene Torres Blas** per a l'obtenció del grau de Doctor en Medicina i Sanitat Animals per la Universitat Autònoma de Barcelona, s'ha realitzat sota la nostra direcció i, un cop considerada satisfactòriament finalitzada, autoritzem la seva presentació per tal que sigui avaluada per la comissió corresponent.

Emmanuel Serrano Ferron

Director

Thomas Sharpton

Director

Santiago Lavín González

Director i tutor

Irene Torres Blas

Doctoranda

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## Agraïments, Agradecimientos, Acknowledgements

*"Les coses importants són les que no ho semblen."*

— **Mercè Rodoreda, Quanta, quanta guerra…**

Com va dir Mercè Rodoreda, "*Escriure bé costa. Per escriure bé entenc dir amb la màxima simplicitat les coses essencials".* Les persones que hagueu tingut la bona (o mala) fortuna de llegir els meus manuscrits segurament us haureu adonat ràpidament de la meva habDit ilitat per explicar les coses en tres línies en comptes d'una. Estareu d'acord amb mi doncs que, com a mínim, el fet que just per la part més bonica i fàcil de redactar de la tesi em manquin les paraules (o potser em sobren tantes que no sé ben bé quines triar) és un gir bastant irònic i alhora graciós de la vida. Igualment, espero que se'm perdoni si al final m'esplaio; al cap i a la fi, l'agraïment és millor en abundància. Però bé, per alguna banda s'ha de començar, i he d'assumir que totes les paraules que puc escriure en el paper es quedaran tan curtes per plasmar el que realment sento que com a màxim podré esbossar un pàl·lid reflex del que m'heu fet sentir durant una caminada que ha durat cinc anys. Si d'alguna forma aconsegueixo fer sentir als presents en aquest text com que us dono una abraçada mentre xiuxiuejo "moltes gràcies" a la orella, em donaré per satisfeta.

Tot i que en la portada de la tesi hi haurà plasmat el nom de l'autor, tots sabem que aquesta tesi no és només fruit meu: és el resultat final d'un mosaic de persones i experiències que han aportat el seu gra de sorra que m'ha ajudat a tirar endavant durant tots els cops

en què se'm va fer difícil. Encara em costa assumir que, d'una forma o una altra, tots els que heu format part del trajecte m'heu conegut i heu decidit que era un digne atuell d'un trosset vostre. Si em deixo algú, no és perquè sigueu menys importants; és perquè he tingut la gran fortuna de tenir a tanta gent meravellosa al meu voltant que les meves neurones són incapaces de recordar-les a totes (la carn és dèbil, diuen). Només espero ser capaç, algun dia, de veure en mi mateixa el que vosaltres veieu, perquè a mi per ara se m'escapa com és possible que decidiu que val la pena tenir-me al costat. Si d'alguna manera me les ha apanyat per no decebre-us, puc dir que major sort no he tingut a la vida.

Però bé, prou petar la xerrada, i comencem pel principi de tot – pels que vau posar la primera pedra dels pilars del projecte i vau decidir, per motius que desconec, apostar per mi. Emmanuel, soy incapaz de describirte de manera sencilla (qué sorpresa, ¿Verdad?), pero para mí, eres como el río que va al lado de un sendero: el agua baja, con más o menos brío, gentil pero imparable, y aunque a veces se le pierde de vista entre el espesor del bosque, siempre está presente con el murmullo de sus aguas. Si se sigue su trayecto, no es posible perderse. Espero no haber sido, sin darme cuenta, un castor que para tu caudal con una presa. Muchas gracias por tu apoyo, paciencia y optimismo eterno que ha conseguido tirar el proyecto hacia adelante a pesar de las dificultades y problemas que han ido surgiendo (que, en una tesis, no suelen ser pocos); por los ratillos en campo y las cervezas que fueron testigo de mil

conversaciones y anécdotas que se quedan en el aire y los suelos de Quintos y Muela.

Seguimos.

Santiago, muchas gracias por tu apoyo incondicional, por estar ahí siempre disponible y responder siempre con “vamos a solucionarlo” cuando aparece alguna piedra en el camino. Por las conversaciones de despacho y tu capacidad de simplificar los problemas cuando parecen montañas e ir dos pasos por delante.

And Thomas, despite being separated by an ocean and several time zones apart, thank you for somehow always being there when I needed your help and sharing your vast knowledge with me; for guiding me and calming my nerves when I thought I’d made a rookie mistake, because your “you will be fine” was somehow the reassurance I needed.

Però aquest grup d’investigació és un ecosistema format per molta més gent que, sense tenir cap tipus d’obligació de fer-ho, han ajudat també d’alguna forma o una altra. Roser, mil gràcies per cuidar-me durant tot aquest temps, per escoltar-me sempre que ho necessitava, i tenir-me en compte per moltes altres coses fora de la tesi. Per totes les conversacions de ferrocarril i recomanacions de llocs que quan ara visito, em fan pensar en tu. Tens l’habilitat d’arropar a qualsevol nouvingut, fins i tot quan tu no estaves passant el teu millor moment. Y Rafi, gracias por tus “Buenos días”

y “Hola, Irene” por las mañanas, y darme consejos esporádicos cuando me veías por el pasillo que demostraban cariño genuino.

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Si miro cap enrere, sé que els primers passos en el món de la ciència els vaig donar amb Jorge, que d’alguna manera vas aconseguir guiar la meva ingenuïtat i energia que tenia jo al 2016 (mare meva...hem tingut una DANA de pel mig i tot des d’aleshores) i donar-me una empenta que em va fer creure que estava feta per aquest món. Moltes gràcies per ser el primer que em va ajudar a obrir camí. Gràcies també a Oscar i Ignasi per continuar alimentant l’espurna investigadora i brindar-me la oportunitat de fer l’internat amb vosaltres, em vaig endur molt bons records, i em sento afortunada que el caminet que vam dibuixar durant aquell temps continuï funcionant més de cinc anys després (i desitjo que molt més). Gràcies també a Gregorio per d’alguna forma trobar temps i preocupar-te per mi i preguntar-me què tal estava i com anava tot si notaves que estava moixa, perquè a vegades un simple missatge

de “Com estàs?” és suficient per recordar que soc afortunada de tenir gent que es preocupa per mi.

Querría también extender mi aprecio y agradecimiento a todas aquellas personas que me han permitido llevar a cabo mi trabajo más allá de la UAB, especialmente a Mónica. Mónica, creo que eres tanto una investigadora como una persona magnífica, y me hiciste sentir como en familia desde el primer día que me acogiste en tu laboratorio en la Universidad de Murcia. Sé que conseguí hacer todo ese duro trabajo gracias a tu apoyo y colaboración, tanto en el ámbito laboral como en el personal. Estoy muy agradecida por tu paciencia y comprensión, por seguir en contacto conmigo y preguntarme qué tal me iba todo, y me alegro mucho de haberte conocido junto a Carlos. Muchas gracias también a todos los compañeros de Madrid: Dani, Alba, Ramón, Juancho…sin vuestra colaboración, constante ayuda y vuestros acertados comentarios, esta tesis tampoco habría salido adelante.

And now it’s the turn of the lovely Combrink Lab, from the University of Arizona. I’m not going to lie, the prospect of spending three months in the United States was thrilling but somewhat scary to me at the same time – and yet it was thanks to all of you that I managed to feel like I’d always been there. You all went above and beyond to turn this into one of the best experiences of my life, and it is clear to me that this lab and its people are the most precious thing that Arizona has to offer. Leigh, thank you so much for your warm welcome, for adopting me as one of your little ducklings – for

asking me first when my birthday was and if I had adapted well. Thank you for all your patience and support and teaching me everything you know about the microbiome, for being a steady line of support from the other side of the world once I returned here and always being available to chat with a smile on your face, well beyond my stay. Half of this thesis would not have come to fruition if it hadn't been for you and your people, and I could not have asked for a better mentor. Natalie, you have also been like a guiding lighthouse while I was trying to navigate the murky waters of learning bioinformatics from scratch, in a completely new research field. You made it look way easier than it is and I am forever grateful to you. I am sure you will shine bright wherever you go. Thanks to Carolina, for showing me around and taking me to explore Tucson's best places and adopting me, quite literally, into her family despite being a stranger, so to speak. And Gracie, even if maybe you're not fully aware, I'll be forever grateful to you for your help and for showing me that people like us (*if you know you know*) have a well-deserved place in the academic world. In that respect, you managed to make me feel less alone. Thank you so much too for sharing with me your love for birds and danger noodles. And to Desiree and all the other members of the Combrink lab, just know you made a Catalan girl not feel like a stranger in a completely different country.

Però ara passarem al moll de l'os que m'ha mantingut en peu durant tot el recorregut, l'equivalent acadèmic d'un cau de hòbbit amb primers i segons esmorzars, el Ying que sosté el Yang perquè sense tots vosaltres, el SEFaS i el WEH no serien el que són: la gent del

327, tant la que hi és ara com la que ha estat. He passat més estona entre les parets d'aquesta oficina que a casa meva, i no podria haver demanat millor companyia. No existeix suficient tinta per poder escriure de vosaltres tot el que m'agradaria, així que intentaré ser breu (tot i que no és el meu punt fort). Vull que quedi clar que l'ordre d'aparició no determina la vostra vàlua, tots heu estat igual d'importants de maneres úniques a cada una de les vostres personalitats. Sou tots irreemplaçables, amb una fortalesa inherent que pasma a qualsevol, i estic segura que arribareu fins on us proposeu.

Marta, tan lluny i tan a prop alhora...podràs estar ara mateix en els Estats Units, però estàs cada dia aquí al despatx, tot i que ja farà més de tres anys que vas marxar (segur que entens a què em refereixo,. Crec que això ja explica sense necessitat de paraules el tipus de persona que ets, per deixar una empremta tan gran per allà on passes. No dubtis mai que vas ser un gran pilar de suport, sempre disposada a ajudar tot i que no t'anés gens bé, amb un sentit del deure digne d'admirar. Et vaig comentar mai que les teves abraçades no només m'alegraven l'any, si no que segurament em van arreglar l'esquena de gamba que tinc?

Mary, la Perseverancia un día despertó y decidió adoptar una forma humana – la tuya. Todos hemos sido testigos de todas las cosas que has vivido y te han pasado, algunas sobre las cuales no tenías voz ni voto; y, aun así, miraste a los ojos lo que parecía imposible, te encogiste de hombros y lo hiciste. A lo mejor por eso se te da tan

bien hacer Trapecio, la Vida te ha entrenado con tantas piruetas. Mil gracias por, a pesar de todo, alegrarnos los días con tu sentido del humor, compartir cenas y excursiones, y dedicarme parte de tu tiempo a hablar de todo y de nada, a dejarme que me desfogara y darme nuevas perspectivas y encontrar en ti otra alma cándida amante del anime. *Shinpai shinaide, daijobuu dayo Mary-chan.*

Helen...ay, Helen. Creo que eres de las pocas personas en que generé una pésima primera impresión (por decirlo suavemente) y de alguna manera me las apañé para convencerte de que fueras mi amiga. Creo que nuestra relación es fruto de cuando un extrovertido (tú) adopta a un introvertido (ah no no…ah bueno sí). Tienes una naturaleza que relaja a cualquiera, y me fascina tu habilidad para empezar conversaciones con extraños como si fueran viejos amigos. Me cogiste de la mano y me acogiste en tu mundo sin tapujos, presentándome a tu gente, y ahora me siento como parte de él. Eres una persona maravillosa, amable, alegre, creativa, prudente y resolutiva como tú sola, con una capacidad de empatía y comprensión que raramente encuentro en otras personas. Ojalá seas consciente de lo mucho que he apreciado siempre tu apoyo y tu amistad, de lo fácil que parece todo cuando tú estás presente, de los paseos por Llinars y las fiestas de tu casa. Nuestro libro de vivencias es como mínimo curioso (“Tira”), pero ojalá vengan muchos más momentos contigo para seguir llenándolo de historietas.

I Josep, el nostre locutor de ràdio preferit, enciclopèdia inacabable de patologia, hemeroteca humana dels Simpson. Sempre disponible per fer un cop demà. No deixaré mai d'admirar la teva diligència i habilitat per fer les coses al moment, el teu inesgotable optimisme i la capacitat de trobar les paraules adients per aixecar la moral i calmar les ments alterades després de jornades inacabables. Sé de primera mà que tu has tingut moments igual de dolents (o fins i tot pitjors) que jo, i tot i així mai has dubtat en donar una empenta i paraules d'ànim quan tu també les necessitaves.

Stef, nos juntó la aleatoriedad de la vida allá por el 2015 y no nos hemos despegado. Es increíble cómo a veces las mejores personas de tu vida aparecen por puro azar. Asusta un poco pensar en la cantidad de cosas bonitas que seguramente no hemos vivido porque la Suerte no lo quiso, ¿No te parece? De anécdotas tenemos varias, algunas tiernas, otras dramáticas. Por suerte creo que estamos en un punto que podemos mirar atrás y reírnos al respecto, al menos con algunas. Incluso desde otro país, eres una fuente constante de cariño y ayuda. Saber que, estés donde estés, puedo contar contigo, es una fuente de alivio, y te aprecio mucho. Creo que, si me preguntan, se quedó buena tarde, ¿No crees?

I per tota la colla que heu anat passant pel despatx: Omar, Genís, Jose, Tonins...tots heu deixat empremta d'una forma o una altra, d'això no tingueu pas cap dubte. Menció d'honor també a Barri i Maña, perquè una bona sessió de "perroterapia" m'ha salvat més d'un cop de la bogeria.

I ara ve el torn de tots els que heu estat allà des de l'inici. Però no em refereixo al doctorat, em refereixo a gairebé la meva vida sencera. M'atreveixo a dir que les nostres són (i espero que continuïn sent) amistats de les que donen enveja, les que es canten en les èpiques, la família triada. Potser tendeixo a tenir mala fortuna perquè ja em gasto tota la meva bona sort mantenint-vos al meu costat. Segurament no soc la persona més difícil de tractar, però tampoc pretenc que sigui feina fàcil, i tot i així continuem buscant-nos els uns als altres per més que se'ns compliqui la vida i se'ns separin els camins. Gemma, ens vam conèixer per primer cop en un Saló del Manga als voltants de 2013; Simó, tu vas tenir la desgràcia de creuar-te amb mi a l'institut, i Gerard i Ariadna teniu la maledicció ja des de pàrvuls. I mireu-nos on som ara; el tros recorregut i les coses que hem anat descobrint a nivell personal que ens han permet entendre'ns millor a nosaltres mateixos. Tots sabem totes les coses que han passat durant tots aquests anys, especialment els últims tres, i sempre heu estat una font inacabable de suport i confiança, fins i tot si estàveu passant pels vostres propis Inferns en vida. Sou unes persones amb les quals no podria sentir-me més còmoda i segura, perquè em deixeu ser tal i com soc realment sense por a perdre-us. Per tots els cops en què he estat absent (a vegades setmanes o mesos) i mai m'heu retirat les invitacions o la paraula, gràcies de tot cor.

Y mis ex compis de cuando aún estaba en clínica, en el hospital de Riera: Sonia, Sofi, Patri, Toni, Maria del Mar. Nos hermanaron los marrones, los cócteles en los Pollos, y el buen humor ácido que nos

ayudó a superar las noches de guardias y los casos imposibles. He conseguido manteneros en mi vida y no podría estar más feliz de conoceros. Muchas gracias por todos los ánimos que me habéis mandado durante todo este tiempo.

I la meva família, tota ella: els meus pares, ma germana, els meus tiets i tietes, els meus avis... No hauria arribat mai on soc si no us hagués tingut al meu costat durant tot aquest temps. El vostre suport incondicional ha pres sempre tantes formes i paraules diferents que em resultaria impossible nombrar-les totes. A ma mare, perquè a part de parir-me a mi, també ha parit aquesta tesi. Perquè sense els cafès pel matí, la pregunta de "Va, avui què vols dinar", els cops que al tornar a casa ja estava tot fet, les tardes de passejos o veure pel·lícules juntes, la forma en què gairebé m'ho toleres tot i poses empeny en comprendre'm...tot aquest esforç que has fet tu per estalviar-me'l a mi és, en la meva humil opinió, un dels actes d'amor més desinteressats que podria fer una persona. Pel meu pare, sempre disponible, sempre preparat per saltar a l'acció si fos necessari. Perquè sé de sobres que series capaç de fer mitja volta amb el camió anant cap a França si et truqués i t'ho demanés. Neus, Newitus, ostentes el títol de germana gran per 20 minuts. No tinc cap dubte que sempre ens tindrem l'una a l'altra. No ho demostrem ja cada dia, quan ho deixem tot de banda per agafar la trucada ben entrada la nit, si és necessari? Quants cops ens enviem bromes i coses que ens fan pensar l'una en l'altra? Et dic que necessito ajuda, i remous cel i terra si fa falta sense ni tan sols dubtar, fins i tot per les coses simples de la vida que se'm fan

muntanya i per tu potser són un dilluns qualsevol. Ets una font d'alegries, anècdotes i de consol. I per tota la resta, pels meus avis, els meus tiets, que tot i que no us veig sovint mai us falta el somriure càlid i l'abraçada, per tots els cops que em pregunteu com va tot i feu un esforç per entendre el que faig, tot i que us pugui quedar lluny. Us estimo moltíssim.

I també, no acabaré aquest text sense fer un homenatge a l'altra part de la meva família, la que va a quatre grapes i parla un idioma que no és el nostre; els que sempre heu estat els meus "nens i nenes", encara que alguns (Selina, Catalina, Totoro, Molly) ja no estigueu entre nosaltres. No hi ha ni un dia en què no pensi en vosaltres.

Nola (Noleta, Nola-Bola), la gata més maca del món (no accepto discussió alguna), no hi ha ni un dia que no m'alegris. El teu ronc i parpelleig lent són els millor antidepressius naturals que tinc, la companya cridanera de les nits i els dies. Tardes senceres barallant-me amb R Studio amb tu a la falda, fins que et cansaves i decidies que ja hi havia prou i m'obligaves a separar-me de l'ordinador per fer-te petons al cap, jugar amb la corda i donar-te menjar; el més gratificant exercici de paciència que podria demanar. Em dones cert tipus de companyia que em manca en les persones, i per això els animals sou el millor que hi ha. Fins i tot la meva Bèstia personal de Monty Pyton: Kokoro. Seràs un conill de menys de dos quilos, però tens dominada a la casa sencera i no hi ha ni un dia en què et miri els ulls i no dubti pas que m'entens. Que no et caic bé no és cap

secret, però oh, és el que hi ha tu. Si Déu existeix tu n'ets la prova, perquè sé (no crec, *sé*) que ets la reencarnació de l'Anticrist; però suposo que el dia que vas néixer va haver una confusió Allà Baix i el pobre Diable va acabar en el cos d'un lagomorf i no un humà. Si ets alguna cosa, és la viva estampa de la resiliència (10 anys i seguim comptant, ets incombustible). I ja sé que no llegiràs mai aquestes paraules, però escolta, si cada matí, quan em mires fixament, puc sentir que penses "not today" i ets "només" una conilla, doncs qui soc jo per tirar la tovallola quan les coses em van malament? El missatge que prediques és clar: si viure és la teva única forma de portar la contrària al món, *fes-ho sense remordiments, carai.*

I bé, ja arriba el moment de lligar i tancar el text. M'enduc moltes coses d'aquesta experiència, i no em fa falta mirar cap enrere per saber que no soc la mateixa persona ara que fa cinc anys. Han passat moltes coses, tant dins com fora de la oficina, de les quals encara estic traient lliçons que m'estan ajudant a descobrir-me a mi mateixa. El que tinc clar és que si ara algú em preguntés si ho tornaria a fer, només acceptaria si m'asseguressin que hi seríeu tots. Gràcies a tots vosaltres, ara continc multituds.

*"Why do you go away? So that you can come back. So that you can see the place you came from with new eyes and extra colors. And the people there see you differently, too. Coming back to where you started is not the same as never leaving."*

— **Terry Pratchett, A Hat Full of Sky**

## List of abbreviations

| | |
|---|---|
| **16s rRNA** | 16S ribosomal RNA |
| **ADF** | Acid detergent fiber |
| **ADL** | Acid detergent lignin |
| **AIC** | Akaike information criterion |
| **ANCOM-BC** | Analysis of composition of microbiomes with bias correction |
| **ANOVA** | Analysis of variance |
| **ASV** | Amplicon sequence variant |
| **AST** | Aspartate aminotransferase |
| **ATP** | Adenosine triphosphate |
| **BKA** | Bacterial killing ability |
| **CAP1 / CAP2** | Constrained analysis of principal coordinates axes 1 (CAP1) and 2 (CAP2) |
| **CFU** | Colony-forming units |
| **CLR** | Centered log-ratio transformation |
| **CP** | Crude protein |
| **CUPRAC** | Cupric reducing antioxidant capacity |
| **DM** | Dry matter |
| **EDTA** | Ethylenediaminetetraacetic acid |
| **FDR** | False discovery rate |
| **FGM** | Fecal glucocorticoid metabolites |
| **FN** | Fecal nitrogen |
| **FRAP** | Ferric reducing ability of plasma |
| **GAMM** | Generalized additive mixed model |
| **GLMM** | Generalized linear mixed model |
| **HD** | High density |
| **HyD** | Hyper-density |
| **IQR** | Interquartile range |

| | |
|---|---|
| **MaAsLin2** | Microbiome Multivariable Association with Linear Models |
| **MFN** | Metabolic fecal nitrogen |
| **NDF** | Neutral detergent fiber |
| **NDVI** | Normalized difference vegetation index |
| **NEFA** | Non-esterified fatty acids |
| **NIRS** | Near infrared spectroscopy |
| **PCA** | Principal component analysis |
| **PC1 / PC2** | Principal Components 1 and 2 |
| **PCoA** | Principal coordinates analysis |
| **PERMANOVA** | Permutational multivariate analysis of variance |
| **PBS** | Phosphate-buffered saline |
| **REML** | Restricted maximum likelihood |
| **TEAC** | Trolox equivalent antioxidant capacity |
| **TEACa** | Trolox equivalent antioxidant capacity area under the curve (a) |
| **TEACh** | Trolox equivalent antioxidant capacity height |
| **TOS** | Total oxidative status |
| **TSB** | Tryptic soy broth |
| **TSS** | Total sum scaling |

# SUMMARY



*Close-up of a red deer's front legs. Photo author: vladimerech in Freepik. Used with permission under a creative commons license.*

*“Sometimes scientists change their minds. New developments cause a rethink. If this bothers you, consider how much damage is being done to the world by people for whom new developments do* not *cause a rethink.”*

— **Terry Pratchett,** **The Science of Discworld**

## Summary

Free-ranging wild herbivores play a fundamental role as keystone species, contributing to maintaining the ecosystem health of the environment they inhabit. However, during the XX century, their numbers started to increase worldwide, to the point that many populations became locally overabundant. The ecosystem effects of herbivore population overabundance have been widely studied, but less attention has been paid to the impacts it has over the overabundant species. This thesis provides an integrative assessment of how population density influences feeding behavior, gut microbiome composition, and physiological status in a free-ranging population of red deer (*Cervus elaphus*).

We first analyzed the role that physiochemical plant composition plays in determining red deer's feeding behavior (i.e. dietary selection) outside of a context of overabundance (study I). Nutritional profile was a stronger selection driver compared to physical components, where animals positively selected plants rich in fibers and crude protein, but avoided plants rich in lignin. In study II, we explored the relationship between gut microbiome composition and host physiological parameters under controlled dietary conditions. Results showed that microbial community composition and taxa turnover, rather than overall diversity, are the main predictors of host physiological status, highlighting the microbiome as a functional ecological unit linked to immune regulation and metabolic balance.

We then evaluated the effects of population overabundance in a highly seasonal environment (study III). Increased population density disrupted the previously observed patterns of feeding behavior, forcing individuals to incorporate plant species that are rich in lignin and anti-herbivory secondary components, decreasing their overall diet quality. This dietary shift was associated with increased physiological stress, indicating that behavioral adaptation to resource limitation entails measurable metabolic costs. Concurrently, in study IV we observed gut microbiome communities underwent compositional restructuring, likely reflecting functional adaptation to poorer diets. While functional redundancy may buffer some metabolic processes, shifts in microbial composition may alter key functions observed in study II, with potential consequences for immune homeostasis and oxidative balance.

Overall, population overabundance acts as a key ecological driver, generating cascading effects from diet to microbiome composition and ultimately to host physiology. Although red deer exhibit considerable behavioral and microbial plasticity, this adaptability appears to involve trade-offs that may compromise long-term physiological stability and population fitness. These findings highlight the importance of integrating ecological, physiological, and microbiological perspectives to better understand and manage wild herbivore populations.

**Keywords:** *Cervus elaphus,* overabundance, diet quality, diet composition, gastrointestinal microbiome, innate immune response

## Resum

Les poblacions d'herbívors de vida lliure juguen un paper fonamental com a espècies clau en el manteniment de la salut de l'ecosistema en el qual habiten. Durant el segle XX, el seu número va augmentar a escala global, fins a tal punt que moltes poblacions es van tornar localment sobreabundants. Els efectes de la sobreabundància sobre els ecosistemes s'han documentat de manera extensa, però s'ha prestat menys atenció a les conseqüències de la sobreabundància sobre l'espècie sobreabundant. Aquesta tesi aporta una visió integrativa sobre les relacions entre la densitat de població, el comportament alimentari, la composició del microbioma gastrointestinal i com aquest afecta l'estat fisiològic en una població salvatge de cérvol vermell (*Cervus elaphus*).

En primer lloc, analitzem el paper de la composició fisicoquímica de les plantes sobre el comportament alimentari del cérvol vermell (estudi I). La palatabilitat de les plantes va influir més que les característiques físiques sobre la selecció de la dieta. De fet, els animals van mostrar preferència per les plantes riques en fibra i proteïna bruta, i van evitar les plantes riques en lignina. En l'estudi II, explorem la relació entre la composició del microbioma intestinal i els paràmetres fisiològics en un grup de cérvols amb dieta controlada. Els resultats van mostrar que la composició de la comunitat microbiana i els canvis taxonòmics, en lloc de la diversitat global de la comunitat, són els principals predictors de l'estat

fisiològic de l'hoste, i suggereixen que el microbioma gastrointestinal actua com una unitat ecològica funcional vinculada a la regulació immunitària i l'equilibri metabòlic del seu hoste.

A continuació, avaluem els efectes de la sobrepoblació en un ambient amb marcada estacionalitat (estudi III). Les cérvoles que es van mantenir a densitats més elevades van ampliar el seu nínxol alimentari incorporant plantes menys palatables, riques en lignina i components secundaris. Aquesta adaptació va repercutir negativament en la qualitat global de la seva dieta i es va associar amb un augment de l'estrès fisiològic. Aquests resultats indiquen que l'adaptació dietària a la limitació de recursos implica un cost fisiològic mesurable. De manera simultània, en l'estudi IV observem que el microbioma intestinal es va adaptar funcionalment a dietes més pobres. A pesar que la redundància funcional pot amortir alguns processos metabòlics, els canvis en la composició microbiana poden alterar funcions clau observades en l'estudi II, amb conseqüències potencials per a l'homeòstasi immunitària, el balanç oxidatiu i la viabilitat a llarg termini de la població en el seu hàbitat natural.

En general, l'excés de població actua com un factor ecològic clau que genera efectes en cascada des de la dieta fins a la composició del microbioma i, finalment, la fisiologia de l'hoste. A pesar que els cérvols exhibeixen una plasticitat conductual i microbiana considerable, aquesta adaptabilitat sembla comprometre l'estabilitat fisiològica a llarg termini i l'aptitud biològica de la

població. Aquestes troballes destaquen la importància d'integrar perspectives ecològiques, fisiològiques i microbiològiques per a entendre i gestionar millor les poblacions d'herbívors salvatges.

**Paraules clau:** *Cervus elaphus,* sobreabundància, qualitat de la dieta, composició de la dieta, microbioma gastrointestinal, resposta immune innata

## Resumen

Las poblaciones de herbívoros de vida libre juegan un papel fundamental como especies clave en el mantenimiento de la salud del ecosistema en el que habitan. Durante el siglo XX, su número aumentó a escala global, hasta tal punto que muchas poblaciones se volvieron localmente sobreabundantes. Los efectos de la sobreabundancia sobre los ecosistemas se han documentado de forma extensa, sin embargo, se ha prestado menos atención a las consecuencias de la sobreabundancia sobre la especie sobreabundante. Esta tesis aporta una visión integrativa sobre las relaciones entre la densidad de población, el comportamiento alimentario, la composición del microbioma intestinal y el estado fisiológico en una población salvaje de ciervo rojo (*Cervus elaphus*).

En primer lugar, analizamos el papel de la composición fisicoquímica de las plantas sobre el comportamiento alimentario del ciervo rojo (estudio I). La palatabilidad de las plantas influyó más que las características físicas sobre la selección de la dieta. De hecho, los animales mostraron preferencia por las plantas ricas en fibra y proteína bruta, y evitaron las plantas ricas en lignina. En el estudio II, exploramos la relación entre la composición del microbioma intestinal y los parámetros fisiológicos del ciervo bajo condiciones controladas. Los resultados mostraron que la composición de la comunidad microbiana y los cambios taxonómicos, en lugar de la diversidad global de la comunidad, son los principales predictores del estado fisiológico del huésped, y sugieren que el microbioma

gastrointestinal actúa como una unidad ecológica funcional vinculada a la regulación inmunitaria y el equilibrio metabólico de su huésped.

A continuación, evaluamos los efectos de la sobrepoblación en un ambiente con marcada estacionalidad (estudio III). Las ciervas que se mantuvieron a densidades más elevadas ampliaron su nicho alimentario incorporando plantas menos palatables, ricas en lignina y componentes secundarios. Esta adaptación repercutió negativamente en la calidad global de su dieta y se asoció con un aumento del estrés fisiológico. Estos resultados indican que la adaptación alimenticia a la limitación de recursos implica un coste fisiológico medible. De manera simultánea, en el estudio IV observamos que el microbioma intestinal se adaptó funcionalmente a dietas más pobres. A pesar de que la redundancia funcional puede amortecer algunos procesos metabólicos, los cambios en la composición microbiana pueden alterar funciones clave observadas en el estudio II, con consecuencias potenciales para la homeostasis inmunitaria, el balance oxidativo y la viabilidad a largo plazo de la población en su hábitat natural.

En general, el exceso de población actúa como un factor ecológico clave que genera efectos en cascada desde la dieta hasta la composición del microbioma y, finalmente, la fisiología del huésped. A pesar de que los ciervos exhiben una plasticidad conductual y microbiana considerable, esta adaptabilidad parece comprometer la estabilidad fisiológica a largo plazo y la aptitud

biológica de la población. Estos hallazgos destacan la importancia de integrar perspectivas ecológicas, fisiológicas y microbiológicas para entender y gestionar mejor las poblaciones de herbívoros salvajes.

**Palabras clave:** *Cervus elaphus,* sobreabundancia, calidad de la dieta, composición de la dieta, microbioma gastrointestinal, respuesta inmune innata

# GENERAL INTRODUCTION



*Typical mediterranean landscape, where red deer thrive (Quintos de Mora, 2021). Photo author: Carlos Hernández-Castellano.*

*"Science is not about building a body of known 'facts'. It is a method for asking awkward questions and subjecting them to a reality-check, thus avoiding the human tendency to believe whatever makes us feel good"*

— **Terry Pratchett,** **The Science of Discworld**

# General introduction

## 1. Importance of wild herbivores

Wild large herbivores and Humankind's history have been intimately intertwined for thousands of years (Bartholomew & Bridsell, 1953; Hewes, 1961), commonly depicted in prehistoric cave art (Northfield, 2022). Wild herbivores are the ancestors of some of the most widespread domestic species. The auroch (*Bos primigenius*) is considered the primal ancestor of cattle *Bos taurus* (Clutton-Brock, 2014), while several different wild species of the *Ovis* genus gave rise to sheep *Ovis aries* (Mereu et al., 2025) and goats (*Capra hircus*) descend from wild bezoar populations *Capra aegragus* (Zeder, 2008). Currently, large wild herbivores are a significant source of meat in poor rural areas (Milner-Gulland et al., 2003) and provide a significant source of income through hunting and ecotourism in different parts of the world (Apollonio et al., 2010; Lindsey et al., 2007; Maciejewski & Kerley, 2014). They also have deep cultural meaning for different human collectives (Devillers & Beudels-Jamar, 2008; Ripple et al., 2016). Most large wild herbivores are ungulates, a group comprising over 400 species that have adapted to many different ecosystems, to the point that they are only absent from Antarctica (Groves & Grubb, 2011; Ripple et al., 2015).

From an ecological perspective, large herbivores play a substantial role in shaping ecosystem dynamics (Figure 1), due to their large body size and feeding behavior (Spitzer et al., 2020).

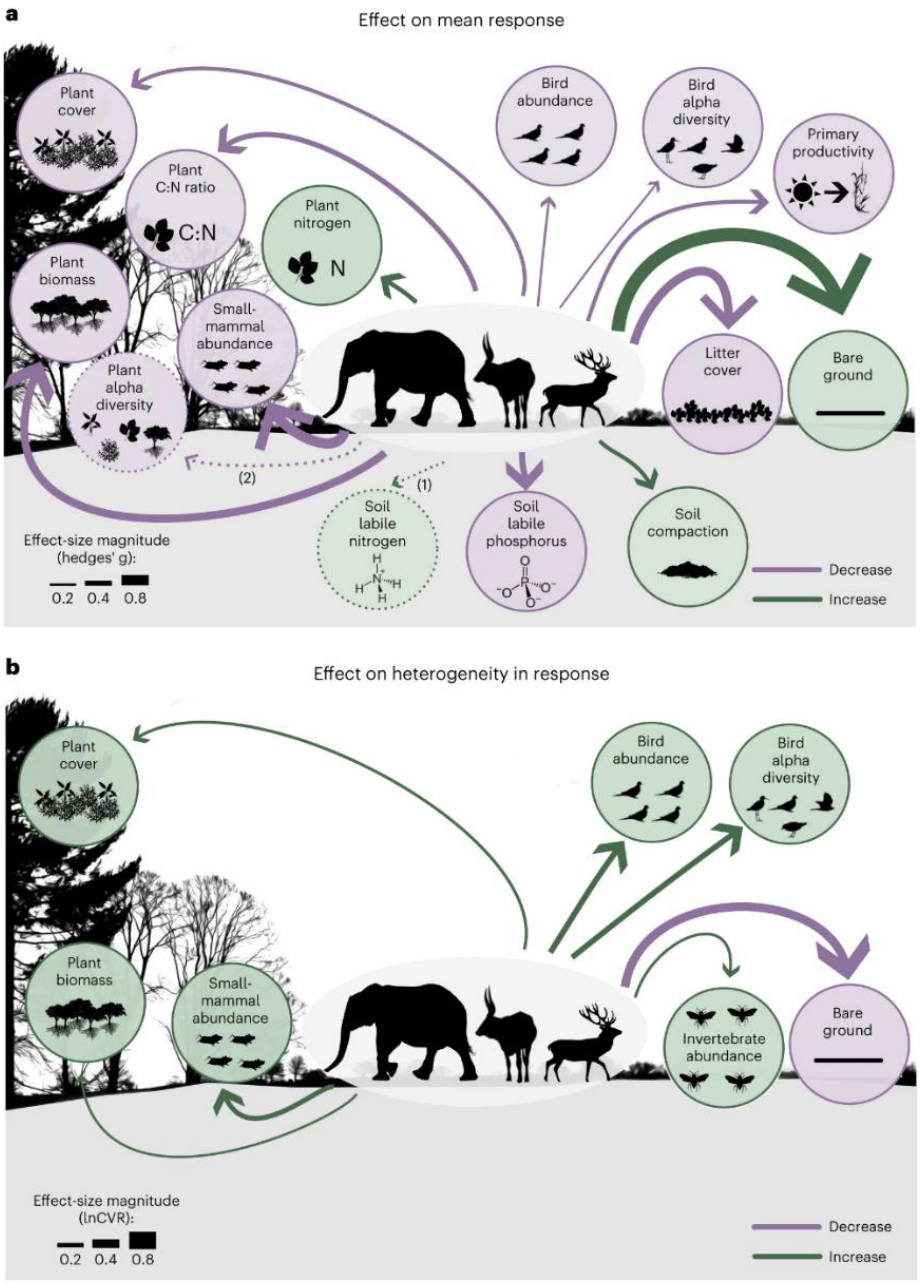



**Figure 1.** Scheme depicting the impacts of herbivore megafauna at different ecosystems levels. Original figure is obtained from Trepel et al. (2024).

There is substantial evidence of the role they play as a keystone species for most of the ecosystems they inhabit (Knapp et al., 1999; Rooney & Waller, 2003), modulating the function and species diversity found in them (Danell et al., 2006). While trampling and feeding off vegetation, large herbivores cause physical damage to plants and modify the soil, maintaining patch heterogeneity and creating recruitment opportunities for other plant species for which the unmodified habitat was not suitable (Hester et al., 2006; Ripple et al., 2015). Large herbivores also have an effect on environmental nutritional cycling. Through urination and defecation, large herbivores return nutrients such as carbon and nitrogen to the soil at faster rates than other natural processes such as decay (Hobbs, 1996; Ripple et al., 2015). The plant damage derived from dietary intake also reallocates nutrients within the plant itself and allows recruitment of other plant species which may change the rate of litter decomposition (Pastor et al., 2006). Even after death, carcass decomposition contributes to nutritional incorporation back into the environment (Doughty et al., 2013; Melis et al., 2007). Their role as seed dispersers is also noteworthy, as their large size allows them to consume more seeds and spread them over larger distances through their feces (Ripple et al., 2015). Finally, they are also an important dietary resource for many apex predators (Ripple et al., 2014). Overall, the different cascading effects that large herbivore populations can have in the environment has led ecologist to consider them “ecosystem engineers” (Danell et al., 2006; Smit & Putman, 2011), as they not only regulate ecosystem services and health (Pringle et al., 2007), but also indirectly affect the plant community, landscape heterogenicity, fire hazard and even modulate populations of smaller

species, either vertebrate (such as birds and micromammals) or invertebrate (Pringle et al., 2023; Trepel et al., 2024).

Arguably, though, the biggest contribution to ecosystem modification is caused by their feeding habits, as stated before. However, vegetation consumption not only impacts vegetation succession and landscape heterogeneity, but also affects photosynthesis, cell respiration, regeneration, resource allocation and growth ability of consumed plants (Gordon & Prins, 2008b).

Despite an increase in the popularity of "rewilding" plans, replacing these wild herbivores with livestock does not have the same effect as maintaining the original free-ranging wild populations (Pringle et al., 2023) , highlighting the importance of conservation efforts to bring back herbivore species facing extinction (Ripple et al., 2015) and managing wild populations to keep population densities at sustainable levels (Putman & Apollonio, 2014).

## 1. Herbivore classification and ruminant physiology

Large herbivores have been traditionally classified based on dietary habits or the morphological adaptations of their digestive system. Despite its limitations (Clauss et al., 2003), large herbivores have been classified into three different groups depending on their main resource use. Therefore, grazers are species that feed mostly on grasses; browsers are species that feed on woody plants and are selective of certain parts of the plant, such as soft shoots, leaves or buds; and intermediate feeders, which are those species that can feed on both woody and herbaceous plants (Hofmann, 1989).

Aside from this resource-based classification, large herbivores can also be classified according to their mechanism of digestion (Feldhamer et al., 2015). Therefore, we have hindgut fermenters, which are further divided into colonic or cecal fermenters (depending on the area of the digestive tract where most of the digestive and fermentation processes take place). Examples of colonic fermenters would include the equids, while cecal fermenters include lagomorphs and rodents (Feldhamer et al., 2015). Colon fermenters retain large particles and digest them over a long period of time, while cecal fermenters select solutes and fine particles from the caecum and discard larger particles that are more difficult to digest (Barboza et al., 2009).

The second group are called foregut fermenters or ruminants. Ruminants (order Artyodactila, suborder Ruminantia) are one of the most successful herbivore groups and make up a large proportion of the known species of wild herbivore ungulates, with more than 200 species included in this group, and with populations of around 75 million wild ruminants and 3.5 billion domesticated ruminants globally (Hackmann & Spain, 2010). The suborder Ruminantia includes Cervidae, Giraffidae, Bovidae, Antilocapridae, Moschidae and Tragulidae (Harfoot, 1981; Nowak & Paradiso, 1983). In Europe, around 90% of the territory hosts one to five wild ungulates; some of the most abundant species are roe deer (*Capreolus capreolus*), red deer (*Cervus elaphus*), fallow deer (*Dama dama*) and moose (*Alces alces*), among other non-ruminant ungulate species such as the wild boar (*Sus scrofa*) (ENETWILD-consortium et al., 2021). Therefore, they are an important factor in shaping ecosystems globally.

Anatomically speaking, ruminants have a series of physical characteristics within their digestive tract that separates them from monogastric species. Their stomach is separated in four different chambers: the rumen, reticulum, omasum and the abomasum (Figure 2, Harfoot, 1981). These compartments work together to decrease food particle size, regulate digestion flow and mix the food content with the ruminal microbial community to ferment the ingested food and get rid of the gases released during the fermentation process (Russell, 2002).

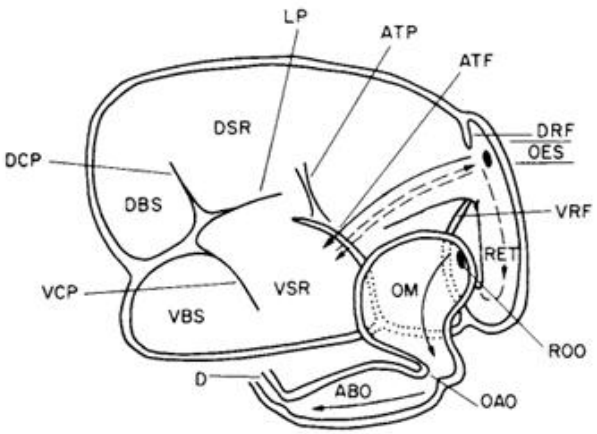



**Figure 2.** Scheme of a rumen anatomy. ABO: abomasum; ATF: anterior transverse fold; ATP: anterior transverse pillar; D: duodenum; DBS: dorsal blind sac; DCP: dorsal coronary pillar; DRF: dorsal reticular fold; DSR; dorsal sac of the rumen; LP: longitudinal pilar; OAO: omaso-abomasal orifice; OES: oesophagus; OM: omasum; RET: reticulum; ROO: reticulo-omasal orifice; VBS; ventral blind sac; VCP: ventral coronary pillar; VRF: ventral reticular fold; VSR: ventral sac of the rumen; Arrows indicate food route through the digestive tract. An intermittent arrow (dotted arrow) indicates rumination route. Image extracted from Harfoot (1981).

The abomasum is the equivalent of the “normal” stomach of non-ruminants. Food digestion in this group of animals relies heavily on fermentation by microorganisms that are found mostly in the rumen and reticulum. Additionally, ingested food goes through a second

mechanical tearing, where some of the contents of the rumen (normally the biggest particles) are regurgitated in the form of a bolus, which is mixed with saliva, re-chewed and swallowed back. This process is called “rumination” (Harfoot, 1981).

The largest compartment of the stomach is the rumen, although its relative size and total volume is species dependent. The rumen is further divided in sub-chambers by different “shelf-like pillars” found in its walls. The internal surface of the rumen is coated with different keratinized papillae that increases the absorption surface. These papillae vary in size and are strategically distributed in different areas of the rumen, with the dorsal and ventral sacs of the rumen containing the biggest papillae. The reticulum is a pouch-like structure, cranial to the rumen and connected to the esophagus, functionally indistinguishable from the rumen. It is covered in a honeycomb-like pattern of keratinized tissue. Digestive content is passed from the reticulum to the rumen to further grind and mix the ingested food with the ruminal liquid, thereby bringing the solid material into close contact with the rumen microorganisms that will further ferment the food. The omasum is connected to both the reticulum and the abomasum and propels food particles from the reticulum to the abomasum. Finally, the abomasum is the tubular part of the stomach that connects to the small intestine, and contains the secretory glands that release hydrochloric acid, pepsin and mucus, therefore carrying out the chemical digestion of the food, mimicking a non-ruminant digestion (Harfoot, 1981).

Ruminants can also be further divided, from a physiological perspective, into a gradient where one extreme is defined as “cattle-type”, and the other side of the spectrum is “moose-type” (Clauss et

al., 2003). The main differences between the two types are both the rumen volume and the stratification of the ruminal content (Figure 3). "Cattle-type" ruminants possess a bigger rumen and marked stratification of its content that allows them to digest food highly rich in fibers, such as grass. Examples of "cattle-type" species would include the European bison (*Bison monaxus*) and are commonly grazers. On the other hand, "moose-type" ruminants have a smaller rumen which lacks the content stratification present in the "cattle-type", and is normally found in browsing species, such as the red deer or roe deer, among others (Clauss et al., 2003).

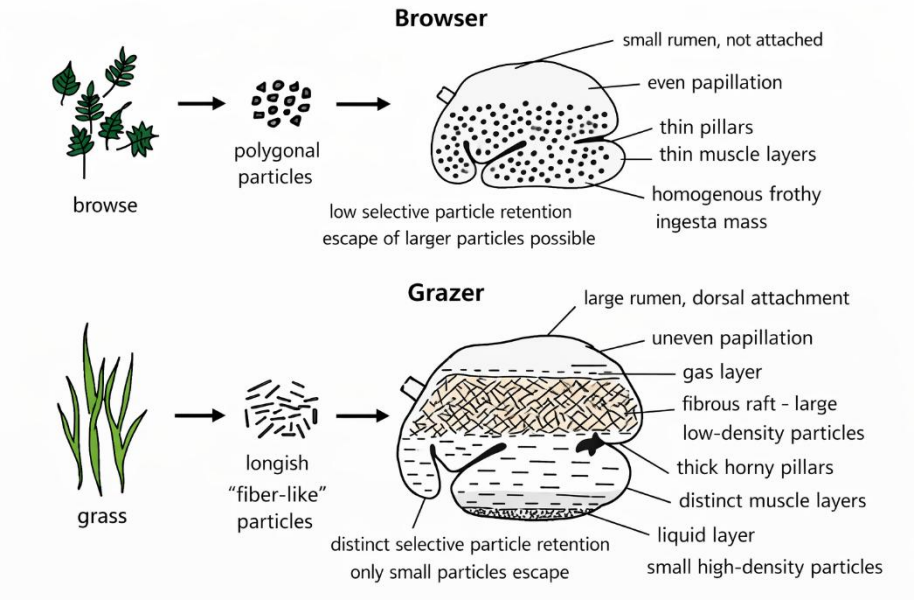



**Figure 3.** Adapted scheme showcasing the difference in rumen content stratification between browsers and grazers, based on Clauss et al. (2003). Author of the image: Emmanuel Serrano.

## 1.1 Diet and feeding behavior: what do wild ruminants eat and why should we care

Feeding behavior is defined as "the selection and ingestion of nutrients required for growth and maintenance" (Le Magnen, 1985). At the same time, feeding behavior is determined by food selection and intake (i.e. amount of food consumed, Ginane et al., 2015). Ruminants spend most of the day feeding (Llonch et al., 2018). Given the vital role they play in shaping plant communities and modifying landscapes, understanding the main drivers of diet selection in free-ranging populations of wild ungulates is crucial to further make population management decisions to optimize ecosystem conservation and restoration and ensure long-term sustainability of the managed populations.

Feeding behavior, and therefore diet selection, is shaped by both host-related and external factors. Wild herbivores have to choose from different plant species that have varying nutritional quality, known as the "foodscape" (Searle et al., 2007). At the same time, this foodscape is conditioned by the herbivore species requirements, foraging behavior, plant availability, environmental seasonality and plant-related traits such as the presence of physical and chemical anti-herbivory defense mechanisms, which determine palatability (Magni et al., 2009). At the same time, feeding generates extra energetic costs to the animal for both food acquisition and digestion. Consequently, wild herbivores need to maximize nutrient and energy intake while minimizing energy expenditure and ingestion of potentially toxic compounds, according to the "optimal foraging" theory (Ginane et al., 2015; Pyke, 1984), choosing not only plant species, but also the most nutritious parts of the plant. Since plant distribution (and thus, food availability, quality

and quantity) is not homogeneous across the landscape, animals might be forced to visit more distant feeding patches when the trade-off between energy expenditure and predation risk with diet quality is worth it (Fryxell, 1999; Searle et al., 2005). Moreover, forage quality and quantity do not only vary spatially, but also seasonally (Albon & Langvatn, 1992; Pettorelli et al., 2005). Therefore, free-ranging wild herbivores inhabiting areas subjected to high variability in resource availability and quality throughout the year may display temporal and spatial changes in diet selection to optimize energy and nutrient obtention (Zweifel-Schielly et al., 2012). To add another layer of complexity to a seemingly simple problem, individual characteristics such as animal species, bite size, physiological status, age, sex or even previous experiences can shape the feeding behavior of an individual (Froy et al., 2018; Ingvartsen & Andersen, 2000; Papachristou et al., 2005; Van Beest et al., 2011). Considering that herbivores through their feeding behavior shape plant communities and succession, it is expected that population densities also play an essential role in shaping a species foodscape. In summary, dietary selection is a complex mechanism in which many species-related and environmental-related factors intervene (Johnson, 1980; Senft et al., 1987; Zweifel-Schielly et al., 2009). However, understanding the drivers of diet selection of a species can provide crucial information to detect key dietary resources, perform habitat suitability assessments, evaluate the potential impact of food scarcity during certain parts of the year on ecosystem viability and long-term resilience of the herbivore population, or predict potential long-term impacts on biodiversity and ecosystem integrity, among others.

## 2. Individuals do contain multitudes: the gut microbiome, role in ruminant digestion and determining individual health and fitness

*The ruminant's microbiome*

The gastrointestinal (GI) microbiome is defined as the microbial community (including bacteria, archaea, protozoa, fungi and viruses) and its collective genome found in the gastrointestinal track of their host (Barko et al., 2018). Although the role that the GI microbiome plays in maintaining its host overall health and fitness is well established, the symbiotic relationship that exists between ruminants and their rumen's microbiome community is even more essential (Russell, 2002). Ruminants do not produce the needed enzymes to properly degrade complex polysaccharides found in plants (Henderson et al., 2015). Functionally speaking, the rumen acts as a big fermentation chamber, creating a microenvironment that is ideal for microbial preservation and growth (Perez et al., 2024; Russell, 2002). Ruminal communities are comprised of a wide variety of bacteria, archaea, protozoa, fungi and bacteriophages, although bacteria make up between 70% to 80% of this microbial population (Church, 1993; McAllister et al., 1994). More than 2500 bacterial species have been isolated from the rumen, and bacterial population density can be as high as $10^{10}$ cells/mL of ruminal fluid (Henderson et al., 2015; Russell, 2002). Despite these numbers, it is calculated that more than 80% of the bacterial species found in the rumen remain unidentified (Cammack et al., 2018; Henderson et al., 2015, 2019). The arrangement is mutually beneficial: on one hand, the rumen microbial population feeds off of the macronutrients found in the food ingested that allow for microbial fermentation and growth

(Hungate, 1966); on the other hand, microbial fermentation generates volatile and short chain fatty acids (VFA and SCFA), microbe-derived crude protein and macronutrients (such as vitamins) that are absorbed by the host and used as the main source of energy and micronutrients (Henderson et al., 2015; Russell, 2002). The GI microbiome also detoxifies plant secondary compounds (Dearing & Kohl, 2017). Since ruminant diets can vary in composition, the available ruminal microbiome needs to have the ability to degrade different substrates and change their metabolic pathways to quickly adapt to potential dietary changes (Perez et al., 2024). Therefore, the rumen microbiome community is made up of different functional groups: cellulolytic, amylolytic, proteolytic and methanogenic, among others (Hungate, 1966).

*Drivers of gut microbiome composition*

Despite sharing a core microbiome community, overall rumen microbiome composition differs between domestic and wild ruminants, both in terms of taxa presence and species richness and diversity (Henderson et al., 2015). Phylogenetically close species tend to have similar gut microbiome composition due to phylosimbiosis (Brooks et al., 2016; Lim & Bordenstein, 2020). Wild ruminants generally display higher species richness compared to either domesticated counterparts or domestic species (Eto et al., 2022). Several host internal and external factors shape gut microbiome communities. Sex (Kim et al., 2019), age (Worsley et al., 2024), genetics (Couch & Epps, 2022), phylogeny (Pansu et al., 2019), gut morphology (O' Donnell et al., 2017; Stevens & Hume, 1998) and even social interactions can affect the gut microbiome (Pfau et al., 2023; Zhu, 2023). Environmental-driven factors, such as

geographical area (Goertz et al., 2019) habitat disturbance (McManus et al., 2021) or environmental stress (Łopucki et al., 2025). However, one of the strongest drivers in shaping GI microbiome is diet (Ross et al., 2024; Youngblut et al., 2019). Carnivore and omnivore species tend to have poorer GI microbiome communities compared to herbivore species, and with complexity in gut morphology and food transit time (Ley et al., 2008; Stevens & Hume, 1998; Zoelzer et al., 2021). At the same time, as discussed in previous sections, diet selection can be influenced by both the landscape features and intrinsic characteristics from the individual, reaching an ecosystem-host equilibrium.

*House of cards: role of the gut microbiome in determining individual's health, fitness and population dynamics*

The gastrointestinal microbiome plays an essential role in shaping its host overall health and fitness status well beyond simply aiding food digestion. Research evidence has established links between GI microbiome and the development and modulation of immunity, maintenance of gut epithelial health, energetic and hormonal balance, metabolism homeostasis, REDOX balance, neurological development and even behavior (Cho & Blaser, 2012; McFall-Ngai et al., 2013; Semenova et al., 2024; Shreiner et al., 2015; Turnbaugh et al., 2007). Under experimental conditions, the gut microbiome has proved to affect host fitness-related factors, such as correct development, longevity and reproduction (Gould et al., 2018). Further evidence collected from free-ranging wild species suggests that gut microbiome diversity and composition at an intra and inter-population level might affect host fitness in wild animals by directly affecting their host phenotype (Lim & Bordenstein, 2020; Suzuki, 2017). The influence that

the gut microbiome has over body homeostasis may reach further than just the host and can also shape the dynamics of populations, communities, the food webs to which the species belongs to and even determine the ability of a population to survive and adapt to new conditions (McKenney et al., 2018; Qin et al., 2020; Worsley et al., 2021; Zhu et al., 2021).

Studying the functional roles of the gut microbiome and its interactions with its hosts can give essential information for conservation and management purposes. For example, gut microbiome diversity can influence the success of reintroduction programs which use animals that have been bred in captivity (Zhu et al., 2021). It has also been suggested that by monitoring and studying the gut microbiome, wildlife managers, biologists and conservationists could obtain valuable information on the overall health, stress, reproduction and finally health of free-ranging populations using non-invasive monitoring methods (Sonnega & Sheriff, 2024).

## 3. About overabundance: what is it, and what consequences does it have?

Overabundance in wild populations has been a topic that has held the interest of the scientific community for decades, particularly in the context of large herbivores. However, the term “overabundance” has a variety of definitions within the scientific literature. Ecologists may define it based on population size exceeding the carrying capacity of an ecosystem, while wildlife managers may consider a species overabundant when it leads to human-wildlife conflict or economic losses. From a conservation standpoint, overabundance may refer to

species whose population growth disrupts ecological balances or threatens biodiversity. Caughley (1981) unionized all these criteria in an effort to establish a more objective definition of when a population is considered overabundant, using both ecological and non-ecological related metrics. Therefore, Caughley (1981) proposed that a population is overabundant when it negatively impacts human lives or livelihoods; when the population density starts to have detrimental effects over the overabundant species by affecting their life history traits; when it has negative consequences over other species, or if the population density causes ecosystem dysfunction. The existence of these criteria implies that overabundance is not just a matter of reaching a certain number of individuals in the environment, but rather a label that can be bestowed upon a specific ecological context (McShea et al., 1997), varying through habitats, affected species or even during certain moments of the year.

The available literature mostly centers its efforts in determining the impacts of overabundance in the environment, and cervids have been central to research on this topic (Côté et al., 2004). Leopold (1933, 1947) already described the negative impacts of white-tailed deer (*Odocoileus virginianus*) over the vegetation in different regions of the USA, and that same concern arose quickly in Europe (Dzieciolowski, 1980). Indeed, overabundant deer can overbrowse available plant species (Donini et al., 2024; Perea et al., 2015), leading to significant damage on highly preferred plants (Rhodes & St. Clair, 2018), affecting seed production (Fattorini et al., 2026; Lecomte et al., 2016) and causing plant biomass loss (Pellerin et al., 2010; White, 2012), potentially compromising long-term viability of the plant species

population and recruitment in that area. Later on, the scope of research expanded, linking deer overabundance not only to changes in vegetation structure and forest succession (Côté et al., 2004), but also to broader ecosystem transformations, cascading effects on different animal species and even on its impact on human activities (Côté et al., 2004). These effects have been widely documented across North America, Europe, and Asia, involving a variety of cervid species (Côté et al., 2004; Suzuki, 2024).

As stated before, overabundance can also have negative consequences over the overabundant species, affecting their life history traits. Overabundant populations have been linked to a decrease from both juvenile and adult members (Clutton-Brock et al., 1987; Festa-Bianchet et al., 2003), a decrease in female fertility (Albon et al., 1983), can affect normal body development (Serrano et al., 2007, 2008) or even change the species feeding behavior and environmental resource use patterns. Mobæk et al. (2009) showed that animals that were using highly productive habitat patches later move to less productive patches when population density increases; additionally, since the environmental resources are limited, high population densities can force the animals to widen their dietary niche by incorporating low-palatable plant resources into their diet (Stewart et al., 2011), potentially negatively affecting nutritional intake (Parker et al., 2009). All these factors can be aggravated if direct competition with another species occurs or in highly seasonal environments, like the Mediterranean, in which landscapes are mainly composed of woody vegetation and highly nutritious plants are available on a very limited timeframe throughout the year (Martínez, 2002; Rueda et al., 2008).

## 4. The red deer (*Cervus elaphus* Linnaeus 1758)

*Taxonomy, distribution and population densities*

The red deer (*Cervus elaphus,* Linnaeus 1758) is a large wild ruminant from the Cervidae family (subfamily Cervinae), widely distributed throughout the Western Palearctic region and most commonly found in areas of North America, Europe and Central Asia (Mattioli et al., 2022). It has also been introduced to other areas outside of their natural range, such as South America and New Zealand for commercial purposes. In Europe, red deer is absent from Finland and Iceland, but widespread across the rest of the continent, where a total of seven subspecies can be found in different regions: *C. e. elaphus* Linnaeus, 1758*,* found in Sweden; *C. e. atlanticus* Lönnberg, 1906*,* in Norway; *C. e. scoticus* Lönnberg, 1906 in the British isles; *C. e. hippelaphus* Erxleben, 1777, the most widespread around continental Europe; *C. e. italicus* Zachos et al., 2014 in Ferrara (Italy); *C. e. corsicanus* Erxleben, 1777 found in Sardinia and Corsica; *C. e. maral* in the Caucasus, and finally *C. e. hispanicus*, also called the Iberian red deer, which inhabits the Iberian Peninsula (Mattioli et al., 2022). Between the XVI and XX centuries, European red deer populations experienced a marked decline and disappeared from historical habitat range due to anthropogenic intervention which led to habitat loss, direct competition with livestock and overhunting; however, numbers started to recover during the second half of the XX century, thanks to new hunting regulations, abandonment of the rural environment, reduction of their natural predators populations, a decrease in livestock competition and the instauration of reintroduction plans, among other factors (Mattioli et al., 2022). The highest numbers of red deer in

Europe can be found in the UK, Spain, Germany and Austria, although population densities vary depending on the region. Average red deer densities range from 2 to 10 red deer/km$^2$ (Clutton-Brock & Albon, 1989; Staines et al., 2008), but can reach values oscillating between 25 to 30 red deer/km$^2$ (Lovari et al., 2007; Torres-Porras et al., 2014). In certain areas of Spain, where red deer populations are kept artificially high for commercial exploitation purposes, densities can reach values of over 30 red deer/km$^2$ (Torres-Porras et al., 2014). Red deer typically thrive in mosaic landscapes made of open forests and grassland clearings; however, their high adaptability allows this species to populate many different regions with specific climatological and vegetation composition conditions. In Spain, red deer is widely spread throughout the territory, only absent in the Balearic and Canary Islands and some parts of Galicia (Figure 4).

*Morphology and social behavior*

The red deer is a species with marked physical and behavioral sexual dimorphism (Figure 5), with sexually mature males typically being heavier and larger than females. Both sexes sport a thick brown-greyish coat during winter, which becomes reddish and shorter during summer, and a cream-colored belly. Stags may display a neck mane that might be underdeveloped or even absent from certain subspecies, like the Iberian and Pannonian subspecies; and a black-stained belly (Mattioli et al., 2022).

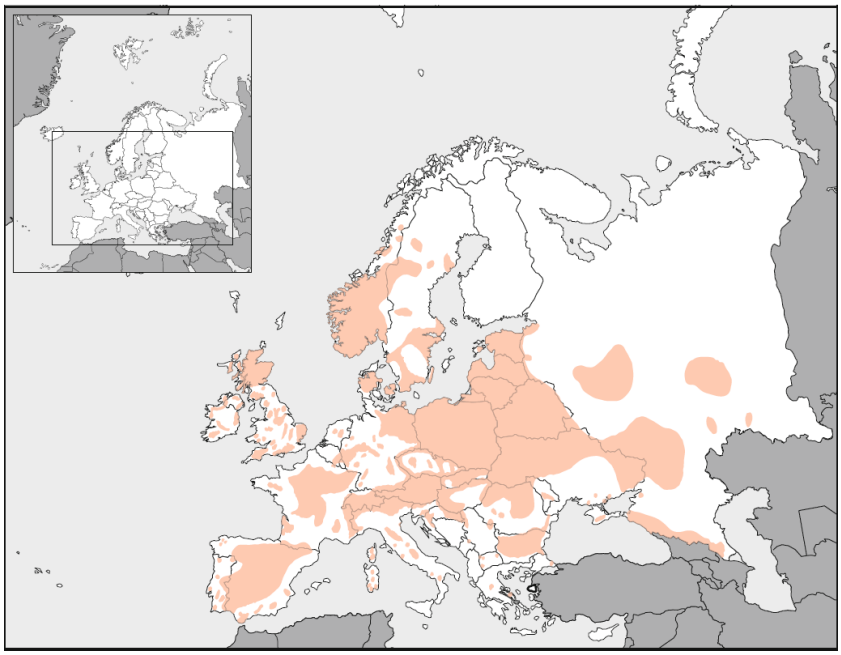


**Figure 4.** Distribution of red deer across Europe. Image extracted from Matiolli (2022).

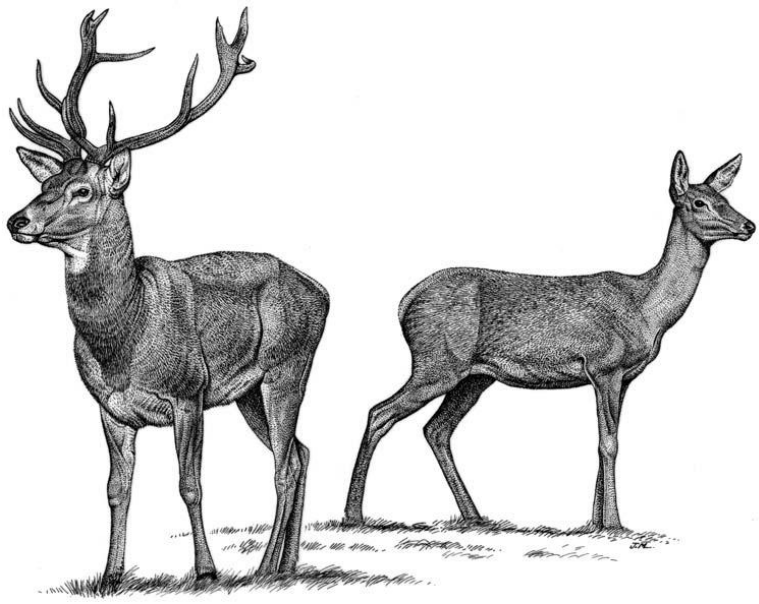


**Figure 5.** Adult male (left) and female (right) red deer. Image extracted from: Carranza (2007).

In Europe, adult males can weigh between 100 to 220 kg, while females oscillate between 55 to 130 kg (Corlatti & Zachos, 2022). The Iberian red deer veers on the smaller side, with males weighing between 80 to 160 kg, and females 50 to 100 kg (Carranza, 2007). Males reach sexual maturity around 2 to 3 years of age, while females do so around 1 to 2 years. Stags over a year of age develop antlers during pre-rutting season (January-March) that are used for territorial and mating rights fights with other males during rutting (September-October). Said antlers are cast around late April (Mattioli et al., 2022).

Both sexes remain segregated throughout most of the year and come together during rutting season (Clutton-Brock et al., 1982). During rut, stags will make harems of up to 50 does and will maintain a small territory of 12 to 14 ha, which will be used by the females to feed. Out of mating season, males tend to either form very small groups with males of similar age or be solitary, while females live in small groups made up of one older female and its descendants from the last couple of years (Carranza, 2007; Clutton-Brock & Albon, 1989). Young does tend to remain within their mother's social group until their first offspring, when they separate from the group and stay in a new home range that overlaps with the former social group. Life expectancy also varies by sex. Under wild, free-ranging conditions, stags tend to have shorter lifespans compared to females (Mysterud et al., 2001). General longevity is quite population-dependent (Mattioli et al., 2022). In the Iberian Peninsula, stags tend to live until 12 to 13 years old, while females live up to 20 years (Carranza, 2007). Due to this discrepancy in life expectancy, unmanaged wild populations tend to have a biased sex ratio towards females of 1:1.2 to 1:1.5 (Mattioli et al., 2022).

*Diet*

Although outdated, red deer have been conventionally considered "intermediate" feeders (Hofmann, 1989) based on their feeding behavior and digestive anatomy. This species displays high dietary plasticity, and in a review carried out by Gebert and Verheyden-Tixier (2001) they identified a total of 145 different plant species that included grasses, shrubs, trees, fruits and seeds. Dietary plasticity in this species is highly environmentally dependent, as red deer adapt their diet according to primary vegetation production (Anderson, 1976; Carranza, 1999), plant phenology and species availability, among other factors (Gebert & Verheyden-Tixier, 2001). Winter and summer tend to be the two critical periods for red deer regarding food availability. In winter, stags need to recover all fat reserves lost during the rutting (Bugalho & Milne, 2003; Torres-Porras et al., 2009) and they tend to feed more on grasses, as woody vegetation becomes rich in lignin and poor in soluble sugars and protein (Verheyden-Tixier et al., 2008). However, in the Mediterranean basin, where vegetation is mainly composed of woody species and grasses and forbs have a highly marked seasonality (meaning they are available for only a couple of months throughout the year), with summers characterized by very low precipitation and high temperatures, red deer act mainly as browsers (Bugalho & Milne, 2003; Casula & Murgia, 2017; Torres-Porras et al., 2009). Food volume consumption and diet composition are also significantly different between sexes and dependent on many external and internal factors, such as season and physiological stage. Males, due to their larger size, tend to eat higher volumes of food compared to females, and their larger ruminal volume allows them to be less selective and use

resources of poorer nutritional quality (Clutton-Brock et al., 1982). Food intake increases during spring and summer, which allows males to accumulate fat reserves before rutting season (when they will experience hypophagia) and females to have enough fat reserves to support pregnancy and lactation. In winter, food intake decreases, yet it is compensated for by an optimization of nutritional absorption (Arnold et al., 2015). Energy requirements of adult stags of average weight (160-185 kg) are double to non-pregnant, non-lactating females of 90-110 kg. Lactating does double their energy requirements compared to their non-lactating metabolism (Clutton-Brock et al., 1982; Jiang & Hudson, 2011; Mitchell et al., 1977).

*Red deer as a provider of ecosystem services and current situation in Europe*

Red deer play an essential role in shaping ecosystem dynamics, due to their large size and feeding behavior. Therefore, they are generally considered ecosystem engineers (Smit & Putman, 2011). Their daily food intake averages 4.5–5 kg/day of dry matter for stags and 3–3.5 kg/day for does (Kay & Staines, 1981; Mulley, 2003; Putman, 1986). This significant amount of volume intake, coupled with their browsing and grazing, shape vegetation dynamics, biodiversity and forest regeneration, as well as nutrient cycling (Fløjgaard et al., 2017; Gill, 1992; Gordon & Prins, 2008a). Red deer can also act as seed dispersers, and sustainable grazing and browsing can promote plant biodiversity, productivity and overall plant species richness (Bakker et al., 2006; Hegland et al., 2013; Schütz et al., 2003). They also help regulate predator and other wild ungulate populations, such as roe deer (*Capreolus capreolus*), alpine chamois (*Rupicapra pyrenaica*), Apennine

chamois (*Rupicapra rupicapra*) and fallow deer (*Dama* dama) through direct competition for resources (Anderwald et al., 2016; Borkowski et al., 2021; Ferretti et al., 2019). Red deer are also a key prey for large carnivores such as wolves (*Canis lupus*) and lynxes (*Lynx lynx*), overall contributing to maintaining ecosystem balance (Belardi et al., 2024; Belotti et al., 2014).

Further than that, red deer also play an important role as a cultural landmark, providing spiritual enrichment and aesthetic value to humans (Garcia et al., 2023; Kumbalsi et al., 2010; Millhäusler et al., 2016), as well as commercial exploitation through hunting and tourism (Csányi et al., 2014). However, most of the effects that red deer have on ecosystem regulation are only positive if population densities do not exceed the carrying capacity of the environment.

The current increase in red deer populations across Europe has generated different conflict scenarios with humans and caused negative consequences for the environment. Some examples include: an increase in car accidents (Apollonio et al., 2010), damage to crops and forest plantations (Corgatelli et al., 2019; Pellerin et al., 2010; Putman & Moore, 1998), biodiversity loss due to overbrowsing (Côté et al., 2004; Putman et al., 2011) and disruption of plant-pollinator biodiversity nets (Hernández-Castellano et al., 2025), among others (Rodríguez-Riaño et al., 2022).

Post-paleolithic rock art (10000-4000 BCE) found in the Iberian Peninsula reveal a part where red deer presence and human settlements and prosperity were deeply bound, with many paintings depicting the whole process of hunting this animal (Merlini, 2025). In

the present day, red deer populations are heavily managed for recreational purposes (mainly hunting for trophy and meat obtention). Therefore, in certain areas of Spain, red deer densities are kept artificially high through the use of supplementary feeding and habitat manipulation, with some areas reaching densities over 30 red deer/km$^2$ (Carpio et al., 2015; Charco García, 2002; Fernández-Olalla et al., 2006; Vicente et al., 2007) or even over 60 red deer/km$^2$ (Acevedo et al., 2008; Azorit et al., 2012), thus affecting their role as ecosystem service providers.

# HYPOTHESES AND PREDICTIONS



*A group of young stags in Quintos de Mora. Photo author: Alfonso San Miguel.*

*"Some questions should not be asked. However, someone always does.*

*'How does it work?' said Archchancellor Mustrum Ridcully, the Master of Unseen University.*

*This was the kind of question that Ponder Stibbons hated almost as much as 'How much will it cost?'*

*They were two of the hardest questions a researcher ever had to face."*

— **Terry Pratchett,** **The Science of Discworld**

## Hypotheses and predictions

The central premise of this thesis is that, under the context of population overabundance, the animals will deplete their preferred resources and thus change their feeding behavior to incorporate less preferred resources, consequently decreasing their overall diet quality. We also expect to see changes in gut microbiome composition linked to these dietary changes, by observing a shift towards species composition that would allow the animals to buffer the consequences of eating plant species richer in toxic secondary compounds and higher in indigestible fiber. We expect to see an increase in physiological stress linked to these observed changes.

From these premises, the following hypotheses, along with associated predictions, are proposed:

**Hypothesis 1:** Feeding selection in red deer is primarily determined by the physicochemical properties of plants, with individuals preferentially selecting resources that maximize nutritional gain while minimizing mechanical and digestive constraints (Study 1).

> **Prediction 1:** Highly preferred plant species will exhibit higher protein content and lower fiber fractions, particularly lignin (the indigestible fiber portion).
>
> **Prediction 2:** Red deer will preferentially consume plant species with lower breaking force.

**Hypothesis 2:** gut microbiome diversity, composition, and temporal stability are positively correlated to host physiological condition and can serve as an indicator of individual health and fitness.

**Prediction 1:** Individuals with higher antioxidant capacity and lower inflammatory status will display higher microbial diversity and greater temporal stability.

**Prediction 2:** stronger innate immune function will be associated with distinct microbial community composition and increased alpha diversity, supporting a link between microbiome structure and host physiological robustness. Given that bacterial taxa can act as pathogens, opportunists or commensals with probiotic capacity, we predict that not all microbial taxa will contribute equally to maintaining innate immunity.

**Prediction 3:** individuals in better nutritional condition would display a more diverse gut microbial community with high temporal stability.

**Hypothesis 3:** population overabundance drives density-dependent shifts in feeding behavior, leading to reduced diet quality and increased physiological stress, particularly under seasonal resource limitation.

**Prediction 1:** individuals at higher population densities will broaden their diet to include less preferred plant species, especially during periods of low primary production. This shift will be associated with stronger depletion of preferred

vegetation, reduced dietary quality, and increased physiological stress, reflected in higher fecal glucocorticoid metabolites.

**Hypothesis 4:** population density–driven changes in diet composition and quality are key drivers of gut microbiome restructuring in red deer, modulated by seasonal environmental variation, as well as increases in physiological stress.

**Prediction 1:** Gut microbiome composition will show temporal turnover associated with vegetation phenology in both herds.

**Prediction 2:** we expect to see changes in gut microbiome in both red deer herds; however, these mentioned changes will differ between populations. The gut microbiome of the herd under higher densities will experience more changes in terms of community diversity and taxa turnover compared to lower densities, linked to the more marked changes in their diet selection to compensate for faster environmental resource depletion.

**Prediction 3:** cortisol concentrations, measured as fecal glucocorticoid metabolites (FGM) will significantly influence changes in gut microbiome communities in terms of diversity metrics and taxa turnover.

# OBJECTIVES



*A mixed group of adult females, juvenile and adult males in Cabañeros National Park. Photo author: Alfonso San Miguel.*

*“Sometimes, the best answer is a more interesting question."*

— **Terry Pratchett,** **The Science of Discworld**

# Objectives

## General objective

Building upon the existing literature presented throughout the introduction, this thesis aims to help fill the potential knowledge gaps regarding the impact that population overabundance (a common scenario found in many different areas of the world) has on host-related parameters in a free-ranging wild herbivore that are essential for determining their overall fitness, such as feeding behavior, nutritional dietary quality and gut microbiome composition; as well as the links between gut microbiome and physiological parameters that regulate nutritional intake, inflammatory status and immune response. We will use the red deer as the model species, given that it is one of the most abundant free-ranging large herbivore species found in the study area (Spain) at extremely high population densities. Our goal is to bridge existing knowledge gaps: while the environmental impacts of overabundance are well documented, far less attention has been paid to its physiological consequences for the overabundant species; therefore, the underlying mechanistic explanations that might tie all these factors together remain understudied. Information regarding highly seasonal environments, such as the Mediterranean ecosystems, is even more scarce.

## Specific objectives

The specific objectives of this thesis are:

**Objective 1:**

To evaluate how plant physicochemical traits influence feeding selection in red deer (Study 1).

**Objective 2:**

To investigate the relationships between gut microbiome characteristics and red deer physiological parameters related to health and fitness (Study 2).

**Objective 3:**

To assess the effects of population overabundance on diet composition, diet quality, and physiological stress in a Mediterranean environment (Study 3).

**Objective 4:**

To determine how density-dependent dietary changes shape the temporal dynamics of the gut microbiome in red deer in a Mediterranean environment (Study 4).

# STUDIES



*Two female red deer during winter in Quintos de Mora. Photo author: PAELLA lab.*

*"A university is very much like a coral reef. It provides calm waters and food particles for delicate yet marvellously constructed organisms that could not possibly survive in the pounding surf of reality, where people ask questions like 'Is what you do of any use?' and other nonsense."*

**— Terry Pratchett,** **The Science of Discworld**

## Study I

# Role of plant nutritional value and physical traits on feeding preferences of red deer (*Cervus elaphus*) in a Mediterranean environment: a multiple-choice test

*Mammalian biology, (2026)*

## Abstract

We evaluate the role of seven Mediterranean plants' physicochemical properties on the feeding selection of female red deer with a multiple-choice feeding test. The tested species included *Brachypodium retusum*, *Cistus albidus*, *Rosmarinus officinalis*, *Thymus vulgaris*, *Juniperus oxycedrus*, *Quercus coccifera* and *Quercus ilex*. We also incorporated data from an observational field study, assessing the browsing degree of plant individuals from the same species to contrast natural feeding with our experimental results. We measured the breaking force, and chemical composition was analyzed for protein, fiber (neutral detergent fiber, acid detergent fiber and acid detergent lignin) and ash content. For the feeding test, we offered fresh shoots of the plants to five adult wild red deer females kept in captivity in the Muela de Cortes Game Reserve (Spain). Plant species varied in mechanical resistance, fiber and crude protein contents mainly. Red deer preferred to feed on *Quercus* spp. and *Cistus albidus* plants while rejecting *R. officinalis* and *T. vulgaris*. These experimental results matched accurately our browsing degree observations in the field. Fiber, protein content and ash were the key drivers of their feeding behaviors, suggesting that chemical composition, rather than physical characteristics, guides deer's feeding choices. Understanding how plant characteristics influence the feeding behavior of red deer can provide valuable insights into their impact on Mediterranean vegetation.

## 1. Introduction

Herbivores are crucial in shaping plant communities through different mechanisms, including diet selection, defined as the behavioral process by which animals choose specific types or parts of available plants to consume (Huntly, 1991; Newman et al., 1995). Feeding behaviors, and thus diet selection, are not only determined by the environmental conditions and the energetic requirements status of the animal, but also by the mechanical and chemical defenses of plants (Magni et al., 2009b). Consequently, when multiple food resources are available, herbivores must choose which plant species to consume to achieve the optimal combination of nutrients while minimizing the intake of anti-nutritional or toxic compounds (Ginane et al., 2015). Therefore, free-ranging large herbivores living in landscapes subjected to great changes in resource dynamics may display spatial and temporal variations in diet selection to fulfill their energetic requirements (Zweifel-Schielly et al., 2012). For example, reindeer (*Rangifer tarandus*) living in tundra habitats, store body reserves to survive in winter feeding on protein-rich herbs and grasses during summer (Klein, 1990; Parker et al., 2009) . During winter, however, the energetic requirements of reindeer are supplied thanks to lichens rich in soluble carbohydrates but very poor in proteins (Heggberget et al., 2002). Along the same lines, ungulates living in Alpine ecosystems may browse on fiber-rich woody plants during the winter but on protein-rich grasses during the summer (Espunyes et al., 2019; Villamuelas et al., 2016; Zweifel-Schielly et al., 2012). In other seasonal

ecosystems where summer limits food availability, such as the Mediterranean (Archibold, 1995), ungulates have a short period to feed on the nutritious grasses (Bugalho & Milne, 2003), being the evergreen woody vegetation the main food resource (Bugalho et al., 2001; Martínez, 2009; Rossiter, 1966). Which factors determine feeding selection during this food limited or rich periods are still being determined for most ungulate species.

Different chemical plant components influence herbivore forage selection and plant palatability (the pleasure derived from food). Nutrients are one of these key chemical factors, especially protein content, mineral profile and fiber, both in quantity and composition of cellulose, hemicellulose and lignin (Cooper et al., 1988; Van, 2006; Van Soest, 1994). Herbivores usually prefer plants with high gross energy, protein, and phosphorus content, but low in fibers and essential oils (Caballero García de Arevalo, 1985). Secondary metabolites (mainly alkaloids, terpenoids and phenyl propanoids) determine plant toxicity and digestibility of plants and thus, palatability (Cooper et al., 1988; Van, 2006; Van Soest, 1994) . Plant morphology and the presence of prickling or sticky structures (spikes, thorns, prickles, specialized trichomes or resin, among others), also drive ungulate consumption (Lev-Yadun, 2021). Moreover, diet choice may be affected by the ease of apprehension of different plant species, which is associated with physical attributes like dry matter content, particle size or resistance to fracture (Van, 2006). Bite size also regulates feeding selection, since animals commonly prefer those plants or parts that allow bigger

bites (Papachristou et al., 2005). The combination of the previously mentioned determinants makes it hard to understand the main drivers of herbivore diet selection and forecast the impact of ungulate populations on ecosystem integrity (Côté et al., 2004).

Most works studying ruminant diet selection have been based on the analysis of fecal or rumen contents. This approach, however, is biased by differences in plant digestibility and the difficulty of identifying vegetal fragments (Gaare et al., 1977; Putman, 1984). Now, DNA metabarcoding techniques overcome this problem, but they are still very expensive and are not free of bias inherent to the methodology (Espunyes et al., 2019). Therefore, free-choice experiments presenting animals with a variety of feed, also known as cafeteria trials, are very useful tools in investigating diet selection directly. Such experiments are typically conducted on domestic animals, like sheep (*Ovis orientalis aries*), goats (*Capra aegagrus hircus*), horses (*Equus caballus*) or donkeys (*Equus asinus*) (Bartolomé et al., 2020; Illius et al., 1999; Meier et al., 2013; Salem et al., 1994; van den Berg et al., 2016). In contrast, trials involving wild herbivores are scarce with few examples such as fallow deer (*Dama dama*) and white-tailed deer *Odocoileus virginianus* (Averill et al., 2016; Bergvall, 2007) .

The red deer (*Cervus elaphus,* Linnaeus 1758) is a large herbivore that has experienced a steady population increase across Europe during the last century, making it a key species shaping European ecosystems through herbivory pressure (Milner et al., 2006).

Previous studies have shown that red deer can quickly adapt and change their feeding habits according to the availability of high-quality food (Hofmann, 1988), as they have a limited ability to digest it. However, the red deer diet can be highly diverse and varies across habitats, environmental conditions, and food availability (Gebert & Verheyden-Tixier, 2001). In a review of studies based on rumen contents, Gebert and Verheyden-Tixier (2001) observed that red deer can feed on at least 145 plant species, ranging from grasses and sedges to shrubs, fruits or seeds. The importance of each of the mentioned factors on red deer diet selection may vary across ecosystems. Forsyth et al. (2005) analyzed the relationship between the chemical traits of leaves and the diet selection of red deer in New Zealand and found that deer prefer to feed on plants with low fiber content. On the other hand, Pollock et al. (2007) found that leaf areas and stem strengths, rather than chemical components, determined the feeding selection of red deer. Therefore, diet selection by red deer has previously been described using observational and analytical methods in different environments, but there is a lack of studies under controlled conditions.

The primary purpose of this study is to evaluate the role of both physical and chemical characteristics of Mediterranean plants in the feeding selection of this herbivore. The objectives of this research are, on one hand, to assess the feeding preferences of red deer in a multiple-choice test and, on the other, to establish a correlation between red deer feeding preferences and plant nutritional composition (nitrogen contents and NDF, ADF and ADL fiber

fractions) and breaking force. Additionally, we integrated field data to assess whether red deer browsing intensity matches the preferences seen in the feeding trial. The main hypothesis of our research is that the chemical and physical traits of plant species affect deer feeding selection. We predict that highly preferred species will be rich in proteins but poor in fibers, particularly lignin. We also expect red deer to prefer plant species with lower breaking force to minimize mastication effort. Finally, we predict that the preference patterns observed in the cafeteria test will be mirrored by browsing intensity data collected in the field.

This study will provide an opportunity to better understand the determinants of diet selection in a wild ruminant (red deer) inhabiting an ecosystem subjected to a highly seasonal environment with periods of food restriction and to predict the potential impact of changes in plant communities.

## 2. Materials and methods

*Study area*

We conducted this study in Muela de Cortes (39° 07’ 21’’ N, 0° 49’ 60’’ W), a limestone massif located in the south-west of the Valencian Community, at the eastern side of the Iberian Peninsula. The topography is highly heterogeneous, encompassing plateaus, ravines, and karst fields that occur across a broad altitudinal range, from 300 up to 1000 meters above sea level at the Caroche massif.

The landscape is mainly dominated by Mediterranean sclerophyllous conifer and broadleaved forests, shrublands, and pine plantations with scattered herbaceous and woody field crops in the lowlands. Plant communities in Muela de Cortes are widely distributed in the Iberian Peninsula (Loidi, 2017) .

Pine forests (*Pinus nigra* and *Pinus halepensis*) and holm oak forests (*Quercus rotundifolia*) are accompanied by sclerophyllous shrublands dominated by *Quercus coccifera* and *Salvia rosmarinus*, together with other evergreen shrubs such as *Chamaerops humilis*, *Erica multiflora* and *Pistacia lentiscus*, and the perennial grass *Brachypodium retusum*.

*Multiple choice test*

In June 2021, we performed a multiple-choice (cafeteria) feeding test on a herd of five adult female red deer with no known health issues. We kept the herd in a 200m$^2$ enclosure with access to water, wheat straw and alfalfa (*Medicago sativa*) *ad libitum*. The herd went through a pre-adaptation period before being formally subjected to the test. During this pre-adaptation period, we provided a combination of pellets and dried alfalfa in hay format. Each plant was presented to the animals in separate buckets which were fixed to the ground and 2 meters away from each other. All plants were presented at the same time to the animals. An operator checked that the red deer had eaten the food offered. Once the herd was adapted to the setting, we placed ~500 g of the test species without any additional food, in line with previous research (Alm et al., 2002;

Bartolomé et al., 2020; Ginane et al., 2005) . Selected plants for the experiment were collected daily in the area during the morning by cutting branches of individual plants. Each test lasted ~11h on average (6h min, 14h max), after which we measured the percentage of food consumed [(offered biomass – rejected biomass)/offered biomass x100]. Each plant item was randomly placed in a new bucket after each trial to avoid any bias due to its location. We also set up two camera traps to check the lack of food spilling or other events. The feeding test was repeated six times. We placed a sample of 150g of each plant species in the enclosure outside the deer range and weighed it at the beginning and end of each cafeteria trial to correct for weight loss by dehydration.

The plants offered to the deer during this multiple-choice test included: *Brachypodium retusum* (Br)*, Cistus albidus* (Ca)*, Juniperus oxycedrus* (Jo), *Quercus coccifera* (Qc)*, Quercus ilex* (Qi)*, Rosmarinus officinalis* (Ro), and *Thymus vulgaris* (Tv). Plant species selection was based in relative abundance of each plant species in the area; description of the presence of these species and/or the species genus in red deer diet, and presence of secondary compounds in the plant species (Table S1).

*Browsing intensity in the field*

The browsing degree of the same plant species was recorded in two enclosures of 7 ha each, hosting two herds of female red deer of 6 to 12 individuals, each herd kept in captivity from June 2021 to July 2022 in the same Muela de Cortes area. The browsing intensity was

assessed using a browsing degree following Perea et al. (2015). Briefly, the scale comprises six categories ranging from 0 to 5, where 0 corresponds to no browsing evidence and 5 to maximum browsing evidence. The data were collected from a total of 287 plants (47 Br, 44 Ca, 21 Jo, 33 Qc, 46 Qi, 47 Ro and 46 Tv) that were surveyed individually in early summer (July 2021) when browse becomes the main food source for deer (Perea et al., 2014).

*Sampling procedure for physicochemical analyses*

Between March and April 2021 we collected 5-cm length samples of terminal shoots of the test species. We collected 91 samples (13 samples per plant species) for measuring the breaking force, defined as the force of fracture that herbivores must exert to cut down the terminal shoots; and 40 (6 Br, 5 Ca, 6 Jo, 5 Qc, 6 Qi, 6 Ro, 6 Tv) for analyzing forage quality. The samples were pre-dried at 60ºC and stored until processing. We measured the breaking force using a TA-XT texturometer attached to Volodekevich bite jaws. Speed was set at 5 mm/s, with the jaws descending until the plant sample was either broken or pressed as far as possible. For each plant sample, we measured three different twigs, performing five equidistant measures alongside each twig, from the basal to the apical end.

Regarding forage quality, samples were milled through a 0.5mm screen, and the chemical analyses were carried out twice according to the AOAC (AOAC, 2005). Thus, DM was determined at 103 °C for 24 h, and ashes were burnt at 550 ◦C for 5 h. Only 0.2 g was used to

determine nitrogen content by combustion, using a LECO CN928 Carbon/Nitrogen Macro Determinator (LECO Instrumentos S.L., Madrid, Spain). Crude Protein was calculated as N x 6.25 and corrected according to Müller (2017).

The rest of the sample was analyzed following the Van Soest method (Van Soest, 1994) to measure plant cell-wall composition, using an Ankom 200 Fiber Analyzer (ANKOM Technology Corporation, 225 Macedon, NY, USA). Neutral detergent fiber (NDF), acid detergent fiber (ADF) and acid detergent lignin (ADL) were sequentially determined on an ash-free basis according to Van Soest et al. (1991) (Van Soest et al., 1991). Hemicellulose content was estimated as the difference between NDF and ADF, and cellulose as the difference between ADF and LDF. Lignin was estimated as the difference between the LDF fraction and the ashes after calcination. Our “minerals” variable corresponds to the sample’s ash content. All values were standardized by percentage of dry matter (DM) content.

*Statistical analysis*

All statistical analyses were carried out using R Studio, version 4.4.1 software (R Core Team, 2024). We performed separate analyses for consumption preferences, breaking force and plant physicochemical traits. Due to the moderate sample size of the experiment, to test differences in species consumption, we ran 1000 bootstrapped simulations of a GLMM including intake percentage as the response variable, species as an explanatory variable, and

cafeteria test number as a random factor. We used the "boot" package (Canty & Ripley, 2024; Davison & Hinkley, 1997). Differences in the mean values of chemical traits (NDF, ADF, ADL, minerals, cellulose, lignin and crude protein) were assessed via ANOVA followed by Tukey's HSD; hemicellulose was analyzed with Kruskal-Wallis and Dunn's tests. We used packages including "dplyr" (Wickham et al., 2023), "car" (Fox & Weisberg, 2019), "FSA" (Ogle et al., 2023) and "multcompView" (Graves et al., 2024).

Breaking force differences among plant species were analyzed using a generalized linear mixed model (GLMM) with a Gamma distribution with logarithmic link function, where species was included as a fixed factor, and individual samples and twig number within the sample as random effects. Significance of random effects was formally tested using restricted maximum likelihood (REML) method and we applied a Likelihood Ratio test for random effects. We validated the model following Zuur et al. (2009b) recommendations and plotted the residuals against fitted values (Fig. S1a) to evaluate homoscedasticity and make sure residuals did not display any systematic pattern; Q-Q plots (Fig. S1b) and histograms (Fig. S1c) to further assess residual distribution. We also applied Tukey's HSD test to see if there were significant differences between the mean value of breaking force for each pair of species. We used the "lme4" (Bates et al., 2015), "multcomp" (Hothorn et al., 2008) and "MuMIn" (Bartoń, 2024) packages to fit the model and extract marginal and conditional $R^2$ values.

To explore the physicochemical characteristics determining feeding selection, we performed two different Principal Component Analyses (PCA) on the Breaking Force (N) and cellular contents (%) of minerals, hemicellulose, cellulose, lignin and crude protein. We used plant species and plant preference as supplementary qualitative variables in the PCAs. Plant preference was calculated using Savage's Index of preference (Delgado et al., 2017) and each species was classified as positive selected (P), not selected (NS) and negatively selected (N). A $\chi^2$ statistic was used to compare the different Savage Index values. The first PCA included all the plant species, while the second excluded *B. retusum,* as it is the only herbaceous plant and therefore its physiochemical profile was different from the other woody species. The goal of this second PCA was to better understand variation among the woody species' profiles and their associated dietary preferences.

For each PCA, we extracted PC1 and PC2 scores and assessed their normality by checking their skewness and kurtosis. We then estimated differences among score means for the supplementary qualitative variables "Species" and "Preference" using an ANOVA test and Tukey's HSD as the post-hoc test, if applicable. We used the R package "missMDA" (Josse & Husson, 2016) to calculate the missing values and the packages "FactoMineR" (Lê et al., 2008) and "factoextra" (Kassambara & Mundt, 2017) to perform the PCA.

## 3. Results

*Multiple choice feeding test*

The consumption of all plant species, except *J. oxycedrus,* significantly varied (Table S2). Deer showed a clear preference for *Q. ilex* and *C. albidus* in all cafeteria trials (Fig. S2), while they avoided feeding on the Lamiaceae plants *R. officinalis* and *T. vulgaris*, eating less than 10% of the total quantity offered in the trials. *B. retusum* intake varied between trials, although its consumption remained medium or low in most of the trials (Fig. S2), with its mean intake being significantly lower than *C. albidus* and *Q. ilex. J. oxycedrus* had a higher mean intake value than *Rosmarinus* and *Thymus* but was not statistically different (Table 1).

*Browsing degree in the field*

None of the surveyed plants exhibited browsing levels above 4 (Table S3). No individuals of *Brachypodium retusum*, *Juniperus oxycedrus*, *Rosmarinus officinalis*, and *Thymus vulgaris* showed no signs of browsing (Table S3). The other species showed browsing indices from 0 to 4, with some interspecific variation (Table S3).

*Breaking force*

Our chosen model explained 75.7% of the observed variance in our data, with random effects accounting for a 34.1% of that observed variance (Table S4). *Q. coccifera* showed the highest breaking force values (Table 1), but also the highest variation in this attribute. *B. retusum* and *J. oxycedrus* showed a significantly lower breaking

force than their counterparts (Table 1). *C. albidus, Q. ilex, R. officinalis* and *T. vulgaris* showed intermediate values in breaking force without statistically significant differences between them (Table 1).

*Forage quality*

NDF was highest in *B. retusum*, while *J. oxycedrus, R. officinalis* and *C. albidus* had the lowest percentage of the NDF fraction (Table 1). ADF was highest, on average, in *T. vulgaris* and in *B. retusum*. The Acid Detergent Lignin ADL fraction was highest in *T. vulgaris* and lowest in *B. retusum*. Regarding hemicellulose, cellulose, and lignin levels, both *Brachypodium* and *T. vulgaris* significantly had the highest values of cellulose content (Table 1). *Thymus* showed the highest values of lignin content, with both species of *Quercus* and *R. officinalis* having lower lignification on average, followed by *C. albidus* (Table 1).

Crude protein contents did not differ between species. Nevertheless, on average *C. albidus* and *Q. ilex* had a higher percentage of crude protein than the rest of the plants (Table 1). Finally, *B. retusum* ash concentration was the highest of all the tested species, and significantly different from the other plant species. *Q. ilex* was the species with the lowest ash content, followed by *Q. coccifera,* while *R. officinalis* had the highest mineral content among the woody plants. However, the mean ash content was not significantly different among woody species (Table 1).

Regarding our PCA results, each PCA explains 66.72% and 64.66% of the total variance, respectively. In both cases, the dimension (PC1) is primarily associated with cell wall composition—especially hemicellulose and cellulose—albeit their contributions vary between PCAs (Table S5). Notably, lignin and breaking force contribute similarly to both PC1 and PC2 (second dimension) in each PCA.

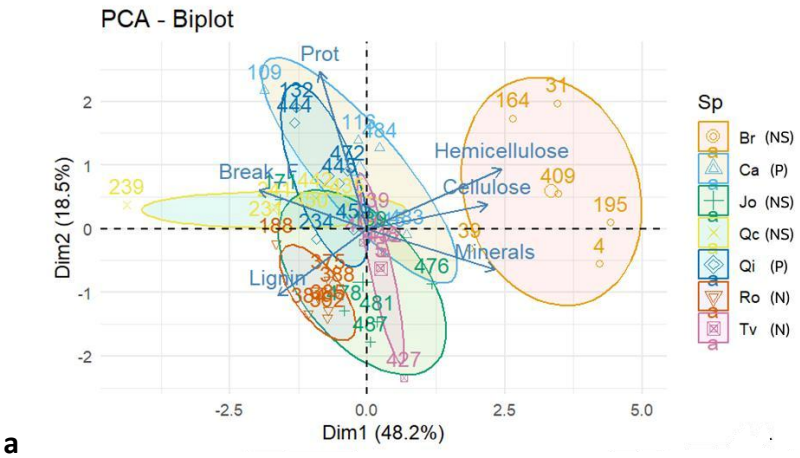
**Table 1.** Feed intake, breaking force (B Force) and nutrient composition (NDF, ADF, ADL, minerals, hemicellulose "Hemicell", cellulose, lignin and crude protein (CP)) of the seven Mediterranean plants used in a multiple-choice test in red deer. Data are displayed on a dry matter basis (%). Super index letters indicate the statistically significant differences among each plant species values for each variable after performing the ANOVA and Tukey's HSD test or Kruskal-Wallis and post-hoc Dunn test.

| Sp | Intake (%) | B Force (N) | NDF (%) | ADF (%) | ADL (%) | Minerals (%) | Hemicell (%) | Cellulose (%) | Lignin (%) | CP (%) |
|---|---|---|---|---|---|---|---|---|---|---|
| **Br** | 21.17 ± 11.10$^{ab}$ (0.00 - 66.95) | 34.99 ± 3.60$^{c}$ (11.60 - 54.65) | 72.50 ± 1.76$^{a}$ (66.64 - 76.30) | 36.19 ± 1.59$^{ab}$ (31.97 - 41.28) | 4.64 ± 0.49$^{d}$ (3.30 - 6.13) | 8.68 ± 1.28$^{a}$ (5.41 - 12.97) | 36.31 ± 0.65$^{a}$ (34.67 - 37.76) | 31.55 ± 1.10$^{a}$ (28.67 - 35.15) | 0.00 ± 0.00$^{d}$ (0.00 - 0.00) | 6.27 ± 0.50$^{a}$ (4.47 - 7.98) |
| **Ca** | 69.43 ± 2.43$^{c}$ (63.91 - 79.82) | 84.43 ± 6.25$^{a}$ (58.39 - 139.90) | 38.94 ± 1.43$^{c}$ (35.69 - 43.30) | 26.23 ± 1.13$^{b}$ (24.28 - 30.05) | 8.50 ± 1.35$^{cd}$ (5.50 - 12.76) | 5.37 ± 1.05$^{b}$ (1.32 - 7.12) | 12.71 ± 0.40$^{a}$ (11.41 - 13.52) | 28.81 ± 0.40$^{b}$ (16.59 - 18.80) | 3.25 ± 1.71$^{cd}$ (0.00 - 8.72) | 7.90 ± 0.72$^{a}$ (6.27 - 9.78) |
| **Jo** | 23.08 ± 11.57$^{b}$ (1.13 - 66.57) | 33.91 ± 2.83$^{c}$ (12.54 - 47.52) | 32.62 ± 7.28$^{c}$ (10.86 - 41.18) | 25.57 ± 6.00$^{b}$ (7.67 - 32.90) | 10.64 ± 2.43$^{bc}$ (3.41 - 13.77) | 5.41 ± 0.43$^{b}$ (4.34 - 6.46) | 7.04 ± 1.28$^{a}$ (3.20 - 8.42) | 14.93 ± 3.57$^{b}$ (4.26 - 19.12) | 5.72 ± 2.01$^{bc}$ (0.00 - 9.44) | 5.91 ± 0.38$^{a}$ (4.83 - 7.40) |
| **Qc** | 44.7 ± 5.50$^{c}$ (27.23 - 63.40) | 147.41 ± 23.05$^{b}$ (65.81 - 312.53) | 45.45 ± 3.41$^{bc}$ (31.88 - 50.25) | 30.30 ± 3.19$^{b}$ (17.63 - 34.19) | 12.24 ± 0.12$^{bc}$ (11.85 - 12.51) | 3.61 ± 0.26$^{b}$ (2.82 - 4.45) | 15.15 ± 0.44$^{a}$ (14.25 - 16.35) | 18.07 ± 3.24$^{b}$ (5.17 - 22.06) | 8.63 ± 0.22$^{ab}$ (8.06 - 9.31) | 6.77 ± 0.29$^{a}$ (5.69 - 7.38) |
| **Qi** | 74.82 ± 3.21$^{cd}$ (67.48 - 84.77) | 82.58 ± 8.99$^{a}$ (46.39 - 148.80) | 42.90 ± 3.23$^{c}$ (32.71 - 46.60) | 28.44 ± 3.05$^{b}$ (21.94 - 31.49) | 9.37 ± 0.33$^{bc}$ (6.90 - 10.74) | 3.28 ± 0.24$^{b}$ (2.94 - 3.64) | 14.46 ± 0.93$^{a}$ (10.77 - 15.72) | 19.07 ± 1.10$^{b}$ (15.05 - 21.00) | 8.33 ± 0.20$^{bc}$ (3.29 - 7.80) | 7.56 ± 0.49$^{a}$ (5.95 - 9.13) |
| **Ro** | 2.07 ± 1.15$^{a}$ (0.00 - 6.88) | 102.91 ± 9.39$^{ab}$ (42.17 - 149.89) | 34.35 ± 1.10$^{c}$ (31.02 - 38.68) | 26.76 ± 0.81$^{b}$ (24.13 - 29.63) | 13.62 ± 0.43$^{ab}$ (12.18 - 15.36) | 5.78 ± 0.19$^{b}$ (4.95 - 6.17) | 7.59 ± 0.34$^{a}$ (6.90 - 9.06) | 13.13 ± 0.66$^{b}$ (10.89 - 15.84) | 7.84 ± 0.51$^{ab}$ (6.01 - 9.46) | 6.03 ± 0.21$^{a}$ (5.48 - 6.94) |
| **Tv** | 2.25 ± 1.29$^{a}$ (0.00 - 6.84) | 79.91 ± 12.04$^{a}$ (34.02 - 179.93) | 57.71 ± 1.98$^{b}$ (52.21 - 65. 32) | 43.88 ± 1.68$^{a}$ (39.02 - 50.11) | 16.68 ± 0.95$^{a}$ (13.94 - 20.39) | 5.43 ± 0.14$^{b}$ (4.96 - 5.98) | 13.83 ± 0.32$^{a}$ (12.99 - 15.21) | 27.20 ± 0.76$^{a}$ (24.59 - 29.72) | 11.25 ± 1.06$^{a}$ (7.97 - 15.43) | 6.83 ± 0.71$^{a}$ (4.09 - 9.48) |

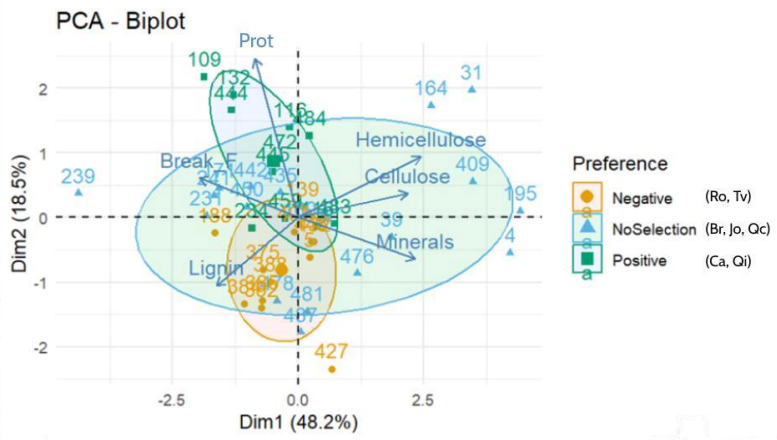
Species negatively selected by red deer, such as *R. officinalis* and *T. vulgaris*, are linked to PC1, especially in the woody-plant PCA (Figures 2a and 2b). *T. vulgaris* exhibits the highest lignin and cell wall content alongside low crude protein, while *R. officinalis* stands out for its high mineral (ash) content. When *B. retusum* is included (Figure 1b), crude protein and lignin are the main variability contributors to PC2; however, removing this herbaceous species shifts PC2 to be more influenced by minerals, crude protein and breaking force (in that order). Species positively selected by red deer such as *C. albidus* and *Q. ilex* remain strongly associated with this second dimension in both PCAs (Figure 1a and Figure 2a), and they are the ones with the highest values of crude protein and breaking force. The rest of the non-selected species, such as *Q. coccifera*, *J. oxycedrus* and *B. retusum* do not display a clear association to any dimension. *B. retusum* has a unique profile with elevated hemicellulose, cellulose, and mineral levels compared to the woody species (Figure 1b).

Figure 1b also shows that selection categories align differently along PCA axes: highly preferred species are associated with higher crude protein content across PC2, while rejected species align with higher lignin and lower protein. Moderately or weakly preferred species tend to vary along PC1, with negatively selected species are mostly located towards the positive side of PC1, which is related to a higher amount of minerals, cellulose, and hemicellulose; while species of a moderate preference rank are closer to the coordinates influenced

by breaking force. These patterns mostly remain even after excluding *B. retusum* (Figure 2b).

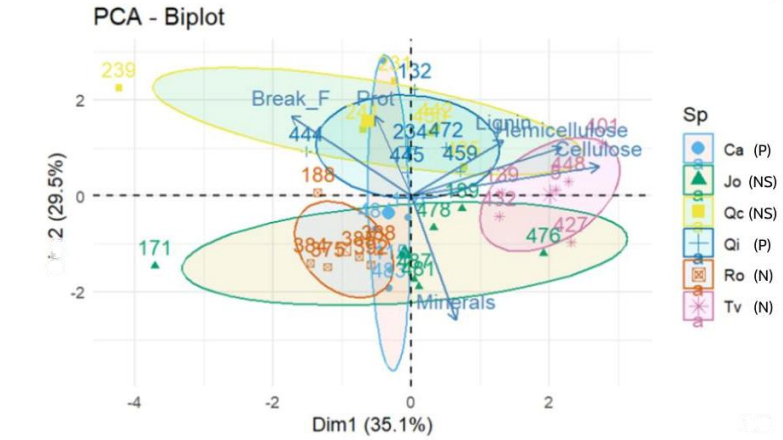



**a**

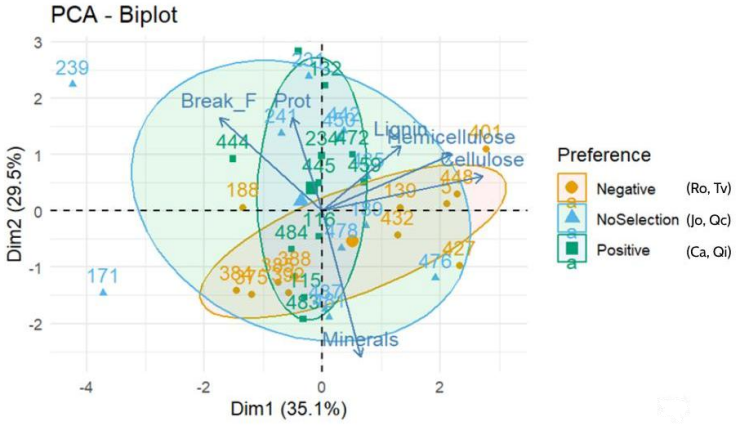



**b**

**Figure 1.** PCA biplots showing the differences in physiochemical characteristics (a) between the seven plant species (Br: *Brachypodium retusum*; Ca: *Cistus albidus*; Jo: *Juniperus oxycedrus*; Qc: *Quercus coccifera*; Qi: *Quercus ilex*; Ro: *Rosmarinus officinalis*; Tv: *Thymus vulgaris*) offered to red deer in a multiple-choice feeding test; and how physiochemical characteristics affected red deer preference (b).




**a**




**b**

**Figure 2** PCA biplots showing the differences in physiochemical characteristics (a) after excluding *B. retusum* (Ca: *Cistus albidus*; Jo: *Juniperus oxycedrus*; Qc: *Quercus coccifera*; Qi: *Quercus ilex*; Ro: *Rosmarinus officinalis*; Tv: *Thymus vulgaris*); and how physiochemical characteristics affected red deer preference (b).

## 4. Discussion

Physicochemical rather than physical traits of our sample of mediterranean plants drove the feeding preferences of female red deer. The consumed plants in both field and controlled conditions were *Q. ilex* and *C. albidus*, which on average had a higher percentage of crude protein than the rest of the plants. Other softer plants with high fiber contents were rejected or moderately consumed, as we have observed in the field and in the cafeteria test. The high preference for *Q. ilex* is consistent with the findings of different authors who have conducted studies analyzing red deer diet in Mediterranean environments (Filella et al., 2024; Martínez, 2009; Miranda et al., 2012). For *C. albidus,* results were slightly different, as monitored individuals were found either unbrowsed, probably because they remained undetected, or completely consumed. Indeed, 54.54% of the plants had a level 0 of browsing degree, while the remaining 45.45% of plants had the highest recorded degree of browsing, with no individuals falling in any of the intermediate browsing degree values. This slight incongruence in the observed browsing results could come from the external factors linked to the natural environment, such as plant coverage, plant species availability or even the location of individual plants, if they are located in areas that can make it difficult for the animals to reach them. Regardless, the positive selection of *C. albidus* observed in the field matches accurately with the preference observed in the cafeteria test.

Additionally, *Q. ilex* and *C. albidus* were also highest in crude protein content. While these results were not statistically significant, this tendency was further reflected in our PCA results, as they indicate that cellulose, hemicellulose contents primarily drive red deer feeding choices, with protein-rich species being preferred. This tendency could indicate selection based on protein content, which is one of the most limiting factors for ruminants (Van Soest, 1994), with high amounts of crude protein usually being positively related to higher digestibility and food intake (Carlier et al., 2009). Crude protein is a generally accepted indicator of food quality (National Research Council, 2007). A 9-15% requirement may be necessary to red deer, depending on body mass and physiological status, which, in Mediterranean ecosystems, is commonly obtained through browsing (Bugalho & Milne, 2003).

*Q. coccifera* was the third most consumed plant in the cafeteria trials; however, its consumption was significantly lower than *C. albidus* and *Q. ilex*. Our browsing data indicate that, although this species is consumed in the wild, the high proportion of individuals classified in categories 1 (54.54%) or 0 (24.24%) of the browsing scale suggests the lack of preference of red deer for this plant. This selection is further supported by our PCA results, where *Q. coccifera* did not show any strong link to either PCA dimension, suggesting its bromatological profile is not especially interesting for an herbivore from a nutritional perspective. Fernández-Olalla et al. (2006) calculated a preference index for species in central Spain using browsing data relative to the plant species percentage cover. Their

results revealed that *Q. coccifera* had an intermediate preference value, in agreement with our findings, in which this woody sclerophyllous species was neither positively selected nor rejected. The results of Fernández-Olalla et al. (2006) also match the rejection of *R. officinalis* and *Thymus* spp. in both our multiple-choice feeding test and the observational study, where none of the monitored individuals were browsed. However, the rejection of *Rosmarinus* in this study contrasts with the findings of Martínez (2009), who identified this species as a primary component of the red deer diet, particularly in south-eastern Spain. These discrepancies suggest that external factors may influence the selection of this species. Nonetheless, determining diet composition through the botanical analysis of stomach content specifically, the microscopic identification of plant species via epidermal fragment examination—entails certain limitations. The accuracy of results is heavily dependent on the researcher's expertise in identifying plant epidermal fragments, potentially under or overestimating the presence of a plant species, and thus, interpretations based on this methodology should be approached with caution. Low consumption of *T. vulgaris* and *R. officinalis* might be better explained by their secondary metabolites: both species are rich in phenolic compounds (Vallverdú-Queralt et al., 2014), which are generally related to a decreased feed intake (Jung & Fahey, 1983), as well as aromatic compounds like terpenes, which tend to be negatively selected by deer (Nolte et al., 2004). Additionally, both *R. officinalis* and *T. vulgaris* had high percentages of lignin, which has

been proven to be a factor negatively affecting herbivore selection (Caballero García de Arevalo 1985), and our PCA results show that species high in lignin and ash, such as these two members of the Lamiaceae, were negatively selected. Ash content contributed significantly to the second PCA axis among woody species, explaining ~45% of its variability and showing a negative correlation with nutritive traits like cellulose and hemicellulose, and was linked to negative preference by red deer in our cafeteria tests. While ash likely reflects silica content in grasses—well known to deter herbivory—this does not have to be the case in woody species, as not all woody species incorporate silica. Furthermore, studies on silica content and herbivore selection of woody species show mixed results, with either no correlation (Fernández Honaine et al., 2019) or a negative one (Hassan et al., 2024). Our findings, however, might suggest high ash content may also reduce palatability in woody plants, aligning with patterns observed in grasses with high silica content.

Finally, the fact that *Q. coccifera* was more consumed than these two species despite having high lignin content might indicate that the presence of aromatic compounds played a more important role determining deer preference for *T. vulgaris* and *R. officinalis.*

Regarding *J. oxycedrus,* despite being the species with the lowest breaking force, was generally rejected, although its consumption by red deer was the highest both in the first and last trials. The fact that breaking force contributed equally to variability in both dimensions

of the PCA that only included the woody species of this experiment supports the idea that breaking force is not a significant factor in explaining plant species selection, which contrasts with the findings by Pollock et al. (2007), where stem strength affected selection. Additionally, we did not detect any browsing pressure in the field study, further supporting the indifferent selection trend observed in several trials. Other authors have described the ingestion of plants from the *Juniperus sp.* genus in other European regions (Dzięciołowski, 1970), and in other species of ungulates, such as moose (Czernik et al., 2013). *Juniperus* is also a plant rich in volatile oils and other herbivore-deterrent components, and several *Juniperus* species are more palatable during autumn and winter because their volatile oil content decreases (Riddle et al., 1996). Our experiment was carried out in July, when summer is starting, and therefore concentration of volatile oils in *J. oxycedrus* would be high. Additionally, from a nutritional perspective, *J. oxycedrus* had the lowest protein content and relatively low cellulose and hemicellulose concentrations; therefore, the red deer may have opted for the other plant species offered in the study that presented a better bromatological profile.

In our analysis, the perennial herb *B. retusum* had higher hemicellulose and cellulose but lower lignin content than the rest of the species. Grasses generally have higher hemicellulose and cellulose wall content and lower lignin than other groups of plants (Hummel et al., 2006; M. Miranda et al., 2012; Renecker & Hudson, 1988; L. W. Smith et al., 1972). We might predict this kind of

composition to correlate with higher intakes, but our experiment did not validate this assumption. Furthermore, the field data corroborated that it was a plant rejected by red deer, as 100% of the *B. retusum* plants displayed no signs of consumption.

*B. retusum* is also the species with the highest ash contents. In grasses, ash is obtained after fiber determination using the Van Soest method is made of inorganic compounds, mainly silica (soil-borne silica) and other minerals (Van Soest, 1994). Silica is known to have a negative correlation with herbivore preference for herbaceous plant species, meaning herbaceous species with higher silica content are less preferred by domestic ruminant species such as sheep or cows (*Bos taurus*). Furthermore, silica has negative effects in rumen digestion (Van Soest, 1994) and is also the main component that causes tooth wear in herbivores. Silica incorporation in leaves is a defense mechanism against herbivory (Van Soest, 1994) well documented in grasses, who can incorporate it into their cell walls to increase their hardness. Therefore, the absence of positive selection for the only herbaceous plant included in this study could be linked to its ash content. The herbaceous layer changes depending on the season, and we performed the test in late spring, when grass plants are scarce and deer positively select woody plants to browse (Bugalho & Milne, 2003). Thus, red deer might increase their intake of these kinds of plants in other seasons like winter, when browse quality decreases (Verheyden-Tixier et al., 2008) or in spring, when *Brachypodium* show new tender leaves. Additionally, previous studies (Gebert & Verheyden-Tixier, 2001)

have shown that red deer may display a preference for woody plants throughout the year, albeit in different parts of Europe.

In summary, the chemical composition had a higher role in the selection by deer in our study than physical traits. However, other physical characteristics which we did not consider might affect the selection, like physical defenses such as spines (Takada et al., 2003) or textural properties and plant architecture (Renaud et al., 2003; Shipley et al., 1999). Moreover, Hartley et al. (1997) found that morphological characteristics can have a stronger impact on selection by deer than the chemical composition of feeds. It is noteworthy, though, that the two *Quercus* species included in this study possess spiky leaf margins and were first (*Q. ilex*) and third (*Q. coccifera*) most consumed plant species included in the experiment, with *Q. coccifera* presenting more prominent spikes than *Q. ilex.* These two species were also the ones with the lowest ash content. Thus, if we also consider that breaking force was not a relevant variable in explaining deer's positive selection for plant species, it reinforces the hypothesis that chemical plant profile might be more important for plant selection than physical defense mechanisms, even though they were not formally tested in this experiment.

Overall, our findings reveal red deer preference for species in a Spanish Mediterranean habitat, in controlled conditions to avoid influences of other factors like food search or hunger. However, environmental factors may also influence the chemical composition and morphology of plants and thus palatability (Hartley et al. 1997).

## 5. Conclusions

Deer preferences in our study were associated with both chemical and physical traits of plants, each playing distinct roles in feeding selection. Preferred species had higher fibre and crude protein contents, and lower concentrations of compounds that reduce digestibility and increase hardness, such as lignin and silica (inferred from ash content). This suggests that deer prioritize protein intake. Plant preferences observed in the multiple-choice feeding test were also mostly reflected in the browsing degree results obtained from our field observations. This knowledge can help predict deer feeding behaviour and design management techniques for wild populations. There is room for further progress in determining the diet selection of red deer by considering environmental and seasonal variability, but our work offers a first insight into this ungulate's preferences and the possible underlying mechanisms.

This study is a step in the direction to understand feeding selection in deer and therefore plan better management strategies to regulate the increasing populations of wild ungulates in Europe, which have a great impact on vegetation and the associated biodiversity. In hunting reserves like Muela de Cortes, wildlife management must be especially well planned considering human activities and understanding the relationship between herbivores and vegetation is key. Cafeteria tests can be complemented with further research using other methods on wild populations, like direct observation or analysis of diet composition.

## 6. Supplementary material

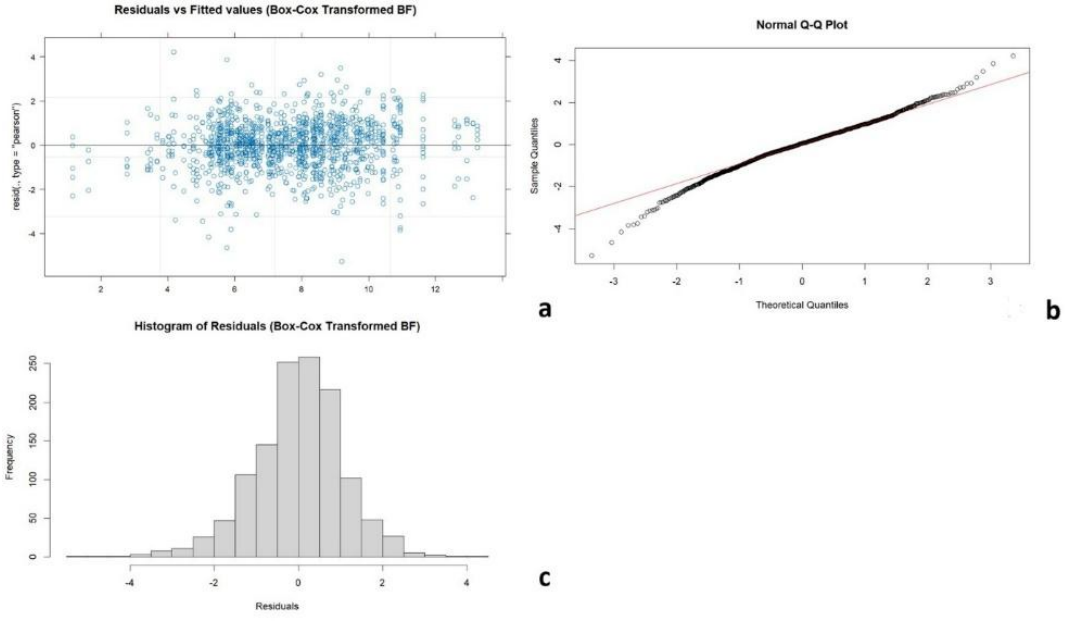



**Figure S1.** Graphics of the residuals from the GLMM model used to assess differences between species in the breaking force. **a.** Plot of the residuals against fitted values; **b.** Q-Q plot with the Q-Q line (red) representing sample residual distribution in each quantile against theoretical quantiles, and **c.** Histogram representing residual distribution to confirm normal distribution

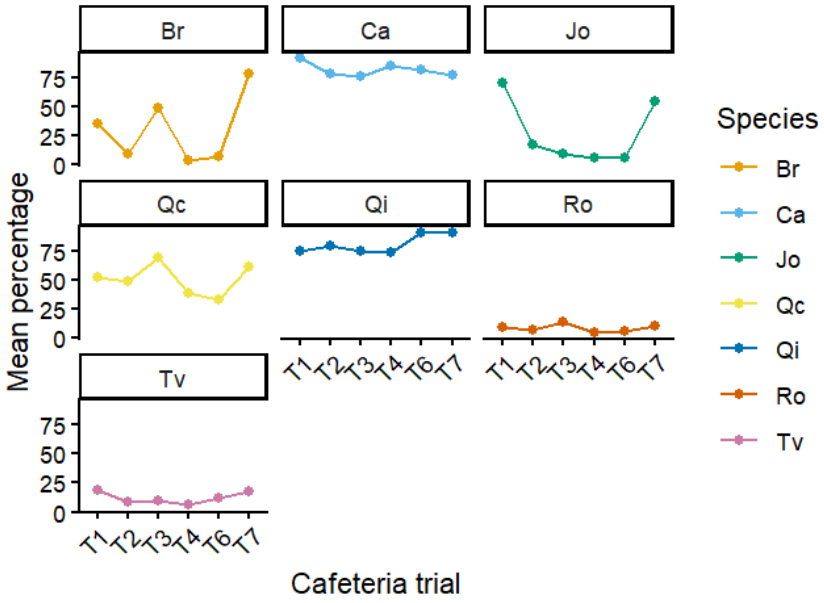



**Figure S2.** Percentage intake (i.e. "Mean percentage") of seven plant species by red deer during six multiple-choice feeding trials (1 to 7, trial 6 was discarded). Values are plant weight differences before and after each trial in percentage. Br: *Brachypodium retusum*, Ca: *Cistus albidus*, Jo: *Juniperus oxycedrus*, Qc: *Quercus coccifera*, Qi: *Quercus ilex*, Ro: *Rosmarinus officinalis*, Tv: *Thymus vulgaris*

**Table S1.** Physiochemical characteristics and dietary inclusion of the selected plant species in red deer feeding habits. Super indexes in the column titles are linked to super indexes in the "References" column, indicating where the information has been extracted from. H: herbaceous; G: graminoid; W: Woody; B: bush; T: tree; P: protein; NDF: neutral detergent fiber; ADF: acid detergent fiber; ADL: acid detergent lignin. P, NFD, ADF and ADL are all expressed in dry matter percentage (% DM).

| **Species** | **Type** | **Nutrients[1]** | | | | **Secondary compounds[2]** | **Described in deer diet?[3]** | **References** |
|---|---|---|---|---|---|---|---|---|
| | | **P** | **NFD** | **ADF** | **ADL** | | | |
| *Brachypodium retusum* | H (G) | 7.0 | 70.7 | 36.5 | 5.2 | None | Yes | [1]Bartolomé et al. 2019<br>[3]Bugalho & Milne 2003 |
| *Cistus albidus* | W (B) | 7.4 | 40.3 | 26.0 | 6.4 | Terpenoids, flavonoids, tannins | Yes, but at genus category | [1]Bartolomé et al. 2019<br>[2]Raus de Baviera et al. 2023. |
| *Juniperus oxycedrus* | W (B) | 4.7 | 36.3 | 34.3 | - | Terpenoids | Yes | [1]Méditerranéennes O 1990<br>[2]Gersbenzon and Croteau 1991<br>[3]Martínez T 2009. |
| *Quercus coccifera* | W (B) | 6.9 | 42.3 | 31.8 | 12.7 | Flavonoids, tannins | Yes, but at genus category | [1]Bartolomé et al. 2019<br>[3]Ito et al. 2002 |
| *Quercus ilex* | W (T) | 9.9 | 45.21 | 30.9 | 11.6 | Terpenoids, flavonoids, tannins | Yes | [1]Bartolomé et al. 2019<br>[2]Karioti et al. 2011<br>[2]Solla et al. 2016<br>[3]Martínez T 2009. |

(Continuation of Table S1 on the next page)

(Continuation of Table S1)

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| *Rosmarinus officinalis* | W (B) | 7.52 | 36.47 | 29.72 | 16.76 | Phenols, aromatic terpenoids, flavonoids | Yes | [1]Bartolomé et al. 2019<br>[2]Chiofalo et al. 2012.<br>[3]Martínez T 2009. |
| *Thymus vulgaris* | W (B) | 7.4 | 56.7 | 40.10 | - | Phenols, aromatic terpenoids, flavonoids, tannins | Yes, but at genus category | [1]Méditerranéennes O 1990<br>[3]Wang et al. 1999 |

**Table S2.** P-values of the bootstrapping testing the feeding intake of seven plants consumed by red deer in eastern Spain. We computed two-sided p-values as the proportion of bootstrap estimates crossing zero. *B retusum* was the reference category in the LM. Significant p-values are marked in bold.

| Species | Estimate | P-value |
|---|---|---|
| *Brachypodium retusum* | 30.535 | **< 0.001** |
| *Cistus albidus* | 51.320 | **< 0.001** |
| *Juniperus oxycedrus* | -3.190 | 0.716 |
| *Quercus coccifera* | 20.332 | **0.010** |
| *Quercus ilex* | 50.402 | **< 0.001** |
| *Rosmarinus officinalis* | -21.747 | **0.014** |
| *Thymus vulgaris* | -18.430 | **0.030** |

**Table S3.** Browsing degree of Mediterranean plants consumed by red deer in eastern Spain. Values represent percentages relative to the total number of individuals per species (column "Total"). Although browsing degree ranged from 0 to 5, none of the plant individuals reached category 4.

| Species | n | Browsing index | | | | |
|---|---|---|---|---|---|---|
| | | **0** | **1** | **2** | **3** | **4** |
| *Bupleurum frutiscensens* | 43 | 43 (100%) | 0 (0%) | 0 (0%) | 0 (0%) | 0 (0%) |
| *Brachypodium retusum* | 47 | 47 (100%) | 0 (0%) | 0 (0%) | 0 (0%) | 0 (0%) |
| *Cistus albidus* | 44 | 24 (54.54%) | 0 (0%) | 0 (0%) | 0 (0%) | 20 (45.45%) |
| *Juniperus oxycedrus* | 17 | 17 (100%) | 0 (0%) | 0 (0%) | 0 (0%) | 0 (0%) |
| *Quercus coccifera* | 33 | 8 (24.24%) | 18 (54.54%) | 6 (18.18%) | 1 (3.03%) | 0 (0%) |
| *Quercus ilex* | 46 | 18 (39.13%) | 11 (18.52%) | 9 (14.81%) | 4 (7.41%) | 5 (3.7%) |
| *Rosmarinus officinalis* | 47 | 47 (100%) | 0 (0%) | 0 (0%) | 0 (0%) | 0 (0%) |
| *Thymus vulgaris* | 46 | 46 (100%) | 0 (0%) | 0 (0%) | 0 (0%) | 0 (0%) |

**Table S4**. Descriptive statistics for the fixed and random terms of a Linear Mixed-Model (LMM) predicting breaking force of plants consumed by red deer in eastern Spain. *B retusum* was the reference category in the LMM. SE: standard error. SD: standard deviation. Bold values indicate statistically significant effects according to likelihood ratio tests ($\chi^2$). NA: Not Applied.

| **Fixed term** | **Estimate** | **SE** | **$\chi^2$** | **P-value** |
|---|---|---|---|---|
| *Brachypodium retusum* (reference) | 5.6448 | 0.3772 | NA | NA |
| *Cistus albidus* | 2.4939 | 0.5401 | **21.324** | **< 0.001** |
| *Juniperus oxycedrus* | -0.3412 | 0.5339 | 0.408 | 0.995 |
| *Quercus coccifera* | 4.2361 | 0.5356 | **62.541** | **< 0.001** |
| *Quercus ilex* | 2.4127 | 0.5345 | **20.378** | **< 0.001** |
| *Rosmarinus officinalis* | 3.1167 | 0.5342 | **34.035** | **< 0.001** |
| *Thymus vulgaris* | 2.0348 | 0.5221 | **15.188** | **0.003** |
| **Random term** | **Variance** | **SD** | **Observations** | **P-value** |
| Individual:Branch | 0.4309 | 0.6564 | 246 | NA |
| Individual | 1.4782 | 1.2158 | 86 | NA |
| Residual | 1.3605 | 1.1664 | NA | NA |

**Table S5.** Contribution to principal components 1 and 2 (PC1 and PC2) of breaking force (Break_F) and cell wall content of minerals, hemicellulose, cellulose, Lignin and Crude Protein (Protein), as well as their correlation to each PC and associated p-value (in bold if significant), for both PCAs. $R^2$ and associated p-value for an ANOVA test for the coordinates explained by each complementary ("Comp.") variable for PC1 and PC2 are also shown. NA: Not applicable. Number displayed between brackets next to the PC1 and PC2 display the eigenvalue percentage of variance for each component. Values marked in bold indicate the parameter was significant. ***: $p < 0.001$; **: $p < 0.01$, *: $p < 0.05$.

**PCA 1 (includes all plant species)**

| | **PC1 (48.23%)** | | **PC2 (18.49%)** | |
|---|---|---|---|---|
| **Active variables** | **Contribution (%)** | **Correlation** | **Contribution (%)** | **Correlation** |
| Break_F | 16.53 | **- 0.69*** ** | 4.14 | 0.21 |
| Minerals | 23.08 | **0.82*** ** | 4.60 | - 0.23 |
| Hemicellulose | 25.59 | **0.86*** ** | 9.84 | **0.33*** |
| Cellulose | 20.31 | **0.77*** ** | 1.55 | 0.13 |
| Lignin | 11.29 | **- 0.57*** ** | 12.33 | **- 0.37**** |
| Protein | 3.20 | - 0.30 | 67.53 | **0.87*** ** |
| **Comp. variables** | **Contribution (%)** | **$R^2$** | **Contribution (%)** | **$R^2$** |
| Sp | NA | **0.79*** ** | NA | **0.52*** ** |
| Preference | NA | NA | NA | **0.36*** ** |

(Continuation of Table S5 on the next page)

| **PCA 2 (discards *Brachypodium retusum*)** | | | | |
|---|---|---|---|---|
| | **PC1 (35.10%)** | | **PC2 (29.50%)** | |
| **Active variables** | **Contribution (%)** | **Correlation** | **Contribution (%)** | **Correlation** |
| Break_F | **17.10** | **- 0.60*** ** | **18.42** | **0.57*** ** |
| Minerals | 2.39 | 0.22 | 44.86 | **- 0.89*** ** |
| Hemicellulose | 27.06 | **0.76*** ** | 6.80 | **0.35*** |
| Cellulose | 41.90 | **0.94*** ** | 2.52 | 0.21 |
| Lignin | 10.07 | **0.46*** ** | 8.93 | **0.40** ** |
| Protein | 1.48 | - 0.18 | 18.47 | **0.57*** ** |
| **Comp. variables** | **Contribution (%)** | **$R^2$** | **Contribution (%)** | **$R^2$** |
| Sp | NA | **0.45** ** | NA | **0.61** ** |
| Preference | NA | NA | NA | NA |

## Study II

# Gut microbiota predicts immune competence and nutritional status in a large wild ungulate

## Abstract

The gut gastrointestinal microbiome plays a pivotal role in maintaining host homeostasis. Experimental evidence has established a link between gut microbiome community and essential physiological functions, including nutrient absorption, oxidative balance and immune modulation. However, these relationships among wild ruminants remain poorly understood. Our experiment aimed to evaluate the link between gut microbiome diversity, composition and temporal stability and the individual's nutritional condition, immune ability and oxidative status in individual female red deer (*Cervus elaphus*) captured in the wild and held in captivity under controlled dietary conditions for eleven months for reintroduction purposes. Overall, the environmental factor and the amount of time spent in the enclosure were the main drivers of changes observed in gut microbiome composition. Alpha diversity indices (Shannon's diversity index and Simpson) proved to be poor indicators of nutritional, immune or oxidative status, while positive significant associations were observed for beta diversity parameters and presence of specific microbial taxa. Overall, our results indicate that gut microbiome composition (e.g., presence and relative abundance of specific taxa), rather than diversity, play a more essential role in modulating host physiological processes that are essential for host survival. In particular, the enrichment of fiber-degrading and short-chain fatty acid–producing taxa may contribute both to efficient nutrient utilization and to the

maintenance of immune homeostasis. Further studies that included metagenomic data could help establish a causative link between the gut microbial community and host overall fitness in wild ruminants.

## 1. Introduction

“All disease starts in the gut” was a statement proclaimed by Hippocrates more than 2000 years ago. Many centuries later, modern research has provided extensive evidence that the gastrointestinal (GI) microbiome plays a central role in maintaining a host's fitness and health status. Studies performed primarily in humans and domestic animals have linked gut microbial composition to host nutrition, energy metabolism, oxidative balance, immune function, gut epithelial integrity and even neurological development through the gut–brain axis (Mayer et al., 2015; Semenova et al., 2024; Shreiner et al., 2015). At the same time, the microbiome is shaped by numerous host and environmental factors, including age, sex, social interactions, parasite burden, diet, host species and geographic location, among others (Barko et al., 2017; Kim & Jazwinski, 2018; Yatsunenko et al., 2012; Zaiss & Harris, 2016) .

These host–microbiome interactions are particularly important in ruminants. Ruminants are one of the most successful and widespread group of herbivorous mammals (Hackmann & Spain, 2010) and are characterized by the presence of a rumen, where a rich gut microbial community ferments complex polysaccharides (different fiber groups) and non-protein nitrogen to obtain volatile fatty acids (VFA) and other micronutrients (Belanche et al., 2021; Henderson et al., 2015). These compounds are essential for fulfilling energy and protein requirements (Bergman, 1990; Flint & Bayer,

2008; Hackmann & Firkins, 2015) but also influence body condition, oxidative balance and disease resistance (Bravo et al., 2012; McKenney et al., 2018). Consequently, variation in microbiome composition may have important consequences for host physiology and potentially for population dynamics in wild herbivores (McKenney et al., 2018; Stothart et al., 2024).

In free-ranging wild animal populations, physiological traits such as oxidative status, innate immunity and energy balance are increasingly used as integrative indicators of health, potential fitness and environmental adaptation (Aleuy et al., 2023; Cram et al., 2015; Dugovich et al., 2019; Kirby et al., 2019). Oxidative stress reflects the balance between the production of reactive oxygen species and antioxidant defenses, while innate immune responses provide the first line of defense against pathogens. Recent work (mainly in humans and domestic animals) reports associations between specific taxa and panels of hematological, inflammatory, biochemical and oxidative stress parameters (Herrera-Quintana et al., 2026). Reviews regarding the relationship between the microbiome–oxidative stress–inflammation axis describe correlations between changes in microbiome composition and alterations in systemic biomarkers of oxidative status and inflammation and reveal that chronic oxidative stress feeds back to change microbial diversity and function (Kunst et al., 2023). However, despite this growing body of evidence from other species showing that these factors are tied to shifts in the gut microbiome, the extent to which variation in gut microbiome composition

covaries with physiological state in free-living wildlife remains poorly understood.

Beyond taxonomic composition, the temporal stability of gut microbial communities may itself provide important information about host physiological state. In many host systems, healthy individuals tend to maintain relatively stable microbial communities through time, whereas physiological stress, disease, inflammation or nutritional imbalance can disrupt host–microbiome homeostasis and lead to microbial instability or dysbiosis (Dominguez Bello et al., 2018; Hooks & O'Malley, 2017; Sommer et al., 2017). Experimental and clinical studies have shown that oxidative stress and immune activation can alter microbial community structure, while microbiome-derived metabolites can, in turn, influence host oxidative balance and immune responses (Kunst et al., 2023; Mostafavi Abdolmaleky & Zhou, 2024). These bidirectional interactions suggest that variation in microbiome diversity, composition and temporal stability may reflect broader aspects of host physiological condition. However, such relationships remain poorly explored in wild animal populations, where repeated sampling of individuals and detailed physiological measurements are not common.

Red deer (*Cervus elaphus*) is a widespread large herbivore, considered a keystone species that provides essential ecosystem services (Garcia et al., 2025). However, due to several anthropogenic factors such as the abandonment of rural areas, re-

introduction initiatives and a decrease in their natural predator populations, among others (Mattioli et al., 2022), their free-ranging wild populations have steadily increased in the last century, especially in Europe, to the point where they have become overabundant in certain countries such as Spain and Portugal (Acevedo et al., 2008, 2011; Vicente et al., 2007). Therefore, it is a particularly interesting species for ecosystem conservation and wild population management (Apollonio et al., 2010). Their gut microbiota is known to respond to diet (Guo et al., 2022, 2024), habitat and supplementary feeding (Menke et al., 2019; Zheng et al., 2024). Discerning through their gut microbiota composition the general fitness status of a population could be useful to determine long-term population viability and influence management decisions of free-ranging red deer populations. However, the strong influence of diet and season in most field studies makes it difficult to disentangle microbiome responses to external conditions from those associated with internal physiological state. Although studies following the same individuals through time are particularly powerful for separating within-individual from among-individual variation, such designs remain rare for wild ruminants, especially when combined with detailed physiological profiling. A controlled setting in which diet is held constant provides a unique opportunity to focus on microbiome–host relationships independent of dietary change.

Our aim was to test how variation in gut microbiome diversity, composition and temporal dynamics and greater compositional

stability (i.e., lower within-individual dissimilarity over time) relates to oxidative status, energetic condition and innate immunity in a herd of red deer under controlled dietary conditions.

We hypothesized that more diverse and more stable gastrointestinal microbiome communities would be positively linked to parameters that determine an individual's overall inflammatory and immunity status. We predict that individuals with higher antioxidant capacity, lower oxidative status and better innate antibacterial capacity would harbor more diverse gut microbial communities and exhibit more stable microbiome profiles through time. We further hypothesized that microbiome composition would not contribute uniformly to host immunity, with specific microbial taxa playing a disproportionate role in determining innate antibacterial capacity. Finally, we predicted that individuals in better nutritional condition would support more diverse and compositionally stable microbial communities. Our study aims to clarify how gut microbial dynamics map onto host fitness-related traits in a large herbivore and to identify microbiome features that may act as potential indicators of physiological robustness.

## 2. Materials and methods

*Deer handling and sample obtention*

We kept a group of 20 female red deer in an enclosure from July 2020 to June 2021. These animals originated from free-ranging wild populations of red deer located in Cabañeros National Park (Toledo,

central Spain) and were captured using corral traps during summer 2020. The herd was then transported to a fenced sheepfold designed to hold cattle, made up of two areas: a 20 x 10 m sheltered building with cement floors covered in straw to provide padding, and an uncovered patio to which the animals had full constant access. Animals were then aged, weighted, measured and identified with individual colored plastic collars and ear tags. Age was estimated by visual inspection of teeth eruption patterns (Azorit et al., 2002). The group was heterogeneous, ranging from 14 months to 8 years old. The herd was supplied with ad libitum wheat straw, alfalfa hay, and a specific fodder for deer (Thurma, Venados Mantenimiento, SA) throughout the duration of the experiment (100 g/kg live weight per animal per day).

The first sampling was conducted upon capture during July 2020. The following sampling events were carried out during October and December 2020, February 2021 and June 2021 (last sampling event) throughout the duration of the experiment. Deer were chemically immobilized in packs of 5-7 individuals, using a mixture of intramuscular Ketamine (1.6 mg/kg) and Xylazine (2 mg/kg). Not all deer could be sampled at all sampling points. Animals at less than ten meters were darted with a blowgun (Marco & Lavín, 1999), while animals at further distances were darted using a rifle (Daninject Dart Guns, USA). Induction time oscillated between 5 and 15 minutes. Once chemically restrained, their limbs were tied and they were blinded with a mask. During each sampling event, blood samples were collected from the jugular vein with an 18G

needle. We placed 2 mL in EDTA tubes; 4 mL in heparin tubes, and 8 mL in serum tubes with gel activator to later obtain serum and plasma samples. Feces directly collected from the rectum using nitrile gloves were stored in zip bags. Samples were stored at 4 $^{o}$C until laboratory processing, which was carried out in less than 24 hours. For plasma and serum obtention, tubes were centrifuged at 3500G for 5 minutes and stored at -80 $^{o}$C until further processing. Feces were stored at -20 $^{o}$C until DNA extraction.

*Haematological and biochemical analysis*

Using the EDTA-preserved blood samples, we determined red blood cell counts (RBC), hemoglobin concentration (HGB), hematocrit (HCT) mean corpuscular volume (MCV), mean corpuscular hemoglobin concentration (MCHC), platelet count (PLT), plateletocrit (PCT), mean platelet volume (MPV), white blood cell count (WBC), lymphocytes (LYMPH), monocytes (MONO), neutrophiles (NEUT), eosinophiles (EO) and basophiles (BASO), using a Sysmex XN-1000 analyzer (Sysmex España S.L, Spain).

We used albumin as an indicator of undernutrition (LeResche et al., 1974); triglycerides as an indirect indicator of body fat reserves (Serrano et al., 2008); and non-esterified fatty acids (NEFA) to evaluate energy balance (Bobe et al., 2004). Urea and creatinine concentrations were also analyzed as indicators of protein catabolism (Phillip et al., 2007). Liver function was assessed by measuring aspartate aminotransferase (AST) activities (Stojević et al., 2005). Finally, oxidative stress was evaluated by determining

total antioxidant capacity using the following parameters: ferric reducing ability of plasma (FRAP), cupric reducing antioxidant capacity (CUPRAC), height-based trolox equivalent antioxidant capacity (TEACh), area-based trolox equivalent antioxidant capacity (TEACa) (Apak et al., 2005; Wang et al., 2004) and total oxidative status (TOS). Determination of these parameters was performed using plasma samples.

Urea, creatinine, triglycerides and AST were quantified using commercially available kits (Beckman Coulter Ireland Inc., County Clare, Ireland) following the manufacturer's instructions. NEFA concentrations were measured using commercial assays (Randox Laboratories Ltd., Ardmore, Crumlin, UK). Albumin was determined using a commercial assay (Albumin OSR 6102, Olympus Life and Material Science Europe GmbH, County Clare, Ireland). Total antioxidant capacity (CUPRAC and TEACh) was determined using the colorimetric method described by Erel (2004), while TOS was measured following Erel (2005). FRAP was determined following the methodology described in Apak et al. (2005). All analytes were quantified using a biochemistry analyzer (Olympus AU600 Automatic Chemistry Analyzer; Olympus Europe GmbH, Hamburg, Germany). Assay precision met analytical validation guidelines, with intra- and inter-assay coefficients of variation below 15%.

To reduce the number of explanatory variables and avoid multicollinearity, principal component analyses (PCA) were performed on groups of biologically related variables using the

"prcomp" function from the "stats" R package version 4.4.1 in R Studio version 4.4.1 (R Core Team, 2024). A total of three PCAs were performed: nutritional status (NEFA, triglycerides, urea, creatinine, albumin, AST), oxidative status (TOS, FRAP, CUPRAC, TEACH, TEACA) and inflammation (WBC, neutrophils, lymphocytes, monocytes). Before each PCA, correlation structure was assessed using Spearman correlation matrices through the "corrplot" function of the "corrplot" package version 0.95 (Wei & Simko, 2024). Afterwards, component retention was based on variance retained and interpretability (Jolliffe, 2002; Jolliffe & Cadima, 2016). We established an empirical threshold where when PC1 explained less than 50% of the variance, both the first and second principal components (PC1 and PC2) were retained as long as the total retained variance was higher than 50%. Thus, final retained dimensions used as explanatory variables were "Nutrition_PC1", linked to energy status (33.2% of retained variance, associated to NEFAs, triglycerides, creating and AST concentrations); "Nutriton_PC2", linked to protein metabolism (27.6% of retained variation, associated to urea, creatinine and albumin levels); "OxStress_PC1", which retained 73.5% of the observed variance, and "Inflam_PC1", which retained 63.3% of the observed variance. Full PCA results can be found in the supplementary information (Figure S1, Table S1).

*Bacterial Killing Assay*

All plasma samples, as well as negative and positive controls, were processed in duplicate to ensure analytical precision. To maintain stable humidity and prevent evaporation, 150 µL of sterile water were added to the outer wells of each plate, resulting in 60 usable wells per plate.

Prior to the assay, the stock solution of *E. coli* was diluted in sterile phosphate-buffered saline (PBS) to obtain a working solution at a concentration of $10^5$ colony-forming units (CFU)/mL in PBS. The selected working dilution for plasma samples to assess antimicrobial capacity was 1:24 in sterile PBS (20 µL plasma in 480 µL PBS). Forty µL of each plasma sample diluted 1:24 were added in duplicate in a sterile, flat-bottom 96-well microplate. In addition, 40 µL of PBS were added in duplicate as positive controls, and 50 µL of PBS was added in duplicate as negative controls. Reference *E. coli* growth curves were established as a 1:10 dilution.

Subsequently, 10 µL of the *E. coli* working solution was added to all wells except the negative controls. Plates were covered and homogenized using a vortex mixer for 1 minute at 150 rpm. After homogenization, the plasma–microorganism mixture was incubated at 37°C for 30 minutes. Following incubation, wells were homogenized again under the same vortex conditions, and 100 µL of tryptic soy broth (TSB) was added to all wells, including controls. Plates were vortexed again, and baseline absorbance (time 0) was measured at 630 nm using a spectrophotometer. Plates were then

incubated at 37°C, and absorbance readings were recorded at 3, 6, 9, 12, 24, and 27 hours. To reduce inter-plate variation, a reference sample was included in duplicate on all plates. This reference consisted of a pooled plasma sample made up of 10 plasma samples from the red deer previously used during plasma dilution optimization.

To calculate plasma antimicrobial capacity (bacterial killing ability, BKA), baseline absorbance values were subtracted from the absorbance readings obtained prior to the onset of the exponential (log) phase of microbial growth. Antimicrobial capacity was calculated as:

$$BKA\ (\%): \left[1 - \left(\frac{Absorbance_{Sample}}{Absorbance_{RefCurve}}\right)\right] \times 100$$

This calculation yields the percentage of bacteria eliminated by the plasma relative to the standard bacterial growth observed in the positive control. Negative controls were not included in the final calculation; they served solely to confirm the absence of contamination in the sample diluents and reagents used in the assay.

*DNA sequencing and microbiome analysis*

A total of 250 mg of each fecal sample was used for DNA extraction. DNA extraction was performed using QIAamp PowerFecal Pro DNA Kits (Qiagen Iberia SL, 08028 Barcelona) according to manufacturer's instructions. Negative controls were added every 46

extractions to account for cross-contamination. After extraction, DNA was quantified for each sample using 2 µL of the final extracted volume using the Qubit dsDNA BR (Broad Range) Assay kit (Fisher Scientific S.L., Madrid, Spain) and an Invitrogen Qubit fluorometer model MP32857 (Fisher Scientific S.L., Madrid, Spain). Extraction aliquots were stored at – 80 $^{o}$C. Library preparation and sequencing were carried out at an external laboratory (CRG-Centre for Genomic Regulation, Genomic Unit 08003 Barcelona, Spain). We requested amplification of the V4 region of 16s rRNA bacterial gene using the 16S Forward primer 515-F 5' – GTGYCAGCMGCCGCGGTAA - 3' and 16S Reverse primer 806RMod-R 5' – GGACTACNVGGGTWTCTAAT – 3' from the Earth Microbiome Project (Walters et al., 2016). Raw sequences were processed and cleaned using QIIME2 v.2024.5 software (Bolyen et al., 2019). Before processing, primers were removed using the "cutadapt" plugin from QIIME2. Sequences were processed using the DADA2 workflow pipeline to trim the adapter sequences using a truncated length of truncLenc = c(240, 170), remove low quality reads and chimeras and identify amplicon sequence variants (ASVs). Taxonomic classification was performed with the unrarefied ASV feature table and Silva 138 classifier as the reference library, using the Klearn machine learning classification method found in "q2-feature-classifier" plugin, following QIIME2 developer's recommendations. Afterwards, non-target sequences were removed from the feature table, including mitochondrial, chloroplast, and unassigned features, using the "q2-taxa" plugin. Features that appeared fewer than 10 times and in fewer than two

samples were removed from downstream analysis. The filtered feature table was used for all subsequent diversity and statistical analyses. Diversity indices were calculated using non-rarefied data, following the recommendations of McMurdie and Holmes (2014). We calculated Shannon's diversity index and Simpson's index as alpha diversity metrics, and Bray-Curtis and Aitchison's distance for beta diversity. We calculated Shannon's diversity index using "qiime diversity alpha" plugin in QIIME2. Rarefaction curves can be found in Supplementary Information (Figure S2). Bray-Curtis dissimilarity index was calculated based on proportional abundance to avoid using rarefaction as a normalization method and not lose valuable data (McMurdie & Holmes, 2014); and Aitchison's distance for evaluating beta diversity were calculated in RStudio version 4.4.1 (R Core Team, 2024) using "phyloseq" version 1.48.0 (McMurdie & Holmes, 2013) and "vegan" version 2.7-2 (Oksanen et al., 2026) packages.

*Statistical analysis*

All statistical analyses were conducted in R Studio version 4.4.1 (R Core Team, 2024). Shannon diversity was used to characterize within-sample microbial diversity by integrating both taxon richness and evenness, allowing us to test whether physiological condition was associated with overall community diversity. Simpson diversity was used as a complementary alpha diversity metric emphasizing dominance patterns and the relative contribution of abundant taxa (Cassol et al., 2025; Qiao et al., 2024). To test associations between

alpha diversity and host physiological traits, we fitted linear mixed-effects models using the packages “lme4” (Bates et al., 2015) and “lmerTest” (Kuznetsova et al., 2017). Shannon diversity was modelled as a Gaussian response, while Simpson diversity, constrained between 0 and 1, was analyzed using beta regression implemented in the “glmmTMB” package version 1.1.14 (Brooks et al., 2017). Prior to modelling, continuous predictors were standardized (mean = 0, SD = 1) to improve model convergence and facilitate comparison of effect sizes.

For both indices, we constructed biologically motivated models to evaluate relationships with oxidative status (OxStress_PC1), inflammatory status (Inflam_PC1), innate immune function (BKA_Ec), and nutritional condition (Nutrition_PC1, Nutrition_PC2), while accounting for time since introduction into the sheepfold (Days_intro). Individual identity (ID_SEfaS) was included as a random intercept to account for repeated measures. Model assumptions were assessed through visual inspection of residuals and using simulation-based diagnostics implemented in the DHARMa package version 0.4.7 (Hartig, 2024). Model fit was quantified using marginal and conditional $R^2$ values obtained using the “MuMIn” package version 1.48.4 (Bartoń, 2024).

Beta diversity was analyzed using both Bray–Curtis and Aitchison distance. Bray–Curtis dissimilarity was used to quantify differences in community composition and assess if compositional turnover and community stability were linked to host physiological traits.

Aitchison's distance was used as a complementary metric to detect broader restructuring caused by less dominant taxa (Fuschi et al., 2025). Bray-Curtis dissimilarity was calculated based on relative abundance data, while for Aitchison distance count data were transformed using a centred log-ratio (CLR) transformation following addition of a small pseudocount to handle zeros.

To test for differences in microbiome composition, we performed permutational multivariate analysis of variance (PERMANOVA) using the "adonis2" function from the "vegan" package, including Days_intro and physiological variables as predictors. To account for repeated sampling of individuals, permutations were restricted within individuals (strata = ID_SEfaS). Marginal effects of predictors were assessed using 9999 permutations. Homogeneity of multivariate dispersion was evaluated using "betadisper" function from the "vegan" package.

Associations between microbial taxa relative abundances and explanatory variables including time spent in the sheepfold, oxidative stress, inflammation status, innate immune capacity and nutritional status were assessed using generalized linear models implemented in microbiome multivariable association with linear models (MaAsLin2) using "Maaslin2" package version 1.18.0 (Mallick et al., 2021). We used a collapsed taxonomy table for the family rank. Rare taxa were filtered out by excluding those present in less than 10% of samples. The same defined immunity and nutritional models applied for alpha and beta diversity analyses

were applied to the MaAsLin2 pipeline, retaining individual identity as a random effect to account for repeated measures. Prior to analysis, data were normalized using total sum scaling (TSS) and log-transformed to improve normality. To account for multiple testing across taxa, p-values were adjusted using the Benjamini–Hochberg false discovery rate (FDR) correction. Associations were considered statistically significant at an adjusted q-value threshold of ≤ 0.05.

## 3. Results

*Alpha diversity*

The 74 samples included in this study contained a total of 4548 ASVs classified to the genus rank. Each sample contained a mean of 860.39 ± 151.16 ASVs (min 336, max 1231). ASV median relative abundance was 3.357e-05 (maximum values of 0.039). The mean genus number per sample was 125.18 ± 11.41 (min 92, max 152). Most of the identified bacterial species belonged to the Firmicutes (66.42%), Bacteroidota (24.70%), Actinobacteriota (4.48%) and Spirochaetota (1.13%) phyla, with the most common groups being *UCG-005* genus (12.35%), Christensenellaceae R-7 group (8.14%), Lachnospiraceae family (6.47%), Rikenellaceae RC-9 gut group (5.40%), *Bacteroides* genus (4.14%), UCG-010 family (4.03%), Clostridia class (3.87%), *Bifidobacterium* genus (3.64%), *Ruminococcus* genus (3.02%) and Oscillospiraceae family (2.91%); although the most common found families varied according to sampling time (Figure 1).

Regarding Shannon's index, antibacterial capacity (BKA) was the only significant variable in the immunity model, displaying a negative association with Shannon's index values (Table 1, Figure S3); while the number of days spent inside the sheepfold was not significant, but displayed a weak positive association (Figure S4). Individual identity explained 9.2% of the observed variance. In the nutritional model, none of the predictors significantly explained variation in Shannon diversity, and individual identity did not explain any percentage of the observed variance (Table 1). Mean Shannon index values were 8.18 ± 0.47 (min 6.41, max 8.82).

Simpson diversity showcased very limited variation (mean Simpson values 0.98 ± 0.017, min 0.88 and max 0.99), resulting in extremely high $R^2$ values and negligible random effects. No significant associations with physiological or nutritional predictors were detected; therefore, the biological interpretability of this index in our present study is quite limited.

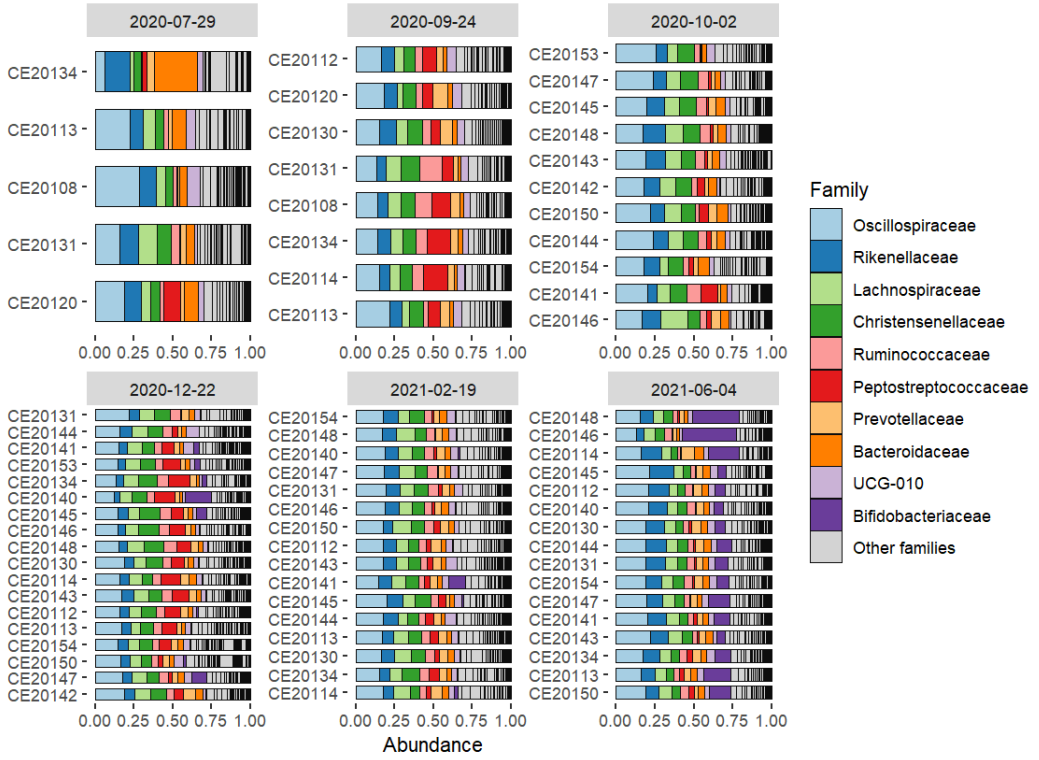



**Figure 1.** Barplot describing gastrointestinal microbiome composition of the top 10 most abundant families found in each sampling date (YYYY/MM/DD), where each horizontal bar represents an individual red deer (CE20XXX).

**Table 1.** Results of the GLMM models for alpha diversity indexes, with sampling size (N) and explanatory variables retained. We report the estimate, standard error (SE) degrees of freedom (DF) and the T-value for each parametric variable. Regarding the random effect, the variance and standard deviation (SD) are reported. The values of the marginal ($R^2$m) and conditional ($R^2$c) $R^2$ for each model are reported. $R^2$: percentage of variation explained by the model. Significant terms are marked in bold and * are used to indicate the degree of significance (· p~0.05; *: p ≤ 0.05; **: p ≤ 0.01; ***: p ≤ 0.001). Shaded cells mean the term is not applicable for that variable.

| **Shannon** | | | | | | | |
|---|---|---|---|---|---|---|---|
| **Model** | **N** | **Explanatory variable** | **Estimate**<br>**Variance** | **SE**<br>**SD** | **DF** | **T-value** | **$R^2$m; $R^2$c** |
| Immunity model | 72 | OxStress_PC1 | - 0.008 | 0.029 | 65.959 | - 0.267 | 0.140; 0.232 |
| | | $(OxStress_PC1)^2$ | - 0.004 | 0.004 | 64.554 | - 0.960 | |
| | | Inflam_PC1 | 0.039 | 0.039 | 65.944 | 0.990 | |
| | | **BKA** | **- 0.005** | **0.002** | **61.824** | **- 2.477*** | |
| | | Days_intro | 0.001 | 0.001 | 65.069 | 1.972 | |
| | | Random effect: ID | *0.022* | *0.150* | | | |
| | | Random effect: residual | *0.189* | *0.434* | | | |
| Nutrition model | 69 | Nutrition_PC1 | - 0.041 | 0.041 | | - 0.991 | 0.036; 0.036 |
| | | Nutrition_PC2 | 0.035 | 0.044 | | 0.799 | |
| | | Days_intro | 0.001 | 0.001 | | 0.982 | |
| | | Random effect: ID | 0.000 | 0.000 | | | |
| | | Random effect: residual | 0.221 | 0.471 | | | |

(Continuation of Table 1 on the next page)

| **Simpson** | | | | | | | |
|---|---|---|---|---|---|---|---|
| **Model** | **N** | **Explanatory variable** | **Estimate** ***Variance*** | **SE** ***SD*** | | **Z-value** | **$R^2$m; $R^2$c** |
| Immunity model | 72 | OxStress_PC1 | - 0.001 | 0.070 | | - 0.070 | 0.998; 0.998 |
| | | Inflam_PC1 | 0.073 | 0.075 | | 0.970 | |
| | | **BKA** | **- 0.159** | **0.084** | | **- 1.880·** | |
| | | Days_intro | - 0.093 | 0.091 | | - 1.020 | |
| | | *Random effect: ID* | *< 0.001* | *< 0.001* | | | |
| Nutrition model | 69 | Nutrition_PC1 | - 0.005 | 0.040 | | - 0.130 | 0.993; 0.993 |
| | | Nutrition_PC2 | 0.020 | 0.043 | | 0.470 | |
| | | Days_intro | - 0.135 | 0.069 | | - 1.950 | |
| | | *Random effect: ID* | *< 0.001* | *< 0.001* | | | |

*Beta diversity*

Bray Curtis dissimilarity index was significantly affected by the number of days spent inside the sheepfold (F = 6.16, p = 0.001) and the bacterial killing ability (F = 4.16, p = 0.001), although the explained variability for each of these factors was low ($R^2$ = 0.074 and 0.050, respectively) (Figure 2a). Oxidative status and inflammation did not significantly explain part of the observed variation (Table S2). In the nutritional model, the number of days spent in the sheepfold remained a significant predictor (F = 6.53, $R^2$ = 0.087, p = 0.001). Additionally, the second nutritional principal component (Nutrition_PC2), linked to protein and nitrogen metabolism, showed a significant but weak effect (F = 1.82, $R^2$ = 0.024, p = 0.009), whereas Nutrition_PC1 (mainly associated to energetic status) was not significant (Figure 2b).

Aitchison distance reflected the same consistent results observed in Bray Curtis (Figure 3a and 3b). The number of days in the sheepfold significantly explained variation in microbiome composition in the immunity (F = 3.62, p < 0.001) and nutritional (F = 3.72, p < 0.001) models, although the percentage of observed variance was very low in each model ($R^2$ = 0.048 and 0.052, respectively). The bacterial killing ability was also significant in the immunity model (F = 2.66, p < 0.001) but only explained 3.4% of the observed variance ($R^2$ = 0.034). Again, no significant associations were observed with the oxidative and inflammation variables (Table S2).

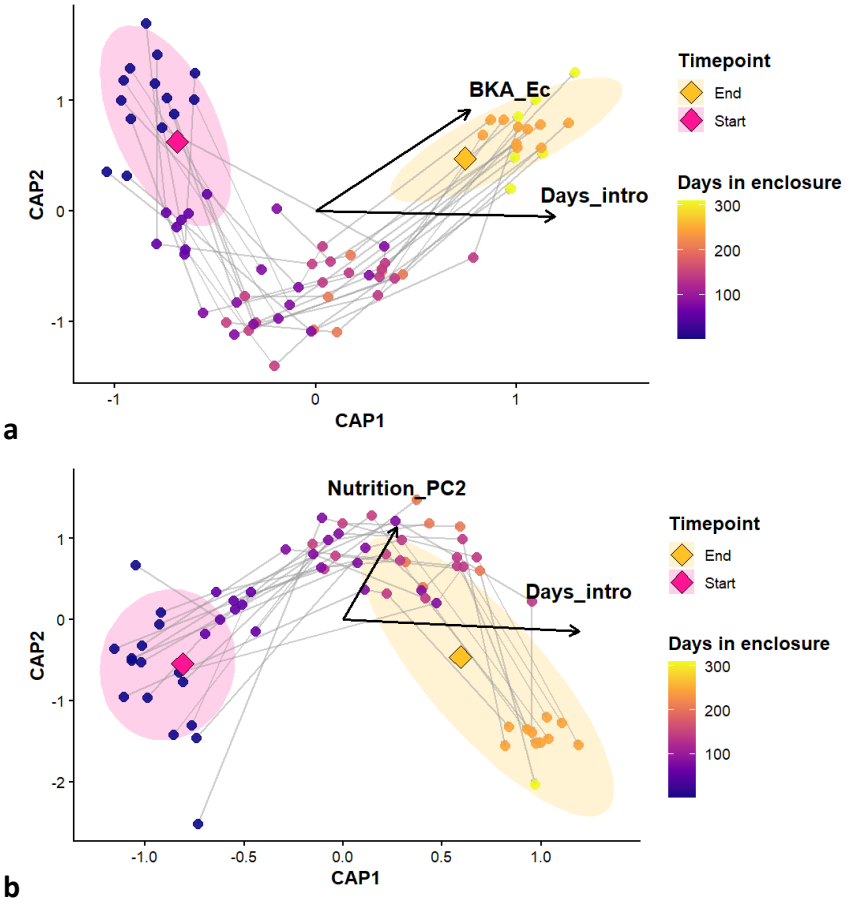



**Figure 2.** Bray Curtis visualization using capscale for the immunity (a) and nutritional (b) models. Each point is a sample. Black arrows display direction and strength of the two significant variables obtained in the PERMANOVA results (days spent in the sheepfold, Days_intro; bacterial killing ability, BKA_Ec; Nutritional component, Nutrition_PC2), with arrow length being proportional to the contribution of each ordination. Each grey line indicates the evolution of the same individual. Shaded ellipses indicate distribution (68% confidence interval) of samples at the start (pink) and end (yellow) of the observation period across individuals. Filled diamonds indicate the corresponding centroids (mean position) for each timepoint group, illustrating the overall shift in community composition. Axes (CAP1 and CAP2) represent the primary constrained dimensions of variation explained by the dbRDA model.

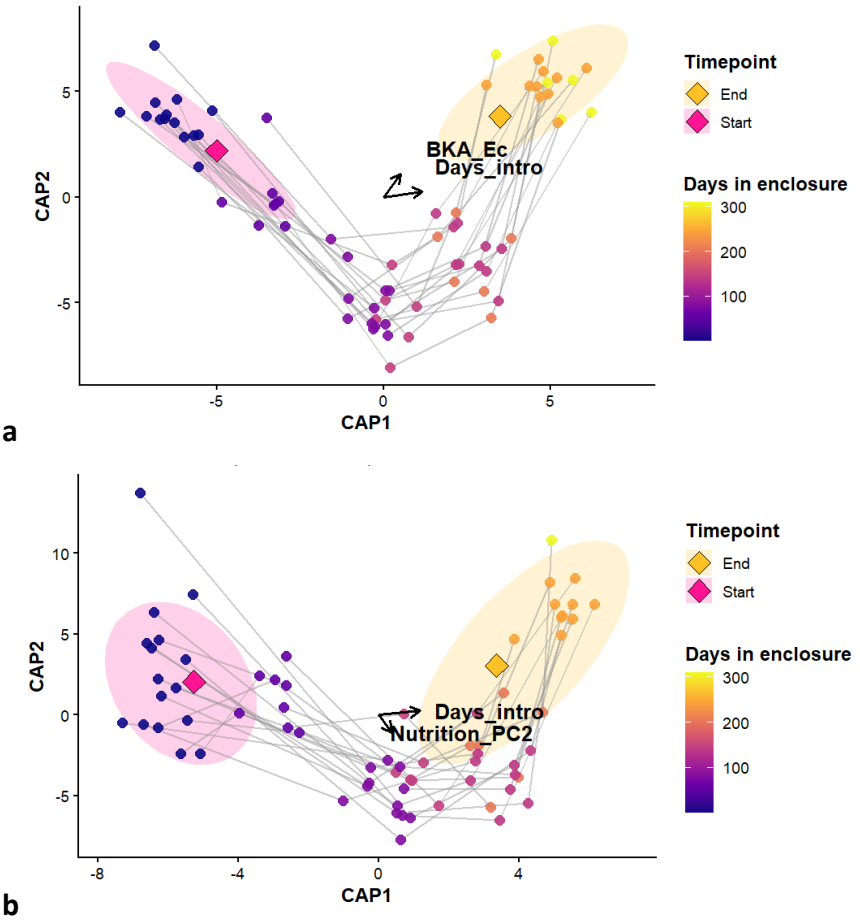



**Figure 3.** Aitchison distance visualization using capscale for the immunity (a) and nutritional (b) models. Each point is a sample. Black arrows display direction and strength of the two significant variables obtained in the PERMANOVA results (days spent in the sheepfold, Days_intro; bacterial killing ability, BKA_Ec; Nutritional component, Nutrition_PC2), with arrow length being proportional to the contribution of each ordination. Each grey line indicates the evolution of the same individual. Shaded ellipses indicate distribution (68% confidence interval) of samples at the start (pink) and end (yellow) of the observation period across individuals. Filled diamonds indicate the corresponding centroids (mean position) for each timepoint group, illustrating the overall shift in community composition. Axes (CAP1 and CAP2) represent the primary constrained dimensions of variation explained by the dbRDA model.

Nutrition_PC2 showed again a significant but weak effect (F = 1.44, p = 0.008, $R^2$ = 0.020). However, tests for homogeneity of dispersion revealed significant differences in multivariate dispersion among individuals in both the immunity (F = 2.74, p = 0.002) and nutritional (F = 2.26, p = 0.011) models, indicating that part of the observed variation in community composition may be influenced by differences in within-individual variability, and therefore PERMANOVA results should be interpreted with caution.

*Taxa association to physiological parameters*

The number of days spent in the enclosure emerged as the main driver of taxonomic variation, with a large number of bacterial families showing significant associations (Figure 4). Several taxa within the phylum Bacteroidota, including Prevotellaceae and other members of the order Bacteroidales, exhibited positive associations with time, indicating an increase in their relative abundance throughout the study period. In contrast, taxa belonging to Proteobacteria, such as Enterobacteriaceae, as well as Campylobacteraceae, showed negative associations with time, suggesting a decline in these groups during the time within the enclosure.

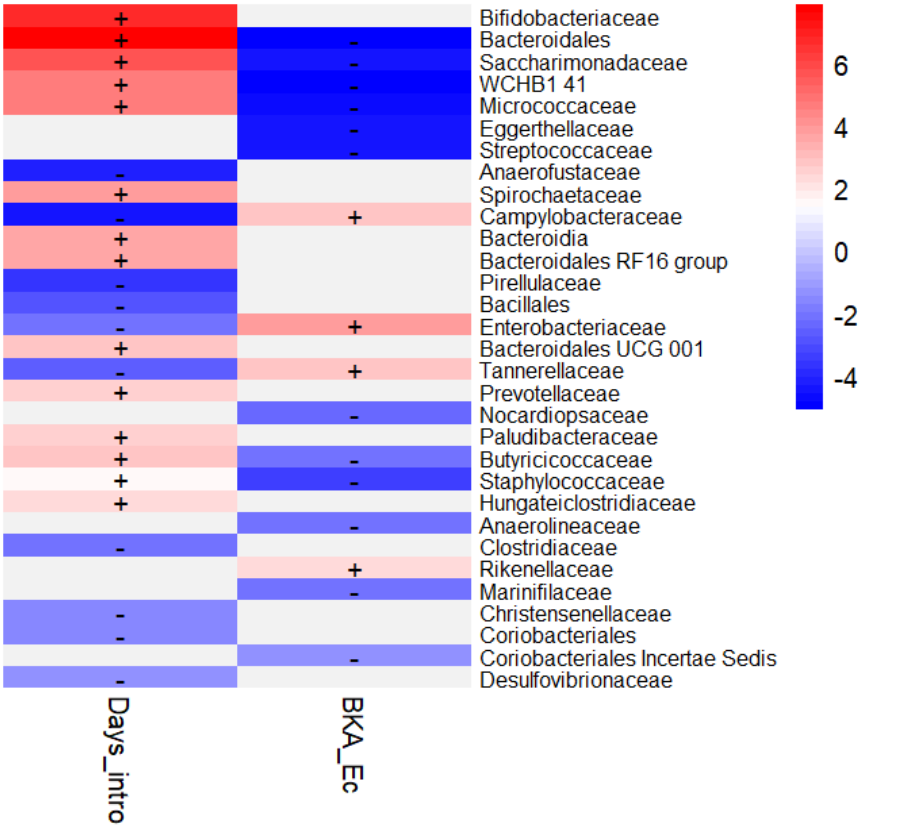



**Figure 4.** Heatmaps displaying microbial taxa with significant associations ($q < 0.05$) to explanatory variables (columns) identified using MaAsLin2 for the immunity model. Rows represent taxa, columns represent metadata variables, and colors indicate the direction and magnitude of associations (red = positive, blue = negative, scaled by -log10(q-value) × sign(coefficient)). Black + and - symbols indicate the effect direction, and taxa are ordered by overall strength of association. Missing values are shown in gray.

Bacterial killing ability was also associated with significant changes in microbiome composition. Most associations were negative, affecting a broad range of taxa including members of Actinobacteriota and Firmicutes, indicating a general reduction in

the abundance of several bacterial groups in individuals with higher antibacterial capacity. However, some taxa, such as Enterobacteriaceae and Campylobacteraceae showed positive associations with BKA (Figure 4). In contrast, there were no significant taxa associations with oxidative stress and inflammatory status.

Differential abundance analysis using MaAsLin2 in the nutritional model revealed patterns largely consistent with those observed in the immunity model (Figure 5). The number of days spent in the enclosure was again the primary driver of taxonomic variation, with a substantial number of bacterial families significantly associated with this variable. In particular, several taxa within the phylum Bacteroidota, including members of the order Bacteroidales such as Paludibacteraceae and other unclassified Bacteroidales groups, showed strong positive associations with time. Additionally, taxa from Bifidobacteriaceae were also positively associated with days spent inside the enclosure. In contrast, several taxa belonging to Firmicutes (e.g., Anaerofustaceae and Clostridiaceae) and Proteobacteria (e.g., Campylobacteraceae and Sutterellaceae) displayed negative associations, indicating a decline in these groups over time. Nutritional principal components showed comparatively fewer and weak associations with microbial taxa (Figure 5).

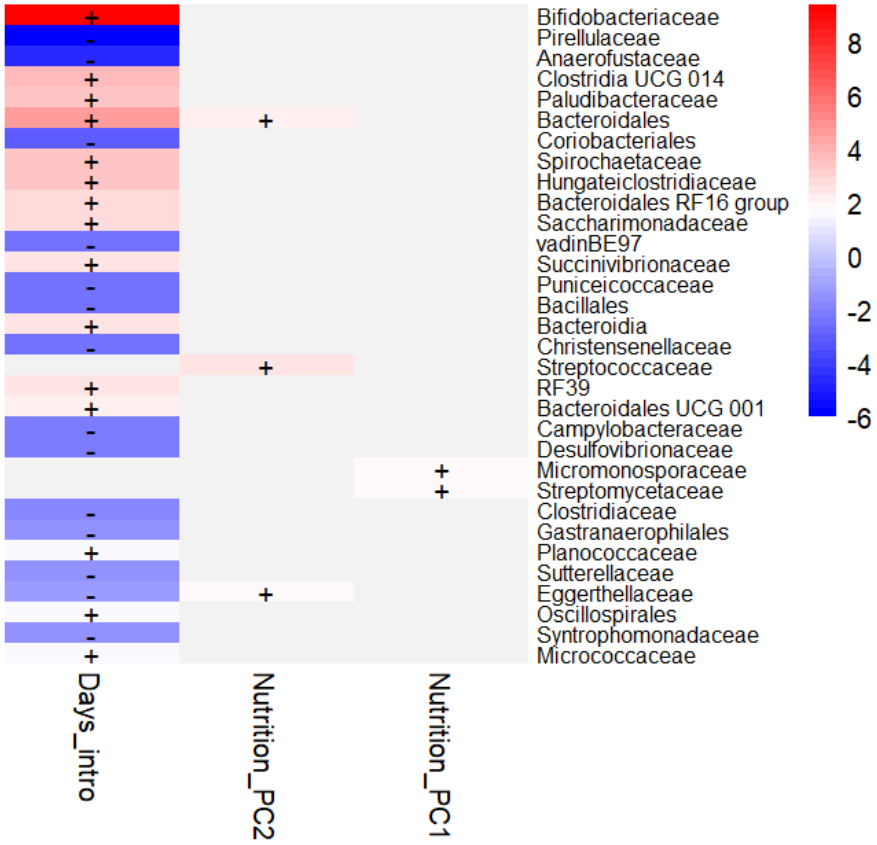



**Figure 5.** Heatmaps displaying microbial taxa with significant associations ($q < 0.05$) to explanatory variables (columns) identified using MaAsLin2 for the nutritional model. Rows represent taxa, columns represent metadata variables, and colors indicate the direction and magnitude of associations (red = positive, blue = negative, scaled by -log10(q-value) × sign(coefficient)). Black + and - symbols indicate the effect direction, and taxa are ordered by overall strength of association. Missing values are shown in gray.

## 4. Discussion

Relative abundances of bacterial phyla within our gut microbiome dataset revealed high proportion of the phyla Firmicutes and Bacteroidetes, which is consistent with previous research carried out in red deer (Guo et al., 2024; Menke et al., 2019; Sun et al., 2023) as well as other cervids, such as roe deer *Capreolus capreolus* (Yuan et al., 2023), elk *Cervus canadensis* (Gruninger et al., 2014) and white-tailed deer *Odocoileus virginianus* (Gruninger et al., 2014). The most abundant taxa described are linked to high-fiber diets (*Christensenellaceae* R-7 group, UCG-010) (Li et al., 2022) and degradation and fermentation of complex polysaccharides and simple carbohydrates (*Clostridium* class) or cellulose (*Ruminococcus*) to later produce short-chain fatty acids and other components (Aristilde, 2017; Robert & Bernalier-Donadille, 2003). *Bacteroides* genus is related to carbohydrate and energy metabolism (Smith & Macfarlane, 1996, 1998). Members of the *Lachnospiraceae* family have been identified as indicators of a healthy gut (Vojinovic et al., 2019) and contribute to homeostatic balance, butyrate production, and pathogen elimination (Mclellan et al., 2013). *Bifidobacterium* genus is also related to carbohydrate metabolization (Milani et al., 2016) but it also promotes intestinal health and optimal immune function (Vlková et al., 2006). The animals were fed with wheat straw, alfalfa hay and deer feed throughout the duration of the experiment, and therefore the observed gut microbiome composition is compatible with this diet rich in fiber and adaptation to captivity (Li et al., 2022).

Shannon and Simpson diversity indices calculate intra-individual gut microbiome diversity but reflect slightly different metrics. Shannon's index is obtained using taxa richness and relative taxa evenness, therefore being sensitive to rare taxa, while Simpson's index reflects dominant species (Cassol et al., 2025; Qiao et al., 2024). Shannon and Simpson indices values in our dataset had a very narrow range, with the mean values being close to the upper range, indicating that gut microbiome community is highly rich and even, and the lack of a significant effect of time suggests that overall intraindividual diversity remained stable throughout the study period.

Contrary to our hypothesis, we detected a significant but weak negative effect of the bacterial killing ability and Shannon diversity index. We also did not find any significant relationship between alpha diversity metrics and oxidative and inflammation status, despite having evidence from human studies that oxidative stress can directly reduce gut microbial alpha diversity (Zünd et al., 2025) and that patients with chronic inflammatory conditions (Inflammatory Bowel Disease, sepsis) have a reduced alpha diversity in their gut microbiome (Alzahrani et al., 2025; Ramalho Guerra et al., 2025).

Conceptually, there is a clear link between gut microbiome diversity and composition and immune function and inflammation. Gut microbes constantly engage pattern-recognition receptors and drive production of antimicrobial peptides, cytokines and other

innate responses that shape pathogen control and tissue homeostasis (Yoo et al., 2020). Microbial metabolites (especially short-chain fatty acids) modulate epithelial barrier integrity, antimicrobial peptide production, and the function of neutrophils, macrophages, and innate lymphoid cells, which directly affects bacterial killing capacity (Cheng et al., 2019; Wiertsema et al., 2021; Yoo et al., 2020). Studies carried out in experimental mice and evidence from human research have linked both low gut microbiome diversity and community alteration caused by expansion of certain taxa (i.e. dysbiosis) to the development of metabolic diseases, such as Diabetes I or Crohn's disease or immune system disfunction by making the host prone to infections (Robertson et al., 2018; Thaiss et al., 2016). It would be expected, then, that individuals with a more diverse gastrointestinal microbiome would have a more competent immune system. However, recent studies found that gross metrics of diversity have a weak or even absent relationship with innate immune response (Habes et al., 2021).

There could be several explanations for the lack of clear correlations found in our study. Firstly, current evidence suggests that the observed effects on the immune function and oxidative status of the host might be more dependent on the presence of specific taxa or functional pathways, rather than in global diversity (Martin et al., 2019). For example, the maintenance of the REDOX equilibrium within a host (which intervenes in oxidative status and immune cell activation) is regulated by the presence of bacterial-derived

metabolites released by commensal bacteria, thus suppressing inflammation and maintaining an anti-oxidative status, while pathogenic bacteria trigger a pro-oxidative state (Kunst et al., 2023). Additionally, the red deer used in our experiment did not display any clinical signs of disease. Studies that have found biological correlations between gut diversity and the mentioned parameters involved individuals that were already in a pro-inflammatory status (Alzahrani et al., 2025; Ramalho Guerra et al., 2025); were experimental settings with germ-free laboratory animals, or focused their efforts into specific populations cohorts (Bosco & Noti, 2021); therefore, biological signals could be stronger. In contrast, relationships between gut microbiome diversity, immune function, and oxidative status in individuals with no apparent homeostatic disruption may be more subtle and therefore harder to detect using coarse diversity metrics.

Importantly, analyses of beta diversity and microbiome stability revealed patterns that were not captured by alpha diversity metrics. This apparent discrepancy is not unexpected, as beta diversity metrics are generally more sensitive than alpha diversity metrics for detecting ecological differences in microbiome datasets (Kers & Saccenti, 2022). Both Bray–Curtis and Aitchison distances indicated significant, albeit weak, shifts in microbiome composition associated with time in captivity and bacterial killing ability. The significant effect of time suggests that, although overall diversity was maintained, the identity and relative abundance of taxa changed over time, reflecting compositional turnover rather than

diversity loss. Together, these results support the idea that the gut microbiome underwent a reassembly process under new environmental conditions, characterized by shifts in community composition and subsequent stabilization, while maintaining overall diversity, and provides further evidence that significant associations between immunity and nutritional metabolism might be more linked to presence of specific taxa than overall diversity parameters. This interpretation is consistent with previous studies showing that host-associated outcomes are often more strongly linked to microbiome composition than to global diversity metrics (Worsley et al., 2021).

Indeed, we found specific taxa correlated to our different explanatory variables, with significant associations found for the time factor, but also for the innate immune response. Again, the days spent in the enclosure were the variable which showed the highest number of correlations for both models, indicating time was the main driver of taxa turnover. However, time should be interpreted as a composite proxy for several unmeasured, time-dependent processes related to changes in environmental and host conditions. Potential factors indirectly tied to the time factor include the establishment of new social hierarchies and associated behavioral interactions, adaptation to captivity, shifts in stress physiology, and changes in environmental factors such as light and temperature. Consequently, the observed temporal patterns cannot be attributed to a single causal factor. Despite this, the direction of microbial changes suggests a strong contribution of dietary

adaptation towards a diet high in carbohydrates. Enrichment of core fibrolytic and saccharolytic ruminant bacteria (Prevotellaceae, Bacteroidales RF16/UCG-001, Hungateiclostridiaceae, Butyricicoccaceae, Paludibacteraceae, Bifidobacteriaceae, some Coriobacteriales and spirochetes) indicates a community adapted to continuous alfalfa and wheat straw digestion, with greater SCFA production and more strictly anaerobic conditions, and reduced representation of potential opportunists like Campylobacteriaceae and Enterobacteriaceae (Henderson et al., 2015; Tovar-Herrera et al., 2026; Wang et al., 2023; Yu et al., 2023; Zhu et al., 2023). Interestingly, Bifidobacteriaceae has been associated with starch fermentation and are known to have anti-inflammatory effects and promote gut health and immune function, especially in young individuals (Milani et al., 2016; Zhuang et al., 2024). The increased presence of this family as time passed, especially at the last stage of captivity, could indicate a shift toward a more stable, less inflammatory hindgut environment after the animals had undergone a period of captivity adaptation, however the nature of our dataset does not allow us to confirm this hypothesis.

Although associations with nutritional principal components were weaker, they were biologically coherent and aligned with known microbial functions. Taxa associated with energetic metabolism included Streptomycetaceae and Micromonosporaceae, which are involved in starch fermentation and cellulose degradation, respectively (Maluszynska & Janota Bassalik, 1974; Ratnakomala et al., 2020). Similarly, the family Streptococcaceae, a group known to

participate in protein degradation (Wang et al., 2019), was associated with protein metabolism.

Families showing strong positive coefficients with the bacterial killing assay included Campylobacteraceae and Enterobacteraceae, indicating that these taxa were more abundant in individuals with higher bacterial capacity. Some members of the Campylobacteraceae family (*Campylobacter* spp.) can cause enteric disease (Man et al., 2010; Wang et al., 2023), and the Enterobacteriaceae family contains many opportunistic and non-opportunistic pathogens (Octavia & Lan, 2014) that generate a pro-inflammatory state in their host and higher abundance often aligns with dysbiosis, barrier stress, and immune activation (Al-Eqabi et al., 2022; Moxley, 2022). Therefore, a positive correlation between these taxa and bacterial killing ability could be reflecting immune activation after exposure to harmful bacteria. On the other hand, most of the taxa that displayed a negative correlation are producers of short-chain fatty acids derived from fiber fermentation, which modulate immune cell activation (Corrêa-Oliveira et al., 2016; Laforest-Lapointe & Arrieta, 2017). Therefore, observed results could suggest that a gut microbiome dominated by fermentative taxa promotes gut barrier integrity and immune regulation, thereby reducing the need for elevated systemic antimicrobial responses. This reasoning is further supported by the lack of significant associations between oxidative and inflammation parameters, which could indicate that proper maintenance of the oxidative and inflammation status is more dependent on the general gut

microbiome composition, rather than specific taxa (Kunst et al., 2023).

## 5. Conclusions

In this study, we show that multiple concurrent, time-dependent processes—including changes in diet, environmental conditions, and host acclimation—play a stronger role in shaping microbiome dynamics than individual's physiological characteristics. Beta diversity parameters proved to be better predictors for determining the immunity and nutritional status of red deer than alpha diversity metrics. Associations between specific microbial taxa and host innate immune function suggest that microbiome composition, rather than diversity per se, plays a key role in modulating host physiological processes. In particular, the enrichment of fiber-degrading and short-chain fatty acid–producing taxa may contribute both to efficient nutrient utilization and to the maintenance of immune homeostasis, suggesting that in wild ruminants, access to a high-quality diet rich in easily digestible fibers might be key for survival in the wild beyond meeting nutritional needs, but also to help keep an optimal physiological balance and immune capacity. These findings highlight the importance of integrating compositional and functional perspectives when studying host–microbiome interactions and suggest that future research incorporating metagenomic and metabolomic approaches will be essential to fully elucidate the mechanisms linking microbial communities to host health in wild ruminants.

## 6. Supplementary material

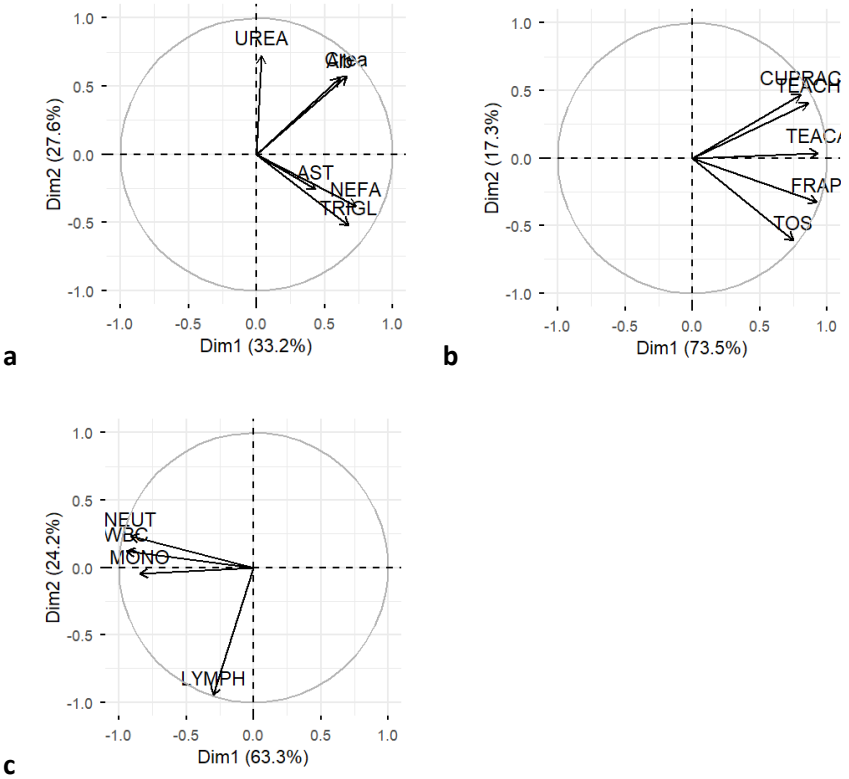



**Figure S1.** PCA results for our nutritional (a), oxidative status (b) and inflammation (c) models. Arrow length is directly proportional to variable contribution to either Dimension 1 (Dim1) or 2 (Dim2) of each PCA. NEFAs: Non-Esterified Fatty Acids; AST: aspartate aminotransferase; TOS: Total Oxidative Status; FRAP: Ferric Reducing Antioxidant Power; CUPRAC: Cupric Ion Reducing Antioxidant Capacity; TEAC: Trolox Equivalent Antioxidant Capacity; WBC: White Blood Cells.

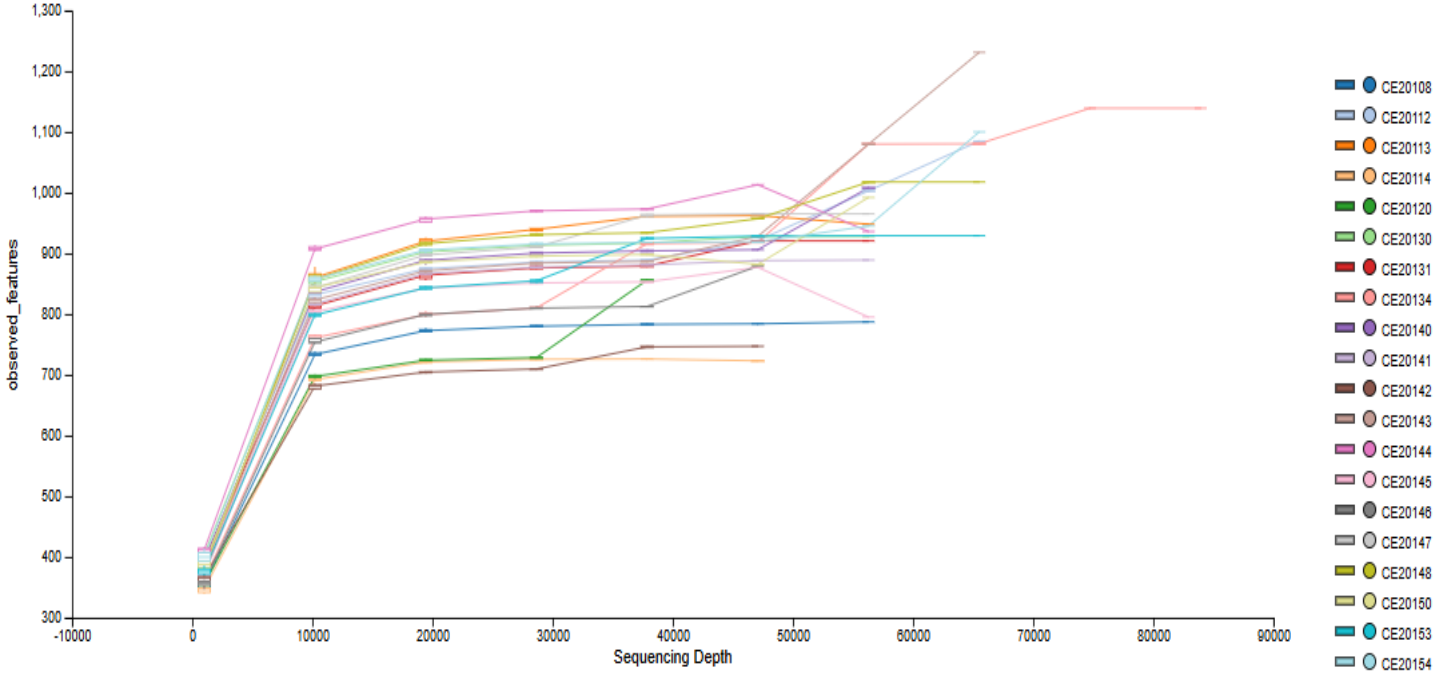



**Figure S2.** Rarefaction curves displaying sequencing depth of the observed features for each individual included in the study, from a total of 74 samples belonging to the same red deer. Each color represents an individual red deer.

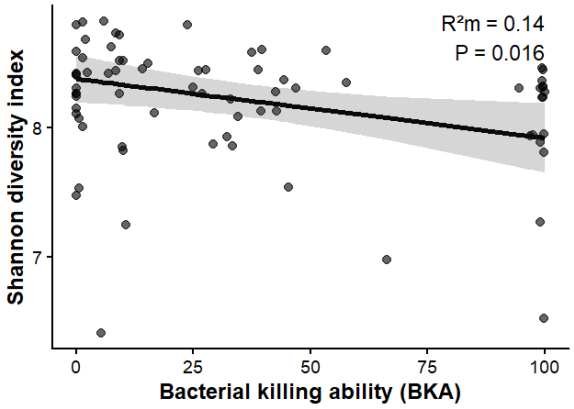



**Figure S3.** Relationship between Shannon diversity index and bacterial killing ability values, including the marginal percentage of observed variance retained by the model (R2m) and p-value (P). Points represent individual samples, while the solid line indicates the fitted relationship from a linear mixed-effects model including individual identity as a random effect. Shaded areas represent 95% confidence intervals.

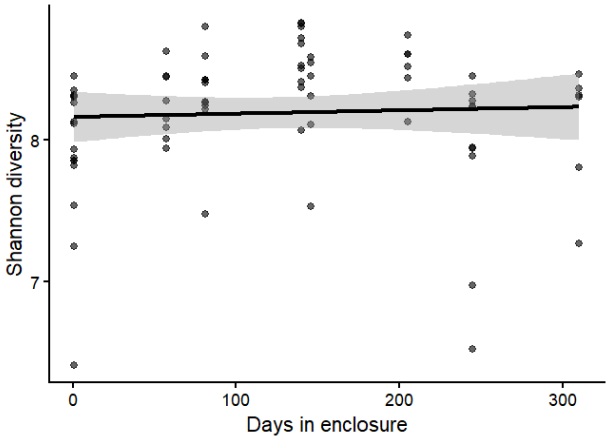



**Figure S4.** Temporal evolution of Shannon diversity index. Each point represents an individual sample, while the solid black line indicates the fitted relationship between the two variables using a linear mixed-effect model which included individual red deer identity as a random effect. Shadowed grey margins indicate 95% confidence intervals.

**Table S1.** Results from each Principal Component Analysis and their respective sample size (n), with contribution and correlation of each explanatory variable to dimension 1 (PC1) and dimension 2 (PC2). NEFAs: Non-Esterified Fatty Acids; AST: aspartate aminotransferase; TOS: Total Oxidative Status; FRAP: Ferric Reducing Antioxidant Power; CUPRAC: Cupric Ion Reducing Antioxidant Capacity; TEAC: Trolox Equivalent Antioxidant Capacity; WBC: White Blood Cells.

| **Nutritional PCA (n=69)** | | | | |
|---|---|---|---|---|
| | **PC1 (33.2%)** | | **PC2 (27.6%)** | |
| **Variables** | **Contribution (%)** | **Correlation** | **Contribution (%)** | **Correlation** |
| NEFAs | 26.72 | 0.73 | 8.84 | - 0.38 |
| Triglycerides | 22.99 | 0.68 | 16.50 | - 0.52 |
| Urea | 0.06 | 0.04 | 31.82 | 0.73 |
| Creatinine | 21.92 | 0.66 | 19.79 | 0.57 |
| Albumin | 18.77 | 0.61 | 18.97 | 0.56 |
| AST | 9.54 | 0.44 | 4.08 | - 0.26 |
| **Oxidative status PCA (n=74)** | | | | |
| | **PC1 (73.50%)** | | **PC2 (17.30%)** | |
| **Variables** | **Contribution (%)** | **Correlation** | **Contribution (%)** | **Correlation** |
| TOS | 15.38 | 0.75 | 42.99 | 0.61 |
| FRAP | 23.27 | 0.92 | 12.35 | 0.33 |
| CUPRAC | 17.56 | 0.80 | 25.27 | - 0.47 |
| TEACh | 20.09 | 0.86 | 19.23 | - 0.41 |
| TEACa | 23.70 | 0.93 | 0.16 | - 0.04 |

(Continuation of Table S1 on the next page)

| **Inflammation PCA (n=74)** | | | | |
|---|---|---|---|---|
| | **PC1 (63.30%)** | | **PC2 (24.20%)** | |
| **Variables** | **Contribution (%)** | **Correlation** | **Contribution (%)** | **Correlation** |
| WBC | 35.14 | 0.94 | 1.49 | - 0.11 |
| Neutrophils | 33.05 | 0.92 | 5.51 | - 0.23 |
| Lymphocytes | 3.54 | 0.30 | 92.76 | 0.95 |
| Monocytes | 28.27 | 0.85 | 0.25 | 0.05 |

**Table S2.** PERMANOVA models and results for Bray Curtis and Aitchison distance. N: sample size. Df: degrees of freedom; SumOfSq: sum of squares. $R^2$ = coefficient of determination. F = F-statistic. Residual = the total amount of variance that is not explained by the explanatory variables included in the model. Total: sum of the residual and explanatory variables. Rows marked in bold indicate that the explanatory variable was significant.

| Model | n | Explanatory variable | Df | SumOfSq | $R^2$ | F |
|---|---|---|---|---|---|---|
| Immunity PERMANOVA | 72 | **Days_intro** | **1** | **0.924** | **0.075** | **6.166*** ** |
| | | **BKA_Ec** | **1** | **0.623** | **0.050** | **4.156*** ** |
| | | OxStress_PC1 | 1 | 0.156 | 0.013 | 1.043 |
| | | Inflam_PC1 | 1 | 0.176 | 0.014 | 1.176 |
| | | Residual | 67 | 10.038 | 0.811 | |
| | | Total | 72 | 12.365 | 1.000 | |
| Nutrition PERMANOVA | 68 | **Days_intro** | **1** | **1.050** | **0.087** | **6.526*** ** |
| | | Nutrition_PC1 | 1 | 0.151 | 0.0126 | 0.940 |
| | | **Nutrition_PC2** | **1** | **0.293** | **0.024** | **1.817** ** |
| | | Residual | 65 | 10.461 | 0.870 | |
| | | Total | 68 | 12.024 | 1.000 | |
| Immunity PERMANOVA | 71 | **Days_intro** | **1** | **57470** | **0.048** | **3.620*** ** |
| | | **BKA_Ec** | **1** | **42222** | **0.035** | **2.660*** ** |
| | | OxStress_PC1 | 1 | 14794 | 0.012 | 0.931 |
| | | Inflam_PC1 | 1 | 16425 | 0.014 | 1.034 |
| | | Residual | 67 | 1063652 | 0.880 | |
| | | Total | 71 | 1208533 | 1.000 | |
| Nutrition PERMANOVA | 68 | **Days_intro** | **1** | **60724** | **0.052** | **3.724*** ** |
| | | Nutrition_PC1 | 1 | 16009 | 0.014 | 0.982 |
| | | **Nutrition_PC2** | **1** | **23557** | **0.020** | **1.445** ** |
| | | Residual | 65 | 1059861 | 0.910 | |
| | | Total | 68 | 1164587 | 1.000 | |

## Study III

# Density-dependent feeding behavior and physiological consequences in overabundant red deer in a Mediterranean environment: an experimental approach

*Journal of Wildlife Management, under review*

## Abstract

Wild herbivore overabundance is increasingly common worldwide. While research has focused on ecosystem effects of high herbivore densities, their impacts on life-history traits of overabundant species remain understudied. This knowledge gap is especially relevant in highly seasonal environments where resources can become seasonally scarce and large herbivores often reach high population densities. This study examines density-dependent feeding selection and diet quality in red deer (*Cervus elaphus*) and evaluates whether these responses are associated with changes in physiological stress. We recreated two overabundant deer densities (high-density, 41 deer/Km$^2$; and hyper-density, 112 deer/Km$^2$) typical of natural and commercial hunting spaces in central and southern Iberia. Faecal samples were collected once a month over a year to assess diet composition and quality. Faecal glucocorticoid concentrations were measured as an indicator of physiological stress. To assess whether changes in feeding behaviour reflected reductions in preferred plant availability, browsing intensity and above-ground biomass of 290 plant individuals were measured at the beginning and end of the experiment. Although population density did not affect diet diversity or evenness, red deer at high and hyper-densities compensated for the depletion of preferred plants (e.g. *Quercus rotundifolia*, *Erica* spp.) by increasing consumption of less preferred species (e.g. *Cistus ladanifer*, *Salvia rosmarinus*), with this shift occurring earlier under hyper-density.

Consumption of non-preferred plants strongly depended on phenological stage, revealing marked differences between preferred and non-preferred woody plants. Despite similar plant cover and composition among enclosures, hyper-density conditions were associated with consistently lower faecal nitrogen concentrations (indicating reduced diet quality) and higher faecal glucocorticoid metabolites (reflecting increased chronic stress) compared to high-density conditions. These results suggest that increasing deer densities alter feeding behaviour, shaped by season and plant preference, and drive resource depletion and physiological stress under overabundant conditions, potentially reducing species fitness and ecosystem sustainability.

## 1. Introduction

Wild herbivore overabundance is an emerging global change driver reshaping terrestrial ecosystems worldwide (Pringle et al., 2023). Large herbivores, i.e., all extant wild terrestrial herbivore species over 45 kg body mass (Wang et al., 2023), exert consistent top-down control on plant communities, affecting soil properties (Wang et al., 2018), nutrient availability (Barbero-Palacios et al., 2023), green biomass (Karp et al., 2024; Meronk & Long, 2025), and species composition (Cuerdo et al., 2025). When herbivore numbers exceed the environment's regenerative capacity, ecological balance is disrupted, biodiversity is threatened, and the risk of human–wildlife conflict or economic loss increases. At this point, the population is considered overabundant (Caughley, 1981).

While herbivore overabundance has been associated with shifts in animal life-history traits, vegetation dynamics, and foraging behavior, these relationships are typically explored independently, leaving the mechanistic explanations among resource depletion, diet quality, and consequences in wild animal fitness and population dynamics poorly understood. Available literature correlates overabundance to changes in body development (Serrano et al, 2007, 2008), female fertility (Albon et al., 1983), and eventually juvenile (Clutton-Brock et al., 1987) and adult survival (Festa-Bianchet et al., 2003); but these studies do not establish the underlying mechanisms driving these patterns, often suggesting that they may result from compromised ecosystem integrity and

reduced resource availability (Hobbs, 1996). Additionally, early detection of overabundance is commonly based on environmental metrics related to vegetation use and resource depletion such as the browsing intensity (Donini et al., 2024; Perea et al., 2015), damage in palatable plants (Rhodes & St. Clair, 2018), effects on seed production (Fattorini et al., 2026; Lecomte et al., 2016), biomass loss (Pellerin et al., 2010; White, 2012) or the increased accumulation of fecal pellets (Limpens et al., 2020), rather than on changes in the life-history traits of the overabundant species, such as feeding behavior. Simultaneous monitoring of herbivore density, diet composition, and plant-level impacts as a sign of resource depletion over time remains rare (Donini et al., 2024; Walker et al., 2023).

Existing research supports potential negative consequences for overabundant herbivores due to competition for food resources. As population density increases, animals tend to move to less productive patches (Mobæk et al., 2009), thereby expanding their dietary niche to include low-palatable resources (Stewart et al., 2011). However, how this bottom-up process varies seasonally, affecting feeding selection, nutrition, and potential physiological stress from consuming lower-quality plants, remains poorly understood. Overall, existing studies have offered valuable insights into density-related changes in herbivore habitat use and foraging behavior, yet they rarely provide an integrated perspective encompassing intra-annual seasonal variation, diet quality, and its effects on physiology and metabolic stress.

Red deer (*Cervus elaphus*) numbers have increased across Europe since 1945, but growth rates and timing differ markedly across regions (Milner et al., 2006; Putman et al., 2011). In Spain and Portugal, red deer populations have grown rapidly in the last century, linked to game-estate management, reintroductions and rural abandonment, currently reaching high densities over 50 deer/km$^2$ in protected reserves and hunting areas (Acevedo et al., 2008, 2011; Vicente et al., 2007). These very high local densities cause a reduction in plant diversity (Perea et al., 2014) and cascading effects on key ecosystem processes such as plant-pollinator networks (Hernández-Castellano et al., 2025), seed dispersal (Muñoz & Bonal, 2007) and impact other vertebrate populations (Barrio et al., 2013; Carpio et al., 2013, 2014).

Across their species-wide distribution, red deer consume a highly diverse diet (Gebert & Verheyden-Tixier, 2001), reflecting their high dietary plasticity and ability to exploit a wide range of plant species year-round in different environmental contexts. In the Mediterranean habitats, red deer rely mostly on evergreen shrubs, particularly in summer, as this dry season becomes the most critical period of the year (Bugalho & Milne, 2003). According to optimal foraging theory (Belovsky, 1984), red deer will optimize energy and nutrient intake by selectively feeding on high-quality plant species or parts, while minimizing the ingestion of fibrous material and toxic secondary compounds (Becker, 2017). Therefore, when overabundant populations overconsume their preferred plant species, their capacity for selective foraging necessarily declines

(Owen-Smith, 2002). This reduction may impose additional physiological stress beyond the natural seasonal constraints associated with changes in plant palatability.

Behavioral adaptations of large herbivores to food shortages can be assessed through the analysis of different types of fecal indicators, with the added advantage that feces are relatively easy to obtain. Fecal nitrogen (FN), used as an indirect indicator of diet digestibility, and fecal fiber content are the two main indicators used to assess diet palatability in ruminants (Ramanzin et al., 2017). Additionally, Fecal glucocorticoid metabolites (FGM) inform about homeostasis of individuals and populations, as FGM are derivatives of cortisol and are known to increase in situations of higher physiological stress (Keay et al., 2006; Santos et al., 2014). In red deer, these fecal indicators have been successfully calibrated and are widely used for population monitoring in Mediterranean ecosystems (Santos et al., 2014, 2018).

This study aimed to experimentally assess annual changes in diet composition, diet quality, and physiological stress to characterize red deer adaptations to overabundance in a seasonal environment. We recreated two overabundant density scenarios and collected fecal samples over 12 months to test how overabundance affects red deer feeding strategies in a Mediterranean Iberian environment, while monitoring plant biomass and browsing intensity to detect resource depletion. Through this field manipulation, we tested the following hypotheses and predictions.

Herbivore overabundance is known to alter plant community composition through the depletion of preferred species (Côté et al., 2004). Accordingly, we predicted density-dependent diet selection (hypothesis 1), whereby red deer under the highest density conditions would expand their dietary niche to include less palatable plant species than individuals at lower densities, particularly during periods of low primary production. This shift in feeding behavior is expected to affect the biomass of consumed plant species, which should decline more markedly under the highest densities. The increased reliance on nutritionally poorer plants under the highest densities is expected to result in a decline in diet quality (hypothesis 2). Finally, reduced diet quality combined with increased foraging effort is predicted to elevate physiological stress (hypothesis 3), leading to higher fecal glucocorticoid metabolite concentrations (Keay et al., 2006; Santos et al., 2014), especially during moments of low primary production.

## 2. Materials and methods

*Study area*

The study was performed in the Valdeyernos valley at ca. 950 m a.s.l., within the public estate of Los Quintos de Mora, in Montes de Toledo rangeland, south-central Iberia (Toledo province, Spain, 4°5057.3800W, 39°26048.5700N). The climate is Continental Mediterranean, with mean annual rainfall of ~600 mm and mean annual temperature of ~14 °C. Quintos de Mora is mainly made up of layers of shale and quartzite. Vegetation consists of a mosaic of

small woodlands dominated by oak trees, mostly holm oaks *Quercus rotundifolia* Lam. (formerly *Quercus ilex* subsp. *ballota*) and *Q. pyrenaica* Willd., together with large scrubland patches dominated by *Cistus ladanifer* L., *Salvia rosmarinus* L., and *Erica* spp. L. (San Miguel et al., 2011). Both scrublands and forests possess scattered herb layers dominated by annual (therophytes) taxa.

*Phenological phase determination methodology*

We characterized the study area vegetation phenology by extracting the NDVI data from 2020 to 2023 using MODIS pixel images, and we used the "phenopix" R package version 2.4.5 (Filippa et al., 2016) to obtain the start and ending dates of the green up, stable peak, senescence and dormancy periods.

We used the Normalized Difference Vegetation Index (NDVI) to describe vegetation activity in the study area from January 2020 to December 2023. NDVI values were obtained from the MODIS-Terra Vegetation Indices collection provided by NASA (MOD13Q1, Collection 5). Specifically, we analyzed 16-day composites of maximum NDVI values at a nominal spatial resolution of 250 meters. Since our sampling period started in April 2021 and finished in May 2022, we extracted the phenological data for the cycles from 2021 to 2023. For the NDVI, we extracted the dates for each phenological phase using the "phenopix" R package (version 2.4.5) in the study area. Briefly, we optimized NDVI value curve fitting to the phenological cycle by empirically setting back the starting database calendar date 181 days. We then performed model fitting

to our database and retained the best model fit and threshold based on the obtained RMSE (root mean square errors) values, where the best fit had the lowest RMSE. We also graphically explored the predicted curve to further confirm model fitting. The model fitting that had the lowest RMSE for the NDVI data was the GU model fitting, with a GU threshold. The GU approach provides the upturn day (UD), stabilization day (SD), downturn day (DD) and restoration day (RD) values. Using their values, we determined the duration and date brackets of the green up (GU) period, stable peak period (SPP), senescence period (SP) and dormant period (DP).

*Field experiment design*

We built three different enclosures of 7.4, 7.1 and 2.6 ha respectively and recreated three different scenarios of contrasted herbivory pressure using female red deer, namely control (2.6 ha enclosure, no deer), high density (7.4 ha enclosure, 63 deer/km$^2$) and hyper-density (7.1 ha, 113 deer/km$^2$). With the control, we recreate a situation of deer exclusion, commonly used to protect emblematic vegetation in protected areas. With the high-density situation, we represent a common situation of many natural spaces from central and southern Spain e.g., 30 deer/Km$^2$ (Carpio et al., 2015; Charco García, 2002; Fernández-Olalla et al., 2006; Vicente et al., 2007). The hyper-density scenario simulates game management conditions in central Spain, where extremely high red deer populations (e.g., 83 individuals/Km$^2$ in Sierra Madrona) are maintained for commercial hunting purposes (Acevedo et al., 2008;

Azorit et al., 2002). The absence of other ungulates (e.g., wild boar *Sus scrofa*, roe deer *Capreolus capreolus*, European mouflon *Ovis aries musimon*) in the enclosures was regularly checked (at least once a month for the duration of the study) through 16 trail cameras (Strikeforce HD ProX 20 MP, motion detection camera trapping) set in the enclosures. We also characterized the woody and herbaceous vegetation present inside each enclosure, determining plant species that were present and their respective vegetation coverage inside each enclosure, using the point-intercept transect method for woody species (Calleja et al., 2019) and following the methodology described in Cuerdo et al. (2025) for herbaceous species; which consisted of describing the herbaceous species occurrence and cover in a total of 90 quadrats that measured 50 x 50 cm.

The most abundant plant species in both treatments were *Cistus ladanifer, Erica arborea* and *Salvia rosmarinus*, while all other species had a coverage of ≤ 2%, approximately. We also compared vegetation cover means for each species found within the enclosures using a Mann-Whitney test to test whether plant coverage differed significantly between treatments, which it did not (Table S2).

We only used female red deer in this experiment to keep deer densities constant by avoiding reproduction, avoiding sex bias (as male red deer are larger than females and tend to eat more than a non-gestating or lactating female) (Dryden, 2011) and for operator safety, as male red deer can be risky to manage during the rut. Red

deer herds were obtained from the Cabañeros National Park (39º 23' 47" N, 4º 29' 14" O), a natural area located 40 km from the study area that shares the same environmental conditions. These females were released into the enclosures in March 2021 at an average age of 21 months (15-32 months) and an average weight of 61 kg (between 47-74 kg). Seven females were released in the high-density enclosure, and 12 into the hyper-density one, creating densities of 95 deer/km$^2$ and 169 deer/km$^2$. As only females were used, these densities are equivalent to 63 and 113 deer/km$^2$ of a population with a 1:1 sex ratio (Peláez et al., 2017).

*Fecal sampling procedure*

Fecal sampling was carried out from April 2021 to May 2022, although June 2021 and April 2022 were not sampled due to logistical reasons. We aimed to collect six fecal samples monthly in each density treatment. We obtained a total of 69 samples from the high-density treatment (monthly mean: 5.7 samples, 5 minimum and 7 maximum) and 68 from the hyper-density treatment (monthly mean: 5.67, 5 minimum and 6 maximum). Each fecal sample corresponded to all the pellets found within the whole fecal pile. We assumed each fecal pile belonged to a different individual. To try to decrease unintentional sampling from the same deer twice, we only picked fecal piles that were more than 10 meters apart from each other and from different areas of the enclosure. Fecal pellets were selected based on freshness, deposited ca. 24–48 h before collection (i.e., pellets with a thin layer of mucus). Pellet samples

were collected with nitrile gloves and stored in a zip log bag and labelled, indicating the sampling site, enclosure and date. Nitrile gloves were changed between each fecal sample. Bags were stored in a cold box (4 to 8 $^{o}$C) during transport to the research station and later kept at -20 $^{o}$C until further processing.

*Diet quality and composition assessment*

We dried the fecal samples at 60 $^{o}$C for 48 to 72 h or until the sample weight remained constant. Subsequently, samples were milled using a CT 293 Cyclotec Labtec Line miller (FOSS Iberia, Barcelona, Spain) with a 1.0 mm particle-size filter. We used a NIRSystems 500 (FOSS, Hillerød, Denmark) to determine fecal nitrogen, fiber composition and fecal glucocorticoid metabolites (FGM) through Near Infrared Reflectance Spectroscopy (NIRS) technique. NIR range was 1100 to 2500 nm with a scanning interval. Each milled sample was placed into a circular capsule with a quartz window of 3.5 cm diameter, and we took two spectral measurements per sample as log 1/reflectance (log 1/R). The calibration models used to predict chemical composition and metabolite concentrations had been previously validated for total fecal nitrogen (Villamuelas et al., 2017), fiber composition (Rossa et al. 2025), and fecal glucocorticoid metabolite levels (Santos et al., 2014). We obtained the fecal nitrogen (FN), acid detergent lignin (ADL) and fecal glucocorticoid metabolites (FGM) for each sample. We used FN as an indicator of diet quality, widely used for wild ruminants (Mandujano, 2014; Portilla Pinzón et al., 2019). Additionally, FN can change over short

time scales, making it useful for detecting seasonal or habitat-driven variation in diet quality (de la Peña et al., 2026). ADL is a non-digestible fiber fraction that is composed of various polyphenols which provide rigidity to the plant as its phenological stage progresses, and higher ADL levels are negatively correlated to nutritional quality (Van Soest, 1967). Finally, FGM was used as an indirect indicator of cortisol concentration to evaluate the adaptation cost of the animal to the environment (Katsu & Baker, 2021).

We used a modified version of Stewart's protocol (D. R. M. Stewart, 1967) for determining diet composition using the cuticle microhistological analysis technique. This technique has been widely used for studying diet composition in both wild (La Morgia & Bassano, 2009; Suter et al., 2004) and domestic species (Bartolomé et al., 2011). A detailed protocol can be found in Espunyes et al. (2019). Briefly, we mixed 10 grams of the sample in a test tube with 5 mL of 65% concentration of $HNO_3$ solution and placed the test tube in a water bath kept at 80 $^{o}$C for 1 min to perform an *in vitro* digestion. The test tube was removed from the water bath and emptied into a glass beaker containing 200 mL of distilled water to stop the chemical reaction. Later, the sample suspension was sieved through 1.00 and 0.25 mm filters. We retained the 0.25 mm filtered fraction and spread it evenly on a glass microscope slide that had an aqueous 50% glycerine solution. We then placed a cover slide over the sample and sealed it using DPX microhistological varnish. We made three replicas per sample. We later examined the slides under

the microscope at x100 and x400 magnification and counted a total of 100 plant fragments per sample. Plant identification was based on the determination of morphological traits from the epidermis, and we used a database of 25 plant species which belonged to Non Leguminous Woody plants (NLW), leguminous woody plants (LW), non-graminoid herbaceous plants (NGH), graminoids (G) and bryophyta (B) functional groups. Fragments that could not be identified as a given species or genre were classified as either "Woody" or "Herbaceous". Using these data, we then calculated Shannon-Wiener's diversity Index, Pielou's Evenness Index for each sample, and Whittaker's dissimilarity index between months to catch dietary shifts as time passed (Whittaker, 1952). The Shannon-Wiener index was calculated:

$$H' = -\Sigma_{i=1}^{S}(P_i \; x \; ln(P_i))$$

where *S* is the number of species and *Pi* is the proportion of *i* species in the sample (Odum, 1974; Shannon & Weaver, 1948). The Shannon-Wiener index ranges from 0 ≤ H' ≤ ln(S). Species evenness (i.e. Pielou's index, E) was calculated as:

$$E = H/ln(S)$$

where *H* is the obtained Shannon index value and *S* is the species number (Pielou, 1969). Pielou's Evenness index ranges from 0 to 1 (Ricotta & Avena, 2003), where 1 means that all species are equally consumed and 0 that animals only feed on one species. Finally, Whittaker's index of dissimilarity calculates shifts in dietary plant composition (Whittaker, 1952), where the dissimilarity index

compares inter-monthly mean proportional similarities of each plant species between two consecutive months, obtaining values that go from 0 (indicating no dietary shift because plant species proportion remains the same between the compared months) to 1 (equivalent of maximum difference in plant proportions).

*Assessment of plant biomass and browsing intensity in the enclosures*

We marked 290 plant individuals from the most abundant woody species found in the enclosures, which are: *Cistus ladanifer, Erica arborea, Erica australis, Erica scoparia, Quercus rotundifolia* and *Salvia rosmarinus.* A total of 126 plants in the high-density treatment, and 165 plants in the hyper-density one. Within the high-density treatment, we marked 18 *C. ladanifer;* 23 *E. arborea*; 19 *E. australis*; 18 *E. scoparia*; 27 *Q. rotundifolia* and 21 *S. rosmarinus.* Regarding the hyper-density treatment, these numbers were 30 *C. ladanifer;* 30 *E. arborea*; 35 *E. australis*; 9 *E. scoparia*; 30 *Q. rotundifolia,* and 31 *S. rosmarinus*. We then did annual recordings of each plant regarding their height (h), minor and major diameters (all in cm), as well as the browsing intensity of each plant using a scale that went from 0 (not browsed) to 5 (maximum browsing), following the methodology described in Perea et al. (2015). Data recording was carried out in early summer during 2021 and 2022 (July), as woody plants become the main food source for red deer during summer (Perea et al., 2014).

*Statistical analysis*

We analyzed three different datasets, to test our hypotheses: (i) diet composition and diet diversity indexes (Shannon's index of diversity and Pielou's index of evenness) obtained from microhistological analyses (hypothesis 1); (ii) changes in plant biomass and browsing degree intensity (hypothesis 1), and (iii) diet quality and physiological stress indicators derived from near-infrared spectroscopy (NIRS, hypotheses 2 and 3).

For testing hypothesis 1, we fitted a set of generalized additive mixed models (GAMMs) using each diet diversity index (Shannon's index, Pielou's index of evenness and Whittaker's index) and the consumption of the most abundant plant species as response variables. We used the "mgcv" package (Wood, 2017) for model fitting. Model selection was based on Akaike's Information Criterion corrected for small sample size (AICc), using the "MuMIn" package (Bartoń, 2024); the model with the lowest AICc and a difference > 2 relative to the second-best model was retained. We inspected the residuals of the final models to assess model adequacy. Prior to model construction, we evaluated correlations among continuous explanatory variables using the "corrplot" package (Wei & Simko, 2024), and highly correlated predictors were excluded. The tested models included the number of days passed since the start of the experiment ("DaysIntro"), phenological stage ("Phenology") and deer density ("Treatment"). "DaysIntro" was always included as a smoothed term with k = 6 to decrease model overfitting. We

transformed the “Phenology” variable to obtain a total of three different phenology periods instead of the original four (green up, stable peak period, senescence period and dormancy period) due to the low sample size (n = 12) retained during the stable peak period; therefore, we pooled the samples from the green up (n = 24) and stable peak (n = 12) period together and made three new levels called green up-stable peak, senescence and dormancy periods. We also included interaction terms between deer density and number of days spent in the enclosure, which represented the main effect of interest, and deer density and phenological stage.

Regarding changes in plant biomass, we summarized the morphometric plant variables (height, maximum diameter, and minimum diameter), through a Principal Component Analysis (PCA) using the “prcomp()” function from the “stats” package in R. We then selected the first principal component (PC1), hereafter called “phytovolume”, as it accounts for 86.3% of the observed variability in our data. We inverted the direction of PC1 by multiplying its scores by −1, as all morphometric variables had negative loadings on this component. Subsequently, we subtracted the minimum PC1 value from all scores to ensure that phytovolume values were strictly positive, thereby facilitating biological interpretation. Dead plants were removed for the PCA analysis and then added to the data with a phytovolume of 0. We evaluated if observed changes in the phytovolume and browsing intensity for each species from 2021 to 2022 within each treatment were statistically significant by

applying a Wilcoxon signed rank test using the “wilcox.test()” function form the “stats” package in R.

For testing hypotheses 2 and 3, we individually fitted FN, ADL and FGM using GAMM models following the same process and explanatory variables described for hypothesis 1.

All analyses were conducted in R Studio version 4.4.1 (R Core Team, 2024).

## 3. Results

*Diet composition and diversity indexes*

Diet mainly consisted of woody species throughout the year, reaching minimum ~75% per month in both treatments (Table S3). Consumption of woody and herbaceous plants was very similar among treatments, and bryophyte consumption was negligible. Regarding diet composition, *Erica spp.* was the most consumed genus, followed by *Quercus rotundifolia, Salvia rosmarinus*, *Cistus ladanifer* and non-graminoid herbaceous plants (Table S3).

Diet diversity (Shannon’s index) and diet evenness (Pielou’s index) followed very similar patterns. The best fitting models for both variables retained the number of days red deer spent inside the enclosure (i.e. “DaysIntro”, smoothed) and plant phenology (“Phenology”, Table 1). Diet diversity and evenness were lowest during dormancy and increased significantly during the green up–

stable peak period, with no significant differences during senescence (Table 1, Figure 1a and 1b).

Temporal changes in diet dissimilarity (Whittaker's index) were influenced by the interaction between days spent inside the enclosures and deer density, as well as plant phenology, explaining 75.8% of the observed variance ($R^2$adj= 0.758, Table 1). Thus, we obtained significant nonlinear temporal patterns that differed between treatments (Table 1). In general, the hyper-density treatment showed smaller changes in diet composition from April 2021 to August 2021, after which values increased until reaching a maximum in December 2021. In contrast, the high-density treatment showed a steady decrease from April 2021 to November 2021, followed by an increase until January 2022 (Figure 1c). Changes in diet composition were significantly greater during senescence than during dormancy, whereas the green up–stable peak period also differed significantly from dormancy.

**Table 1.** Results of the final retained GAMM models for diet indexes ("Resp. var" column). N: model sample size. In the "explanatory variable" (Exp. variable) column, smoothed terms are marked with an S() and interactions are marked using ":". Shaded cells indicate that parameter/category is not applicable to that specific variable. Column "level" refers to explanatory variables with more than 2 levels. "Estimate": coefficient for parametric variables; Edf: Estimated degrees of freedom, applicable only to a smoothed term; SE: Standard Error; "T-test/F-test": statistical test applied to parametric variables (T-test) or smoothed terms (F-test); $R^2$: percentage of observed variability explained by the model. Bold numbers indicate the parameters were significant, and * indicate significance degree (*: $p < 0.05$; **: $p < 0.01$, ***: $p < 0.001$). GUSPP: green up-stable peak period; SP: senescence period.

| Resp. var | N | Exp.variable | Level | Estimate Edf | SE | T-value F-value | $R^2$ |
|---|---|---|---|---|---|---|---|
| Shannon index | 136 | S(Days) | | 4.83 | | **16.05*** ** | 0.396 |
| | | Phenology | GUSPP | 0.624 | 0.117 | **5.33*** ** | |
| | | | SP | 0.065 | 0.090 | 0.73 | |
| Pielou's index | 136 | S(Days) | | 4.81 | | **14.57*** ** | 0.403 |
| | | Phenology | GUSPP | 0.134 | 0.032 | **4.12*** ** | |
| | | | SP | - 0.020 | 0.025 | -0.81 | |
| Whittaker's index | 126 | S(Days:$Treat_{HD}$) | | 3.87 | | **31.64*** ** | 0.758 |
| | | S(Days:$Treat_{HyD}$) | | 4.80 | | **16.41*** ** | |
| | | Treatment | | 0.007 | 0.021 | 0.304 | |
| | | Phenology | GUSPP | - 0.008 | 0.025 | - 0.321 | |
| | | | SP | 0.177 | 0.019 | **9.40*** ** | |

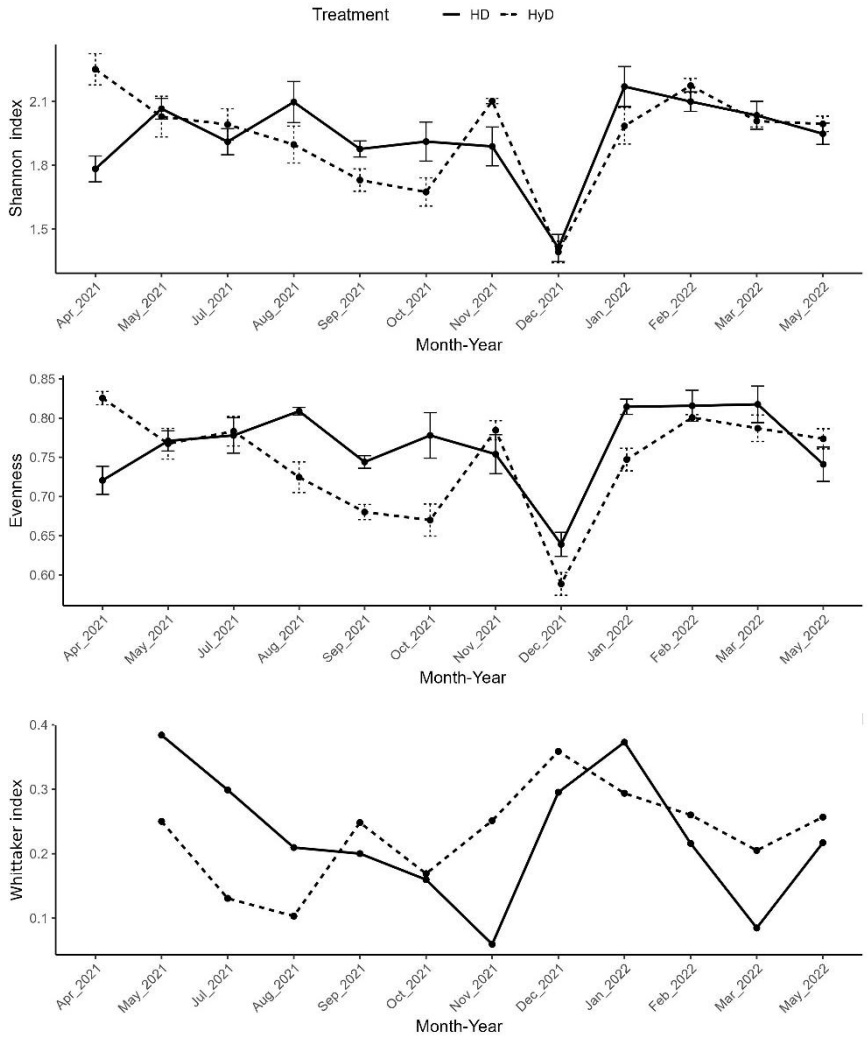



**Figure 1.** Monthly dynamics of Shannon's diversity index (a), Pielou's index of evenness (b) and Whittaker's dissimilarity index (c) for the sampling period (April 2021 to May 2022), for both deer densities HD: high density, HyD: hyper-density) throughout the experiment (from April 2021 to May 2022).

*Plant species consumption*

*Erica* spp.*, Quercus rotundifolia, Cistus ladanifer* and *Salvia rosmarinus* were the most consumed plants (Supplementary Information). *Erica* spp. followed the same consumption pattern in both treatments, although the hyper-density treatment displayed basal levels that remained higher throughout the duration of the experiment, compared to high density (Figure 2a). *Erica* consumption was influenced by the phenological cycle and the interaction term between treatment and days spent inside the enclosures (Table 2). *Erica* spp. consumption peaked during December 2021 (SP period) and then went down and remained at stable levels for both treatments for the rest of the experiment.

For *Quercus rotundifolia*, consumption rose from April to August 2021 in both treatments, but then remained very low under hyper-density, while under high density it peaked in summer and declined gradually toward winter before slightly recovering in spring 2022 (Figure 2b). The difference in *Q. rotundifolia* consumption between treatments was most marked during the Green Up Stable Peak period (August 2021 to November 2021), and the start of the Senescence period (November 2021 to December 2021), while consumption levels remained similar for the rest of the sampling cycle. For *Q. rotundifolia,* the best model retained the interaction between the smoothed term “DaysIntro” and the treatment variable and phenology (Table 2).

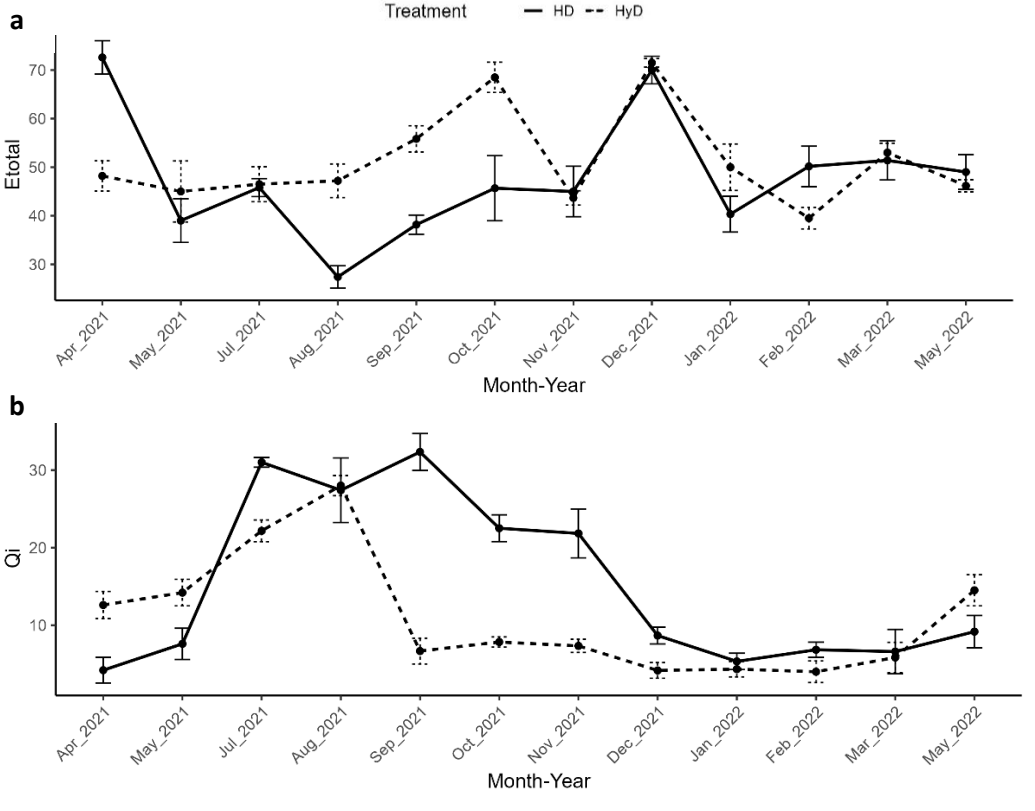



**Figure 2.** Monthly dynamics of the percentage (%) of *Erica* sp. "Etotal" (a) and *Quercus ilex* "Qi" (b) consumption for the sampling period (April 2021 to May 2022), for both deer densities "TreatmentOrder" (HD: high density continuous line, HyD: hyper-density, dashed line).

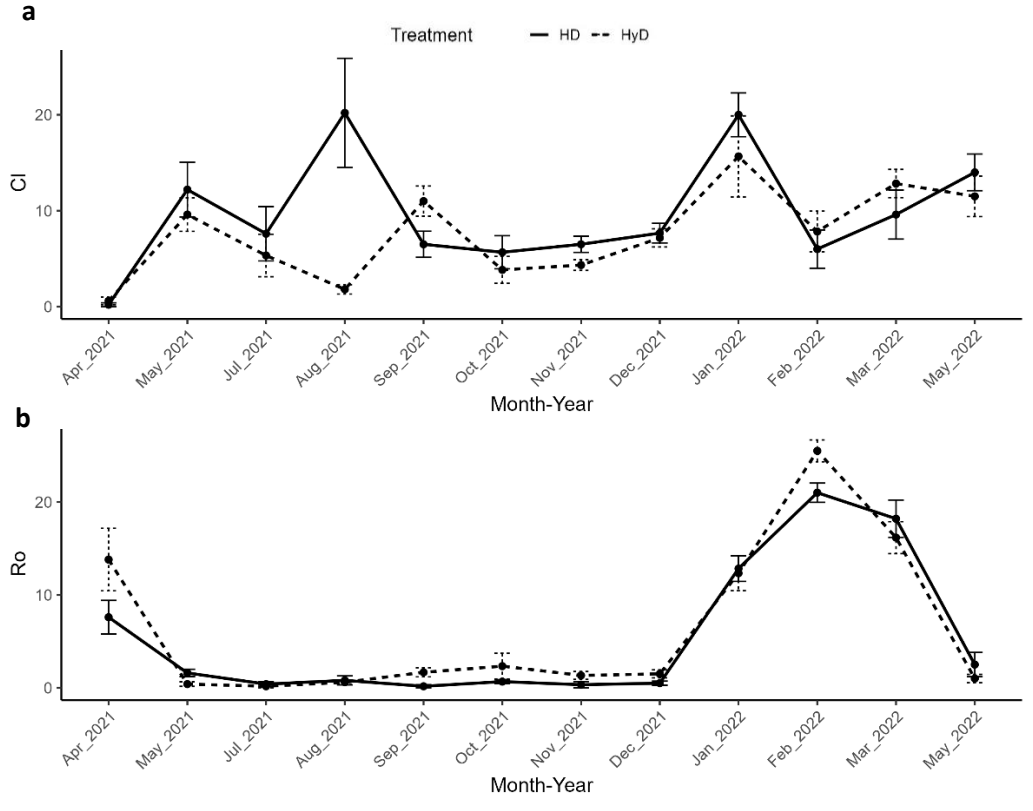



**Figure 3.** Monthly dynamics of the percentage (%) of *Cistus ladanifer* "Cl" (a) and *Salvia rosmarinus* "Ro" (b) consumption for the sampling period (April 2021 to May 2022), for both deer densities "TreatmentOrder" (HD: high density continuous line, HyD: hyper-density, dashed line).

**Table 2.** Results of the final retained GAMM models for plant species consumption ("Response variable" column). N: sample size (number of observations included in the model). In the "explanatory variable" column, smoothed terms are marked with an S() and interactions are marked using ":". Shaded cells indicate that parameter/category is not applicable to that specific variable. Column "level" refers to explanatory variables with more than 2 levels. "Estimate": coefficient for parametric variables; Edf: Estimated degrees of freedom, applicable only to a smoothed term; SE: Standard Error; "T-test/F-test": statistical test applied to parametric variables (T-test) or smoothed terms (F-test, numbers in cursive); $R^2$: percentage of observed variability explained by the model. GUSPP: green up-satable peak period; SP: senescence period. "Treat": treatment; "Phen": phenology.

| Response variable | n | Explanatory variable | Level | Estimate Edf | SE | T-value F-value | $R^2$ |
|---|---|---|---|---|---|---|---|
| *Erica* consumption | 136 | S(Days:Treatment$_{HD}$) | | 4.75 | | **14.06*****| 0.417 |
| | | S(Days:Treatment$_{HyD}$) | | 4.72 | | **9.48***** | |
| | | Treatment | | 3.544 | 1.651 | **2.15*** | |
| | | Phenology | GUSPP | - 27.205 | 5.646 | **- 4.82***** | |
| | | | SP | - 6.561 | 4.401 | - 1.49 | |
| *Quercus rotundifolia* consumption | 136 | S(Days:Treatment$_{HD}$) | | 4.636 | | **35.97***** | 0.720 |
| | | S(Days:Treatment$_{HyD}$) | | 4.205 | | **10.99***** | |
| | | Treatment | | - 4.904 | 0.815 | **- 6.015***** | |
| | | Phenology | GUSPP | - 4.27 | 2.781 | - 1.537 | |
| | | | SP | - 4.53 | 2.280 | **- 1.938*** | |

(Continuation of Table 2 on the next page)

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| *Cistus ladanifer* consumption | 136 | S(Days) | | 2.468 | | **6.31*** | 0.156 |
| | | Treatment | | 3. 619 | 1.589 | **- 2.277*** | |
| | | Phenology | GUSPP | - 6.633 | 2.703 | **- 2.454**** | |
| | | | SP | - 2.306 | 2.612 | - 0.883 | |
| | | $Treat_{HyD}$:$Phen_{GUSPP}$ | | 3.800 | 2.647 | 1.435 | |
| | | $Treat_{HyD}$:$Phen_{SP}$ | | 2.619 | 2.647 | 0.989 | |
| *Salvia rosmarinus* consumption | 136 | S(Days) | | 4.951 | | 109.9 | 0.847 |
| | | Treatment | | 0.066 | 0.794 | 0.083 | |
| | | Phenology | GUSPP | 4.402 | 2.064 | **2.133*** | |
| | | | SP | 1.570 | 1.619 | 0.970 | |
| | | $Treat_{HyD}$:$Phen_{GUSPP}$ | | 1.485 | 1.322 | 1.123 | |
| | | $Treat_{HyD}$:$Phen_{SP}$ | | 1.601 | 1.321 | 1.211 | |

Consumption of *Cistus ladanifer* was generally lower in the hyper-density treatment compared to high-density and was lowest from September to December 2021 (during the green up-stable peak period) and later peaked in January 2022 for both treatments (Figure 3a). For *Salvia rosmarinus,* consumption values between both treatments overlapped and followed an extremely similar pattern over time (Figure 3b), where the plant was absent from the diet from May 2021 to December 2021 and then started to increase until reaching a peak during February 2022, to then slowly decrease over time until reaching levels close to 0 by May 2022. For both these plants, the best model included the days spent inside the enclosures as a smoothed term; and the interaction between the "Treatment" and the Phenology variable (Table 2).

*Plant biomass (i.e. phytovolume) and browsing intensity*

Changes in phytovolume were quite species-specific. The mean phytovolume of all plant species in the high-density treatment increased from 2021 to 2022 (Figure 4), although the mean increase was greater for the control. On the other hand, except for *Silvia rosmarinus*, plants found within the hyper-density enclosure experienced a decrease in their phytovolume from 2021 to 2022, with *Quercus rotundifolia* being the most affected species losing more than 50% of their original phytovolume; while the *Erica* spp. lost almost 10% and *Cistus ladanifer* 3.32%. Except for *C. ladanifer,* all the observed changes in the hyper-density treatment were significant (Figure 3). Similar patterns were observed in browsing

intensity, which was generally higher under the hyper-density treatment than under high density, except for *Salvia rosmarinus*. The most significant results were found in *Quercus rotundifolia,* where the mean browsing degree increased from 1 to 2 in the high-density treatment and from 1 to 5 in the hyper-density treatment (Figure 3).

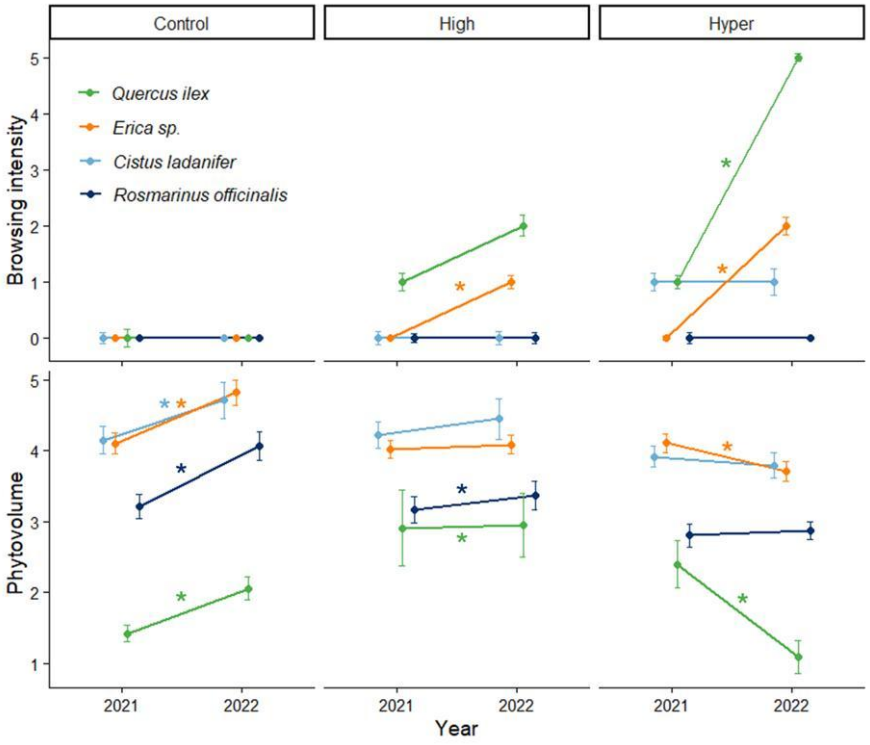



**Figure 4.** Inter-annual changes in mean browsing intensity and phytovolume within each treatment ("Control", "High": high-density, "Hyper": hyper-density) from 2021 to 2022. Asterisks (*) indicate whether observed changes were significant between years ( $p < 0.05$).

*Fecal nitrogen (FN), acid detergent lignin (ADL) and fecal glucocorticoid metabolites (FGM)*

FN values (Figure 5a) peaked at the start of the experiment (May 2021), during winter (December 2021) and at the end of the experiment (May 2022). The best model for this variable retained Treatment, and the interaction between treatment and the days that red deer spent inside each treatment, indicating non-linearity and that the pattern of FN evolves differently over time between the two enclosures (Table 3, Figure 5a). The hyper-density treatment had lower FN levels compared to the high-density treatment throughout the sampling cycle.

Regarding ADL, the best-fitting model included phenology and the interaction between the (smoothed) number of days post-release and treatment (Table 3). ADL values peaked during the first sampling month and displayed a marked decrease from May to August 2021 (Figure 5b). ADL values were consistently higher in the hyper-density treatment, compared to high density. ADL levels started to increase from August 2021 to the following months and remained stable from October 2021 until the end of the sampling period.

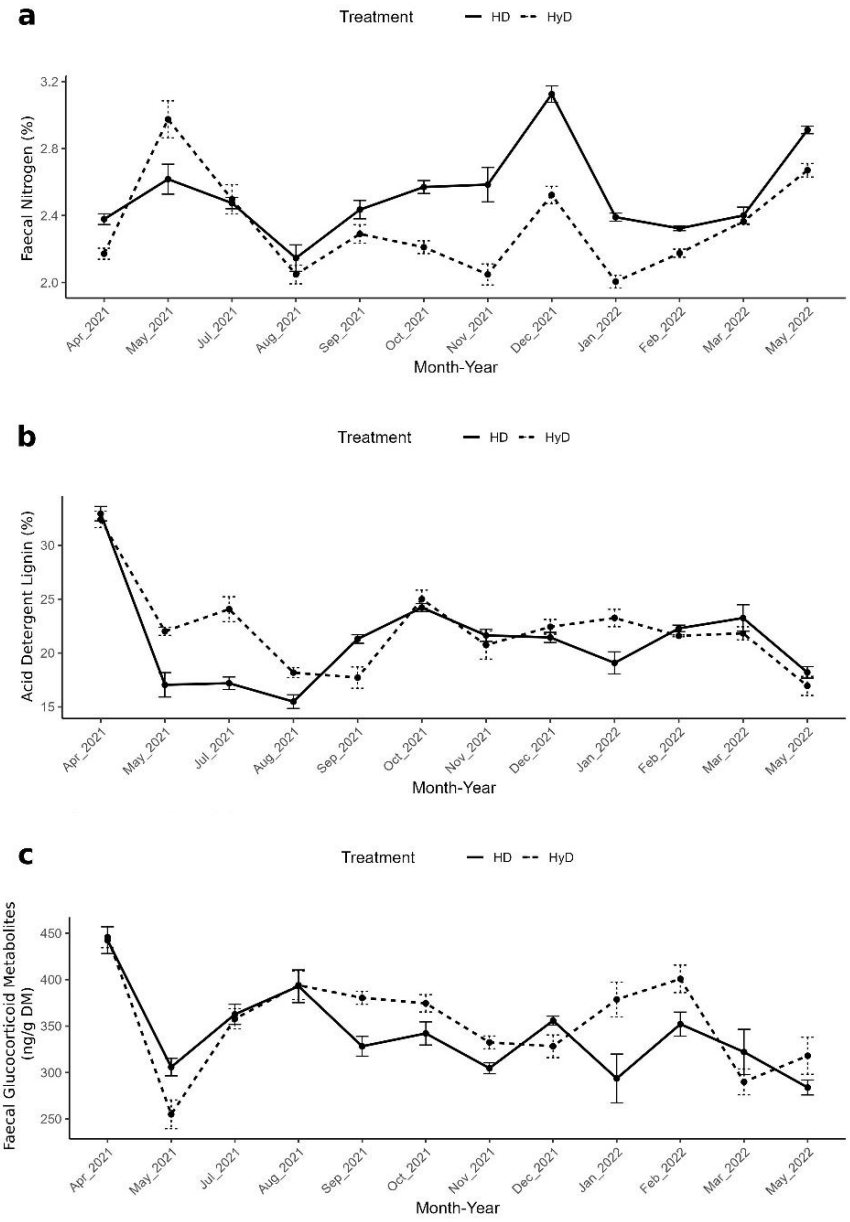



**Figure 5.** Monthly dynamics of fecal nitrogen (a), acid detergent lignin (b) and fecal glucocorticoid metabolites (c) for the sampling period (April 2021 to May 2022), for each treatment ( HD: high density, HyD: hyper-density).

**Table 3.** Results of the final retained GAMM models for diet quality parameters ("Response variable" column). N: sample size (number of observations included in the model). In the "explanatory variable" column, smoothed terms are marked with an S() and interactions are marked using ":". Shaded cells indicate that parameter/category is not applicable to that specific variable. Column "level" refers to explanatory variables with more than 2 levels. "Estimate": coefficient for parametric variables; Edf: Estimated degrees of freedom, applicable only to a smoothed term; SE: Standard Error; "T-test/F-test": statistical test applied to parametric variables (T-test) or smoothed terms (F-test); $R^2$: percentage of observed variability explained by the model. . GUSPP: green up-satable peak period; SP: senescence period. "Treat": treatment; "Phen": phenology.

| Response variable | n | Explanatory variable | Level | Estimate Edf | SE | T-value F-value | $R^2$ |
|---|---|---|---|---|---|---|---|
| Fecal nitrogen (FN) | 135 | S(Days:$Treat_{HD}$) | | 4.86 | | **14.69*** ** | 0.5027 |
| | | S(Days:$Treat_{HyD}$) | | 4.69 | | **8.56*** ** | |
| | | Treatment | | - 0.201 | 0.038 | **- 5.368*** ** | |
| Acid detergent lignin (ADL) | 134 | S(Days:$Treat_{HD}$) | | 4.897 | | **41.38*** ** | 0.696 |
| | | S(Days:$Treat_{HyD}$) | | 4.474 | | **27.62*** ** | |
| | | Treatment | | 1.003 | 0.417 | **2.402*** | |
| | | Phenology | GUSPP | - 2.428 | 1.412 | - 1.719 | |
| | | | SP | - 2.435 | 1.128 | **- 2.160*** | |
| Fecal glucocorticoid metabolites (FGM) | 134 | S(Days) | | 4.852 | | **16.50*** ** | 0.398 |
| | | Treatment | | - 6.141 | 10.818 | - 0.568 | |
| | | Phenology | GUSPP | - 85.362 | 27.645 | **- 3.088** ** | |
| | | | SP | 9.826 | 22.202 | 0.443 | |
| | | $Treat_{HyD}$:$Phen_{GUSPP}$ | | 38.627 | 18.138 | **2.130*** | |
| | | $Treat_{HyD}$:$Phen_{SP}$ | | 41.526 | 17.937 | **2.315*** | |

FGM concentrations (Figure 5c) fluctuated and overlapped between the two treatments, and treatment on its own was not a significant variable in the chosen GAMM model. FGM values also did not show any marked peak during the sampling period; however, FGM concentrations tended to stabilize and slightly decrease as time passed by. The retained model for FGM included the interaction between treatment and phenology and the smoothed term of days spent inside the enclosures (Table 4). The change of FGM tendency between treatments linked to phenology can be observed in the graphic, where FGM values remained consistently higher in the hyper-density treatment from August 2021 to November 2021 (green up-stable peak) and then from December 2021 February 2022 (senescence period).

## 4. Discussion

Overall, deer density affected the temporal dynamics of resource depletion and diet composition, in particular diet shifts and the sequence of forage exploitation. Under higher densities, key resources were depleted earlier, forcing deer to consume previously avoided plants with a decline in diet quality and increased physiological stress. These patterns highlight the detrimental effects of overabundance on red deer fitness and feeding behavior.

Contrary to our first hypothesis, deer density did not affect diet diversity or evenness. Instead, diet structure was mainly explained by time since release and vegetation phenology. Overall diet diversity was moderate, and plant species were consumed

unevenly, with *Erica* spp. and *Quercus rotundifolia* dominating the diet, as described in other ecosystems(Fernández-Olalla et al., 2006; Martínez, 2002, 2009; Perea et al., 2014). Diet diversity and evenness patterns were similar during the Green-Up and Senescence period. During Green-Up, increasing plant biomass (Pettorelli et al., 2005) likely allowed deer to selectively feed on new growth, reducing overall evenness. The greater variability in diet diversity during senescence per month suggests that some animals may keep feeding selectively on residual green species, while others diversify again. In contrast, diet diversity and evenness were highest during the dormant phase, suggesting a broad and balanced consumption of available plant species, likely reflecting the need to exploit a wide range of resources when overall forage availability is low or of poorer nutritional quality.

Whittaker's dissimilarity index showed overall diet stability, although patterns diverged between treatments during the Green Up-Stable Peak phase and the early part of the Senescence phase. Under hyper-density conditions, differences in diet increased steadily, reaching a maximum around early winter (December 2021), indicating a diversification in diet composition. This likely reflects resource depletion due to higher browsing pressure, forcing deer to include alternative or less-preferred species in their diet such as *Cistus ladanifer* (Fernández-Olalla et al., 2006; Perea et al., 2014).

Conversely, in the high-density treatment, Whittaker's index values continued decreasing until mid-fall (November 2021), indicating that deer maintained a relatively stable and homogeneous diet during this period, possibly because resource availability was still sufficient. However, a sharp increase from November 2021 to January 2022 suggests a sudden dietary shift, likely related to phenological transitions in vegetation such as oak masting (Azorit et al., 2012; Picard et al., 1991) or delayed resource depletion relative to the hyper-density treatment. Later in the experiment, diet composition stabilized under hyper-density, suggesting that animals had already converged on a restricted set of available resources, whereas the high-density herd continued showing greater dietary variability.

Taken together, the patterns observed in the dietary indexes indicate that increased deer density accelerates dietary change and broadens the range of consumed plant species, particularly during times of the year when high-quality food becomes limited. Under hyper-density conditions, deer adjust their diet more frequently throughout the year, whereas under lower density, dietary shifts are less frequent but more abrupt, likely reflecting delayed responses to vegetation depletion. The significant effect of phenological phase in all models underscores the key role of vegetation dynamics in shaping temporal diet composition.

The results in our diet indexes can be better understood by looking into the temporal dynamics of plant species consumption. Species-

specific modelling revealed distinct consumption trajectories between species depending on deer density and time. Consumption of the highly preferred *Quercus rotundifolia* increased initially in both treatments but declined sharply under hyper-density after summer, indicating depletion of this key resource. Its low initial cover (<2%), combined with increased browsing intensity and declining phytovolume strongly suggests resource exhaustion under intense herbivory. In contrast, reliance on *Erica spp*., often described as a buffer forage for red deer (Caballero García de Arevalo, 1985; Clutton-Brock et al., 1987; Jayakody et al., 2011; Milne et al., 1978), increased particularly under hyper-density, likely compensating for the reduction of preferred oak resources. Overall, these results support the hypothesis that deer in hyper-density conditions depleted highly preferred resources earlier than those in high-density conditions, which likely experienced fewer resource constraints. The disappearance of plant resources can trigger cascading ecological effects on plant diversity. Under hyper-density conditions, preferred plant species are depleted earlier and more frequently due to increased herbivory, leading to biotic homogenization of plant communities (Perea et al., 2014) and a parallel homogenization of herbivore diets. These results could also suggest that diet simplification can work as an early warning of biotic homogenization and unsustainable levels of herbivore populations.

The low browsing intensity and phytovolume increase for *Cistus ladanifer* and *Salvia rosmarinus*, confirm that deer have a have low

dietary preference for these two species in the studied area (Perea et al., 2014). However, each species presented a distinct consumption pattern. *C. ladanifer* consumption peaked in late summer in the high-density treatment, possibly linked to fruit availability (Malo & Suárez, 1996), whereas this peak was absent under hyper-density, potentially reflecting reduced flowering and fruit production under intense herbivory pressure (Hernández-Castellano et al., 2025). Regardless, consumption tended to increase in both treatments as time passed. In contrast, *S. rosmarinus* was absent from red deer diets in both treatments until December 2021 to May 2022. Both are plant species with anti-herbivory metabolites, mainly terpenoids in the case of *S. Rosmarinus* (Dietz, 1968; Gershenzon & Croteau, 1991), and flavonoids and diterpenes in the case of *C.* ladanifer (Alías et al., 2012; Sosa et al., 2004), which display marked seasonal patterns (Alías et al., 2012; Valares Masa et al., 2016). Seasonal variation in these compounds likely explains winter increases in *C. ladanifer* consumption and the delayed exploitation of *S. rosmarinus*, as C*. ladanifer* consumption peaks again during January, when flavonoid contents are the lowest in this species but their protein content increases, thus making it a more attractive resource (Alías et al., 2012). Regarding *S. rosmarinus,* its flowers are the part of the plant with the least concentration of secondary metabolites (Del Baño et al., 2004). The marked consumption increase of this plant around the flowering period and after red deer have spent more than 6 months inside the enclosures might suggest that deer adjusted their

foraging strategy to exploit these species when their nutritional payoff was temporarily enhanced and after exhausting other resources, consistent with optimal foraging theory.

Taken together, these findings reveal a density-dependent shift in foraging behavior and resource use that agrees with our hypothesis. Under hyper-dense conditions, red deer displayed earlier and more pronounced depletion of highly preferred resources (*Q. rotundifolia*) and increased reliance on buffer species (*Erica* spp.). The dietary convergence observed between both population densities likely reflects a homogenization of resource availability in the enclosures after prolonged browsing pressure, revealing overabundance conditions.

Importantly, fecal nitrogen showed similar seasonal trends in both treatments but remained generally lower under hyper-density. FN peaked for both treatments in December 2021, coinciding with the lowest values of both Shannon and Pielou's indexes and an increase in Whittaker's index, suggesting a marked dietary shift to more digestible species (such as *Erica* spp.). Fecal nitrogen remained higher in the high-density treatment, suggesting that animals in this treatment still had a better diet quality than the hyper-density herd, aligning with our second hypothesis. Similarly, acid detergent lignin reflected seasonal plant phenology, decreasing during periods of active plant growth (when the plant is also rich in protein and simple sugars) and increasing during lignification (Azorit et al., 2012; Martínez, 2009). ADL values were initially higher under hyper-

density but converged between treatments over time, suggesting increasing constraints on dietary optimization under intense browsing pressure. Additionally, stable ADL levels found in both treatments, with no sharp increase during the dry months when lignification happens, could indicate that both deer densities were high enough to disrupt the plant species natural lignification cycle (Gordaliza et al., 2025).

Finally, patterns of FGM indicated that cortisol dynamics were shaped by time spent inside the enclosures and the interaction between red deer density and phenology. FGM are expected to increase during times of environmental or endogenous-driven physiological stress (Santos et al., 2014). Overall, FGM values appeared higher during the green up-stable peak and senescence periods in the hyper-density treatment, despite the expectation that glucocorticoid output should decrease during the Green Up-Stable Peak period, when forage quality peaks (Martínez, 2009). Individual factors that affect FGM levels can include diet, sex and metabolic rate (Santos et al., 2014). Because all animals were similar in sex and age and experienced comparable reproductive conditions, these differences are most likely explained by changes in diet. Deer at hyper-density displayed higher monthly FGM mean values than high-density deer during periods of naturally occurring food scarcity (August 2021 to February 2022), suggesting a cumulative physiological cost of sustained high herbivory pressure and more rapidly declining forage availability. This interpretation is supported by the strong reduction in phytovolume and the

increased browsing intensity observed in the hyper-density treatment, especially for highly selected species such as *Quercus rotundifolia*, whose consumption decreased sharply only in the hyper-density group once its availability was presumably exhausted. Overall, the observed patterns indicate that hyper-density deer were forced to adjust their diet earlier, turning increasingly to less preferred or more chemically defended species (e.g. *Cistus ladanifer*, *Silvia rosmarinus*) as high-quality resources diminished.

## 5. Conclusions

These results collectively indicate that deer density not only influences overall resource depletion but also shapes the temporal dynamics of diet composition, mainly in terms of diet shifts. Hence, the sequence of forage exploitation, whereby key resources are depleted earlier, force animals to incorporate previously avoided plants that may have detrimental effects on red deer physiology and ecosystem sustainability. These changes observed in red deer subjected to higher population densities resulted in an overall diet that had poorer quality and increased stress, showcasing the detrimental effects that overabundance has on red deer feeding behavior and potentially fitness.

## 6. Supplementary information

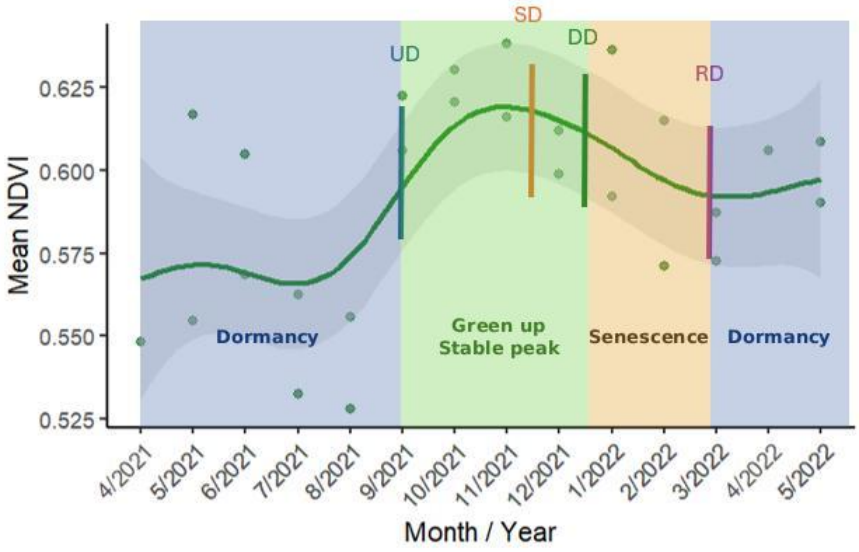



**Figure S1.** Monthly mean normalized difference vegetation index (NDVI) values in Quintos de Mora during the sampling period comprised from April 2021 to May 2022. The band around the NDVI line indicates the 95% confidence interval. The numbers of the X axis correspond to the months, going from 1 (January) to 12 (December), followed by the year (2021 or 2022). UD: upturn day; SD: Stabilization day; DD: downturn day; RD: restoration day. The colored squares indicate the duration of each phenological phase.

**Table S1.** Definitions and obtained dates of main vegetation phenological phases and their corresponding durations, as estimated from NDVI trends corresponding to the sampling period in Quintos de Mora (April 2021 to May 2022).

| Parameter | Definition | 2021 | 2022 |
|---|---|---|---|
| **Upturn day (UD)** | The day marking the start of green-up, when vegetation index values begin a sustained increase after winter dormancy | 16/08/2020 | 06/09/2021 |
| **Stabilisation day (SD)** | The day marking the end of the green-up phase and the start of the stable high (peak) period of vegetation activity. | 22/09/2020 | 14/11/2021 |
| **Downturn day (DD)** | The day marking the start of senescence, when vegetation index values begin a consistent decline. | 1/12/2020 | 15/12/2021 |
| **Restoration day (RD)** | The day marking the return to baseline or minimum greenness, indicating the end of the growing season. | 07/02/2021 | 26/02/2022 |
| **Green Up (GU)** | Duration (in days) of canopy development. SD-UD. | 16/08/2020 from 22/09/2020 (37 days) | 06/09/2021 from 14/11/2021 (69 days) |
| **Stable peak period (SPP)** | Duration (in days) of high greenness plateau. DD-SD. | 22/09/2020 from 1/12/2020 (70 days) | 14/11/2021 from 15/12/2021 (30 days) |
| **Senescence period (SP)** | Duration of vegetation decline. RD-DD. | 1/12/2020 from 07/02/2021 (68 days) | 15/12/2021 from 26/02/2022 (73 days) |
| **Dormancy period (DP)** | Duration (in days) of non-growing season, minimum greenness period. RD-UD. | 07/02/2021 from 06/09/2021(211 days) | 26/02/2022 from 06/09/2022 (192 days) |

**Table S2.** Woody vegetation coverage, expressed in percentage, for the species found inside the transects, divided by deer density (enclosure) treatment ("Treatment", HD: high density, HyD: hyper-density). ND: Not Detected. Plant species that appear as Not Detected are plants that were not present in the vegetation transects. The coverage result for both *Erica spp.* and *Quercus spp.* is a sum of the coverage from all the species from that genus. NAs within the p-value cells from the Mann-Whitney test indicate that the sample size was too low to perform the statistical test. A dash (-) indicates that mean comparison was not carried out. "P-adj": P-adjusted.

| ***Quintos de Mora*** | ***Treatment*** | | ***Mann-Whitney results*** |
|---|---|---|---|
| **Plant Species** | **HD** | **HyD** | **P-value** |
| *Arbutus unedo* | 0.12 | 0.24 | NA |
| *Cistus ladanifer* | 26.03 | 29.35 | 0.97 |
| *Cistus populifolius* | ND | 3.08 | NA |
| *Daphne gnidium* | 0.32 | 0.35 | NA |
| *Erica arborea* | 14.52 | 18.24 | 0.81 |
| *Erica australis* | 0.92 | 5.92 | 0.86 |
| *Erica scoparia* | 0.38 | 1.29 | NA |
| *Erica spp* | 15.82 | 25.44 | - |
| *Phyllirea angustifolia* | 0.07 | 0.15 | NA |
| *Pinus pinaster* | 1.09 | 0.48 | 0.47 |
| *Quercus faginea* | ND | 0.03 | NA |
| *Quercus ilex* | 1.55 | 1.83 | 0.47 |
| *Quercus pyrenaica* | ND | 0.49 | NA |
| *Quercus spp* | 1.86 | 2.03 | - |
| *Rosmarinus officinalis* | 13.54 | 13.22 | 0.26 |
| *Thymus mastichina* | 0.13 | 0.17 | NA |

**Table S3**. Red deer diet monthly characterization for both treatments throughout the duration of the study. Data are displayed as "Mean (Min-Max)" values (in percentage). *Erica* spp and *Quercus* spp. rows collects fragments that belonged to the *Erica* or *Quercus* genus, but species could not be determined. "Woody" row contains fragments that were characterized as belonging to a woody species, but neither the species or genus could be determined. Species marked in bold letters are the most consumed ones. Sp: plant species; can include non leguminous woody species (purple cells); graminoids (orange cells), forbes  graminoids; F: forbes (green cells). .

| | | Dormancy period | | | | Green-up and Stable Peak Period | | | Senescence period | | | Dormancy period | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | April | May | July | August | September | October | November | December | January | February | March | May |
| | | 2021 | | | | | | | | 2022 | | | |
| Sp | Treatment | | | | | | | | | | | | |
| *A. telonensis* | HyD | 1.2 (1-4) | 1.6 (0-3) | 0.16 (0-1) | 0 | 0 | 0 | 0 | 0 | 0.16 (0-1) | 0.66 (0-2) | 0.33 (0-1) | 1.5 (0-4) |
| | HD | 0.2 (0-1) | 4.6 (0-7) | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0.16 (0-1) | 2 (0-7) | 2.16 (0-6) |
| *A. unedo* | HyD | 0 | 1.2 (0-2) | 2 (0-4) | 3.2 (0-6) | 0.66 (0-2) | 1.33 (0-3) | 1.33 (1-2) | 0 | 0.16 ( 0-1) | 0 | 0 | 0 |
| | HD | 0.4 (0-2) | 1.2 (0-3) | 0.2 (0-1) | 2.8 (0-6) | 0.5 (0-1) | 0 | 0 | 0 | 0.33 (0-2) | 0 | 0 | 0.16 (0-1) |
| ***C. ladanifer*** | **HyD** | **0.6 (0-2)** | **9.6 (5-14)** | **5.33 (0-14)** | **1.8 (1-3)** | **11 (6-16)** | **3.83 (0-8)** | **4.33 (3-6)** | **7.16 (5-10)** | **15.66 (5-27)** | **7.83 (2-15)** | **12.83 (8-18)** | **11.5 (5-17)** |
| | **HD** | **0.2 (0-1)** | **12.2 (7-23)** | **7.6 (0-16)** | **20.2 (4-33)** | **6.5 (3-12)** | **5.66 (0-10)** | **6.5 (3-9)** | **7.66 (5-11)** | **20 (9-25)** | **6 (1-13)** | **9.6 (3-17)** | **14 (8-20)** |

| | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *C. populifolius* | HyD | 0.4 (0-1) | 0 | 0.66 (0-3) | 0.6 (0-1) | 0.33 (0-1) | 0.5 (0-2) | 0 | 0 | 0 | 0 | 0 | 0 |
| | HD | 0 | 0 | 0.6 (0-1) | 1 (0-3) | 0.33 (0-1) | 0 | 0.83 (0-2) | 0.16 (0-1) | 0 | 0 | 0 | 0.16 (0-1) |
| *D. gnidium* | HyD | 1 (0-4) | 0 | 0.16 (0-1) | 0.2 (0-1) | 0.66 (0-2) | 0 | 0 | 0.33 (0-2) | 0.33 (0-1) | 0.16 (0-1) | 0.16 (0-1) | 0 |
| | HD | 0 | 0 | 0 | 0.2 (0-1) | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| ***Erica spp*** | **HyD** | **27.8 (22-35)** | **36.2 (27-48)** | **37 (26-45)** | **40.2 (35-58)** | **49.33 (40-61)** | **60.5 (50-67)** | **40.66 (35-46)** | **65.16 (62-67)** | **41 (31-50)** | **27 (21-32)** | **37.33 (26-51)** | **37.67 (34-45)** |
| | **HD** | **46 (40-51)** | **31.2 (20-37)** | **36.4 (33-44)** | **22 (17-24)** | **34.5 (31-45)** | **40.5 (35-67)** | **41.33 (27-56)** | **64.66 (58-77)** | **31.67 (24-49)** | **29.5 (21-41)** | **32.6 (25-40)** | **38.83 (28-43)** |
| *E. arborea* | HyD | 8.2 (6-11) | 8.4 (4-13) | 9.13 (5-12) | 5.4 (2-9) | 5.16 (3-6) | 7.5 (6-9) | 3 (0-5) | 5.5 (4-8) | 5.83 (3-9) | 5.33 (3-7) | 7.66 (3-14) | 8.17 (3-11) |
| | HD | 12.4 (9-16) | 7 (3-14) | 8.6 (6-11) | 4.6 (3-7) | 3.66 (2-5) | 4.83 (2-15) | 3.5 (1-8) | 5.16 (2-9) | 7.66 (6-9) | 9.16 (7-12) | 7.60 (6-9) | 6.66 (5-9) |
| *E. australis* | HyD | 12.2 (10-16) | 0.4 (0-2) | 0.16 (0-1) | 1.6 ( 0-3) | 1.33 (0-2) | 0.5 (0-1) | 0 | 0.83 (0-2) | 3.16 (1-6) | 7.16 (4-11) | 8 ( 4-14) | 0.33 (0-1) |
| | HD | 14.2 (9-19) | 0.8 (0-2) | 0.8 (0-2) | 0.8 (0-4) | 0 | 0.33 (0-1) | 0.16 (0-1) | 0.16 (0-1) | 1 (0-3) | 11.5 (4-16) | 11.2 (6-16) | 3.5 (0-9) |
| *P. angustifolia* | HyD | 1.2 (0-3) | 2.00 (0-6) | 0.66 (0-3) | 0.6 (0-1) | 0 | 0.16 ( 0-1) | 11 (10-13) | 0 | 1.83 (0-4) | 0.66 (0-1) | 0 | 0.33 (0-1) |

| | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | HD | 0 | 5.00 (0-7) | 2.2 (0-4) | 1.2 (0-3) | 0.33 (0-1) | 7.5 (0-12) | 4.33 (0-13) | 0 | 2.16 (0-4) | 0 | 0 | 0 |
| *P. pinaster* | HyD | 0.4 (0-1) | 0.20 (0-1) | 0 | 0.2 (0-1) | 0.16 (0-1) | 0.83 (0-3) | 3.33 (1-5) | 0 | 0.5 (0-1) | 1.33 (0-3) | 0.5 (0-3) | 0.16 (0-1) |
| | HD | 0 | 0.40 (0-2) | 0.2 (0-2) | 0.8 (0-1) | 1.66 ( 0-3) | 0.66 (0-1) | 1.83 (1-3) | 0.5 (0-2) | 1 (0-2) | 0.16 (0-1) | 0 | 0.16 (0-1) |
| *Quercus spp* | HyD | 1.8 (0-5) | 0 | 0.83 (0-2) | 0.4 (0-1) | 0 | 0.33 (0-1) | 0.66 (0-1) | 0 | 0 | 0 | 0 | 0.33 (0-1) |
| | HD | 0 | 0 | 0.4 (0-1) | 0.4 (0-2) | 0.33 (0-1) | 0 | 0.83 (0-3) | 0 | 0 | 0 | 0 | 0.16 (0-1) |
| ***Q. rotundifolia*** | **HyD** | **12.6 (9-19)** | **14.20 (9-18)** | **22.16 (18-27)** | **28 (23-30)** | **6.66 (2-12)** | **(6-10)** | **7.33 (6-10)** | **4.16 (2-9)** | **4.33 (1-8)** | **4 (0-8)** | **5.83 (2-14)** | **14.5 (9-22)** |
| | **HD** | **4.2 (0-9)** | **7.60 (1-10)** | **31 (29-32)** | **27.4 (21-43)** | **32.33 (23-38)** | **22.5 (14-25)** | **21.83 (8-30)** | **8.66 (4-12)** | **5.33 (2-10)** | **6.83 (4-10)** | **6.6 (0-16)** | **9.16 (4-16)** |
| *Q. pyrenaica* | HyD | 1 (0-3) | 1.00 (0-3) | 1.5 (0-4) | 0 | 0.66 (0-2) | 2.66 (0-8) | 7.66 (7-9) | 0 | 2.5 (0-5) | 0.16 (0-1) | 0.33 (0-1) | 2.67 (1-4) |
| | HD | 0.2 (0-1) | 0.80 (0-3) | 0.6 (0-2) | 4.2 (0-7) | 4.5 (1-8) | 5 (0-14) | 4 (0-11) | 0.16 (0-1) | 2.33 (0-3) | 0.66 (0-2) | 0.2 (0-1) | 1 (0-2) |
| *S. rosmarinus* | HyD | 13.8 (8-27) | 0.40 (0-1) | 0.16 (0-1) | 0.6 (0-1) | 1.66 (0-3) | 2.33 (0-8) | 1.33 (0-2) | 1.5 (0-3) | 12.33 (5-19) | 25.5 (21-28) | 16.16 (8-20) | 1 (0-3) |
| | HD | 7.6 (4-14) | 1.60 (1-3) | 0.4 (0-1) | 0.8 (0-2) | 0.16 (0-1) | 0.66 (0-1) | 0.33 (0-2) | 0.5 (0-1) | 12.83 (7-16) | 21 (18-24) | 18.2 (14-24) | 2.5 (1-9) |

| | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *T. mastichina* | HyD | 3 (0-8) | 2.00 (0-4) | 2.33 (0-6) | 0.8 (0-1) | 0.66 (0-2) | 0.66 (0-2) | 2 (1-3) | 0 | 1.33 (1-2) | 4.83 4-6) | 1.66 (0-5) | 0.33 (0-1) |
| | HD | 2 (0-5) | 0.60 (0-1) | 1.8 (0-5) | 0.4 (0-1) | 1.16 (0-2) | 1 ( 0-2) | 0.16 (0-1) | 0 | 1.16 (0-2) | 4 (1-8) | 1.2 (0-2) | 0.32 (0-1) |
| *T. guttata* | HyD | 0 | 0 | 0 | 0.2 ( 0-1) | 0 | 0 | 0 | 0 | 0.33 (0-1) | 0.16 (0-1) | 0.66 (0-3) | 0.5 (0-3) |
| | HD | 0 | 0.20 (0-1) | 0.2 (0-1) | 1 (0-2) | 0 | 0 | 0.16 (0-1) | 0 | 1.33 (0-3) | 0.16 (0-1) | 0.2 (0-1) | 0.5 (0-2) |
| Woody | HyD | 5.6 (3-7) | 6.00 (5-10) | 7 (4-9) | 9.6 (2-12) | 9 (1-16) | 6.33 (0-10) | 14 (8-22) | 3.66 (0-9) | 0.83 (0-3) | 4.33 (1-6) | 2.5 (0-8) | 10.16 (4-16) |
| | HD | 5.4 (3-9) | 5.80 (3-11) | 3.8 (2-6) | 8.2 (3-13) | 4.66 (2-6) | 8.33 (2-13) | 7 (0-14) | 2.5 (0-6) | 4.66 (2-10) | 2.66 (0-4) | 5.2 (0-12) | 10 (3-18) |
| *L. stoechas* | HyD | 2 (1-4) | 0.20 (0-1) | 1.16 (0-2) | 0.8 (0-1) | 0 | 1.16 (0-4) | 0.66 (0-1) | 0 | 0.16 (0-1) | 0 | 0 | 1.16 (0-3) |
| | HD | 0 | 0.20 (0-1) | 0.6 (0-3) | 0.4 (0-1) | 0 | 0 | 0 | 0 | 0 | 0.16 (0-1) | 0 | 0 |
| *Aira sp.* | HyD | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | HD | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *B. madritensis* | HyD | 0 | 0.6 (0-1) | 0.66 (0-2) | 0 | 0.33 (0-1) | 0 | 0 | 0.5 (0-1) | 0 | 0.33 (0-1) | 0.33 (0-1) | 1 (0-2) |
| | HD | 0.2 (0-1) | 0.8 (0-2) | 0 | 0 | 0 | 0 | 0.16 (0-1) | 0 | 0 | 0.16 (0-1) | 0.2 (0-1) | 0.5 (0-2) |
| *G. parisiense* | HyD | 2.8 (0-6) | 0.8 (0-2) | 0 | 0 | 0 | 0 | 0 | 0 | 0.16 (0-1) | 0.16 (0-1) | 0 | 0 |

| | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *P. bulbosa* | HD | 1.6 (0-5) | 2.6 (0-6) | 0 | 0.2 (0-1) | 0 | 0 | 0.16 (0-1) | 0 | 0.33 (0-1) | 0 | 0 | 0.16 (0-1) |
| | HyD | 0.2 (0-1) | 1 (0-4) | 0.16 (0-1) | 0 | 0.33 (0-2) | 0 | 0 | 1.33 (0-3) | 0 | 1.66 (0-6) | 0.16 (0-1) | 0.16 (0-1) |
| *V. membranacea* | HD | 0.4(0-1) | 0.2 (0-1) | 0 | 0 | 0 | 0 | 0.5 (0-2) | 0.5 (0-1) | 0.33 (0-1) | 0.33 (0-1) | 0.6 (0-1) | 0.83 (0-1) |
| | HyD | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *Graminoid* | HD | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | HyD | 0.4 (0-2) | 3.6 (0-8) | 2 (0-5) | 0.8 (0-2) | 1 (0-2) | 0.16 (0-1) | 0 | 2.16 (1-4) | 0.5 (0-1) | 1 (0-5) | 0.5 (0-2) | 0.16 (0-1) |
| *Asphodelus spp* | HD | 0.6 (0-1) | 10.2 (2-20) | 0.2 (0-1) | 0 | 0.5 (0-1) | 0.16 (0-1) | 0.66 (0-2) | 1.83 (0-3) | 0.33 (0-1) | 0.5 (0-2) | 0 | 0.83 (0-2) |
| | HyD | 0.2 (0-1) | 0.4 (0-1) | 0.83 (0-2) | 0.8 (0-2) | 0.5 (0-1) | 0.33 (0-1) | 0 | 0.33 (0-1) | 1.83 (0-4) | 3.5 (2-6) | 1.16 (0-3) | 0.5 (0-2) |
| *HNG* | HD | 1 (0-2) | 0 | 1.2 (0-5) | 0.2 (0-1) | 0.66 (0-4) | 0 | 0.83 (0-1) | 0.33 (0-2) | 2.66 (0-6) | 1.66 (0-3) | 0.6 (0-2) | 0.16 (0-1) |
| | HyD | 3.4 (0-7) | 6.8 (0-12) | 5 (4-7) | 3.6 (0-5) | 9.66 (6-14) | 2.16 (1-5) | 2.66 (1-5) | 5.83 (2-11) | 3.5 (1-8) | 3.83 (2-6) | 2.5 (0-5) | 5.67 (3-10) |
| *Brassicaceae* | HD | 2.6 (0-7) | 6.2 (1-12) | 2.8 (1-6) | 2.8 (2-3) | 7.5 (3-10) | 2.66 (1-4) | 4.16 (0-7) | 7.16 (2-12) | 3.83 (1-10) | 3 (1-5) | 2.2 (1-4) | 4.83 (2-8) |
| | HyD | 0.2 (0-1) | 3.4 (2-6) | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0.5 (0-2) | 1.5 (0-3) |
| BR (TOTAL) | HD | 0.4 (0-1) | 2.6 (0-10) | 0.2 (0-1) | 0.4 (0-2) | 0 | 0 | 0 | 0 | 0.33 (0-1) | 0.16 (0-1) | 0.2 (0-1) | 1.66 (0-4) |
| | HyD | 0 | 0 | 0.66 (0-3) | 0.6 (0-2) | 0.83 (0-1) | 0.83 (0-3) | 0 | 1.5 (0-3) | 3.5 (1-6) | 0.33 (0-2) | 0.83 (0-4) | 0.66 (0-2) |
| | HD | 0.4 (0-1) | 0.4 (0-2) | 0.2 (0-1) | 0 | 0.66 (0-3) | 0 | 0.66 (0-4) | 0 | 0.66 (0-2) | 2.16 (0-6) | 1.6 (0-4) | 1.66 (0-5) |

## Study IV

# From microbiome to micro*bye*ome: overabundance modulates and depletes the gastrointestinal microbiome community in red deer

## Abstract

The gut microbiome plays a fundamental role in the optimal digestion of plant material, the obtention of essential nutrients and the maintenance of the overall health status of ruminants. One of the main factors that modulate gut microbiome composition is diet, particularly plant diversity and nutritional quality. The foraging behavior of wild herbivores is influenced by population density, which can be especially important in highly seasonal environments that undergo natural periods of food scarcity and lower dietary quality. However, literature establishing direct links between herbivore density, shifts in diet quality and, thus, gut microbiome community composition, is scarce. Our goal in this work is to determine whether population density shapes the structure and diversity of gut microbiome communities in two herds of red deer (*Cervus elaphus*), and whether these responses are associated with changes in diet quality and diversity. We designed two enclosures mimicking scenarios of deer overpopulation densities (high-density, 41 deer/$Km^2$; and hyper-density, 112 deer/$Km^2$) and collected fecal samples over a 12-month sampling period. From these samples, we determined gut microbiome composition, diet quality (metabolic fecal nitrogen and acid detergent lignin) and the fecal glucocorticoid metabolites, as proxy for physiological stress. Alpha and beta diversity metrics varied over the year and were density dependent, where lower deer densities displayed diversity depletion, while higher densities had marked phylogenetic turnover and higher

community richness. Interestingly, plant phenology rather than diet or deer density were the main driver of microbial turnover. The observed changes in relative abundance of specific microbial taxa were also density-dependent, with the hyper-density herd showing an increase in bacterial taxa adapted to diets rich in digestible fiber, while simultaneously suffering depletion from taxa linked to the ability to adapt to harsher environments. All in all, our results suggest a bottom-up gut microbiome adaptation in red deer driven by changes in diet composition with potential negative consequences for the animal's fitness in the medium term.

## 1. Introduction

The mammalian gut microbiome has been an intense subject of research in recent years, and a growing body of literature supports its influence on health, nutrition, phylogenetic evolution and ecology in mammals (Kartzinel et al., 2019). The gut microbiome has been proven to significantly influence different physiological functions of their hosts (McFall-Ngai et al., 2013), including the development and regulation of the host immune response (Levy et al., 2017; Round & Mazmanian, 2009), nutrient obtention (Krajmalnik-Brown et al., 2012) and synthesis (Rowland et al., 2018), neurotransmitter and hormone modulation (Strandwitz 2018; Martin et al., 2019) or behavior (Heijtz et al., 2011), among others. Evidence stemming from laboratory animals and observational studies carried out in wildlife also suggests that the gut microbiome influences host fitness by affecting host development, longevity and reproduction (Gould et al., 2018; Lim & Bordenstein, 2020; Suzuki, 2017a).

However, the gastrointestinal microbiome is an adaptive community that responds to internal host and external environmental factors (Flynn et al., 2023; Wang et al., 2023). In ruminants, the gut microbiome maximizes the digestion efficiency of plants, especially allowing fiber fermentation for energy and nutrient extraction, vitamin synthesis and the neutralization of plant toxic compounds (Dearing & Kohl, 2017). Therefore, herbivore's gut microbiome communities are more diverse and

complex than those belonging to carnivore or omnivore species (Ley et al., 2008; Stevens & Hume, 1998; Zoelzer et al., 2021) and are essential in maintaining host fitness and their ability to adapt to changes in their environment (McKenney et al., 2018).

Available studies in both captive and free-ranging wild herbivores show that diet composition and quality are key drivers of gut microbiome diversity (Guo et al., 2024a; Huang et al., 2026a; Nishida & Ochman, 2018a; Wu et al., 2023) of both browsers and grazers (Kartzinel et al., 2019; Krizsan et al., 2018; Rojas et al., 2021). The feeding behaviour of wild ruminants is adapted to plant availability and palatability, which in turn is influenced by the season of the year and population density (Cuerdo et al., 2025; Pettorelli et al., 2002, 2005). When population density increases, animals are forced to feed in less productive patches, increasing intra- and interspecies competition, causing a bottom-up regulation (Mobæk et al., 2009) and resource depletion (Donini et al., 2024; Walker et al., 2023). To compensate for this limitation, animals expand their dietary niche by feeding on less preferred plant species to meet their nutritional requirements (Stewart, 1967). Therefore, changes in the gut microbiome of wild ruminants may be expected when population density exceeds the carrying capacity of a given ecosystem. This adaptation could improve the host adaptive abilities of individuals to broaden their dietary niche (Moeller & Sanders, 2020). However, despite the increasing evidence of how population densities can affect foraging behavior, little evidence directly ties population density to changes in gut microbiome

composition. The need to broaden our knowledge on this subject is becoming more relevant recently, as populations of large wild herbivores, particularly deer (Cervidae), have increased across both Europe and North America (Carpio et al., 2021).

The red deer (*Cervus elaphus*) is a widely spread ruminant species, with a high dietary plasticity, allowing them to quickly adapt to different landscapes and change their feeding habits throughout the year (Gebert & Verheyden-Tixier, 2001). They are also considered a keystone species due to the effect they have on ecosystem dynamics and ecosystem services, particularly through their feeding behavior (Garcia et al., 2025). Red deer are one of the most common ungulate species in Europe, and in recent decades, their numbers have increased across almost all European regions (Milner et al., 2006). Due to changes in game estate management, reintroduction programs, and rural abandonment, among other factors (Acevedo et al., 2008, 2011; Vicente et al., 2007), red deer populations in some areas of Spain and Portugal have reached densities exceeding 50 individuals/km$^2$. These high densities are particularly evident in protected reserves and hunting estates, where populations are now considered overabundant.

In cervids, existing research on gut microbial communities has largely focused on the effects of seasonality, geographic variation, and captivity-related dietary changes (Hu et al., 2018; Li et al., 2022; You et al., 2022; Zhang et al., 2018), while studies on red deer have primarily examined environmental drivers of microbial variation

(Menke et al., 2019), interspecific differences (Wang et al., 2019) or the effect of diet quality (Guo et al., 2024). Although recent work emphasizes the importance of identifying key microbial taxa involved in maintaining deer health (Wang et al., 2023), the relationship between gut microbiota and nutritional condition in red deer remains poorly understood, particularly under conditions of overabundance. Addressing this gap is critical for understanding how population density may indirectly affect host health through diet–microbiome interactions.

The aim of this work is to add to the available literature by exploring how population overabundance might modulate the gastrointestinal microbiome composition in red deer. This experiment is a continuation of previous research exploring the effects of overabundance on diet selection and quality in two red deer herds held at two different overabundant population densities (Cuerdo et al., 2025; Rello et al., 2023; Torres-Blas et al., 2023), where we observed that animals subjected to higher densities adapted by expanding their dietary niche and including plant species normally avoided (Study III). Now, we aim to investigate if this shift in the feeding selection due to the resource depletion induced by the overabundance scenario has driven changes in the gut microbiome community. We expect that both herds would experience microbiome composition turnover linked to changes in the phenological phases of the vegetation. We also expect that changes in microbiome composition will follow different trajectories, as the animals held under higher density conditions

depleted their environmental resources faster and therefore changed their feeding behavior more markedly throughout the experiment.

As a result, we expected to see a significant increase in gastrointestinal microbiome alpha diversity and microbial taxa that would favor the digestion of plant resources that are richer in fibers in the higher density setting as part of a physiological adaptation to the resource limitation. Finally, given that individuals subjected to higher population densities exhibit elevated physiological stress (fecal glucocorticoid metabolites FGM concentration), and that glucocorticoids have been linked compositional microbiome changes in other ungulates (Łopucki et al., 2025), we predicted that FGM would significantly contribute to explaining changes in taxa turnover.

## 2. Materials and methods

*Experimental design and study area characteristics*

This study was carried out in Quintos de Mora (Toledo, Spain). Quintos de Mora (39º 25' 08" N, 4º 03' 36" W) is in the central part of the Oretano system, on the southern slope of the Montes de Toledo. Its relief is made up of a series of mountain ranges with heights between 1000 and 1200 meters, with a total area of 6864 ha of Mediterranean forest (San Miguel et al., 2011).

Quintos de Mora consists mainly of layers of shale and quartzite. The landscape is a mosaic of shrublands and forested patches. The shrublands are mainly integrated by rockrose *(C. ladanifer, C. populifolius*), rosemary (*Silvia rosmarinus*) and different species of heather (*E. australis, E. scoparia* and *E. arborea*). The forests are dominated by holm oaks (*Quercus rotundifolia*), often interspersed with multi-stemmed individuals and gall oaks (*Quercus faginea* subsp. *broteroi*). Their understories mainly comprise *Erica arborea* and *Arbutus unedo*. Both shrublands and forests possess scattered herb layers dominated by annual (therophytes) taxa (*Bromus, Crepis, Filago, Hipochaeris, Tolpis, Tuberaria* and *Vulpia,* among others).

We built two enclosures in an area that had been free from herbivory pressure for 20 years in central Spain. The enclosures measured 7.4 and 7.1 ha and were used to simulate two different scenarios of herbivory pressure: hyper- (113 deer/km$^2$) and high-density (62 deer/km$^2$), respectively. These population densities were selected to reflect common densities found in different areas of Spain, e.g., 30 deer/Km$^2$ (Carpio et al., 2015; Charco García, 2002; Fernández-Olalla et al., 2006a; Vicente et al., 2007), as well as on hunting game reserves that specifically manage wild game populations for commercial hunting, e.g., 83 deer/km$^2$ (Acevedo et al., 2008; Azorit et al., 2002, 2012). For this experiment, we only used adult female red deer in order to maintain animal densities,

avoid reproduction and sex bias and to keep operators safe; male red deer are more difficult to manage, especially during rutting season. All animals were obtained from wild herds found in Cabañeros National Park (39° 34' 49'' N, 4° 40' 43), a natural area that is only 40 km away from the study area and has the same environmental conditions and landscape. Animals were released into the enclosures in March 2021 and kept until May 2022, with an average age of 21 months (15-32 months) and average weight of 61 kg (47-74 kg). We released 12 red deer in the 7.4 ha enclosure and 7 in the 7.1 ha, which would be the equivalent of an herbivory pressure of 63 and 113 deer/km$^2$, respectively, with a 1:1 sex ratio population (Peláez et al., 2017). These numbers would also accurately reflect the social behavior of a big herd group under natural conditions (Jedrzejewski et al., 2006). We also characterized enclosure vegetation (presence and percentage cover of woody and herbaceous species) to make sure both herds had the same dietary resources available, using the point-intercept transect method for woody species (Calleja et al., 2019) and 50 x 50 cm quadrats (a total of 90) for herbaceous species, following Cuerdo et al. (2025). Deer survival and the presence of other herbivores were monitored using a network of 16 trail cameras (Strikeforce HD ProX 20 MP). These were checked monthly for the duration of the study. Finally, we characterized the phenological cycle of each area by extracting Normalized Difference Vegetation Index (NDVI) values using MODIS-

Terra datasets (Solano et al., 2010) obtained through satellite information at a spatial resolution of 250 m and applying the "phenopix" package from R (Filippa et al., 2016) to obtain the dates and duration of the main phenological phases in each area (dormancy period, senescence period, green up and stable peak period).

*Sample collection and storage*

Fecal sampling was carried out monthly, with the goal of obtaining 6 samples for each herd per month. Sampling took place from April 2021 to May 2022, although sampling was not possible during June 2021 and April 2022 for logistical reasons. We collected a total of 69 samples for the high-density treatment (monthly mean: 5.75 samples ($5_{min}$ – $7_{max}$) and 68 from the hyper-density treatment (monthly mean: 5.67 samples ($5_{min}$ – $6_{max}$). One fecal sample consisted of an entire fecal pile. Only fecal piles that were more than 10 meters away from each other and looked fresh (even surface with mucus and easily crushable between fingers, with no fungal growth present) were considered for sampling to decrease the risk of sampling the same individual twice. Nitrile gloves were changed between the collection of each fecal sample. Bags were stored in a cold box (4 to 8 $^{o}$C) during transport to the lab and frozen at -20 $^{o}$C until further processing.

*Diet quality and composition*

Samples were dried at 60 $^{o}$C until the weight remained constant (accomplished after 72 hours) and milled using a 1.0 mm particle

filter with a CT 293 Cyclotec Labtec Line miller (FOSS Iberia, Barcelona, Spain). Fecal nitrogen (Metabolic fecal nitrogen, MFN, which indicates protein digestibility and the efficiency of microbial protein synthesis in the gut (Hironaka et al., 1970; Schwarm et al., 2009);, fiber composition (Acid detergent lignin, ADL, the most recalcitrant part of plant cell walls and is not fermented by ruminant microbes (Hindrichsen et al., 2006; Van Soest, 1967) and fecal glucocorticoid metabolites (FGM, non-invasive integrative indicators of adrenocortical activity, a proxy for physiological stress (Santos et al., 2014)) were determined using the near infrared spectroscopy technique (NIRS) with a NIRSystems 500 (FOSS, Hillerød, Denmark) set to 1100 to 2500 nm wave range. Samples were loaded into a 3.5 cm diameter round capsule with a quartz window and two spectral measurements of log 1/reflectance were taken for each sample. The calibrated models to predict fecal nitrogen (Villamuelas et al., 2017) fiber composition (Rossa et al., 2025) and FGM (Santos et al., 2014) have already been validated and can be found in the literature.

We assessed dietary composition by applying the cuticle microhistological analysis technique, using a modified version of Stewart's protocol (Stewart, 1967). A complete description of this modified version can be found in Espunyes et al. (2019). Briefly, we mixed 10 milled sample grams with 5 mL of $HNO_3$ 63% concentration solution in a test tube to perform an in vitro artificial digestion. The test tube was placed in an 80 $^{o}$C water bath for 1 min, removed from the bath and then its contents were spilled in a glass

beaker that contained 200 mL of distilled water to stop the chemical reaction. Afterwards, the mixture was sieved through two different filters: first through 1.00 mm and then 0.25 mm. The fraction retained in the 0.25 mm was then distributed evenly over the surface of a glass microscope slide with a 50% aqueous glycerine solution, covered with a glass cover and sealed using DPX microhistological varnish. Three replicas were done per sample. The slides were then assessed under the microscope at x100 and x400 magnification. We counted and identified a total of 100 cuticular fragments per sample. Plant cuticle fragment identification was performed using a reference library of 25 plant species. Using these data, we obtained diet diversity metrics by calculating the Shannon-Weiner diversity Index for each sample (Odum, 1974; Shannon & Weaver, 1948).

*Microbiome sample DNA processing and sequencing*

We extracted sample DNA using QIAamp PowerFecal Pro DNA Kits (Qiagen Iberia SL, 08028 Barcelona) following the manufacturer's instructions and used up to 250 mg of each sample for DNA extraction. We added a negative control after every 46 extractions. We used 2 µL for DNA quantification, which we performed with the Qubit dsDNA BR (Broad Range) Assay kit (Fisher Scientific S.L., Madrid, Spain) and an Invitrogen Qubit fluorometer model MP32857 (Fisher Scientific S.L., Madrid, Spain). Extraction aliquots were stored at – 80 $^{o}$C. Sequencing was carried out at an external laboratory (CRG-Centre for Genomic Regulation, Genomic Unit

08003 Barcelona, Spain). Requested region amplification was region V4 from the 16S rRNA bacterial gene using the following primers: 16S Forward primer 515-F 5' – GTGYCAGCMGCCGCGGTAA - 3' and 16S Reverse primer 806RMod-R 5' – GGACTACNVGGGTWTCTAAT – 3' from the Earth Microbiome Project (Walters et al., 2016).

*Bioinformatic analysis*

We processed the raw sequence data using QIIME2 (version 2024.5) software (Bolyen et al., 2019). Primers were removed from the sequences before starting sequence processing using the "cutadapt" plugin from QIIME2. We implemented the DADA2 workflow pipeline to remove low-quality reads and chimeras and identify amplicon sequence variants (ASVs, i.e., unique DNA sequences from amplified genetic regions to distinguish microorganisms), using a truncating length of 240 for forward and 153 for reverse reads respectively (truncLen = c(240, 135)). The resulting feature table contained unrarefied ASV counts per sample, and representative ASV sequences were retained to perform ASV taxonomic classification using the sKlearn machine learning classification method found in *q2-feature-classifier* plugin. We used the Silva 138 classifier as the reference library. Following taxonomic assignment, the feature table was filtered to remove non-target sequences, including mitochondrial, chloroplast, and unassigned features, using the *q2-taxa* plugin. Features that appeared less than 10 times and in less than two samples were removed from downstream analysis. The filtered feature table was used for all

subsequent diversity and statistical analyses. Rarefaction was set at 42000 for obtaining alpha (ASV richness, Shannon's diversity index and Faith's phylogenetic diversity) and beta diversity indexes (Bray-Curtis, Weighted and Unweighted UniFrac).

*Statistical analysis*

Gastrointestinal microbiome alpha diversity was quantified using the Shannon-Weiner diversity index and Faith's Phylogenetic Diversity (PD, Faith & Baker, 2006). Faith's PD index measures intra-sample phylogenetic diversity, so the value increases when the community includes lineages that are more distantly related. The goal of our statistical analysis was to evaluate if deer density ("Treatment"), the amount of time spent by the red deer inside the enclosures ("DaysIntro"), plant phenological phase ("Phenology"), diet quality and diversity (MFN, ADL and dietary Shannon-Wiener's diversity index) and physiological stress (FGM) were related to changes in microbiome diversity using generalized additive mixed models (GAMMs). We previously checked for collinearity using Pairwise Pearson correlation coefficients, avoiding including variables with a correlation coefficient over 0.60 in the same model (Young, 2018). Then, we compared with Akaike's information criterion (AIC). The model with the lowest AIC was retained as the best-supported model (Zuur et al., 2009a). GAMMs for each alpha diversity metric were fitted using the "mgcv" version 1.9-4 (Wood, 2017) and the "lme4" package version 1.1-35.5 (Bates et al., 2015)

in R Studio version 4.4.1 (R Core Team, 2024). Candidate models are summarized in Table 1.

**Table 1.** Statistical models (M) representing a set of biological hypothesis to explain the role of the time spent inside the enclosures ("DaysIntro"), population density (high and hyper density, coded as Treatment), plant phenology, physiological stress (fecal glucocorticoid metabolites, FCM), diet quality (metabolic fecal nitrogen, MFN, and acid detergent lignine, ADL), and diversity (Shannon_diet) on the gut microbiome diversity of two female red deer herds kept in captivity in two enclosures with natural Mediterranean vegetation in Quintos de Mora, Central Spain. The * indicates an interaction term. The "S()" indicates a smoothed term and the Shannon_diet is the Shannon-Wiener's diversity index or red deer diets.

| Model | Model structure | Biological hypothesis |
|---|---|---|
| M1 | S(DaysIntro)*Treatment | Alpha diversity over time depends on deer density (Kartzinel et al., 2019). |
| M2 | M1 + Phenology | The plant phenological stage contributes to the previous M1 model, explaining the temporal variation in the treatment-specific alpha diversity (Y. Wang et al., 2023) |
| M3 | M1 + MFN + ADL | Diet quality, evaluated by MFN and ADL, contributes to explaining the temporal variation in the treatment-specific alpha diversity (Guo et al., 2024a) |
| M4 | M1 + Shannon_diet | Dietary diversity contributes more than phenology (M2) or diet quality (M3) to explaining the temporal variation in the treatment-specific alpha diversity (Kartzinel et al., 2019) |
| M5 | M1 + FGM | In this case, the physiological stress added to M1 is the main influence of alpha diversity (Łopucki et al., 2025) |

All models included “DaysIntro” as a smoothed term to capture non-linear temporal trends, with maximum basis dimension set to k = 5 to avoid model overfitting. Models were fitted using the restricted maximum likelihood (REML). Model assumptions were evaluated using gam.check() function from the mgcv package and graphical inspection of residuals to verify normality and homoscedasticity.

Our hypotheses predict temporal turnover and divergent community trajectories between density treatments; beta diversity metrics were used to assess treatment- and time-dependent compositional differences beyond what alpha diversity alone can capture. Beta diversity was assessed using the Bray–Curtis dissimilarity index (Somerfield, 2008), weighted and unweighted UniFrac distances. Bray–Curtis dissimilarity quantifies differences in taxa relative abundances, whereas UniFrac distances incorporate phylogenetic information, with unweighted UniFrac reflecting differences in community membership (presence–absence) and weighted UniFrac additionally accounting for taxa relative abundances (Chang et al., 2011; Lozupone et al., 2011). The combined use of these metrics allows us to assess compositional shifts (Bray-Curtis) and if they stem from a loss of phylogenetically diverse taxa (Unweighted UniFrac) and/or changes in their relative abundance (Weighted UniFrac). Distance matrices were calculated using the “phyloseq” version 1.48.0 (McMurdie & Holmes, 2013), “vegan” version 2.7-2 (Oksanen et al., 2026), and “ape” version 5.8-1 (Paradis & Schliep, 2019) packages in R. We first performed a

permutational multivariate analysis of variance (PERMANOVA) using the “adonis2()” function in vegan for each beta diversity index using only “Treatment” (deer density) as an explanatory variable to see if there would be significant differences between treatments. FGM and ADL concentrations presented a moderate correlation (r = 0.62), so they were not included together in the same PERMANOVA. As phenology was nested within the sampling month variable, we decided to retain the sampling month variable, assuming it would represent the effect of cumulative time spent in the enclosures as well as phenology. As stress has been identified as a factor that can affect gut microbiota composition (Łopucki et al., 2025), we ran two marginal PERMANOVA models stratified by treatment to account for FGM. The first PERMANOVA included sampling month, MFN, FGM values and diet diversity; the second PERMANOVA included sampling month, MFN, ADL values and diet diversity. All PERMANOVA tests were run with 9999 permutations. Homogeneity of multivariate dispersion was assessed for sampling month using the “betadisper()” function followed by ANOVA (PERMDISP). In cases where multivariate dispersion differed significantly among groups, we conducted a sensitivity analysis to evaluate whether PERMANOVA results were driven by extreme samples. Outliers were identified using a 1.75× interquartile range (IQR) criterion based on distances to group centroids (“betadisper”), removed, and analyses were repeated. Finally, community patterns were visualized using Principal Coordinates Analysis (PCoA).

Differential abundance testing was conducted using ANCOM-BC (analysis of composition of microbiomes with bias correction), which estimates bias-corrected log-fold changes while accounting for compositionality and sampling fraction differences. Models were implemented separately for each treatment to accommodate treatment-specific temporal trajectories identified in diversity analyses. This analysis was carried out in QIIME2.

Sampling month was intrinsically associated with vegetation phenology, as each sampling event matched a specific phenological phase. This resulted in structural confounding between calendar time and phenology, precluding their simultaneous independent inclusion in the same model. To reduce parameterization while preserving biological interpretability, sampling events were aggregated into four discrete phases of our experiment ("IntroPhase") representing major phenological periods across the study that also accounted for the time that had passed since the start of the experiment. These four phases were phase 1 (April 2021 to July 2021, linked to Dormancy Period); phase 2 (September to October 2021, linked to green up-stable peak period); phase 3 (November 2021 to January 2022, senescence period) and phase 4 (February to May 2022, the second dormancy period).

All statistical analyses were carried out in R Studio version 4.4.1 (R Core Team, 2024) and QIIME2 version 2024.5 (Bolyen et al., 2019).

## 3. Results

*Alpha diversity results*

We identified a total of 5425 ASVs out of 102 samples classified to the genus level. Out of 5425, only 397 (7.32% approximately) appeared less than 10 times. Median relative abundance of ASVs was 1.89e-05 (max 0.058). Rarefied samples contained a median relative ASV abundance per sample of 730 ± 165.974 (min 115, max 1033). The median number of genera identified per sample was 124 ± 12.281 (min 68, max 148). Most of the identified ASVs belonged to the Firmicutes (77.46%) and Bacteroidota (15.54%) phyla, followed by Actinobacreriota (2.01%) and Proteobacteria (1.95%). The top 10 most abundant taxa across all samples were *UCG-005* (18.07%), Lachnospiraceae family (12.78%), Christensenellaceae R-7 group (5.96%), Oscillospiraceae family (4.83%), Bacilli class (4.66%), Rickenellaceae RC9 gut group (3.74%), Eubacterium coprostanoligenes group (3.46%), UCG 010 family (3.26%), Bacteroides (2.96%) and Ruminococcaceae family (2.41%).

The final selected models for Shannon's diversity and Faith's PD differed in the retained explanatory variables (Table 2). For Shannon diversity, the best-ranked model included the interaction between deer density and number of days spent in the enclosures, along with the effect of diet diversity (Table 2).

**Table 2.** Generalized additive mixed model (GAMM) selection for Shannon and Faith's phylogenetic diversity (PD) indices based on Akaike's Information Criterion (AIC). The best-supported model for each response variable is marked in bold. ΔAIC represents the difference between each model's AIC value and that of the null-model. Df: degrees of freedom. AICc: corrected AIC value. Wi reflects the approximate probability that model *i* is the best model in the set. Model and model structure follow Table 1's pattern. All models included 102 observations. "Treat": population density treatment.

| Index | Model | Model structure | df | AIC | AICc | ΔAIC | Wi |
|---|---|---|---|---|---|---|---|
| Shannon | **M4** | **M1 + Shannon_diet** | **9** | **336.11** | **338.07** | **0.00** | **0.56** |
| | M1 | S(Days)*Treat. | 8 | 337.29 | 338.84 | 0.72 | 0.39 |
| | M3 | M1 + MFN + ADL | 10 | 340.17 | 342.59 | 4.53 | 0.06 |
| | M2 | M1 + Phenology | 10 | 340.75 | 343.17 | 5.11 | 0.04 |
| | M0 | Null model | 3 | 344.24 | 344.48 | 6.38 | **0**.02 |
| | M5 | M1 + FGM | 9 | 346.29 | 348.25 | 10.18 | 0.00 |
| Faith's PF | **M2** | **M1 + Phenology** | **10** | **634.70** | **637.12** | **0.00** | **0.74** |
| | M3 | M1 + MFN + ADL | 10 | 637.55 | 639.97 | 2.85 | 0.18 |
| | M4 | M1 + Shannon_diet | 9 | 640.25 | 642.21 | 5.08 | 0.06 |
| | M1 | S(Days)*Treat. | 8 | 642.12 | 643.67 | 6.49 | 0.03 |
| | M5 | M1 + FGM | 9 | 645.24 | 647.20 | 10.07 | 0.00 |
| | M0 | Null model | 3 | 654.62 | 654.86 | 17.70 | 0.00 |

For the phylogenetic diversity (Faith's PD), the best modes included the same "deer density and number of days spent in the enclosure" interaction and the phenological phase of the year (Table 2). The model, including FGM concentrations, received little support as a candidate for explaining variation in either Shannon diversity or Faith's phylogenetic diversity (Table 2).

Temporal trends of Shannon's diversity index differed between treatments. In HD ($Estimate_{Days_HD}$ = 2.925, T = 9.309, $p < 0.001$), microbial diversity displayed a progressive decrease from day 300 onwards, while Shannon values remained constant in HyD ($Estimate_{Days:HyD}$ = 1.000, T = 0.009, $p > 0.05$, Figure 1a). On the other hand, diet diversity (Estimate = - 0.788 ± 0.453, T = -1.742, $p > 0.05$) was negatively associated with the Shannon diversity index of the microbiome, suggesting that more diverse diets were linked to less diverse microbial communities. This model explained 21.6% ($R^2$ = 0.216) of the observed variance.

Along the same lines, Faith's PD showed different trends between treatments (Fig. 1b), with a progressive decrease in the HD treatment ($Estimate_{Days:HD}$ = 1.000, T = 6.242, $p < 0.05$) and an increase in the HyD treatment ($Estimate_{Days:HyD}$ = 1.000, T = 3.427, $p > 0.05$). Phenology was also associated with Faith's PD (Fig. 1c), with higher values observed during the green-up–stable peak period (GUSPP, Estimate = 4.130 ± 1.214, T = 3.403, $p < 0.001$) than during dormancy or senescence (SP, Estimate = - 0.797 ± 1.459, T = - 0.547, $p > 0.05$). The retained model explained 18.0% ($R^2$ = 0.180) of the observed variance.

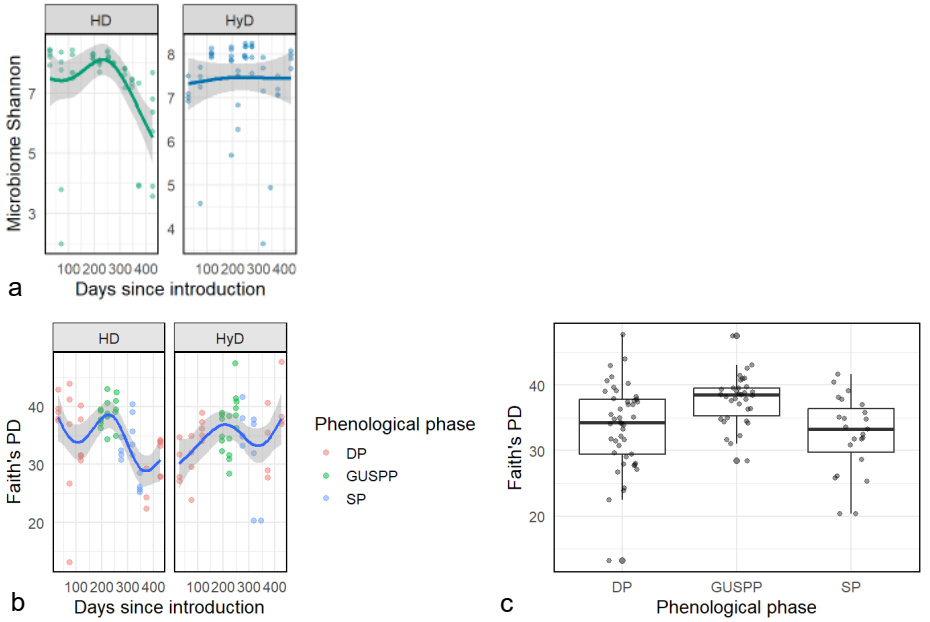



**Figure 1.** Trends in the Shannon's diversity index ("Microbiome Shannon", a) and the Faith's phylogenetic diversity (PD, b) of the gastrointestinal microbiome of female red deer herds kept at high density (HD, 62 deer/km$^2$) and hyper-density (HyD, 113 deer/km$^2$) in two enclosures with Mediterranean vegetation in Quintos de Mora, Central Spain. In c we represent changes in PD across the phenological phases of the vegetation called dormancy period (DP), green up-stable peak period (GUSPP), and senescence period (SP).

*Beta diversity results*

Bray Curtis dissimilarity index did significantly differ between deer density populations ($F = 4.34$, $p < 0.001$), although the explained variability was modest ($R^2 = 0.041$) (Figure 2a). The PERMANOVA model including FGM levels showed that they do not have any significant effect in observed Bray Curtis variation ($F = 1.16$, $p = 0.208$). Therefore, reported results come from the PERMANOVA model that does not include it.

The month of sampling explained 25.39% of the observed variation in Bray-Curtis distance ($F = 3.34$, $p < 0.001$, Figure 2b). Lack of overdispersion in the sampling month indicates that the observed effects are robust. The ADL and MFN concentrations were also significant ($F_{ADL} = 1.68$, $p = 0.03$; $F_{MFN} = 2.54$, $p = 0.0016$), but with a lower effect size than the sampling month (1.27% and 1.29%, respectively). Diet diversity did not contribute significantly to Bray-Curtis variation ($F = 1.29$, $p = 0.108$).

Deer density also had a significant effect on unweighted ($F = 3.46$, $p < 0.001$) and weighted ($F = 3.60$, $p = 0.039$) UniFrac distances, although it only explained a modest proportion of the observed variation (3.47% and 3.51%, respectively, Figures 3a and 3b).

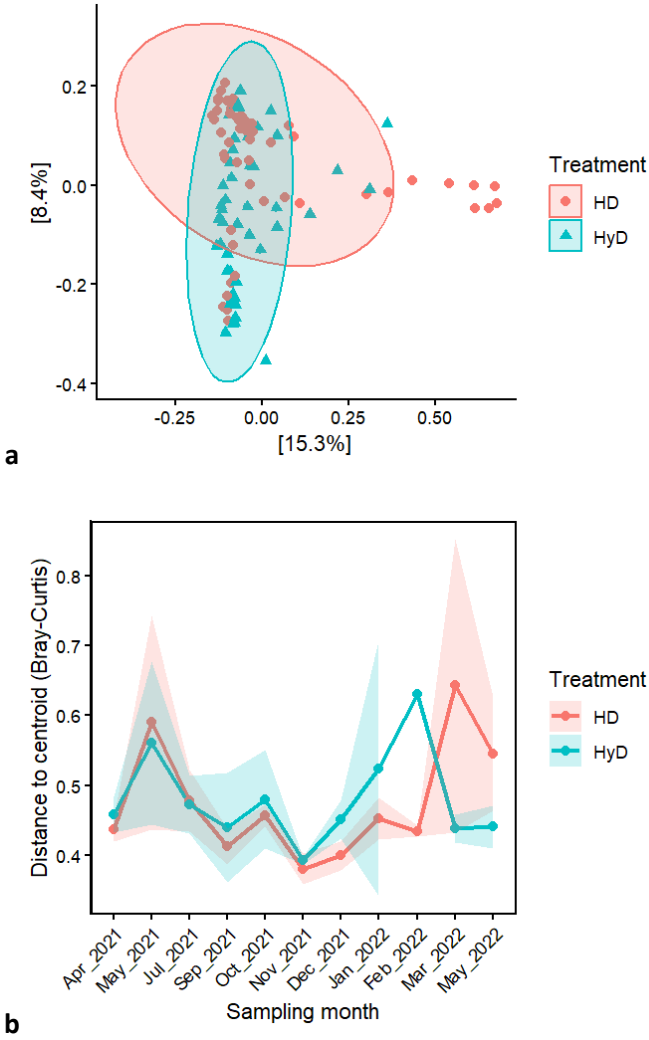



**Figure 2.** Principal Coordinate Analysis (PCoA) plot (**a**) of differences in the relative abundance of gut microbial communities of red deer based on Bray-Curtis dissimilarity. Colored ellipses represent statistically significant differences in microbial taxa between deer density treatments (HyD: hyper-density, HD: high density); in **b** temporal trend in the distances to the centroid (e.g., spread out of the microbial community from their group's average position) across months spent in hyper- and hy-density enclosures.

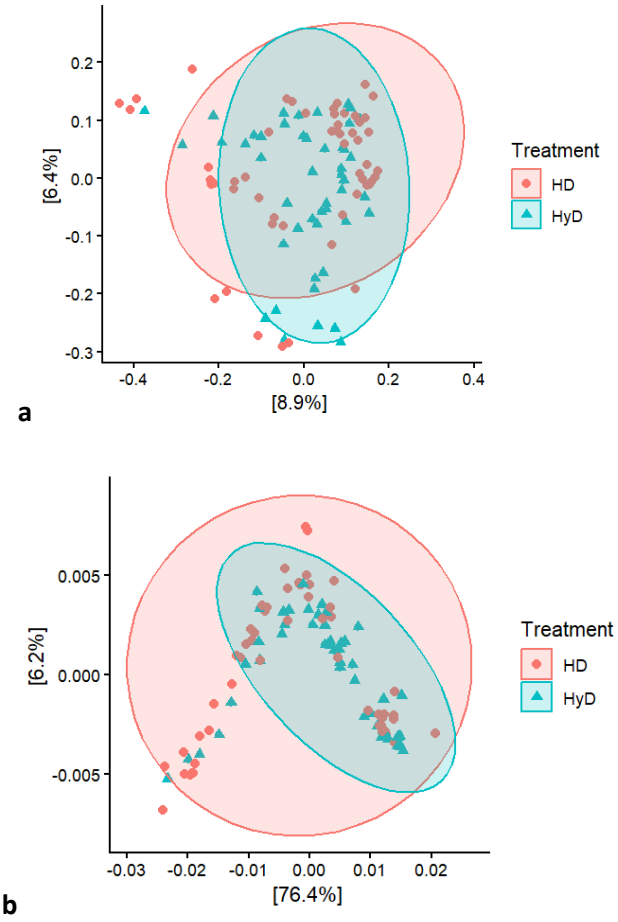



**Figure 3.** Principal Coordinate Analysis (PCoA) plots based on Unifrac distances representing unweighted (presence -absence of microbial taxa, 3a) and weighted (accounting for the relative abundance, 3b) differences in the gut microbial communities of red deer based on Bray-Curtis dissimilarity with phylogenetic correction. Colored ellipses represent statistically significant differences in microbial communities between deer density treatments (HyD: hyper-density, HD: high density).

Both indexes displayed overdispersion for the month of sampling (unweighted UniFrac F = 2.09, p = 0.032; weighted UniFrac F = 3.77, p < 0.001). FGM was not significant for either index ($F_{UnweightedUniF}$ = 1.14, p = 0.202; $F_{WeightedUniF}$ = 0.24, p = 0.850). When permutations were restricted within treatment, sampling month explained 23.42% (F = 2.92, p < 0.001) of variation in unweighted, and 21.63% in weighted (F = 2.82, p < 0.001) UniFrac distances.

Regarding unweighted Unifrac, ADL and MFN significantly contributed to explaining observed variation ($F_{ADL}$ = 1.82, p = 0.0018; $F_{MFN}$ = 2.39, p < 0.001), although their contribution was modest (1.46% and 1.91%, respectively). Diet diversity barely explained 1% of the observed variation (F = 1.32, p = 0.058). After checking and removing outliers (n = 2) from the sampling month variable to perform the robustness test, the observed effects remained the same; except that diet diversity increased its level of significance (F = 1.34, p = 0.0437). In contrast, ADL, MFN and diet diversity did not significantly explain the observed variation in Weighted UniFrac and contributed less than 1% of the observed variation to this index ($F_{ADL}$ = 0.41, p = 0.659; $F_{MFN}$ = 1.18, p = 0.307; $F_{Diet}$ = 1.24, p = 0.252). These results still hold after eliminating outliers (n = 4) and performing the robustness test.

*Relative abundance changes (ANCOM-BC)*

In density treatments, the microbiome community of our deer herds experienced significant restructuring compared to the initial phase of the experiment (April to July 2021). During September to October 2021 (phase 2), HD communities appeared to favor fermentation of more readily available carbohydrates with reduced representation of canonical fiber degraders, whereas HyD communities maintained or enhanced structural fiber breakdown and secondary fermentation pathways. Under HD, the microbiome was characterized by enrichment of Muribaculaceae, Erysipelatoclostridiaceae, Rickettsiales, and Carnobacteriaceae, alongside depletion of Enterobacteriaceae, Clostridiaceae, and the Bacteroidales RF16 group (Figure 4a). In contrast, HyD did not display significant depletion of taxa; rather, it exhibited enrichment of taxa more directly linked to structural polysaccharide degradation and secondary fermentation, including Hungateiclostridiaceae, Anaerofuscaceae, and Gastranaerophilales (Figure 5a).

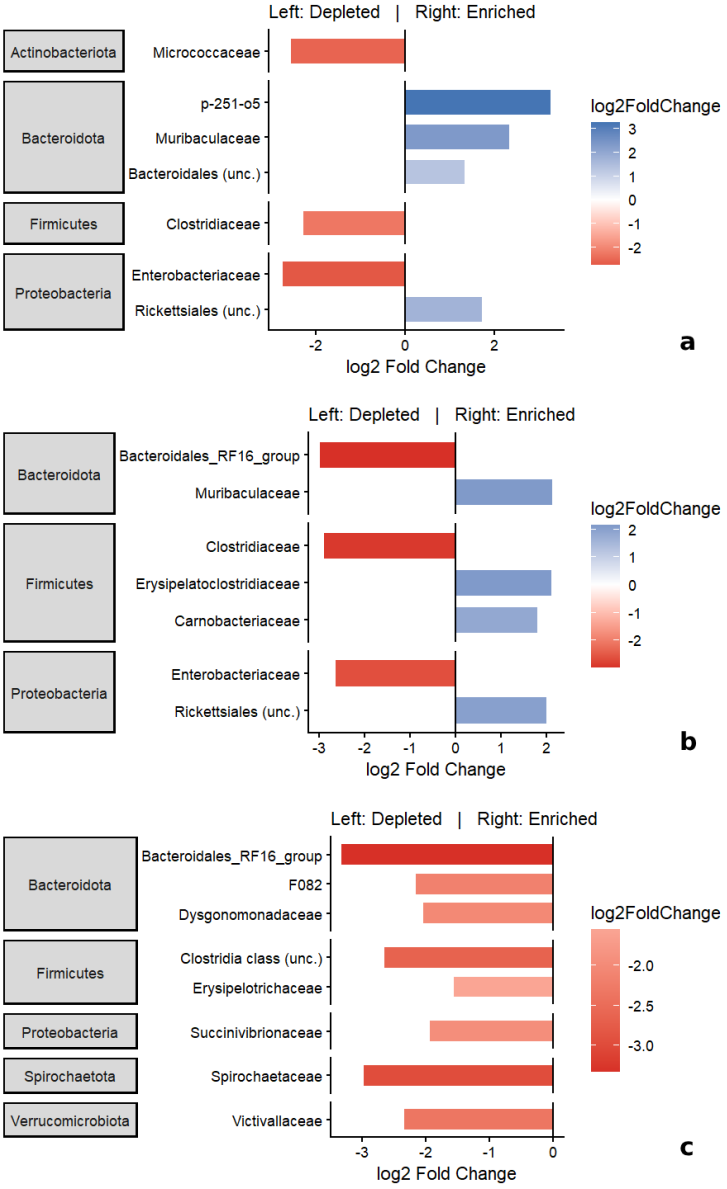



**Figure 4.** ANCOM-BC barplots show significant ($p < 0.05$) family-level taxa changes in the high-density herd across three periods. Bars indicate relative depletion (negative, red, left) or enrichment (positive, blue, right) compared to the reference period (April–July 2021). Grey labels denote phylum affiliation.

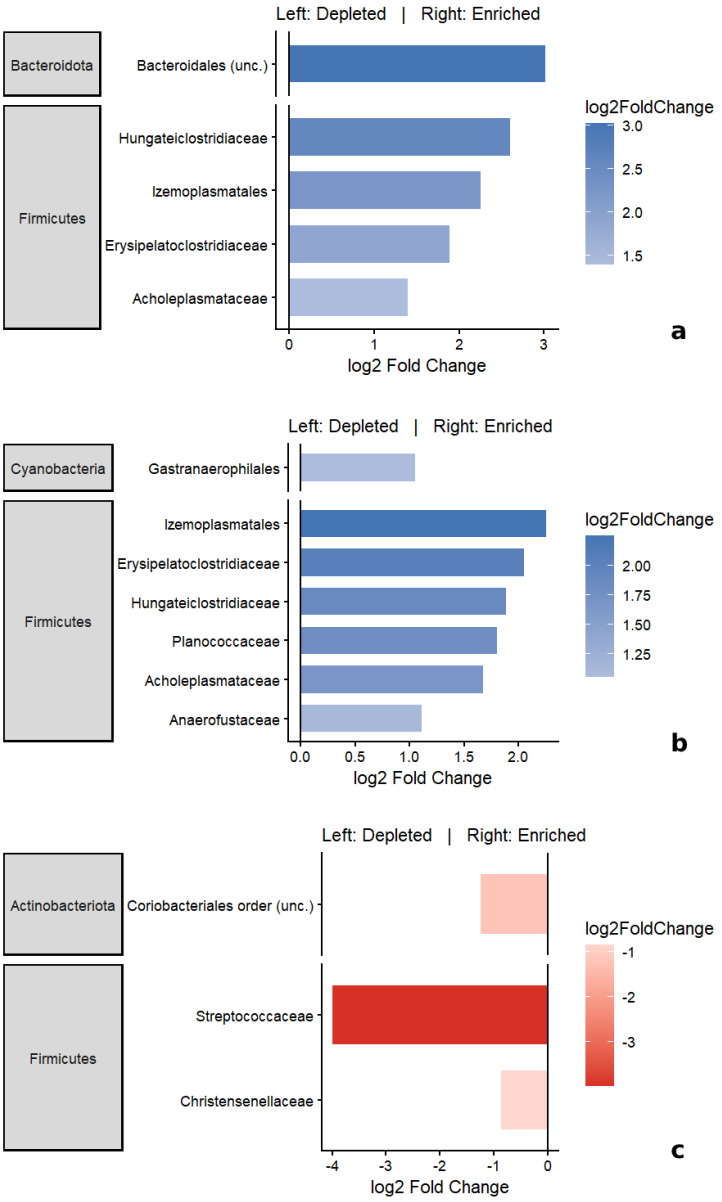



**Figure 5.** ANCOM-BC barplots show significant ($p < 0.05$) family-level taxa changes in the hyper-density herd across three periods. Bars indicate relative depletion (negative, red, left) or enrichment (positive, blue, right) compared to the reference period (April–July 2021). Grey labels denote phylum affiliation.

From November 2021 to January 2022, changes highlight density-dependent divergence in microbial energy-harvesting strategies. Under HD, enrichment of Muribaculaceae, Erysipelatoclostridiaceae, Rickettsiales, and Carnobacteriaceae, combined with depletion of Enterobacteriaceae, Clostridiaceae, and Bacteroidales RF16 group, suggests a shift toward fermentation of soluble and readily digestible substrates rather than structural polysaccharides (Figure 4b). In contrast, HyD displayed enrichment of taxa more directly associated with structural fiber degradation and secondary fermentation, including Izemoplasmatales, Erysipelatoclostridiaceae, Hungateiclostridiaceae, Planococcaceae, Acholeplasmataceae, Anaerofuscaceae and Gastranaerophilales (Figure 5b).

Finally, from February to May 2022, microbial restructuring was characterized predominantly by taxonomic depletion rather than enrichment, but HD treatment suffered depletion of more taxa than HyD and additionally, different taxa were depleted in each treatment. HD experienced depletion of Erysipelotrichaceae, Succinivibrionaceae, Dysgomonadaceae, F082, Victivallaceae, the Bacteroidales RF16 group, and members of Spirochaetaceae and uncultured Clostridia (Figure 4c). HyD exhibited depletion of taxa more closely associated with nitrogen metabolism and community stability, including Christensenellaceae, Coriobacteriales, and Streptococcaceae (Figure 5c).

## 4. Discussion

In this study, we examined how overabundance acts as a bottom-up force shaping the gut microbiome of red deer. We have seen how the density effects depended strongly on time since release and were further influenced by plant phenology. Differential abundance analyses showed density-dependent shifts in microbial composition, with hyper-density favoring fiber-degrading taxa and high-density taxa linked to more digestible substrates.

Most of the identified microbial taxa in our herds belonged to the Firmicutes and Bacteroidota phyla, in agreement with previous results described in this species in other European regions (Menke et al., 2019), other wild cervids (Gruninger et al., 2014; Li et al., 2017; Łopucki et al., 2025) and domestic ruminants (Andrade et al., 2020). Under high-density conditions, both Shannon diversity and Faith's PD declined over time, reflecting a progressive loss of diversity. In contrast, under hyper-density, Shannon diversity remained stable while Faith's PD increased, suggesting maintenance of abundance structure alongside phylogenetic turnover through the incorporation of more divergent lineages. In our experiment, the greater oscillation in diet diversity observed in the hyper-density treatment compared to high density potentially reflects the need for larger dietary shifts (in terms of plant species composition and proportional intake) to meet nutritional requirements. These more marked changes in feeding behavior could support why gastrointestinal microbiome Shannon's index remained stable

under hyper-density conditions, since evidence obtained from cattle and sheep indicate that expanded plant dietary diversity affect taxa turnover rather than changes in alpha diversity indexes per se, at least in the rumen (Forcina et al., 2022; Xu et al., 2025).

On the other hand, phenology emerged as a key driver of phylogenetic diversity. Seasonal shifts in plant availability and quality are known to shape free-ranging wild herbivore gut microbiomes (Brown et al., 2022), particularly in systems dominated by woody vegetation where nutritional quality declines outside the growing season, such as the Mediterranean environment (Azorit et al., 2012; Martínez, 2009). The increase in Faith's PD under hyper-density may therefore reflect broader dietary niches during periods of scarcity, promoting phylogenetic restructuring without major changes in overall abundance patterns. Taken together, these results indicate that lower population densities are associated with intra-sample gradual diversity erosion, while higher densities display phylogenetic restructuring without disruption of abundance stability. These findings suggest that a gastrointestinal microbiome community that is phylogenetically diverse could be crucial for the long-term health and adaptability of red deer populations to highly degraded environments or habitats with marked seasonality, where the availability of higher quality dietary resources is restricted to specific periods of the year. Our results are consistent with a global meta-analysis showing that diet is a major driver of gut microbiome diversity and structure in vertebrates, and that shifts in microbial diversity represent a key

mechanism by which hosts adapt to local environmental conditions (Xie et al., 2024).

Regarding beta diversity, not only the time elapsed since release but also of plant fenology, explained the largest proportion of variation across all distance metrics, indicating that seasonal processes act as a dominant ecological filter shaping both taxonomic composition and phylogenetic structure. Such strong temporal effects likely integrate shifts in plant development and maduration, environmental microbial exposure, and host physiological state, thereby driving broad restructuring of the microbial community. In contrast, deer density exerted a more modest but consistent influence (~3–4% of variation) across abundance-based and phylogeny-based metrics, suggesting that density-related processes affect not only taxonomic composition but also the evolutionary structure of the microbiome, potentially through mechanisms such as altered contact rates, environmental transmission, or density-dependent stress. Contrary to our expectations, diet quality (ADL and MFN) and diversity contributed comparatively little (<2%) to between-sample variation, despite diet being one of the main drivers of changes in gut microbiome in wild ruminants (Ma et al., 2019). Although the red deer has a high dietary plasticity (Gebert & Verheyden-Tixier, 2001), the red deer in our study system relied mostly on woody plants (Study III), which have similar nutritional profiles (Torres-Blas et al., 2026), which could potentially explain why diet qualiy contributed marginally to observed changes, indicating that while diet may fine-tune microbial composition,

broader seasonal and density-related processes are the primary determinants of gut microbiome structure in this system.

Additionally, stress has been described as an important factor in determining gut microbiome composition in different species, including deer (Wang et al., 2023). However, contrary to available literature (Łopucki et al., 2025), FGM did not play a role in explaining part of the variation observed in either alpha or beta diversity indexes. This discrepancy may reflect differences in the specific metabolites measured, as well as potential species-specific responses to stress. Furthermore, the strong effect of time elapsed since release ("month") likely captures substantial environmentally driven variation, potentially masking more subtle stress-related effects. Free-ranging cervids are also regularly exposed to diverse environmental stressors (Łopucki et al., 2025); therefore, it would be expected that this group of mammals might have developed adapted mechanisms which mitigate the potential effects that stress has over the gastrointestinal community. As a result, stress-induced microbial changes may be transient and therefore not detectable, however the nature of our dataset does not allow us to test this potential explanation.

The ANCOM-BC results further support the hypothesis of population density causing changes in gut microbiome due to dietary shifts. Using the period from April to July 2021 as the reference phase, which coincided with the dormancy phenophase (i.e. the period within the year where plant growth is suspended

and metabolic activity is at a minimum), the changes in taxa enrichment from September to October 2021 were characterized by a Bacteroidota-dominated shift toward enhanced polysaccharide degradation and propionate production to exploit more digestible green browse fractions (Guo et al., 2024a; O' Donnell et al., 2017; Zheng et al., 2024). This suggests that during this time the animals had a diet rich in protein and easily digestible fiber portions. These months would correspond to the Green Up phenophase, where plants start to grow and leaves and young shoots become rich in protein and soluble carbohydrates and low in indigestible fiber such as lignin (de la Peña et al., 2026; Villamuelas et al., 2016). From November 2021 to January 2022 further Bacteroidales RF16 depletion contrasted with sustained Muribaculaceae gains and a Firmicutes dominance, implying intensified secondary fermentation of a fiber-rich diet and lactate utilization amid declining forage quality (Guo et al., 2024; Qi et al., 2024; Zhu et al., 2024), coinciding with the onset of the Senescence period, when plant nutritional quality starts to decline (Azorit et al., 2012). By the second dormancy period (February to May 2022), changes in relative abundance were characterized by depletion of several key families (RF16 group, F082, Dysgonomonadaceae, Spirochaetaceae, Victivallaceae) that hydrolyze cellulose, hemicellulose, and other plant polysaccharides into simple sugars that are later fermented into VFA, which provide around 70% of the metabolizable energy in ruminants (Armentano, 1992; Van Soest, 1994). On the other hand, members of the Clostridiaceae family ferment proteins and amino

acids (Liu et al., 2020). These shifts indicate a reduced capacity for primary fiber degradation at a time when forage quality is already low, potentially compromising the ability to efficiently extract energy from nutritionally poor resources. Among the taxa affected, the decline of Succinivibrionaceae and the RF16 group is particularly informative. Members of the Succinivibrionaceae specialize in producing succinate and propionate, helping support efficient energy harvest (Liu et al., 2020; Qi et al., 2024), while RF16 has been associated with modulation of endotoxins and the inflammatory response in domestic cattle (Fonseca et al., 2023). Reduction in Succiinivibrionaceae has been associated to abrupt dietary changes and periods of food restriction, which are often associated to changes in their host immune response (Castillo-Lopez et al., 2024; McCabe et al., 2015), and therefore, their decrease has been proposed as a biomarker of environmental-related metabolic stress in the host (Łopucki et al., 2025). Together, the depletion of these functionally important groups suggests not only reduced fiber degradation and energy extraction efficiency, but also a shift in host physiological state. These microbial changes are consistent with increasing nutritional stress under poor-quality diets and may indicate a diminished capacity to maintain homeostasis under resource-limited conditions, potentially accompanied by immune dysregulation or a pro-inflammatory state.

The changes observed in the hyper-density treatment are also consistent with progressive adaptation to a diet that increasingly

becomes poorer in quality but varies seasonally. However, the composition, number, and direction of significantly affected taxa differed from the high density herd, indicating that comparable functional shifts were achieved through distinct taxonomic pathways. In September to October 2021, taxa shifts were characterized by the enrichment of uncultured Bacteroidales together with several Firmicutes families (Hungateiclostridiaceae, Erysipelatoclostridiaceae, Izemoplasmatales), suggesting an upregulation of hindgut fiber and protein fermentation (Acholeplasmataceae) and intensified nitrogen recycling (Viquez-Umana et al., 2025), compatible with having good diet quality. Notably, members of the Erysipelatoclostridiaceae family have been identified as part of healthy core gut microbiome in cattle (Ma et al., 2020). From November 2021 to January 2022, taxa enrichment was further expanded with additional increases in hemicellulose-degrading taxa (Anaerofustaceae) (Liu et al., 2025) that also synthetize important micronutrients (Gastranaerophilales) (Dai et al., 2024; Ma et al., 2020), indicating a diversified network of primary and secondary fermenters able to exploit low-quality browse rich in complex polysaccharides while maintaining short-chain fatty acid production. During the last dormancy phase from February to May 2022, only three families (Coriobacteriales, Streptococcaceae, Christensenellaceae) were significantly depleted, all of which are associated with simple sugar degradation (Gupta et al., 2013, 2017; Nagaraja & Titgemeyer, 2007). From these families, the decrease in Christensenellaceae is particularly

noteworthy. This family contributes to the production of short-chain fatty acids such as butyrate and propionate from simple substrates and cellulose, and are vital in yak to adapt to harsher and higher altitudinal environmental conditions (Fan et al., 2020), suggesting that it might be a key family to allow its host to adapt to poorer quality feed and harsher meteorological conditions. Moreover, Christensenellaceae has been recognized as highly heritable family in humans, domestic and free-ranging wild species (Stothart et al., 2024) that can act as a biomarker of overall gut health (Waters & Ley, 2019) and gastrointestinal community resilience in humans and wild horses (Ruaud et al., 2020; Stothart et al., 2024). Therefore, its depletion can reflect a potential loss of a key microbial taxon linked to host resilience under nutritionally poor and environmentally challenging conditions, reflecting a diminished capacity of individuals to cope with low-quality forage. In this context, and given the proven heritability of this family, the observed Christensenellaceae family loss might negatively impact the long-term population viability in harsh environments.

The fact that we observed significant changes in taxonomical abundance for both population densities between the first (April to July 2021) and last (February to May 2022) experimental phases, despite sharing the same phenophase further supports the hypothesis that these adaptations are both density and also time (season) dependent. Finally, regardless of density treatment, it is interesting to note that during the last experimental phase, both herds experience taxa depletion of key families that may ultimately

limit energy acquisition and compromise host resilience in highly seasonal environments.

## 5. Conclusions

In conclusion, our results demonstrate that red deer population density interacts with temporal dynamics to shape intra- and inter-individual gut microbiome diversity, structure, and taxonomic composition. While seasonal processes emerged as the primary driver of microbial turnover, deer density promoted distinct restructuring trajectories. Lower densities were associated with gradual diversity loss, whereas hyper-density conditions promoted phylogenetic restructuring, suggesting alternative microbial configurations under intensified resource competition. Observed taxa shifts for both densities revealed a reduced capacity for fiber degradation and energy harvest under prolonged resource limitation, with depletion of microbial taxa that act as a key component for immune and inflammatory host regulation, as well as overall gut microbial resilience. Taken together, our findings highlight that sustained high population densities may compromise microbiome-mediated nutritional efficiency and host resilience in highly seasonal environments, with potential consequences for the long-term adaptability and viability of wild ungulate populations.

# GENERAL DISCUSSION



*Adult red deer doe with her offspring in a dehesa from Central Spain. Photo author: Alfonso San Miguel.*

*"'I like that explanation,' said Ridcully. 'It is elegant, Mister Stibbons.'*

*'It's only a guess, sir.'*

*'Good enough for physics,' said Ridcully."*

**— Terry Pratchett,** **The Science of Discworld**

## General discussion

Free-ranging wild ruminants exist in a delicate balance between their habitats and their own individual and population traits. Over time, they shape the ecosystems they inhabit and help maintain their sustainability when their numbers remain within the environment's carrying capacity (Ripple & Beschta, 2005).

This thesis has been an attempt to provide an integrated view on the effects that population overabundance has on the basic pillars that determine a wild ruminant's population fitness and long-term viability: feeding behavior and gastrointestinal microbiome composition, using the red deer, one of the most widespread wild ruminants, as the experimental model. Throughout this thesis, we first explored feeding behavior under conditions that were non-related to population density (Study I) and explored the correlation between host physiochemical parameters and gut microbiome composition under controlled dietary conditions to further determine the influence of gut microbiome in an adult ruminant's fitness status beyond food digestion (Study II). Later on, we have determined how a scenario of population overabundance impacted both diet selection (Study III) and gut microbiome changes (Study IV) in a highly seasonal environment. In this section, we will discuss the results obtained in the previous chapters in a more integrative way.

**Role of physiochemical plant characteristics in diet selection of red deer**

In **study I,** we have seen that a plant's nutritional profile was a bigger determining factor in influencing red deer's feeding behavior, rather than physical characteristics such as breaking force. Particularly, although both protein content and fiber proportion were significantly related to positive selection, crude protein concentrations played a bigger role in plant selection than fiber contents, despite fiber fermentation providing more than 80% of the energy requirements in ruminant species (Bergman, 1990) and also generating the ATPs needed by the ruminal microbial community to generate microbial cellular protein (Sanjorjo et al., 2023). Crude protein, however, is considered an essential factor in ruminant nutrition because it provides the main source of nitrogen supply to the rumen microbiome, consequently modulating microbial protein synthesis, digestibility and animal performance (Gallardo et al., 2010; Miranda et al., 2012). Rumen fermentation synthetizes SVFA and microbial crude protein, which in turn is responsible for providing up to 85% of amino acid supply that reaches the animal's small intestine for later absorption (Hackmann & Firkins, 2015). Ruminant intake is also closely tied to palatability, bite size and forage density among other factors, and animals tend to select diets high in digestible organic matter rather than simply high in protein. Therefore, the fact that preferred species had high crude protein and fiber content could indicate that crude protein influences selection positively only when the rest of the nutrition

profile is “interesting” from a dietary perspective (low in lignin, high in complex fibers).

Additionally, the plant species that were positively selected matched accurately with on-field results. It is worth mentioning, however, that both datasets (the multiple-choice test and the field browsing dataset) came from herds integrated by only female red deer. Differences in feeding behavior between both sexes have been described in many different Cervidae species, including red deer (Beier, 1987; Garcia et al., 2023; Koga & Ono, 1994), as males and females have different digestive capabilities, ruminal capacity and energetic and nutritional requirements derived from their marked sexual dimorphism (Barboza & Bowyer, 2000; Bowyer, 1984). These results could indicate that cafeteria tests are a relying experimental design to accurately predict preference under natural conditions as long as the demographic characteristics (in terms of studied species and sex ratio) between the multiple-choice test and the wild population match.

**Associations between gut microbiome composition and physiological parameters**

Although gut microbiome’s primary role in ruminants is to carry out digestion and fermentation to turn indigestible forages into essential nutrients, we cannot underestimate its influence in regulating other important metabolic pathways that are essential for determining the host's overall health and fitness status. However, diet is known as one of the main drivers of gut

microbiome diversity in herbivores (Guo et al., 2024; Huang et al., 2026b; Nishida & Ochman, 2018; Wu et al., 2023). Because of this, in **study II** we hypothesized that associations between physiological parameters and gut microbiome would become more evident in red deer under controlled dietary conditions, where diet composition remained the same for a lengthy period.

We did not find any meaningful associations between alpha and beta diversity parameters and oxidative stress, and the available literature provides mixed results. Some studies found evidence on the effects of a lower alpha diversity, disease occurrence (Kriss et al., 2018), decrease in host survival (Bestion et al., 2017) and low body condition (Stoffel et al., 2020); while others did not find it (Goertz et al., 2019; Worsley et al., 2021), or even found a positive relationship (Härer et al., 2024). This disparity may be attributed to several factors. First, not all the studies use the same metrics to measure alpha diversity, yet each alpha-related index provides different information (Cassol et al., 2025). Therefore, diversity indexes must be carefully selected to accurately answer the stated research question, also hindering direct result comparison between studies. Second, factors like species, age, sex and disease status are also relevant in detecting effects. Carnivores and omnivores have less diverse communities than herbivores, and younger animals (especially before switching to their final adult diet) tend to have more unstable gastrointestinal microbiome communities (Yin et al., 2023). Overall, the limited availability of studies linking host fitness to alpha diversity, coupled with the mixed results, could indicate

that the relationship between intraindividual microbial diversity and host fitness is species-specific.

Available literature also points to positive associations between beta diversity (i.e., the degree of dissimilarity between communities) and host performance via the Anna Karenina principle (Zaneveld et al., 2017). This concept proposes that "all healthy microbiotas are similar; each dysbiotic microbiota is dysbiotic in its own way," implying that individuals experiencing dysbiosis may exhibit more variable microbial community compositions, and therefore higher beta diversity, than healthy individuals. This variability likely arises from a reduced capacity of the host experiencing dysbiosis to regulate and maintain a stable gut microbial community. Therefore, stable gastrointestinal microbiome communities are expected to have lower beta diversity values, as communities would be more similar. Our alpha and beta diversity results were compatible with a community that does not lose diversity, but experiences taxa turnover, therefore suggesting that the red deer in our experiment had the ability to keep a healthy microbiome community while at the same time adapting to the captivity setting. One of the main limitations of this study is that we could not control social interactions or identify the dominant females of the herd. Studies carried out in wild animal populations revealed that social interactions can facilitate microbial exchange between individuals (Tung et al., 2015). Therefore, individuals belonging to the same social group in species with complex social structures are expected to have similar gut microbiome

composition. Interindividual relationships and dominant and submissive roles could therefore be another underlying mechanism driving gastrointestinal microbiome community changes.

One of the most significant findings in **study II** is that specific bacterial taxa and community turnover (beta diversity), rather than alpha diversity metrics, are the true predictors of a deer's physiological status. Ruminants possess one of the most complex gastrointestinal microbiome communities (Sanjorjo et al., 2023). The gastrointestinal community in this group of animals is not just a health associate but a core part of the digestive system, making host fitness especially sensitive to microbial composition. Therefore, it is biologically plausible that, in red deer as wild ruminants, the overall status of key fitness-related physiological traits, such as innate immunity and nutritional condition, is driven more by the overall composition and equilibrium of the gastrointestinal microbial community than by the presence of specific taxa alone. From this perspective, the microbiome acts as a functional ecological unit, in which community structure and turnover better capture host–microbe interactions relevant to fitness than traditional diversity metrics or single-taxon approaches.

**Population density-dependent effects on feeding behavior and gut microbiome composition**

In **study III** and **study IV,** we have evaluated the influence that overabundance has over diet selection and gastrointestinal microbiome composition, and its potential impact on red deer's

physiological stress, which we used as an indirect assessment of metabolic costs.

In **study III** we found that population density shaped diet selection, with marked differences in feeding behavior between the two population densities. In **study I,** we observed that in a context of resource abundance and nonexistent intra and interspecies competition, red deer showed positive selection for plants that were rich in fibers and crude protein (*Quercus rotundifolia,* which we also refer to as *Quercus ilex* in study I), while plants that had higher content of lignin were negatively selected (*Salvia rosmarinus*, formerly *Rosmarinus officinalis*; and *Thymus vulgaris*). However, the selection patterns in **study III** got partially disrupted under a scenario of overpopulation. Density-dependent effects were more linked to dietary turnover and effect on consumption of certain species, rather than changes in overall diet diversity and species evenness. Under higher population density conditions, red deer depleted the preferred resources observed in **study I** (*Quercus rotundifolia*) sooner and showed higher dietary adaptation through an increase in diet diversification (dietary Whittaker's index) and decrease in evenness (meaning the animals relied on a higher consumption of selected plant species). These changes were linked to an incorporation in their diet to plant species negatively selected in **study I,** such as *Salvia rosmarinus.* Species that were negatively selected in **study I** had higher lignin and ash content, indicating that those plants are of poor nutritional quality, since lignin is the indigestible portion of fiber content (Van Soest, 1994). Additionally,

deer relied heavily on phenological phase to adjust their diet, as showcased by the changes in dietary plant diversity, but also on evenness (i.e. relative plant proportions) and the incorporation of the non-preferred plant species into their diet when their concentrations of anti-herbivory secondary compounds were at their lowest, emphasizing the impacts of overabundance in the feeding behavior of herbivores living in highly seasonal environments, such as the Mediterranean climate (Bugalho & Milne, 2003).

Overall, **study III** revealed that under overabundance conditions, herbivores end up disrupting the optimal foraging theory, expanding their dietary niche and incorporating previously avoided plants with poor nutritional quality to meet daily energy and nutrient requirements that come at a greater physiological cost, showed by the higher concentrations of fecal glucocorticoid metabolites. In summary, then, red deer had the ability to adapt to an environment that became gradually poorer in resource ability, but at an extra metabolic cost.

**Study IV** allows us to give a potential mechanistic explanation on how this adaptation to consuming plants of poorer nutritional profile and higher concentrations of secondary compounds takes place. Red deer are known to have a high dietary plasticity, feeding on woody vegetation, grasses, seeds and fruits, switching between food resources throughout the year and sometimes even migrating to areas with better resources. Red deer already possess

evolutionary adaptations that inhibit part of the herbivory deterrent secondary compounds found in some plants, such as tannins (Austin et al., 1989). Additionally, the gastrointestinal microbiome of herbivore mammals can detoxify plant compounds (Dearing & Weinstein, 2022), thus allowing its host to use a wider range of plant resources. Moreover, recent research has provided evidence that gastrointestinal microbiome plasticity and quick ability to adapt to new dietary substrates may be a key element for their hosts to adapt to suboptimal habitat conditions (Amato et al., 2013; Baniel et al., 2021).

In our results, we observed that the lower population densities experienced intraindividual diversity erosion, while the herd subjected to higher densities displayed phylogenetic restructuring without intraindividual diversity loss. More diverse diets normally provide a higher diversity of nutritional substrates to the gastrointestinal microbiome community, therefore promoting the maintenance of a wider range of microbial taxa (Heiman & Greenway, 2016). In herbivores, functional redundancy is a common pattern observed in gastrointestinal microbiome composition, where different taxa perform the same metabolic task. Therefore, functionally redundant communities may buffer functional loss despite taxonomic turnover, allowing key metabolic processes to be maintained even when specific taxa are depleted or even lost (Fässler et al., 2025). Taken together, these results suggest that a highly diverse gastrointestinal microbiome in terms of species number, phylogenetic diversity and functional redundancy is key to

allowing red deer to adapt to harsher environmental conditions characterized by resource loss and a potential increase in resource competition. Moreover, as observed in the results of **study II,** in red deer, physiological processes that are essential for determining an individual's general fitness status (and, consequently, the population it belongs to) can depend on the overall community composition, both in terms of richness and species abundance and even on the presence of specific taxa. Therefore, the impact of overabundance-driven changes in the gastrointestinal microbiome linked to feeding behavior may extend beyond facilitating dietary adaptation. As shown in **study II**, host physiological status in red deer is not primarily determined by overall microbial diversity, but rather by shifts in community composition and the presence or absence of specific functional taxa. In particular, taxa involved in fiber degradation and the production of short-chain fatty acids (SCFAs) play a central role in maintaining gut homeostasis, regulating inflammation, and modulating oxidative balance.

In this context, microbiome restructuring observed under higher density conditions (**study IV**) may have important physiological consequences. Although an increase in dietary diversification could, in principle, promote microbial functional diversity, the concurrent shift toward lower-quality diets rich in lignin and secondary compounds may alter the balance of microbial taxa. This could result in a reduced relative abundance of key fermentative and SCFA-producing bacteria, or a restructuring of the community that prioritizes detoxification over efficient fermentation.

Such changes may compromise the production of beneficial microbial metabolites involved in maintaining epithelial integrity and immune regulation, potentially promoting a pro-inflammatory and pro-oxidative physiological state. At the same time, the loss or reduced abundance of taxa associated with efficient nutrient extraction (particularly those linked to protein metabolism) or the ability to adapt to harsher environmental conditions could exacerbate the nutritional constraints imposed by a low-quality diet and compromise the long-term population viability in their natural habitat.

Taken together, these results suggest that although microbiome plasticity may enable red deer to cope with suboptimal diets under overabundant conditions, these changes are not necessarily beneficial. Instead, they may reflect a functional reorganization that prioritizes short-term adaptation to resource scarcity at the expense of long-term physiological stability, contributing to increased metabolic stress and reduced overall fitness.

**Study limitations and considerations**

There are different limitations in the studies included in this thesis. Some of them have already been discussed throughout this section, while others are inherent to the methodologies used and the nature of wildlife studies that we believe are worth mentioning.

Diet composition in **studies III and IV** was assessed using the microhistology technique. Despite being known as a reliable

technique for obtaining relative plant species abundance and diet composition (Holechek et al., 1982; Stewart, 1967), its use can have several biases that need to be considered. Species identification accuracy heavily relies on observer experience and epidermal integrity (Alipayo et al., 1992). Some species may not possess enough unique traits to accurately identify their presence; consequently, reaching the species rank, or even the genus, is not always feasible (Holechek et al., 1982). There is also a tendency to overestimate less digestible species, and species that are consumed sporadically, yet important from an ecological perspective, might remain undetected. (McInnis et al., 1983). Some studies have found discrepancies between ruminal content and fecal content regarding plant presence (Anthony & Smith, 1974). Despite this, it is assumed that fecal plant content is a good approximation of ruminal content. Despite these limitations, the microhistology technique is still a reliable method to quantify relative consumption of plant species, as it provides proportions. In this context, molecular identification techniques are a useful tool if the final goal of the study is to really investigate total diet diversity and not relative plant proportions, since it does not allow for taxa quantification (Kartzinel et al., 2015). Overall, available literature shows that both techniques are complementary, but one does not fully substitute the other. Relative cost is also another important factor to consider, as microhistology is a cheaper technique. In our studies, our goal was to study diet selection both in terms of plant species presence but also relative dietary abundance; hence, we used the microhistology technique.

The further obtention of browsing data on the field helped provide more robustness to the obtained results.

Most studies aiming to characterize the gastrointestinal microbiome of free-ranging wild animals must contend with a series of inherent methodological limitations. Samples are typically collected from the environment, where they may have already undergone some degree of DNA degradation due to climatic conditions. Subsequent handling, transport, and storage can further contribute to DNA breakdown, potentially affecting sequencing depth and taxonomic resolution, and thereby limiting direct comparability with other studies. In addition, environmental sampling often precludes individual identification, constraining inference to the population level rather than allowing for fine-scale, individual-based analyses.

Despite these constraints, such limitations are not unique to this study but are shared across most research on wild, free-ranging populations. Importantly, methodological advances in sequencing technologies, together with increasingly robust bioinformatic pipelines, have substantially improved the reliability and interpretability of microbiome data derived from environmental samples. As a result, these approaches continue to provide valuable ecological insights, particularly when the objective is to detect broad patterns in community composition, taxa turnover, and population-level responses to environmental variation. Therefore, while results should be interpreted with appropriate caution, they

remain highly informative for understanding host–microbiome interactions in natural systems where more controlled sampling designs are not feasible.

Finally, our experimental design did not allow us to include the social interaction factor in our studies as a potential source of microbiome diversity and community homogenization (Murillo et al., 2022). Given the structured social organization of red deer, particularly among females, future studies should explore how social grouping and sex-specific behavior contribute to microbiome variation within populations.

**Future research perspectives**

Future research should aim to build on the integrative framework proposed in this thesis by combining advances in dietary, microbiome, and ecological monitoring. The application of DNA metabarcoding could be a promising non-invasive tool to accurately characterize plant consumption in free-ranging populations, allowing researchers to identify key dietary species and monitor the use of nutritionally important or conservation-relevant plants over time. At the same time, despite the growing body of literature on gastrointestinal microbiomes in wild herbivores, important knowledge gaps remain (Combrink et al., 2023). Current evidence linking microbiome composition to host fitness is largely correlative (Suzuki, 2017; Worsley et al., 2024), and longitudinal studies following the same individuals through time are still scarce due to logistical constraints (Combrink et al., 2023). Expanding such

designs would be essential to understand how microbiome communities respond to environmental fluctuations, identify core and transient taxa, and detect periods of increased susceptibility to physiological stress or disease.

Additionally, the field of metagenomics and multiomics allow researchers to identify the metabolic function of certain taxa, directly linking microbiome community composition to certain animal traits (Wei et al., 2025). Although this technology has already been applied to domestic animals to improve animal productivity (Wei et al., 2025), the application of this methodology to potentially discern the relationship between the presence of certain taxa to activation of metabolic pathways that have a direct impact on the host overall health status remains understudied in wild species.

Finally, future studies should aim to integrate microbiome dynamics with individual and population traits, including social structure, survival, fecundity, and recruitment. Such an approach would provide a more comprehensive understanding of how diet–microbiome–physiology interactions scale up to influence population resilience and long-term viability under changing environmental conditions.

**Management implications and practical recommendations**

Our findings provide important insights for the management of wild red deer populations, particularly in Mediterranean ecosystems where seasonal resource limitation is already a defining ecological

constraint. Traditionally, population management has relied heavily on visible indicators such as body condition, vegetation damage, or population counts. However, our results suggest that physiological and microbiome-mediated responses to overabundance may occur well before these overt signs become apparent.

From a management perspective, the observed density-dependent dietary shifts indicate that high population densities can force individuals to abandon optimal foraging strategies and incorporate lower-quality, lignin-rich plant species. Although red deer display a high degree of dietary plasticity, this flexibility comes at a cost, as reflected by increased physiological stress and the need to rely on suboptimal nutritional resources. Therefore, wildlife managers should aim not only to prevent habitat degradation but also to maintain population densities below thresholds that trigger these costly dietary adaptations.

Importantly, our results highlight the gastrointestinal microbiome as a sensitive and integrative indicator of population health. Changes in microbiome composition, particularly shifts affecting fiber-degrading, short-chain fatty acid–producing taxa or the periodical increase of opportunistic taxa such as *Escherichia* spp. or *Campylobacter* spp., may signal early metabolic disruptions in nutritional balance or immune regulation. This suggests that microbiome-based metrics could complement traditional monitoring tools by providing early-warning signals of population-level stress before declines in body condition or survival becomes

evident, with the added advantage that it would be a non-invasive, easily obtainable sample from the environment.

Additionally, our findings emphasize that not all plant resources contribute equally to herbivore health and that, when the population surpasses the carrying capacity of the environment, red deer have the ability to incorporate previously unwanted species to their diet. This phenomenon has several different implications. First, monitoring of browsing and plant biomass loss of non-palatable species could be an easy indicator that overabundance has been reached in that habitat and therefore, population management measures have to be put in place to return the species to sustainable levels. Second, management strategies aimed at improving habitat quality—such as promoting plant communities rich in high-quality forage species—could help maintain both optimal diet selection and a functionally beneficial microbiome composition. In Mediterranean systems, this may involve adaptive habitat management practices that consider plant phenology, browsing pressure, interspecies competition and vegetation recovery dynamics.

Finally, the interaction between diet, microbiome composition, and physiological status suggests that overabundance may have cryptic effects on population fitness that are not immediately visible. Microbiome restructuring that prioritizes detoxification over efficient fermentation may allow short-term persistence under poor conditions but could reduce long-term resilience by impairing

immune function and increasing oxidative stress. Consequently, management decisions based solely on population size or vegetation impact may underestimate the true biological cost of overabundance.

Ultimately, our findings suggest that overabundance is not only an ecological problem but also a physiological one, with consequences that are mediated through diet–microbiome interactions and may remain undetected without integrative approaches to wildlife monitoring. Taken together, our results support a more integrative approach to wildlife management, where population density, habitat quality, and host–microbiome interactions are considered jointly. Such an approach may improve the ability to maintain sustainable populations while preserving both ecosystem integrity and animal health.

# CONCLUSIONS



*A lonely adult red deer doe, staring into the camera. Photo author: PAELLA lab.*

*"To live in harmony with nature, we must know how to sing the same song as nature. To do that, we must understand nature. Good intentions aren't enough. Science might be – if we use it wisely."*

**— Terry Pratchett,** **The Science of Discworld**

## Conclusions

1. Red deer feeding behavior is primarily driven by plant nutritional quality, favoring plants with high crude protein content and low in lignin, while physical traits such as breaking force do not play a significant role in plant selection.
2. Microbiome composition and taxa turnover, rather than overall diversity, are the main factors associated with host physiological status, including immune function and nutritional condition. Taxa involved in fiber degradation and short-chain fatty acid production are key to maintaining gut homeostasis, immune regulation, and oxidative balance.
3. Population overabundance disrupts optimal foraging patterns, forcing individuals to expand their dietary niche and incorporate previously avoided, lower-quality plant species, with detrimental effects to red deer physiology and ecosystem dynamics.
4. Adaptation to resource limitation in red deer is mediated by both behavior and microbial symbiosis, but this adaptability may conceal underlying physiological costs with important implications for long-term population resilience.

# GLOSSARY



*Close-up of a red deer eye. Photo authors: Jens & Renheimer in Pexel. Used with permission under a creative commons license.*

*“Particles of raw inspiration sleet through the universe all the time. Every once in a while, one of them hits a receptive mind, which then invents DNA or the flute sonata form or a way of making light bulbs wear out in half the time. But most of them miss. Most people go through their lives without being hit by even one.*

*Some people are even more unfortunate. They get hit by them all.”*

**— Terry Pratchett,** **Wyrd sisters**

# Glossary

## 0

- **16s rRNA: a** component of the 30S small subunit of prokaryotic ribosomes (bacteria and archaea) that acts as a universal molecular marker for identifying and classifying microorganisms.

## A

- **Area-based rolox equivalent antioxidant capacity (TEACa):** an index of antioxidant capacity calculated from the area under the reaction curve over time, reflecting both the magnitude and kinetics of radical scavenging activity.
- **Acid detergent fiber (ADF):** a measure of plant fiber including cellulose and lignin; indicates low digestibility—higher ADF means poorer forage quality. Obtained by subtracting hemicellulose values from neutral detergent fiber values.

$$ADF = NDF - hemicellulose$$

- **Acid detergent lignin (ADL):** a fraction of non-digestible fiber that serves as an indicator of poor diet quality in herbivores. Obtained by subtracting the cellulose portion from ADF values.

$$ADL = ADF - cellulose$$

- **Aitchison's distance:** Euclidean distance between two compositions after Centered-Log Ratio (CLR) transformation. It measures dissimilarity between compositional data.
- **Alpha diversity:** metrics used to quantify the internal diversity of a single microbiome sample.
- **Amplicon sequence variant (ASV):** high-resolution DNA sequence units representing unique microbial taxa derived from sequencing data.
- **Ana Karenina effect:** ecological principle stating that healthy microbiomes are similar, while unhealthy (dysbiotic) ones differ widely between individuals.
- **Analysis of Compositions of Microbiomes with bias correction (ANCOM-BC):** statistical analysis used to identify differentially abundant taxa in a microbiome dataset, which corrects for biases originating from unequal sampling fractions using a log-linear model.
- **Ash content:** the inorganic residue remaining after combustion of plant material, reflects mineral content and may reduce palatability when high.

# B

- **Bacterial killing ability (BKA):** a laboratory method used to measure innate immune competence by the ability of the plasma sample to kill bacteria and obtain plasma antimicrobial capacity, expressed as the percentage of bacteria eliminated relative to a bacterial growth control curve.
- **Beta diversity:** measures of compositional variation between samples, indicating how much two microbial communities differ from one another.
- **Bite size:** The amount of plant material an herbivore can consume in one bite; it influences feeding efficiency and selection.
- **Bray-Curtis dissimilarity index:** quantitative index used to measure the compositional differences between two communities based on relative species abundance, ranging from 0 (completely identical composition) to 1 (completely dissimilar). One of beta diversity most commonly used indexes.

$$BC_{UV} = \frac{\sum_{j=1}^{S}\left|x_{Uj} - x_{Vj}\right|}{\sum_{j=1}^{S}\left(x_{Uj} + x_{Vj}\right)}$$

- **Breaking force:** the physical force measured in Newtons, required to cut terminal plant shoots.

- **Browsing degree:** a categorical scale (0–5) used to quantify the intensity of herbivore consumption on plants in the field.
- **Browsing intensity:** a measure of herbivore pressure on plants, typically scored from no browsing to maximum browsing, used as an indicator of resource use and depletion, normally quantified using the browsing degree.
- **Bryophyta:** a group of non-vascular (lack specialized tissue for transporting water and nutrients), seedless land plants comprising mosses, liverworts, and hornworts.

# C

- **Cafeteria test (multiple-choice feeding test):** an experimental design where animals are simultaneously offered a variety of plant species to directly determine dietary preferences under controlled conditions.
- **Carrying capacity:** the maximum population size that an environment can sustain without suffering negative consequences.
- **Cellulose:** a structural carbohydrate in plant cell walls; partially digestible and contributes to fiber content.
- **Centered log-ratio (CLR) transformation:** normalization method of composition data (like percentages or proportions) by dividing each part by the geometric mean of all components and taking the natural logarithm.

- **Crude protein (CP):** an estimate of protein content derived from nitrogen concentration. High concentrations are correlated to higher diet quality.
- **Cupric ion Reducing Antioxidant Capacity (CUPRAC):** colorimetric method used to measure the total antioxidant capacity (TAC) of biological samples, based on how effectively a sample can reduce copper(II) to copper(I).

# D

- **Diet composition:** presence of plant species and their relative proportions found in the diet.
- **Diet quality:** an assessment of the nutritional value of consumed forage. In herbivores, protein content indirectly measured through nitrogen content and levels of lignin are the two main components used to assess diet quality, where diets rich in protein and poor in lignin are considered to be of higher quality.
- **Diet selection:** The behavioral process by which animals choose among available food resources to meet nutritional needs.
- **Dietary plasticity:** the ability of an organism to adapt its diet based on resource availability and environmental conditions.

- **Digestibility:** the extent to which food can be broken down and absorbed by an organism. In herbivores, it is influenced by fiber and lignin content.
- **Dormancy period:** phenological stage during the annual cycle when plant's metabolic activity is at its minimum and growth is stopped.
- **Dysbiosis:** imbalance in the bacterial composition of a microbiome community, usually linked to a homeostatic disruption of the community. Dysbiosis can be characterized by loss of beneficial bacteria, opportunistic bacteria overgrowth and an overall loss of microbial diversity.

# E

- **Enhanced vegetation index (EVI):** an optimized satellite-derived index used to quantify vegetation greenness and health, with superior sensitivity in high-biomass areas compared to NDVI. Compared to the NDVI, EVI also incorporates the blue band in its formula, therefore decreasing atmospheric interference and enhancing sensitivity in areas with dense vegetation. The mathematical formula is found below, where C1 and C2 are coefficients for correcting atmospheric resistance; NIR is near-infrared spectrum; red and blue light indicates reflectance in the red and blue light band, respectively; L is canopy background adjustment, and G is an additional correction factor. Values

range from -1 to 1, with positive higher values correlated to dense and green vegetation canopy.

$$EVI = G \times \frac{(NIR - Red\ light)}{(NIR + C1 \times Red\ light - C2 \times Blue\ light + L)}$$

# F

- **Faith's phylogenetic distance (Faith's PD):** an alpha diversity metric that represents the diversity contained in a sample from a phylogenetic perspective, therefore higher values indicate higher taxonomical richness. Since it is calculated using an algorithmic approach, it does not have any specific formula. It is calculated by summing the dataset phylogenetic tree branch lengths.
- **False discovery rate (FDR):** expected proportion of false positives among all significant results in multiple testing scenarios as a method of controlling for Type I errors.
- **Fecal glucocorticoid metabolites (FGM):** a metabolite derived from cortisol found in feces used as an non-invasive indicator of physiological stress.
- **Fecal nitrogen (FN):** The proportion of nitrogen in fecal samples, used as a proxy for the amount of nitrogen available in the diet for digestion. It is a standard non-invasive indicator of diet quality.

- **Feeding behavior:** actions, patterns, and mechanisms organisms use to seek, obtain, and consume nutrients to survive, driven by physiological hunger or external cues.
- **Ferric reducing ability of plasma (FREP):** laboratory technique used to measure the total antioxidant capacity of plasma, by measuring the ability of plasma antioxidant components to reduce ferric-tripyridyltriazine ($Fe^{3+}$-TPTZ) complex to its ferrous ($Fe^{2+}$) form at low pH, which produces an intense blue color measured at 593 nm.
- **Foodscape:** the spatial and temporal distribution of food resources with varying nutritional qualities available to herbivores.
- **Forage quality:** The nutritional value of plant material, typically determined by protein, fiber, and mineral content.
- **Functional redundancy:** ability of a microbiome community to retain its physiological functions after suffering microbial biomass and diversity loss.

# G

- **Gastrointestinal microbiome:** community of microorganisms (including bacteria, viruses, fungi and archaea) found in the gastrointestinal tract of individuals.
- **Generalized additive mixed models (GAMM):** statistical model that models nonlinear correlations between the

response and explanatory variables using smoothing functions while accounting for repeated measures of the same sampling unit.

- **Generalized linear mixed models (GLMMs):** statistical models used to analyze non-normally distributed data that include both fixed (general trends) and random effects (accounting for variance within the same group or individuals), suitable for non-independent repeated measures data of the same sampling unit.
- **Green up period:** period of a plant's life cycle where dormancy is disrupted and vegetation begins new leaf growth.

# H

- **Height-based trolox equivalent antioxidant capacity (TEACh):** an index of antioxidant capacity based on the maximum reduction (peak change) in absorbance during the reaction, reflecting the maximal antioxidant effect.
- **Hemicellulose:** a plant cell wall component that is more digestible than cellulose; calculated as NDF minus ADF.
- **Herbaceous plant:** a vascular plant with soft, flexible, green stems that lacks persistent woody tissue above ground.
- **Herbivory pressure:** the impact of herbivore feeding on plant communities and vegetation structure.

- **Host fitness:** the overall health, survival, and reproductive success of an organism.

# I

- **Innate immunity:** the first line of host defense against pathogens. Composed by physical (mucosal and epithelial junctions) and chemical barriers (enzymes, antimicrobial peptides), inflammation serum proteins and immunity cells like phagocytes and natural killer cells, among others.

# L

- **Lignin:** a complex, indigestible polymer in plant cell walls that reduces digestibility and palatability.

# M

- **Metabolic fecal nitrogen (MFN):** proportion of fecal nitrogen from bacteria and endogenous losses, considered as an indirect measure of the amount of nitrogen found in the diet and available for digestion and assimilation. Obtained by subtracting the residual food nitrogen (defined as the proportion of nitrogen bound to food particles and unavailable for ruminal digestion, RFN) fraction from the overall fecal nitrogen (FN) value.

$$MFN = FN - RFN$$

- **Microbiome multivariable association with linear models (MaAsLin2):** a R Package designed to determine multivariable association between metadata and microbial meta-omics features such as taxa, genes, or metabolic pathways, among others, using general linear models with a false discovery rate control to handle cross-sectional, longitudinal and sparse data.
- **Microbiome plasticity:** the ability of a microbial community to adapt to exogenous and endogenous changes.

# N

- **Near-infrared reflectance spectroscopy (NIRS):** a technique that measures transmission and absorption of near-infrared light in biological samples used in our studies to predict the chemical composition (nitrogen, fibers) and metabolic markers (FGM) in fecal samples.
- **Neutral detergent fiber (NDF):** residual portion after extraction with hot neutral detergent solutions that contain cellulose, hemicellulose, lignin and ash content, normally used as an indicator of diet quality (higher values implicate lower quality).
- **Normalized difference vegetation index (NDVI):** remote sensing metric used to assess the presence, density and health of green vegetation by measuring the difference

between the reflected (near-infrared, NIR) and absorbed (red light) by plants. Values range from -1 to 1, where positive values close to one (> 0.5) indicate green and healthy vegetation, while lower values (< 0.5) indicate stressed or barren vegetation. NDVI values are calculated using the following formula:

$$NDVI = \frac{(NIR - Red\ light)}{(NIR + Red\ light)}$$

# O

- **Ordination:** multivariate techniques used to visualize patterns in community composition constrained by explanatory variables.
- **Oxidative status:** The balance between the production of reactive oxygen species (free radicals) and antioxidant defenses.
- **Oxidative stress:** a physiological condition caused by an imbalance between free radicals and antioxidants.
- **Optimal foraging theory:** an ecological theory that hypothesizes that foraging behaviors have evolved through natural selection to maximize energy returns while considering environmental and physiological constraints and costs.

# P

- **Palatability:** the attractiveness or acceptability of food to animals, influenced by their physicochemical properties such as taste, texture, nutrients, and secondary compounds, among others.
- **Permutational multivariate analysis of variance (PERMANOVA):** a non-parametric statistical test used to evaluate differences in multivariate data, determining if their centroids or dispersions differ significantly.
- **Phenology:** the study of cyclic and seasonal natural phenomena, specifically the growth phases of vegetation (e.g., green up, stable peak period, senescence, and dormancy).
- **Phenophase:** life cycle stage within a recurring biological cycle.
- **Phylogenetic diversity:** a measure of biodiversity that considers evolutionary relationships between species.
- **Phylogenetic redundancy:** phenomenon where different, distantly related (taxonomically distinct) microbial species carry out similar metabolic functions or ecological roles within a community.
- **Phylosimbiosis:** an ecological and evolutionary pattern where the microbial community composition (microbiome)

of a host species mirrors its evolutionary history or phylogenetic relationships.

- **Physicochemical traits:** the combination of mechanical defenses (e.g., hardness, breaking force) and chemical composition (e.g., protein, lignin, secondary metabolites) that determine a plant's palatability and nutritional value.
- **Phytovolume:** a metric derived from plant height and diameter used to estimate above-ground biomass. Reductions in phytovolume were used to track resource depletion in your experimental enclosures.
- **Pielou's index of evenness (J'):** measures distribution evenness across species included in the sample. Its values range from 0 to 1, with 1 indicating that all species are equally abundant. Shannon's index is the basis of calculating Pielou's. Pielou's formula is displayed below, where H' is Shannon's diversity index and S is the number of observed species.

$$J' = \frac{H'}{ln(S)}$$

- **Principal component analysis (PCA):** a statistical method used to reduce data dimensionality and identify key variables explaining variation.

- **Principal coordinate analysis (PCoA):** visualization technique that maps high-dimensional bacterial community data onto a 2D or 3D plot to show sample similarities.

# R

- **Random effect:** a statistical term included in mixed models to account for variability of the same sampling unit.
- **Rarefaction:** normalization technique that randomly subsamples sequencing reads to a uniform depth across all samples, correcting for uneven sequencing effort. It allows for "fair" comparisons of alpha and beta diversity by ensuring that differences in diversity are not simply due to some samples having more reads than others.

# S

- **Secondary metabolites:** chemical compounds (e.g., alkaloids, terpenes, phenolics) that can deter herbivory by reducing palatability or being toxic.
- **Senescence:** plant phenological phase characterized by being the final stage of leaf development, characterized by a progressive degradation of leaf cellular components.
- **Shannon's diversity index (H'):** an alpha diversity metric that measures the richness (number of different taxa present in the sample) and evenness (relative abundance of

each taxa present) within a sample. Value range depends on the total number of taxa present. Higher values indicate a more diverse and even community, while lower values reflect lower diversity and evenness. This index is more sensitive to species richness. The formula is displayed below, where $p_i$ represents the likelihood of observing the *i* taxa.

$$H' = -\sum(p_i \times ln(p_i))$$

- **Short volatile fatty acids (SVFAs) or short chain fatty acids (SCFA):** short-chain carboxylic acids made up of less than six carbons and produce microbial-mediated fiber anaerobic fermentation in the gut. Acetate ($C_2$), propionate ($C_3$) and butyrate ($C_4$) are the main ones; other less common SVFAs include valeric acid ($C_5$) and caproic acid ($C_6$), among others. They are the main energetic source for ruminants and act as anti-inflammatory and immune modulator molecules.

- **Silica:** an abrasive inorganic compound in plant tissues (especially grasses) that reduces digestibility and increases tooth wear.

- **Simpson's diversity index:** an alpha diversity index that calculates the probability that two randomly selected microbes from a sample belong to the same species. Values range from 0 to 1, where values closer to 0 represent infinite diversity (0 probability that the two randomly selected microbes belong to the same species) while values closer to

1 indicate no diversity (same probability of the two microbes belonging to the same species). This index is more sensitive to species evenness. Simpson's formula is displayed below, where $S$ is the number of observed taxa and $p_i$ is the proportion of taxa.

$$1 - \sum_{i=1}^{S} p_i^2$$

- **Stable peak period:** phenological phase where plant activity is at its highest.

# T

- **Taxa turnover:** The process by which the identity and relative abundance of specific bacterial groups in the gut change over time or in response to different ecological filters, such as population density or diet shifts.
- **Total sum scaling (TSS):** a normalization method where counts are converted to relative abundances.
- **Trolox equivalent antioxidant capacity (TEAC**): assay that measures the ability of antioxidants to reduce the ABTS radical cation. Indicative of antioxidant capacity.

# U

- **Unweighted UniFrac distance:** a beta diversity metric that measures the difference between two microbial communities based on phylogenetic branch lengths, but considering only the presence and absence of taxa instead of relative abundances.

# V

- **V4 region of 16S rRNA:** a ~254 base pair highly variable segment used in microbial community profiling. It is popular for NGS (Next-Generation Sequencing) due to its high taxonomic resolution, balanced length, and compatibility with Illumina sequencing.

# W

- **Weighted UniFrac distance:** beta diversity index that measures the phylogenetic dissimilarity between two microbial communities that consider both phylogenetic tree length branches and relative taxonomic abundance.
- **Woody plant:** a perennial plant that develops hard lignified tissues for structural support, with stems that survive and continue to grow in diameter above ground for several years. Normally includes trees and shrubs.

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*“Morning With the Wild Red Deer”, 1876. Samuel John Carter. Photo author: Tate museum.*

*"This book was written using 100% recycled words."*

**— Terry Pratchett, Wyrd sisters**

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